

THE IMPORTANCE OF UNCOUPLING TROPONIN I PHOSPHORYLATION
FROM Ca^{2+} SENSITIVITY IN THE PATHOGENESIS OF CARDIOMYOPATHY

by

Maria Papadaki

Thesis submitted for the degree of

Doctor of Philosophy

Myocardial Function
National Heart and Lung Institute
Faculty of Medicine
Imperial College London

September 2015

Abstract

Heart muscle contraction is regulated via the β adrenergic response that results to phosphorylation of Protein Kinase A (PKA), which in turn decreases the Ca^{2+} sensitivity of the cardiac myofilament, which is very important for the heart muscle to relax. Mutations in the thin filament that cause Dilated Cardiomyopathy (DCM) and some that cause Hypertrophic Cardiomyopathy (HCM) abolish this relationship, so that the Ca^{2+} sensitivity becomes independent of Troponin I (TnI) phosphorylation (uncoupling). The aim of the thesis is to unravel the molecular mechanism of the uncoupling phenomenon. It is known that there is a specific interaction between the phosphorylatable TnI N terminal peptide and the Ca^{2+} binding site on TnC, that is weakened by phosphorylation and we hypothesize that it is disrupted in case of a DCM or HCM mutation, giving rise to the uncoupling phenomenon. Ca^{2+} sensitisers and desensitisers change the Ca^{2+} sensitivity of the cardiac muscle like mutations do but their relationship with TnI phosphorylation has never been studied before.

Using the *in vitro* motility assay I showed that the Ca^{2+} sensitisers EMD 57033 and Bepridil increased the Ca^{2+} sensitivity of donor thin filaments and additionally they uncoupled the Ca^{2+} sensitivity from the TnI phosphorylation. Epigallocatechin-3-gallate (EGCG) decreased the Ca^{2+} sensitivity of donor thin filaments whilst retaining the coupling. On the other hand, EGCG reduced the Ca^{2+} sensitivity of phosphorylated but not dephosphorylated mutant thin filaments restoring the Ca^{2+} sensitivity change to TnI phosphorylation. EGCG re-coupled 5 DCM (*TPMI* E54K and E40K, *TNNI3* K36Q, *TNNC1* G159D, *ACTC* E361G) mutants and 3 HCM (*TPMI* E180G, *TNNT2* K280N, *ACTC* E99K) mutants which were originally uncoupled.

We were given 30 analogue compounds structurally similar to EGCG and nine of them were able to re-couple uncoupled *TPMI* E180G HCM mutant thin filaments. The working compounds re-coupled DCM mutation *TPMI* E54K and HCM mutation *ACTC* E99K.

I show for the first time that it is possible to mimic and reverse the effect of DCM and HCM mutations on troponin pharmacologically. EGCG and its analogue compounds might have significant implications for the effective treatment of thin filament cardiomyopathies that uncouple the Ca^{2+} sensitivity from TnI phosphorylation.

In a separate study I investigated 11 mutations in skeletal muscle tropomyosin associated with various myopathies. I found that 7 mutations cause a gain of function that could be accounted for at the molecular level due to destabilising specific actin-tropomyosin interactions. Gain of function at the molecular level correlates with a hypercontractile phenotype in patients.

Declaration of originality

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Table of Contents

Abstract.....	2
Declaration of originality	4
Copyright declaration.....	5
Table of Contents	6
List of Figures.....	12
List of Tables	17
List of Abbreviations	20
Acknowledgements	23
1 Introduction	24
1.1 General Introduction	24
1.2 Muscle structure.....	25
1.2.1 The skeletal muscle.....	25
1.2.2 The heart muscle and the cardiovascular system.....	27
1.2.3 The sarcomere	29
1.2.4 The thick filament.....	31
1.2.5 The thin filament.....	32
1.3 Muscle contraction.....	42
1.3.1 The cardiac cycle	42
1.3.2 The action potential.....	43
1.3.3 The excitation-contraction coupling	44
1.3.4 The sliding filament theory and the cross-bridge cycle	46
1.3.5 Thin filament switch	48
1.3.6 The three state model	50
1.3.7 The regulation of the cardiac muscle	52
1.4 Primary cardiomyopathies	55
1.4.1 Dilated Cardiomyopathy (DCM)	55
1.4.2 Hypertrophic Cardiomyopathy (HCM).....	57
1.4.3 Methodology to study familial cardiomyopathies	60

1.4.4	The molecular mechanisms of DCM and HCM mutations	62
1.5	The uncoupling of Ca^{2+} sensitivity to TnI phosphorylation and its importance.....	64
1.5.1	TnI phosphorylation by PKA.....	64
1.5.2	The discovery of the uncoupling phenomenon.....	64
1.5.3	Uncoupling as a mechanism of cardiomyopathies with thin filament mutations.....	65
1.5.4	The physiological importance of the uncoupling phenomenon.....	68
1.6	Aims.....	69
2	MATERIALS AND METHODS.....	71
2.1	Sources of sample proteins	71
2.1.1	Sources of human heart samples.....	71
2.1.2	Sources of mouse heart samples	72
2.1.3	Tropomyosin samples	73
2.2	Protein preparation.....	75
2.2.1	Purification of myosin from rabbit muscle	75
2.2.2	Preparation of Heavy meromyosin (HMM).....	76
2.2.3	Preparation of skeletal F-actin	76
2.2.4	Purification of the troponin complex	79
2.2.5	Further manipulation of the troponin complex	81
2.3	Analysis of proteins	83
2.3.1	Determination of protein concentration.....	83
2.3.2	Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	85
2.3.3	Protein staining	86
2.3.4	Western Blot	86
2.4	Sources of drugs and compounds	88
2.4.1	Ca^{2+} sensitisers.....	88
2.4.2	Ca^{2+} desensitisers	88
2.4.3	EGCG analogue compounds.....	88
2.5	The in vitro motility assay (IVMA).....	90
2.5.1	Buffers for IVMA	90
2.5.2	Fluorescent labelling of F-actin	90

2.5.3	Affinity precipitation of HMM	90
2.5.4	Preparation of the flow cells	91
2.5.5	Reconstitution of the thin filaments	92
2.5.6	Loading of the flow motility cell	93
2.5.7	Preparation of Ca^{2+} -EGTA buffers	95
2.5.8	Experimental strategy	96
2.5.9	Recording Apparatus	97
2.5.10	Analysis of Filament Movement.....	99
2.5.11	Statistical analysis	104

3 Mutations in tropomyosin that cause gain of function in skeletal muscle myopathy patients 106

3.1	Introduction.....	106
3.2	Tropomyosin mutations in <i>TPM2</i> and <i>TPM3</i> that cause gain of function....	109
3.2.1	In vitro motility analysis of <i>TPM2</i> and <i>TPM3</i> mutations	110
3.3	Tropomyosin mutations in <i>TPM3</i> that cause a loss of function.....	116
3.3.1	<i>In vitro</i> motility assay analysis of R167H and R244G	117
3.4	Two novel <i>TPM3</i> deletions also cause a hypercontractile phenotype with gain of function.....	121
3.4.1	<i>In vitro</i> motility analysis of ΔE224 and ΔE218 <i>TPM3</i> mutations	122
3.5	Discussion.....	128
3.5.1	IVMA Advantages and Limitations.....	128
3.5.2	Structure of the tropomyosin-actin interface predicts mutations that cause gain of function in skeletal muscle diseases	129
3.5.3	Linkage between the Ca^{2+} sensitivity caused by skeletal muscle myopathy and patient phenotype	131
3.5.4	Structural analysis of tropomyosin-actin regulation.....	134

4 Investigation of the effect of Ca^{2+} sensitisers on reconstituted human heart thin filaments 137

4.1	General Introduction	137
4.2	Investigation of the effects of EMD 57033 on human heart thin filaments..	139
4.2.1	Introduction.....	139
4.2.2	<i>In vitro</i> motility analysis on EMD 57033 on donor thin filaments.....	140

4.2.3	The effect of EMD 57033 on the coupling between TnI phosphorylation and Ca^{2+} sensitivity on donor thin filaments.....	145
4.2.4	Investigation of the effects of EMD 57033 on DCM E54K mutant human thin filaments.	149
4.3	The effect of Bepridil on human heart thin filaments.....	152
4.3.1	Introduction.....	152
4.3.2	Dose response curve of Bepridil on donor human heart thin filaments.....	153
4.3.3	The effect of Bepridil on the coupling between phosphorylated and dephosphorylated donor thin filaments.....	154
4.3.4	The effect of Bepridil on DCM E54K mutant thin filaments	157
4.4	Discussion.....	161
4.4.1	Studying the mechanism of the uncoupling phenomenon using pharmacological agents.....	161
4.4.2	Ca^{2+} sensitisers mimic the effects of HCM mutations.....	161
4.4.3	Mechanism of action of the Ca^{2+} sensitisers.....	162

5 Investigation of the effect of EGCG on reconstituted human thin filaments..... 166

5.1	Introduction.....	166
5.2	Investigation of the effect of EGCG on donor thin filaments.....	167
5.2.1	<i>In vitro</i> motility assay results of donor phosphorylated and dephosphorylated thin filaments treated with EGCG.	167
5.2.2	Dose response curve of EGCG on donor thin filaments.....	170
5.2.3	Reversibility of the action of EGCG.....	172
5.3	The effect of EGCG on <i>TPMI</i> mutations that cause DCM or HCM.....	173
5.3.1	The effect of EGCG on <i>TPMI</i> E54K DCM mutation mutant thin filaments.....	173
5.3.2	The effect of EGCG on <i>TPMI</i> E40K DCM mutant thin filaments	176
5.3.3	The effect of EGCG on <i>TPMI</i> E180G HCM mutant thin filaments	179
5.3.4	Dose response curve of re-coupling by EGCG.....	182
5.4	The effect of EGCG on three further DCM mutations	183
5.4.1	The effect of EGCG on <i>TNNC2</i> G159D DCM mutant thin filaments...	183
5.4.2	The effect of EGCG on <i>TNNI3</i> K36Q DCM mutant thin filaments.....	187
5.4.3	The effect of EGCG on <i>ACTC</i> E361G DCM mutant thin filaments	190

5.5	The effect of EGCG on two HCM mutations	193
5.5.1	The effect of EGCG on <i>TNNT2</i> K280N HCM mutant thin filaments ...	193
5.5.2	The effect of EGCG on <i>ACTC</i> E99K HCM mutant thin filaments	197
5.6	DISCUSSION	202
5.6.1	The uncoupling phenomenon and its relationship to EC ₅₀	202
5.6.2	The Ca ²⁺ desensitiser EGCG restores coupling to thin filaments with HCM and DCM mutations.....	204
5.6.3	Mechanistic insights on the action of EGCG.....	207
5.6.4	Clinical relevance of re-coupling by EGCG	210
6	The effect of EGCG analogues on human heart thin filaments.....	211
6.1	Introduction.....	211
6.2	Rapid screening assay to identify re-couplers	212
6.2.2	Developing a screening methodology.....	212
6.2.3	Results of the rapid screening assay	214
6.3	Evaluation of best candidates on <i>TPM1</i> E180G HCM mutant thin filaments.....	217
6.4	Evaluation of the best drug candidates in <i>TPM1</i> E54K DCM mutant thin filaments.....	223
6.5	Evaluation of the best drug candidates on <i>ACTC</i> E99K HCM mutant thin filaments.....	227
6.6	The effect of the compounds on donor thin filaments.	231
6.7	The re-coupling effect of known Hsp90 inhibitors.....	233
6.8	Discussion.....	235
6.8.1	Rapid screening methodology.....	235
6.8.2	Further analysis of potent re-couplers allows for insights about the uncoupling phenomenon.....	235
6.8.3	Limitations	239
6.8.4	Clinical perspectives	239
7	FINAL DISCUSSION	241
7.1	Aims of the thesis.....	241
7.2	Uncoupling by mutations as a disease-causing process.....	242
7.3	Small molecules can modulate coupling.....	244

7.4	Ca ²⁺ desensitisers like EGCG can reverse the uncoupling phenomenon.	245
7.5	Insights for troponin regulatory mechanism	248
7.6	Clinical implications of the uncoupling phenomenon	252
7.7	Future work.....	253
BIBLIOGRAPHY		257
APPENDIX.....		274

List of Figures

Figure 1-1: Skeletal muscle structure	26
Figure 1-2: The anatomical structure of the heart.....	28
Figure 1-3: The structure of a sarcomere	30
Figure 1-4: Structure of myosin II	32
Figure 1-5: Thin filament structure.....	32
Figure 1-6: Structure of actin.....	33
Figure 1-7: Structure of tropomyosin	36
Figure 1-8: Structure of troponin	37
Figure 1-9: Structure of TnC.....	38
Figure 1-10: Cartoon structure of cTnI.....	40
Figure 1-11: The integration of cTnT into the thin filament	41
Figure 1-12: The cardiac cycle	42
Figure 1-13: The action potential propagation in the heart.....	44
Figure 1-14: Ca^{2+} cycle in the cardiomyocyte	45
Figure 1-15: The cross-bridge cycle	47
Figure 1-16: The structure of the core domain of the troponin complex.....	48
Figure 1-17: The thin filament switch	49
Figure 1-18: The three state model and the tropomyosin block	51
Figure 1-19: β adrenergic response of the cardiac muscle.....	54
Figure 1-20: Section of a DCM vs donor heart.....	56
Figure 1-21: Heart from an HCM patient	58
Figure 1-22: The distribution of sarcomeric mutations in HCM	59
Figure 1-23: Methodology used to study HCM and DCM.....	62
Figure 1-24: The uncoupling phenomenon in DCM and HCM.....	67
Figure 2-1: Troponin extraction steps.....	81
Figure 2-2: Troponin extraction steps.....	83
Figure 2-3: The calibration curve for the Bradford assay.....	84
Figure 2-4: Phosphate affinity SDS PAGE showing phosphorylated and dephosphorylated troponin.....	87
Figure 2-5: Densitometric analysis	88
Figure 2-6: Affinity precipitation of HMM	91

Figure 2-7: Construction of the flow cells	92
Figure 2-8: Flow motility assay protocol.....	94
Figure 2-9: Recording apparatus.....	97
Figure 2-10: Image acquisition	99
Figure 2-11: Automatic filament tracking	101
Figure 2-12: Histograms from the tracking software to construct the Ca^{2+} activation curves	103
Figure 2-13: Ca^{2+} activation curves of thin filaments reconstituted from proteins extracted from donor samples	104
Figure 2-14: Distribution of the EC_{50} ratios Donor/Failing troponin	105
Figure 3-1: Tropomyosin- actin interactions	108
Figure 3-2: Location of <i>TPM2</i> and <i>TPM3</i> mutations.....	110
Figure 3-3: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with ΔK49 mutant tropomyosin vs WT	112
Figure 3-4: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with R91G mutant tropomyosin vs WT	113
Figure 3-5: Ca^{2+} activation curves for motile velocity and percentage motility of thin filaments reconstituted with K168E mutant tropomyosin vs WT	114
Figure 3-6: Ca^{2+} activation curves for motile velocity and percentage motility of thin filaments reconstituted with ΔE139 mutant tropomyosin vs WT	115
Figure 3-7: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with mutant R167H tropomyosin vs WT	118
Figure 3-8: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with R244G mutant tropomyosin vs WT	119
Figure 3-9: Location of the novel mutations in <i>TPM3</i>	122
Figure 3-10: Ca^{2+} activation curve for percentage motility and motile velocity of thin filaments reconstituted with ΔE218 mutant tropomyosin vs WT	124
Figure 3-11: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with ΔE224 mutant tropomyosin vs WT	125
Figure 3-12: Synopsis of the effect of skeletal muscle myopathy mutations on Ca^{2+} sensitivity	131
Figure 3-13: Tropomyosin and actin complex open and closed states	135
Figure 4-1: Chemical structure of EMD 57033	139

Figure 4-2: <i>In vitro</i> motility measurements comparing the addition of troponin and tropomyosin to actin filaments.....	141
Figure 4-3: The effect of EMD 57033 on thin filaments containing donor troponin	143
Figure 4-4: Dose response curve for EMD 57033 on donor thin filaments.....	144
Figure 4-5: TnI Western blot of Phos-tag gels.....	145
Figure 4-6: Ca ²⁺ activation curves comparing donor thin filaments containing phosphorylated or dephosphorylated troponin treated with 30 µM EMD 57033... ..	147
Figure 4-7: Ca ²⁺ activation curves comparing thin filaments containing phosphorylated or dephosphorylated troponin and E54K mutant tropomyosin treated with EMD 57033	150
Figure 4-8: Chemical structure of Bepridil.....	152
Figure 4-9: Bepridil dose response curve on reconstituted donor thin filaments	153
Figure 4-10: The effect of Bepridil on phosphorylated and dephosphorylated donor thin filaments	155
Figure 4-11: Ca ²⁺ activation curve of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 µM Bepridil	159
Figure 4-12: Binding of the Ca ²⁺ sensitisers to TnC.....	163
Figure 5-1: Chemical structure of EGCG	166
Figure 5-2: Ca ²⁺ activation curve of phosphorylated or dephosphorylated donor thin filaments treated with 100 µM EGCG.....	168
Figure 5-3: EGCG dose response curve for phosphorylated and dephosphorylated thin filaments.....	171
Figure 5-4: Reversibility of EGCG.....	172
Figure 5-5: Ca ²⁺ activation curve comparing phosphorylated or dephosphorylated E54K mutant thin filaments treated with 100 µM EGCG	174
Figure 5-6: Ca ²⁺ activation curve comparing phosphorylated or dephosphorylated E40K mutant thin filaments treated with 100 µM EGCG	177
Figure 5-7: Ca ²⁺ activation curve comparing phosphorylated and dephosphorylated E180G mutant thin filaments treated with 100 µM EGCG	180
Figure 5-8: Dose response curve for EGCG on E180G mutant thin filaments	182
Figure 5-9: Ca ²⁺ activation curves for phosphorylated and dephosphorylated G159D mutant thin filaments treated with EGCG	185

Figure 5-10: Ca^{2+} activation curves for phosphorylated and dephosphorylated K36Q mutant thin filaments treated with 100 μM EGCG.....	188
Figure 5-11: Ca^{2+} activation curves comparing phosphorylated and dephosphorylated E361G mutant thin filaments treated with 100 μM EGCG	191
Figure 5-12: Ca^{2+} activation curves for phosphorylated and dephosphorylated thin filaments reconstituted with exchanged K280N HCM mutant troponin treated with 100 μM	195
Figure 5-13: Heart morphology of 21-week-old E99K mouse.....	198
Figure 5-14: Ca^{2+} activation curve comparing phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG	200
Figure 5-15: The relationship between EC_{50} and uncoupling.....	204
Figure 5-16: The effect of EGCG on the coupling between TnI phosphorylation and Ca^{2+} sensitivity on DCM and HCM mutations	205
Figure 5-17: The effect of EGCG on myofibrils from E361G transgenic mice	207
Figure 6-1: Chemical structure of Silybin	211
Figure 6-2: Drug screening assay concept and possible outcomes.....	213
Figure 6-3: A screening example of two different compounds using the E180G HCM model.....	215
Figure 6-4: Screening summary of all the compounds tested with IVMA	216
Figure 6-5: Small molecule dose response data for percentage motility in <i>TPMI</i> E180G mutant thin filaments	218
Figure 6-6: Small molecule dose response data for motile velocity of <i>TPMI</i> E180G mutant thin filaments	219
Figure 6-7: Dose response data for percentage motility difference between phosphorylated and dephosphorylated <i>TPMI</i> E180G mutant thin filaments treated with different compounds	220
Figure 6-8: Dose response data summary for percentage motility difference between phosphorylated and dephosphorylated <i>TPMI</i> E180G mutant thin filaments treated with different compounds	221
Figure 6-9: Small molecule dose response data for motile velocity and percentage motility of phosphorylated and dephosphorylated <i>TPMI</i> E54K DCM mutant thin filaments.....	224

Figure 6-10: Percentage motility difference between phosphorylated and dephosphorylated <i>TPM1</i> E54K DCM mutant thin filaments dose response curves	225
Figure 6-11: Summary of percentage motility difference between phosphorylated and dephosphorylated <i>TPM1</i> E54K DCM mutant thin filaments treated with different compounds	226
Figure 6-12: Small molecule dose response summary for percentage motility and motile velocity of <i>ACTC</i> E99K HCM mutant thin filaments	228
Figure 6-13: Small molecule data for percentage motility difference between phosphorylated and dephosphorylated <i>ACTC</i> E99K HCM mutant thin filaments..	229
Figure 6-14: Summary of the percentage motility difference between phosphorylated and dephosphorylated <i>ACTC</i> E99K HCM mutant thin filaments treated with different compounds	230
Figure 6-15: Ca^{2+} activation curves for phosphorylated and dephosphorylated donor thin filaments treated with different compounds	232
Figure 6-16: Percentage motility of Tpm1 E180G phosphorylated and dephosphorylated thin filaments treated with different known Hsp90 inhibitors...	234
Figure 7-1: The physiological consequences of uncoupling in DCM	243
Figure 7-2: Uncoupling in E99K myofibrils.....	244
Figure 7-3: EGCG binding to TnC	246
Figure 7-4: Structure of phosphorylated and unphosphorylated troponin determined by MD simulations.....	250
Figure 7-5: Amino acid changes in troponin subunits upon phosphorylation	251

List of Tables

Table 1-1: Examples of the uncoupling phenomenon in DCM and HCM	68
Table 2-1: Table of human heart samples used in this study	72
Table 2-2: Summary of the Tropomyosin mutations used in this study	74
Table 2-3: EGCG and Silybin analogues.....	89
Table 2-4: 10 x thin filament preparation for the IVMA	93
Table 2-5: 10 x troponin preparation for Buffers C and D	94
Table 2-6: The Ca^{2+} - EGTA Buffers	95
Table 3-1: Summary of tropomyosin mutations in <i>TPM2</i> and <i>TPM3</i> which give gain of function.....	116
Table 3-2: Summary of the mutations in <i>TPM3</i> that cause loss of function.....	120
Table 3-3: Summary of motility data for motile velocity of thin filaments reconstituted with ΔE218 mutant tropomyosin vs WT	126
Table 3-4: Summary of motility data for percentage motility of thin filaments reconstituted with ΔE218 mutant tropomyosin vs WT	126
Table 3-5: Summary of motility data for motile velocity of thin filaments reconstituted with ΔE224 mutant tropomyosin vs WT	127
Table 3-6: Summary of motility data for percentage motility of thin filaments reconstituted with ΔE224 mutant tropomyosin vs WT	127
Table 3-7: Summary of tropomyosin mutations causing gain of function	132
Table 4-1: Ca^{2+} sensitivity data for motile velocity of phosphorylated vs dephosphorylated thin filaments treated with 30 μM EMD 57033	148
Table 4-2: Ca^{2+} sensitivity data for percentage motility of phosphorylated vs dephosphorylated thin filaments treated with 30 μM EMD 57033	148
Table 4-3: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E54K mutant thin filaments treated with EMD 57033.....	151
Table 4-4: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 30 μM EMD 57033	151
Table 4-5: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated donor thin filaments treated with 100 μM Bepridil.....	156

Table 4-6: Ca ²⁺ sensitivity data for percentage motility of phosphorylated and dephosphorylated donor thin filaments treated with 100 µM Bepridil.....	156
Table 4-7: Ca ²⁺ sensitivity data for motile velocity of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 µM Bepridil..	160
Table 4-8: Ca ²⁺ sensitivity data for percentage motility of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 µM Bepridil..	160
Table 5-1: Ca ²⁺ sensitivity data for motile velocity of donor thin filaments treated with 100 µM EGCG.....	169
Table 5-2: Ca ²⁺ sensitivity data for percentage motility of donor thin filaments treated with 100 µM EGCG.....	169
Table 5-3: Ca ²⁺ sensitivity data for motile velocity of phosphorylated or dephosphorylated E54K DCM mutant reconstituted thin filaments treated with 100 µM EGCG.....	175
Table 5-4: Ca ²⁺ sensitivity data for percentage motility of phosphorylated or dephosphorylated E54K DCM mutant reconstituted thin filaments treated with 100 µM EGCG.....	175
Table 5-5: Ca ²⁺ sensitivity data for motile velocity of phosphorylated and dephosphorylated E40K thin filaments treated with 100 µM EGCG.....	178
Table 5-6: Ca ²⁺ sensitivity data for percentage motility of phosphorylated and dephosphorylated E40K mutant thin filaments treated with 100 µM EGCG....	178
Table 5-7: Ca ²⁺ sensitivity data for motile velocity of phosphorylated and dephosphorylated E180G mutant thin filaments treated with EGCG.....	181
Table 5-8: Ca ²⁺ sensitivity data for percentage motility of phosphorylated and dephosphorylated E180G mutant thin filaments treated with EGCG.....	181
Table 5-9: Ca ²⁺ sensitivity data for motile velocity of phosphorylated and dephosphorylated G159D DCM mutant thin filaments treated with 100 µM EGCG.....	186
Table 5-10: Ca ²⁺ sensitivity data for percentage motility of phosphorylated and dephosphorylated G159D mutant thin filaments treated with 100 µM EGCG .	186
Table 5-11: Ca ²⁺ sensitivity data for motile velocity of thin filaments reconstituted with phosphorylated or dephosphorylated exchanged K36Q troponin treated with 100 µM EGCG.....	189

Table 5-12: Ca^{2+} sensitivity data for percentage motility of thin filaments reconstituted with phosphorylated and dephosphorylated exchanged K36Q troponin treated with EGCG	189
Table 5-13: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E361G mutant thin filaments treated with 100 μM EGCG..	192
Table 5-14: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E361G thin filaments treated with 100 μM EGCG	192
Table 5-15: Ca^{2+} sensitivity data summary for motile velocity of phosphorylated and dephosphorylated thin filaments reconstituted with K280N HCM mutant troponin treated with 100 μM EGCG	196
Table 5-16: Ca^{2+} sensitivity data summary for percentage motility of phosphorylated and dephosphorylated thin filaments reconstituted with K280N HCM mutant troponin treated with 100 μM EGCG	196
Table 5-17: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG	201
Table 5-18: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG	201
Table 5-19: Ca^{2+} sensitivity data for DCM and HCM thin filaments treated with EGCG.....	206
Table 5-20: Effect of E180G mutation, phosphorylation and EGCG on myofibrillar contractility	209
Table 6-1: Data summary for the difference between phosphorylated and dephosphorylated <i>TPMI</i> E180G HCM mutant thin filaments treated with different compounds	222
Table 6-2: Data summary for the percentage motility difference between phosphorylated and dephosphorylated <i>TPMI</i> E54K DCM mutant thin filaments treated with different compounds	226
Table 6-3: Data summary of the percentage motility difference between phosphorylated and dephosphorylated <i>ACTC</i> E99K HCM mutant thin filaments treated with different compounds	230
Table 6-4: Summary of Ca^{2+} sensitivity for percentage motility of phosphorylated and dephosphorylated donor thin filaments treated with different compounds	233
Table 6-5: Small molecule screening summary	238

List of Abbreviations

ACEX- Actin Extraction Buffer

ADP- Adenosine Diphosphate

ATP- Adenosine Triphosphate

AV- Atrioventricular

BSA- Bovine Serum Albumin

cAMP- cyclic Adenosine Monophosphate

CICR- Calcium induced calcium release

CFTD- Congenital Fibre type Disproportion

DCM- Dilated Cardiomyopathy

DMSO- Dimethyl Sulfoxide

DTT- 1-4- Dithiothreitol

E-64- L-trans-Epoxysuccinyl-leucylamide-(4-guanido)-butane

EC coupling- Excitation-Contraction coupling

ECG- Echocardiograph

ECL- Enhanced chemiluminescence reagents

ECO- Echocardiography

EGCG- Epigallocatechin-3-gallate

EGTA- Ethylene glycol-bis (2-amino-ethylether), N, N, N', N'- tetraacetic acid

EHT- Engineered Heart Tissue

ELC- Essential Light Chain

EM- Electron Microscope

F-actin- Filamentous Actin

fDCM- familial DCM

fHCM- familial HCM

Fmax- Maximum force

G-actin- Monomeric actin

HCM- Hypertrophic Cardiomyopathy

HOCM- Hypertrophic Obstructive Cardiomyopathy

HMM- Heavy Meromyosin

Hsp90- Heat Shock Protein 90
 i_{Ca} - Calcium current
 i_F - Pacemaker current
IVMA- *in vitro* Motility Assay
 K_{ACT} - Rate of force development
 K_{REL} - Rate of fast relaxation phase
LMM- Light Meromyosin
LVEF- Left Ventricular Ejection Fraction
LVOTO- Left Ventricular Outflow Tract Obstruction
MCA- Medial Cerebral Artery
MD- Molecular Dynamics
MHC- Myosin Heavy Chain
MOPS- 3-(N-Morpholino) propanesulphonic acid
MyBP-C- Myosin Binding Protein C
 n_H - Hill Coefficient
NTG- Non transgenic
PAGE- Polyacrylamide Gel Electrophoresis
PBS- Phosphate Buffered Saline
Phos-tag- Phosphate Affinity SDS PAGE
PKA- Protein Kinase A
PLB- Phospholamban
PMSF- Phenylmethylsulfonyl Fluoride
Pi- Inorganic Phosphate
PIPES- Piperazine-N,N'-bis (2-ethanesulphonic acid)
PV- Pressure Volume
RLC- Regulatory Light Chain
RMSF- Root Mean Square Fluctuation
S1- Subfragment 1
SA- Sinoatrial
SAH- Subarachnoid Haemorrhage
SAP- Shrimp Alkaline Phosphatase
SCD- Sudden Cardiac Death
SD- Standard Deviation
SDS- Sodium Dodecyl Sulphate

SEM- Standard Error of Mean
SERCA- Sarco- Endoplasmic Reticulum Calcium ATPase
SR- Sarcoplasmic Reticulum
TG- Transgenic
 T_{LIN} - Duration of slow relaxation phase
Tn- Troponin
TnC- Troponin C
TnI- Troponin I
TnT- Troponin T
Tpm- Tropomyosin
Triton X- Polyethylene glycol tetr-octylphenyl ether
Tween 20- Polyoxyethylenesorbitan monolaurate
unP- unphosphorylated
WT- Wild Type
 ϕ - Tetramethyl Rhodamine Isothiocyanate (TRITC) -phalloidin

Acknowledgements

I would like to thank my main supervisor, Professor Steve Marston for giving me the opportunity to work on this stimulating project and most of all for teaching me and guiding me throughout the PhD. A particular thanks goes to my secondary supervisor, Dr Andrew Messer for his help and his patience with me. I would like to thank Mr O'Neal Copeland for his assistance in the lab and for all the "silly" talks. The other members of Steve Marston group: Dr Petr Vikhorev and Dr Natalia Smoktunowicz and also former members who unfortunately made a shorter appearance in my work life: Dr Weihua Song and Mr Ross Wilkinson. Throughout my time in the lab I had the opportunity to start a very exciting collaboration with the molecular dynamics group who gave a different touch to the project so thanks to Dr Ian Gould, Mr Juan Eiros Zamora and Mr David Wilson. Special thanks to Miss Alice Sheehan for her help with the compounds and for making motility pleasant during her time in our lab. Finally I would like to thank from the bottom of my heart my colleague, Mr Thomas Owen for "making science happen".

I would like to mention my gratitude to British Heart Foundation for funding me and also to our collaborators for giving me samples. Specifically these are: Professor Cris Dos Remedios for the human heart samples, Dr Kristen Nowak and her team for the skeletal tropomyosin samples, Dr Mohammed El-Mezgueldi for providing wild type cardiac tropomyosins and Dr Charles Redwood for kindly donating the recombinant mutant cardiac tropomyosin and troponin samples. Sandra Donkervoort and Dr Carsten Bonnemann for the $\Delta E218$ and $\Delta E224$ tropomyosins and for the excellent collaboration to produce the paper.

I would also like to take this opportunity to thank everyone who has been part of my life throughout these three amazing years and always supporting me; the pineapples, 2 Skelwith road, "kouklarines", my two dearest friends Alex and Anti, the computer science crew, my Biochem friends Zeki and Jamie and special thanks to my wife for always believing in my powers. Lastly I am eternally grateful to my family in Greece and especially my parents for giving me the best support in all the aspects of my life.

1 Introduction

1.1 General Introduction

The main subject studied in this thesis is the “uncoupling” of troponin I (TnI) phosphorylation to the calcium (Ca^{2+}) sensitivity in the heart muscle and its importance in health and disease. Heart muscle contraction allows for the heart to successfully pump blood around the human body. This is achieved in the molecular level by thick filaments in the sarcomere composing mainly of myosin to slide along thin filaments composing of actin, tropomyosin and troponin. The contraction is initiated through intracellular Ca^{2+} concentration, which is released inside the muscle cells and binds to troponin C (TnC), the calcium-sensing component of the troponin/tropomyosin switch. This allows for structural changes to occur and actin can now bind to myosin so contraction can occur (Kobayashi and Solaro, 2005).

As opposed to the skeletal muscle contraction, which is modulated through the nerve impulses, cardiac contraction is modulated by the effect of two hormones, adrenaline and noradrenaline. The response of the heart muscle to adrenaline is regulated via the β adrenergic signaling, which leads to phosphorylation of Protein Kinase A (PKA) that acts on many proteins, which in turn regulate heart muscle contraction. One of these proteins is TnI and when it gets phosphorylated by the PKA, the Ca^{2+} sensitivity of the sarcomere decreases, meaning that the rate of Ca^{2+} dissociation from TnC is higher, and the muscle can then relax.

The response of the heart to adrenaline has been found to be disrupted in heart diseases (El- Armouche and Eschenhagen, 2009). For example, in inherited cardiomyopathies Dilated Cardiomyopathy (DCM) and Hypertrophic Cardiomyopathy (HCM), which I am studying, it has been recently discovered that Ca^{2+} sensitivity does not change upon TnI phosphorylation, and we have named this phenomenon “uncoupling” (Memo et al., 2013, Bayliss et al., 2012, Messer and Marston, 2014).

Opposing to the structural mechanism of regulation of the heart muscle by Ca^{2+} , which has been elucidated mainly due to crystallographic studies (Takeda et al.,

2003), the structural mechanism of the regulation of Ca^{2+} sensitivity upon phosphorylation still remains unknown, mainly due to the fact that the structure of the part of the TnI peptide that gets phosphorylated still remains unresolved (Hwang et al., 2014). This brings us difficulties in unraveling the mechanism behind the uncoupling phenomenon and how the different mutations in any of the sarcomeric proteins can lead to uncoupling of the Ca^{2+} sensitivity to TnI phosphorylation. The aim of the thesis is to shed light upon the uncoupling phenomenon and its importance; in a structural, physiological and clinical perspective.

1.2 Muscle structure

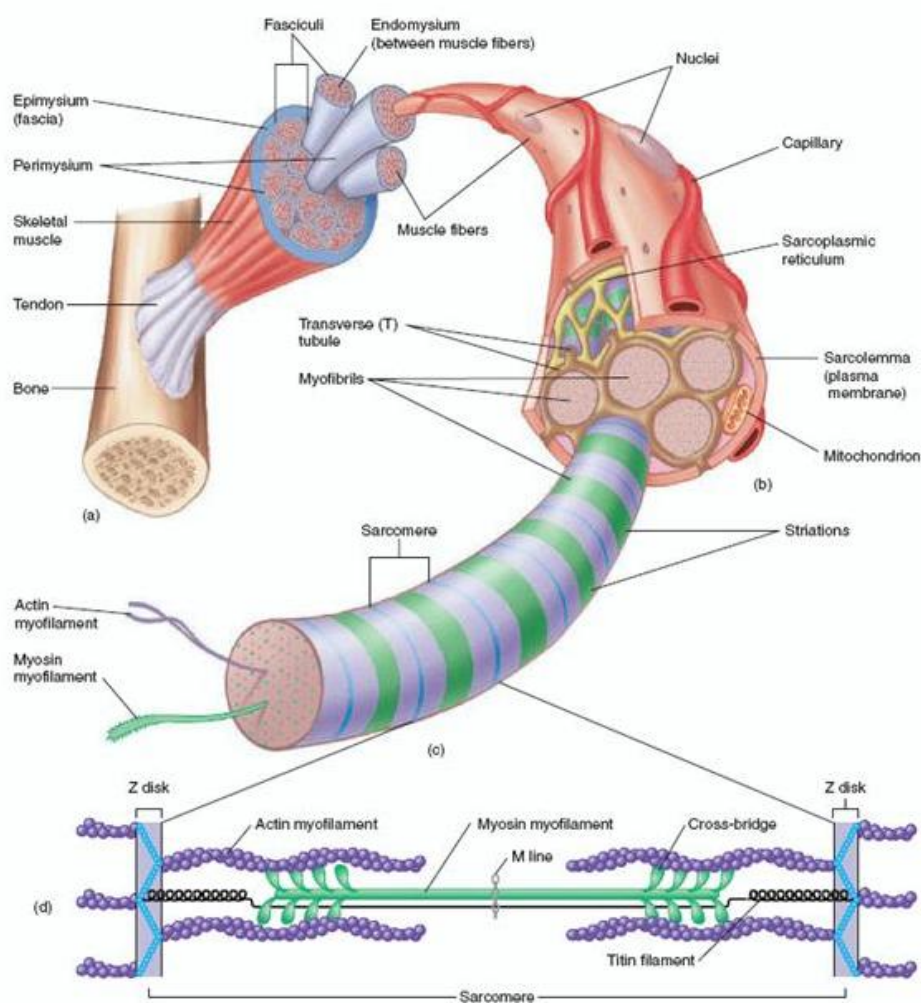
There are three types of muscles in the human body: smooth muscle, skeletal muscle and cardiac muscle. Skeletal muscle is anchored to the skeleton of an organism and cardiac muscle is analogous to the skeletal muscle but it is only found in the heart. Cardiac and skeletal muscles are structurally very similar but they have many differences in contractile protein isoforms and regulatory proteins, which will be described later on in the chapter.

1.2.1 The skeletal muscle

The primary function of the skeletal muscle is to support and move the skeleton of an organism. The skeletal muscle also supports soft tissue, maintains body temperature and guards body entrances and exits. Usually it is attached to bones through tendons, which are made of collagen and has the ability to contract and this produces movement. The skeletal muscle is a voluntary muscle and it is under the control of the central nervous system. Skeletal muscle contains neurons, blood vessels, connective tissue and specialized muscle cells called myocytes. A diagram of the structure of the skeletal muscle is shown in **Error! Reference source not found.** The skeletal muscle is separated from the surrounding organs through a layer of collagen fibres called the epimysium. The skeletal muscle is organised in bundles of muscle fibres called fascicles, which are separated from each other by the perimysium containing blood vesicles and nerves. Each fascicle is itself constructed by single muscle fibres, separated by endomysium. Throughout single fibres specialized cells called satellite cells are found, which are skeletal muscle precursor cells that differentiate to myocytes in case of injury.

Skeletal muscle fibres are massive single cells that contain many nuclei inside the same cell membrane called the sarcolemma. The sarcolemma contains structures called the transverse tubules (T-tubules) that contain many ion channels, important for the membrane potential depolarization that initiates contraction (See Section 1.3.2). The main organelle of the myocytes is the sarcoplasmic reticulum, which releases Ca^{2+} ions important for contraction (See Section 1.3.3). Inside the muscle fibres hundreds to thousands myofibrils are located, that are themselves composed of thin and thick filaments (See Section 1.2.3). Different genetic diseases of the skeletal muscle can occur in case of a mutation in any of the skeletal muscle genes. These will be discussed more in Chapter 3.

Figure 1-1: Skeletal muscle structure



Structure of the skeletal muscle and its different components. Taken from <http://www.shoppingtrolley.net/skeletal%20muscle.shtml>

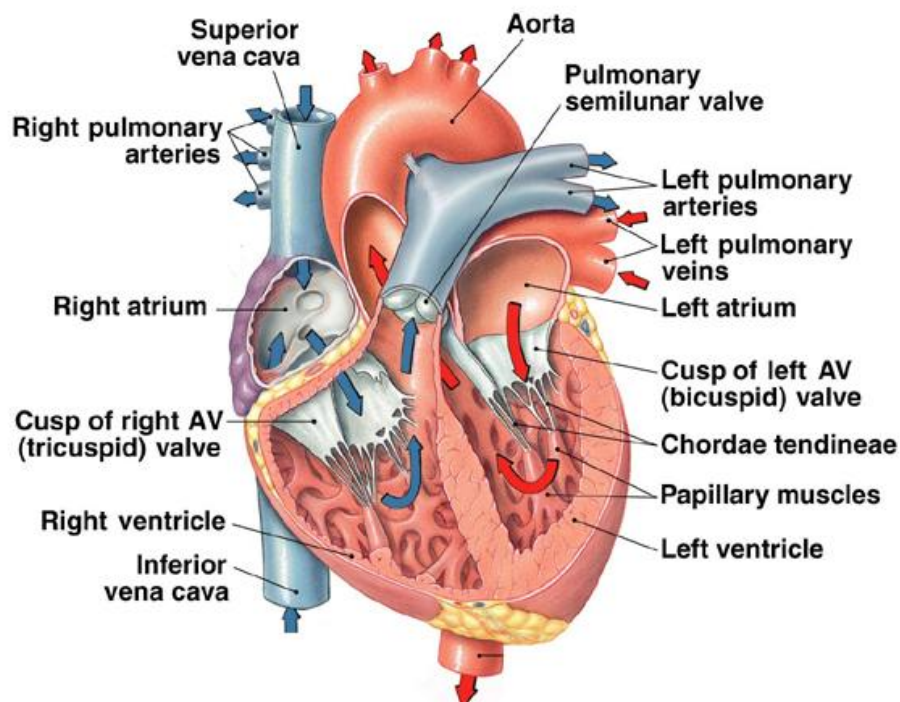
1.2.2 The heart muscle and the cardiovascular system

The function of the heart muscle is to pump blood around the human body for the delivery of oxygen and nutrients and also for the excretion of CO₂. The cardiovascular system consists of two circulatory systems: the pulmonary system which pumps blood from the right side of the heart to the lungs and then back to the left side of the heart and the systemic system which pumps blood from the left side of the heart to the rest of the body and then back to the right side of the heart. The first person that described in detail the anatomy of the heart was William Harvey (1578-1657).

1.2.2.1 The structure of the heart

The heart can be divided into four chambers. Left and right side of the heart consist of a mechanical pump, which is called the ventricle and the atrium, which is located above the ventricle and is responsible for filling the ventricle. The heart is divided into the upper chamber (containing the atria) and the lower chamber (containing the ventricles) separated by the septum. Atria and ventricles are separated by the mitral valves, which carry the blood from the atria to the ventricles only one way preventing backflow. The valve on the right side is called the tricuspid valve and the one on the left side is called the mitral valve. The semilunar valves are located between the arteries that carry the blood away from the heart and the ventricles. The semilunar valve on the right side of the heart is called pulmonary valve and the semilunar valve on the left side of the heart is called aortic valve. Figure 1-2 shows the structure of the heart.

Figure 1-2: The anatomical structure of the heart



Anatomy of the human heart with its different components. Arrows indicate the blood flow in the heart. Taken from <http://www.colorado.edu/intphys/Class/IPHY3430-200/image/14-7.jpg>

The main type of cells in the heart are the cardiomyocytes, which are the muscle cells that contain the contractile apparatus and are responsible for the beating of the heart (see Section 1.2.3). Other heart cell types found in the heart are cardiac pacemaker cells and cells of the vasculature such as fibroblasts, smooth muscle cells, pericytes, epithelial cells, endothelial cells and other connective tissue cell types, that make up 70% of the heart cells (Katz, 2006). The cardiomyocytes are found in the myocardium, which is the muscle layer of the heart and are arranged in fibres consisting of four to six myocytes separated from each other by collagen (LeGrice et al., 1995).

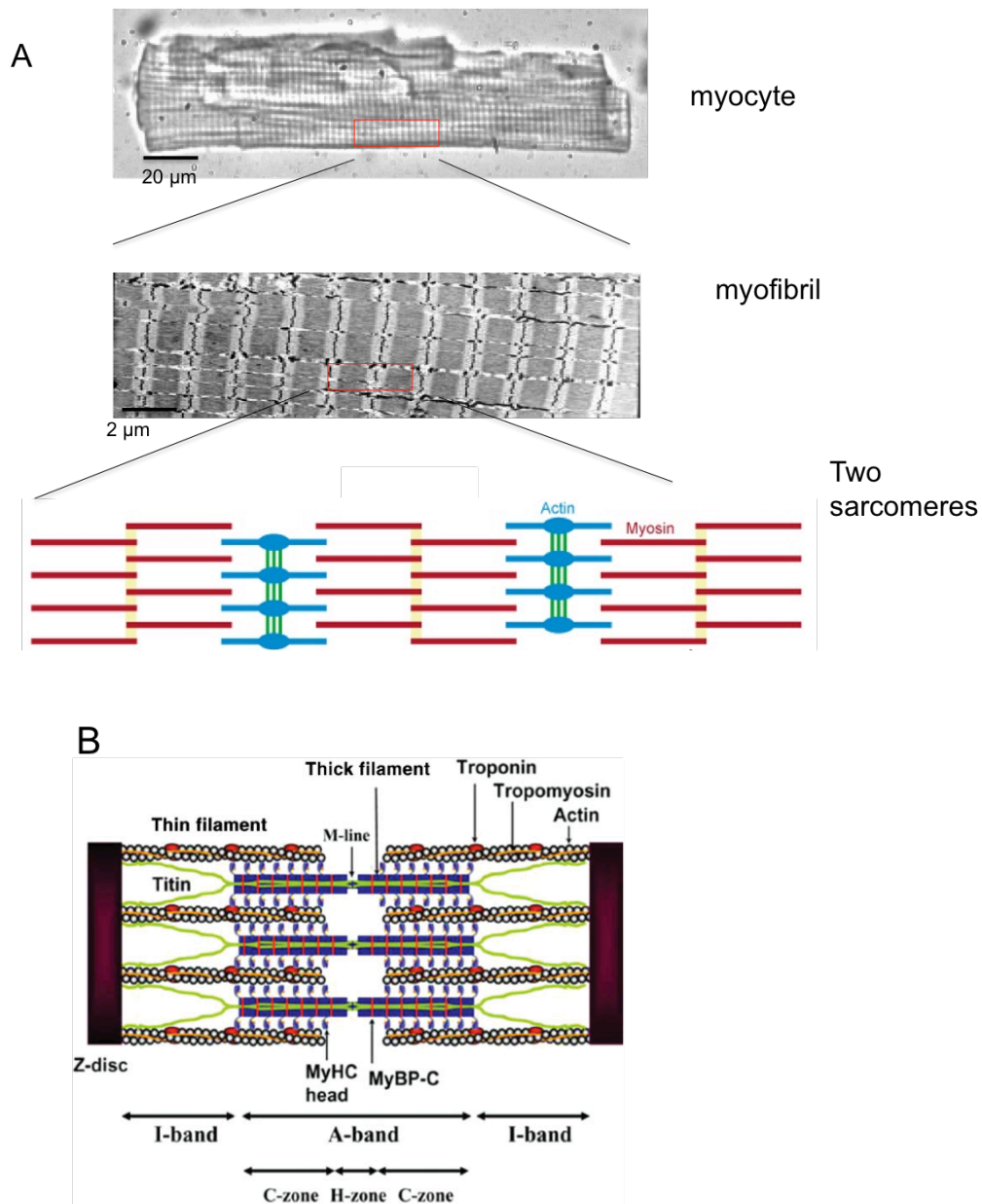
1.2.3 The sarcomere

Skeletal muscle and cardiac muscle are also called striated muscles, because they have striations when they are looked at under the phase contrast microscope. Striated muscle contains sarcomeres, which is the basic unit of contraction. Sarcomeres are composed of parallel thick and thin filaments. Contraction of the muscle occurs through the sliding of thin filaments upon the thick filaments. Sarcomeres are repeated in series to form a myofibril, which in turn is repeated in parallel to form a myocyte, which is the muscle cell. The myocytes, apart from myofibrils also contain cell nuclei, mitochondria to provide energy and other organelles.

A sarcomere is defined as the section between two Z lines. Actin thin filaments are anchored in the Z lines with opposite polarity either side. The thick filaments are also bipolar with a bare zone in the centre with no cross-bridges. The M line is located in the middle of the thick filament that contains the protein myomesin, which holds the thick filaments together. The area where the thick filaments are present is called anisotropic or A band from its appearance in the phase contrast microscope. Within this region there is the C zone, which is the area where the C protein is present and where the thick filaments interact with the thin filaments. The region that contains only thick filaments is called the H zone. The isotropic or I band is the region whereby only thin filaments are found. Upon contraction, the I band shortens, while the A band remains constant.

Thin filaments are composed of actin, troponin and tropomyosin, which will be described in detail later on. Thick filaments are composed mainly of myosin (see section 1.2.4), myosin binding protein C (MyBP-C) that is found in the C zone and titin, which is the largest protein in the human body. Other sarcomeric proteins include nebulin, obscurin, and tropomodulin that play a role in assembly of the sarcomere. Figure 1-3 B shows the structure of a sarcomere.

Figure 1-3: The structure of a sarcomere



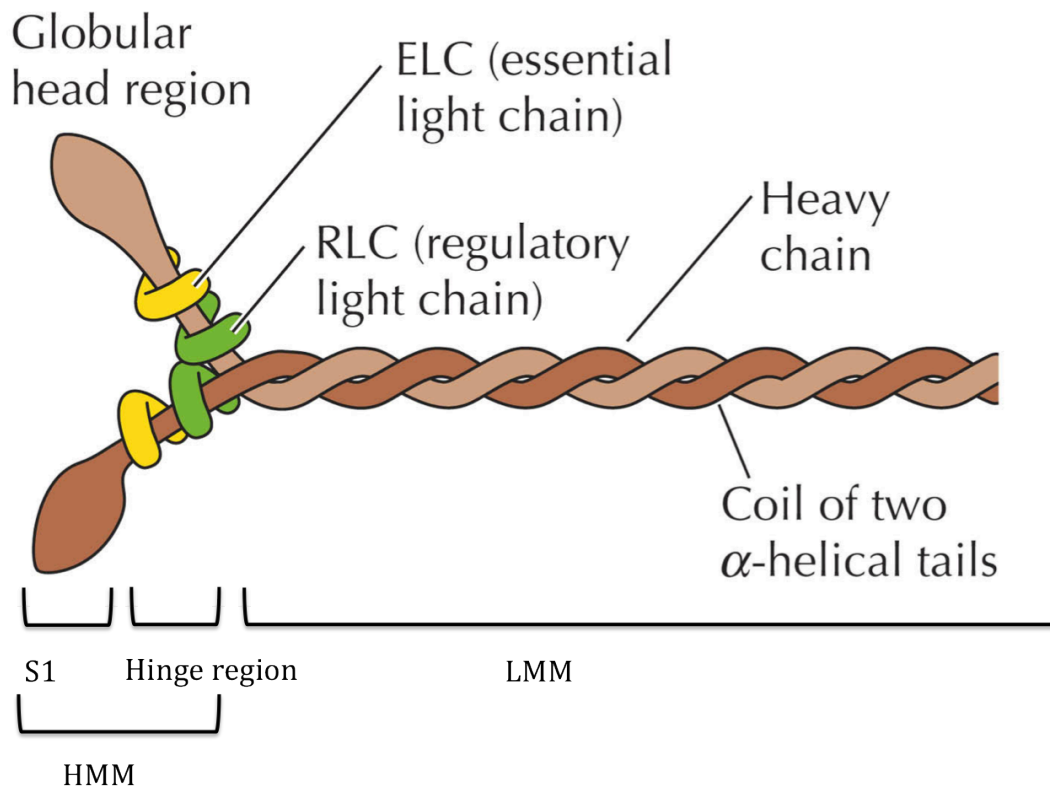
A) A cardiac myocyte is composed of repeated myofibrils, which in turn consist of repeated sarcomeres. B) Structure of one sarcomere: Thin filaments are composed of actin (white spheres), troponin (red spheres) and tropomyosin (orange strands). Thick filaments are composed of myosin (purple rectangles) and MyBP-C (red rectangles). Titin is also shown in green. Adapted from Morimoto (Morimoto, 2008).

1.2.4 The thick filament

The thick filament is composed mainly of the motor protein myosin, and also of myosin associated proteins such as MyBP-C and titin. Titin is a giant protein that spans the whole sarcomere and is responsible for controlling the passive tension (Neiva-Sousa et al., 2015). The role of MyBP-C is not yet fully understood but it is essential for the sarcomeric organisation and for the actin-myosin cross-bridges to occur (Moss et al., 2015).

Myosin is encoded by thirteen classes of genes in total. There are different types of myosin, such as myosin I, II and V. Myosin II is involved in muscle contraction and it consists of two heavy chains and four light chains, the heavy chain being 200 kDa each and the light chains being 17-25 kDa (Gordon et al., 2000). Different genes encode myosin heavy chains in skeletal and cardiac muscle. Skeletal muscle myosin heavy chain is encoded by six genes in mammals (*MYH*-1, -2, -3, -4, -8, -13). Cardiac myosin is encoded by two genes, *MYH*-6 and -7 that encode the two isoforms of cardiac myosin heavy chains, α and β (Jaenicke et al., 1990). The ratio between the two isoforms change during development and the β isoform is the predominant ventricular form in human adult (Lompre et al., 1984). The light chains are themselves subdivided into two categories, regulatory (RLC) and essential (ELC) and each heavy chain is associated with one of each of the light chains. A myosin heavy chain contains a myosin head called Subfragment 1 (or S1), which contains an actin binding site and an ATP binding site, essential for muscle contraction. The rest of the myosin heavy chains form a dimeric coiled-coil structure, which forms the filament and is called the myosin rod. The rod is connected to the S1 via the hinge region. A pair of each of the light chains binds to the hinge region. Treatment of myosin II with α -chymotrypsin leads to a cleavage in the hinge region, separating the myosin head from the rods. The myosin head together with the hinge region and the light chains is called the heavy meromyosin (HMM) fragment and the other fragment, containing just the myosin rod is called light meromyosin (LMM) (Weeds and Taylor, 1975). Figure 1-4 shows the structure of myosin. HMM and S1 are soluble and are commonly used for *in vitro* studies, including the main method used in this thesis, the *in vitro* motility assay (IVMA).

Figure 1-4: Structure of myosin II

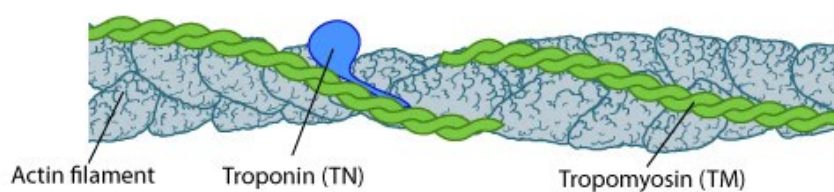


Myosin structure and its domains. ECL essential light chain, RLC regulatory light chain, S1 subfragment 1, HMM heavy meromyosin, LMM light meromyosin. Adapted from Alberts et al (Alberts et al., 2002)

1.2.5 The thin filament

The thin filament consists of actin, tropomyosin and troponin. A complex is formed by actin polymerisation to form a long filamentous structure (Figure 1-5).

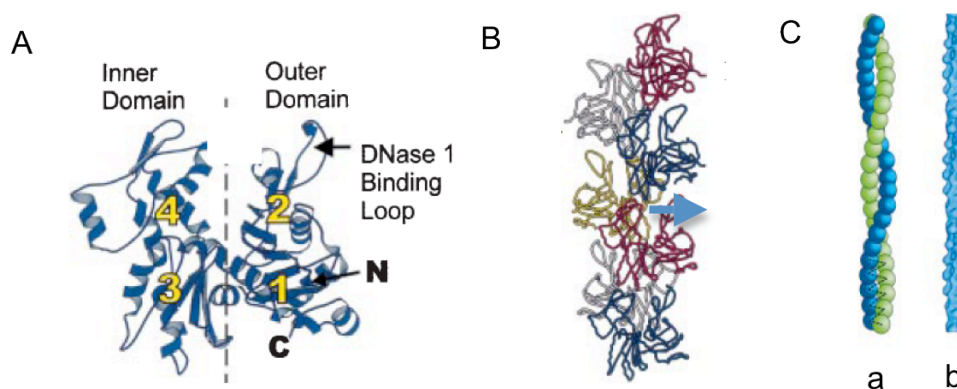
Figure 1-5: Thin filament structure



The structure of the thin filament consisting of actin, troponin and tropomyosin. Taken from <http://www.mechanobio.info/topics/cytoskeleton-dynamics>

Actin is the major component of the thin filament, because it is the molecule that makes the contractile interaction by interacting with myosin. There are two forms of actin, globular (G-actin) and filamentous (F-actin). A G-actin monomer consists of a single polypeptide chain with 375 amino acid that folds into two domains. The large (inner domain) and the small (outer domain) are linked together by two loops and as a result, two clefts are formed between the domains. The upper cleft contains the nucleotide site where ATP binds and a divalent ion binding site where Ca^{2+} and Mg^{2+} bind (Dominguez and Holmes, 2011). G-actin polymerises to form the F-actin by a small re-arrangement of the domains with the aid of ATP hydrolysis (dos-Remedios and Moens, 1995). The F-actin is stabilised together by hydrophobic and electrostatic interactions between the actin monomers. The DNase 1 binding loop (D loop) plays a major role in the formation of these interactions and also for the polymerisation of G-actin to F-actin (von der Ecken et al., 2015). The structure of actin has been resolved by electron microscopy (EM) and has been shown to be a double helix with 13 actin molecules per half turn (Holmes et al., 1990). Figure 1-6 shows the structure of actin.

Figure 1-6: Structure of actin



A) Structure of G actin monomer, showing the inner domain (3 and 4) and the outer domain (1 and 2). B) Structure of the F actin C) structure of actin thin filament a) actin monomers presented as green and blue spheres to form the double helix and b) the shape of the F actin as discovered by EM. Adapted from Hodgkinson (Hodgkinson, 2000).

1.2.5.2 Tropomyosin

Tropomyosin is the second component of the thin filament and its role is to regulate contraction. It is a dimer, consisting of two tropomyosin molecules of around 33 kDa each. Tropomyosin is encoded by four genes, *TPM1*, *TPM2*, *TPM3* and *TPM4* and all of them have alternatively spliced variants that result in many tropomyosin isoforms. The splicing of each gene can produce up to 40 different mRNA variants (Vindin and Gunning, 2013). Because of great variety of tropomyosin variants, the naming of the variants has led to confusion. Recently, Geeves et al have devised a new system for tropomyosin nomenclature which takes into account the gene and exon structure (Geeves et al., 2015). This new nomenclature has been adapted from all the major labs working with tropomyosin and should be used globally (Marston, 2015).

Different isoforms of tropomyosin are found in different types of muscle and in different combinations. The most predominant form in the heart muscle is the Tpm1.1, at 97-99%. In skeletal muscle Tpm1.1, Tpm2.2 and Tpm3.12 are all expressed in both slow and fast fibres, but in different proportions depending on the muscle type. When Tpm2.2 and Tpm1.1 or Tpm3.12 are found in the same tissue, then tropomyosin prefers to form heterodimers (for example Tpm1.1 with Tpm2.2 or Tpm2.2 with Tpm3.12). Tpm1.1 is expressed in higher levels in fast twitch type 2 fibres and less in slow switch type 1 fibres. Tpm3.12 is only expressed in slow switch type 1 fibres but Tpm2.2 is expressed in all skeletal muscles (Perry, 2001).

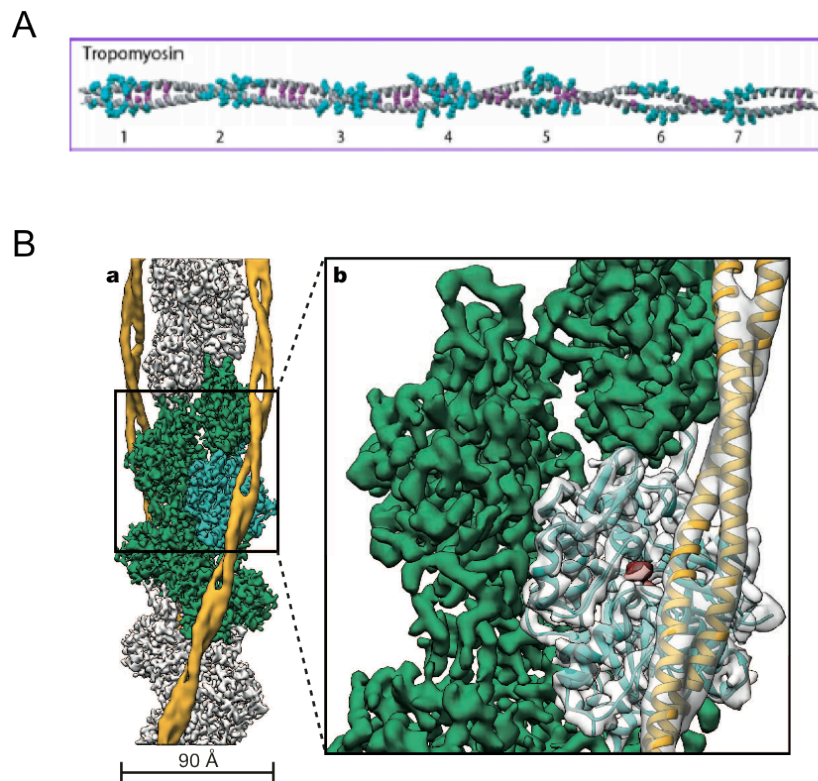
The key function of tropomyosin is to regulate muscle contraction in a co-operative manner. This is successfully achieved by tropomyosin's structure and its incorporation into the thin filament. Tropomyosin dimers form a semi-rigid curved coiled coil that fits itself around the actin helix. Each tropomyosin dimer forms head to tail interactions with another tropomyosin dimer in order to form a long continuous polymer along the F-actin. N terminal acetylation of tropomyosin is crucial for binding of the two monomers together as well as for the formation of complex with troponin T (Palm et al., 2003).

Recently the 3D structure of F-actin in complex with tropomyosin was determined using cryo-electron microscopy at a resolution of 6.5 Å. Tropomyosin is negatively charged and can interact with a positively charged group in F-actin (von der Ecken et al., 2015). One tropomyosin dimer interacts with seven actin monomers and tropomyosin contains seven pseudo-repeating units at the site of interaction with actin

(Hitchcock DeGregori and Varnell, 1990). Tropomyosin has an organization of seven structural motifs of 40 amino acids each, that are found at the place where tropomyosin interacts with the seven actin molecules. The structural motif at the site of interaction with actin can be divided in two regions, the α band (consisting of 20 amino acids) and the β band (consisting of the rest 20 amino acids). The α band is thought to be involved in the actin-tropomyosin interaction in the “closed ” state whereas the β band is thought to be involved in the actin-tropomyosin interaction in the “open” state (Holthauzen et al., 2004) although this is challenged by recent structural analysis (von der Ecken et al., 2015). There is a specific amino acid motif in tropomyosin at the site of interaction with actin, with two basic or acidic amino acids next to each other that make contact with actin. The actin-tropomyosin contacts are very important in health and disease, because a series of mutations in this interface in Tpm2.2 and Tpm3.12 has been found that cause a hypercontractile phenotype in patients, and can be explained on a basis of disrupting this structure (Memo and Marston, 2013). This structure-function relationship will be examined in Chapter 3.

The affinity by which tropomyosin interacts with one actin monomer is relatively weak, (K_d 0.1-1 mM) because the majority of the interactions between actin and tropomyosin are mainly electrostatic (Lehman and Craig, 2008). The total sum of weak interactions between actin and tropomyosin justifies the strong interaction between the two proteins. The weak interactions allow for the flexibility of tropomyosin binding to actin that is important for the muscle contraction (Li et al., 2010). As will be described in Section 1.3.4, tropomyosin sterically blocks actin/myosin interaction in the absence of Ca^{2+} . In the presence of Ca^{2+} the position of tropomyosin on the actin filament changes, allowing for the interaction of actin with myosin to occur. The mechanism by which tropomyosin moves over actin is uncertain, but a sliding mechanism has been proposed (Lehman and Craig, 2008). The tropomyosin polymer is relatively rigid, which is important for the formation of the thin filament and also for the transduction of the Ca^{2+} signal to the thin filaments (Li et al., 2010). Figure 1-7 shows the structure of tropomyosin and actin complex.

Figure 1-7: Structure of tropomyosin



A) Ribbon showing the dimer coil coiled structure of tropomyosin. The numbers indicate the seven positions of actin binding and actin binding sites are shown in cyan. (Hitchcock- DeGregori and Singh, 2010) B) a) Cryo-electron microscopy of F-actin interacting with tropomyosin. Actin subunits are in grey with five of them in green and one in cyan. Tropomyosin in yellow. b) close view of the actin-tropomyosin interactions. (Von der Ecken et al., 2015)

1.2.5.3 Troponin

Troponin is the regulatory protein of the thin filament and it is the mediator for the different signals (for example Ca^{2+} binding and phosphorylation in the case of the cardiac muscle) to regulate muscle contraction. Troponin forms a complex of three different subunits. These are Troponin T (TnT), Troponin I (TnI) and Troponin C (TnC). For each of the subunits there are multiple isoforms that are expressed according to different tissue types and also different developmental stages. There are three genes that encode for TnI, three genes that encode for TnT and two that encode

for TnC (Barton et al., 1997). Figure 1-8 shows the structure of the troponin complex with the different subunits.

Figure 1-8: Structure of troponin

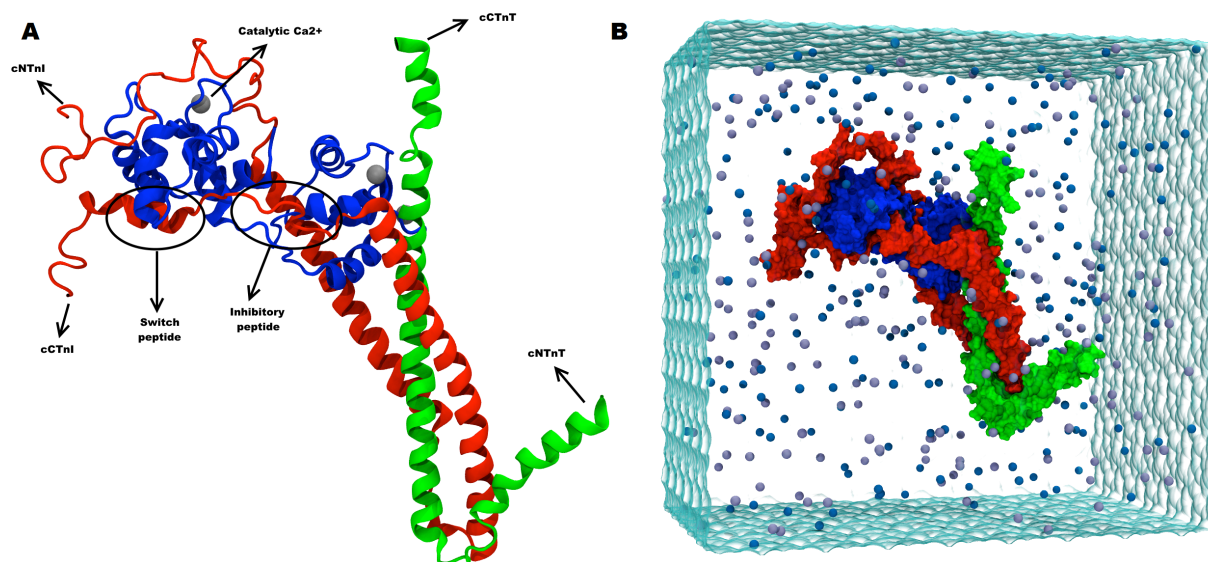


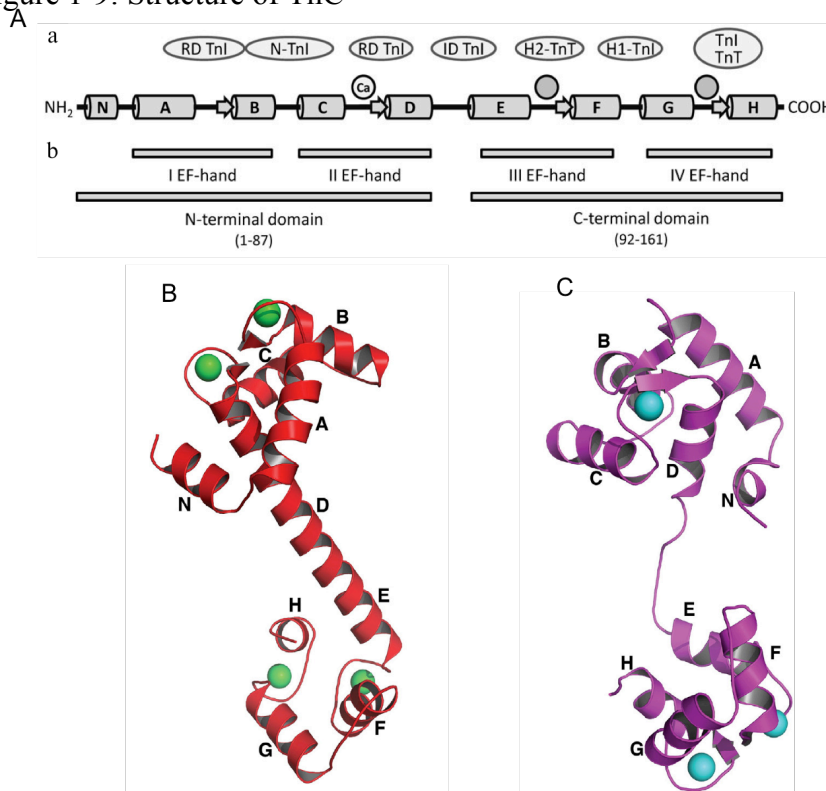
Figure showing the structure of cardiac troponin as determined by Molecular Dynamics (MD) simulations using the crystal structure of Takeda (Takeda et al., 2003) and modeling the missing peptides. Blue represents TnC, red represents TnI and green represents TnT. A) Ribbon diagram of troponin and its different subunits. Catalytic and structural Ca²⁺ are shown, and also the inhibitory and the switch peptide of TnI (see Section 1.2.5.3.2). B) Troponin surface rendering in a water molecule box. Images were kindly provided by Mr Juan Eiros Zamora as part of the MD project.

1.2.5.3.1 Troponin C

Troponin C (TnC) is around 18 kDa and is encoded by two genes: *TNNC1* which encodes the TnC subunit in the fast skeletal muscle and *TNNC2* which encodes the TnC subunit in the slow skeletal and the heart muscle (Townsend et al., 1997b, Townsend et al., 1997a). TnC forms the core of the troponin complex and it is a dumb-bell shape composed of two globular regions at the N terminal and the C terminal respectively that are linked together by a link chain consisting of hydrophobic amino acids (Herzberg and James, 1985). The linker chain forms a stable α helix in the crystal structure form, but in solution it can adopt different structures

(Li and Hwang, 2015). The function of the TnC subunit is the binding of Ca^{2+} , which regulates muscle contraction. Apart from Ca^{2+} , the divalent cation Mg^{2+} also binds to TnC. The divalent cations can bind to either of the two globular regions at an EF hand motif (Kawasaki et al., 1998). Two of the sites, III and IV have high affinity for Ca^{2+} ($K_{\text{Ca}} \sim 10^7 \text{ M}^{-1}$) and these two sites are called structural sites, because almost always divalent cations are bound there. Sites I and II are selective to Ca^{2+} but with a lower affinity for Ca^{2+} ($K_{\text{Ca}} \sim 10^5 \text{ M}^{-1}$). They are called regulatory sites because they are responsible for the regulation of the muscle to Ca^{2+} . In the cardiac muscle there is only one regulatory site, which is site II (Gordon et al., 2000). Comparison of the two domains of TnC showed that skeletal TnC N terminus was in a closed state while skeletal TnC C terminus was in an open state, suggesting that binding of Ca^{2+} in TnC N terminus results in a “closed” to “open” transition exposing a hydrophobic cleft. In the case of cardiac TnC, both Ca^{2+} occupation at site II and the presence of TnI are required for the “open” conformation of TnC, having implications for the regulation of the cardiac muscle (see Section 1.3.7).

Figure 1-9: Structure of TnC



A) a) Secondary structure of TnC. Cylinders represent helices and arrows represent β strands. Ca^{2+} in a circle represents the regulatory Ca^{2+} site and the circles without designation represents structural site. Proteins of the thin filament that interact with TnC are also indicated (Katrukha, 2013) b) Domain structure of the TnC. B) Structure of skeletal TnC. Ca^{2+} is represented by green spheres C) Structure of cardiac TnC. Ca^{2+} is represented by cyan spheres (Li and Hwang, 2015)

1.2.5.3.2 Troponin I

Troponin I (TnI) is the inhibitory subunit of troponin and is around 24 kDa in size. There are three isoforms of TnI that are expressed in different tissues. The slow skeletal isoform is encoded by *TNNI1* gene, fast skeletal isoform is encoded by *TNNI2* gene and the cardiac specific isoform of TnI is encoded by *TNNI3* gene. The structure of the different isoforms is highly conserved, the only difference being the cardiac specific isoform that contains an extra N terminal peptide (Barton et al., 1997). In the cardiac muscle, TnI isoform switching occurs during development. Slow skeletal TnI is the predominant form during foetal development. The levels of slow skeletal TnI start declining at birth and have completely switched to cardiac specific TnI isoform 9 months after birth (Marston and Redwood, 2003).

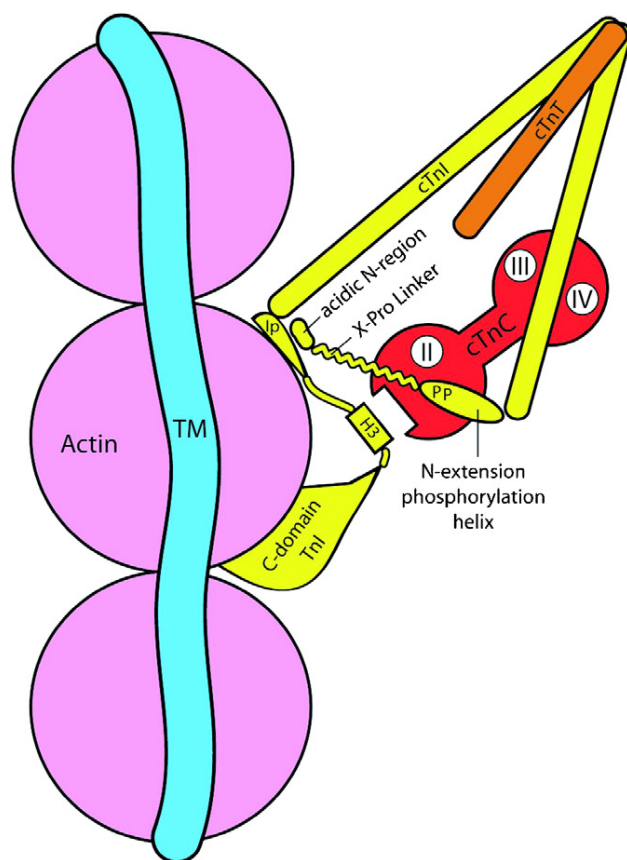
TnI has six regions, each of them has a specific function: 1) an N terminal extension, unique to the cardiac TnI, 2) the region binding to the C terminal lobe of TnC 3) a region that binds TnT in the C Terminus 4) the first region that binds to actin that includes the inhibitory peptide 5) a switch region and 6) a second actin binding region (Kobayashi and Solaro, 2005).

The main function of TnI is to bind to actin and inhibit its interaction with myosin. In the absence of tropomyosin and the other troponin subunits, TnI is able to inhibit ATPase activity. The region that is responsible for the inhibitory action of TnI is called the inhibitory peptide and it involves the residues 96-116 in rabbit skeletal muscle. The inhibitory region alone can inhibit myosin ATPase activity and can bind to actin *in vitro* (Perry, 1999). The second inhibitory region is also important for the Ca^{2+} regulation of the muscle, as it was shown from the first crystal structure of cardiac TnI (Takeda et al., 2003).

Most of the functional domains of troponin can be found in the crystal structure apart from the N terminal extension of the cardiac TnI (TnI₁₋₃₁). This is a very important region of the TnI, because it contains the phosphorylatable Serines 22 and 23. These are the only phosphorylatable sites on troponin and are phosphorylated by PKA under physiological conditions (Ayaz-Guner et al., 2009). The function of the phosphorylation of Serines 22 and 23 is a decrease in the Ca^{2+} sensitivity of the thin filament, which ultimately modulates the response of the thin filament to adrenaline (Solaro et al., 2008). When TnI gets phosphorylated, the rate of Ca^{2+} dissociation

from TnC increases by 2-3 fold (Robertson et al., 1982). More information about the regulation of the cardiac muscle by phosphorylation can be found in Section 1.3.7. Howarth et al have proposed the structural changes occurring in the TnI-TnC that lead to a change in Ca^{2+} dissociation upon TnI phosphorylation in the N terminal extension. This model suggests that when TnI is dephosphorylated the N terminal extension is in contact with the TnC region where Ca^{2+} binds and this increases the affinity of Ca^{2+} to TnC. Phosphorylation of the Serines 22 and 23 results in the N terminal extension moving away from the TnC and that affects Ca^{2+} binding (Howarth et al., 2007). This model has now been updated with more recent findings using molecular dynamics simulations of whole troponin showing that the changes that occur upon phosphorylation of TnI are subtle (Cheng et al., 2014). Figure 1-10 shows a simplified model of cardiac TnI with its different regions and how it interacts with its neighbouring proteins.

Figure 1-10: Cartoon structure of cTnI



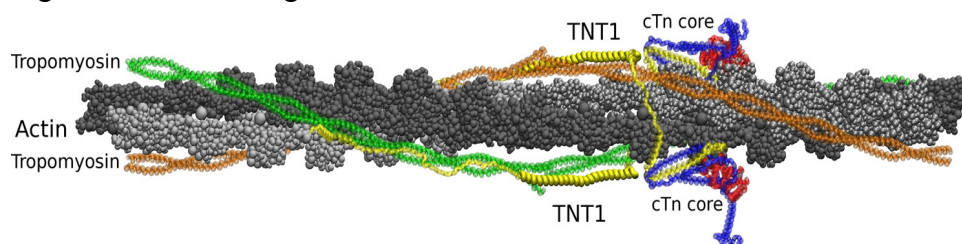
Cartoon diagram showing the structure of TnI and its interaction with actin (purple), tropomyosin (light blue) and TnC (red) in the presence of Ca^{2+} . The Ip region is the inhibitory region and H3 indicates the switch peptide that binds to a hydrophobic patch on TnC. The structural and regulatory Ca^{2+} sites on TnC are also shown (Solaro et al., 2008)

1.2.5.3.3 Troponin T

Troponin T (TnT) is 35 kDa and it is important to anchor the troponin complex onto the actin filament. It has been described as the glue that holds the regulatory system together (Tobacman, 1996). TnT is encoded by the genes *TNNT1*, *TNNT2* and *TNNT3*. Different genes express TnT according to the tissue type. The cardiac specific form of TnT is encoded by *TNNT2* and the gene is alternatively spliced to produce different isoforms (Marston and Redwood, 2003). Fast skeletal TnT is expressed by *TNNT3* and slow skeletal TnT is expressed by *TNNT1*. There are no cardiac isoforms of TnT present in the skeletal muscle and the different isoforms are expressed in different stages of development.

Only a small part at the C terminus is present in the first crystal structure of troponin (Takeda et al., 2003) and the other parts of the molecule have been gathered by the structure of fragments. TnT can be subdivided into two regions after enzymatic cleavage, TNT1 (N terminal) and TNT2 (C terminal). TNT1 binds to tropomyosin and its association is not sensitive to Ca^{2+} . This region, which is not found in the crystal structure, is very important for the regulation of the thin filament together. A study by Manning et al has shown that the flexibility of this region is correlated to the Ca^{2+} sensitivity by studying different mutants in this region (Manning et al., 2012b). A crystal structure containing the N terminus and C terminus of tropomyosin together with a small fraction of cardiac TNT1 showed the formation of a four helix bundle containing two tropomyosin C terminal chains, one tropomyosin N terminal chain and one of TnT (Murakami et al., 2008). The integration of cTnT into the thin filament is shown in Figure 1-11. The TNT2 region of TnT anchors the TnT to TnC/TnI complex through interaction with the N terminus of TnI in a Ca^{2+} independent manner (Malnic et al., 1998). The integration of TnT in the rest of the thin filament is of particular importance, as the function of TnT is to co-ordinate muscle contraction.

Figure 1-11: The integration of cTnT into the thin filament



The orientation of cTnT to the thin filament: TnT is in yellow with TNT1 thickened, cTnI is in blue, cTnC in red, actin in light/dark grey, tropomyosin in green and orange (Manning et al., 2012a)

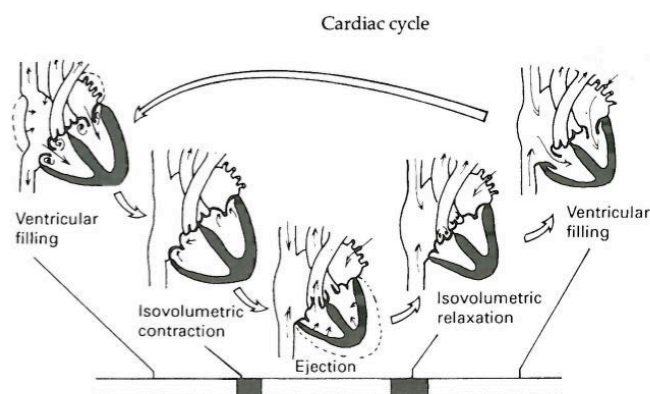
1.3 Muscle contraction

1.3.1 The cardiac cycle

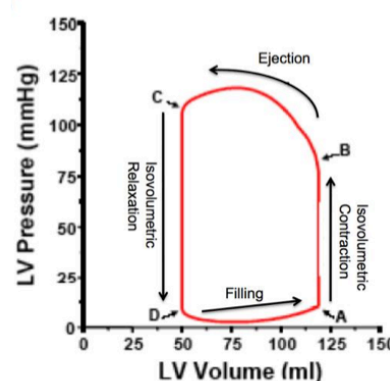
The cardiac cycle is the series of events that occurs in the whole heart for it to beat and be able to circulate blood around the body (Figure 1-12). The cardiac cycle has an average duration of 0.9 sec (67 beats per min). It contains two phases: systole, which occurs when the ventricles contract, and diastole, which occurs when the ventricles relax. One cardiac cycle can be described as a “Pressure and Volume” loop (PV loop). It consists of four phases: Ventricular filling (D), Isovolumetric contraction (A), Ejection (B), Isovolumetric relaxation (C). Ventricular filling is the initial phase of the cycle in which blood flows from the atria to the ventricles. Ventricular pressure then begins to rise, and in order to further fill the ventricles the atria contract. As soon as the ventricular pressure rises above atrial pressure, atrioventricular valves close and the ventricle becomes a closed chamber. This results in a steep rise in the pressure but the volume remains the same. The next step is the ejection phase, which occurs when ventricular pressure exceeds arterial pressure. Then, the outflow valves are open and ejection can initiate. The pressure then begins to decline as cardiac muscle begins to relax. A period of isovolumetric relaxation occurs then, whereby the pulmonary valves close and the ventricles become again a closed chamber. The pressure falls very quickly and when ventricular pressure falls below the levels of the atrial pressure the atrioventricular valves open and blood can flood through from the atria, which has now been refilled during ventricular systole.

Figure 1-12: The cardiac cycle

A



B



A) The stages of the cardiac cycle. B) Left ventricular pressure- volume loop (PV loop) for one cardiac cycle. The loop has an anti-clockwise direction (Levick, 2000).

1.3.2 The action potential

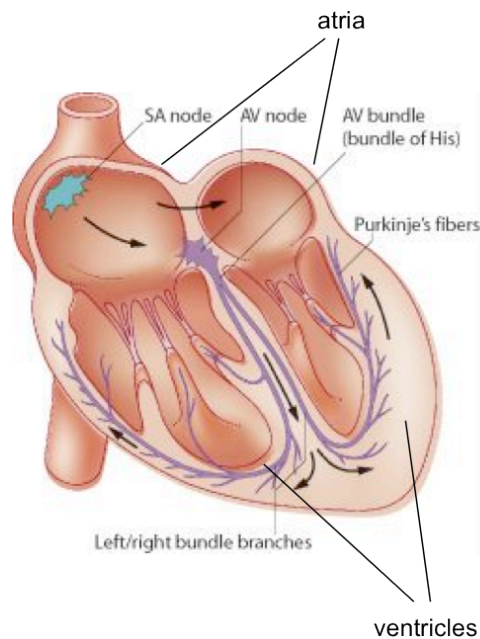
The signal that triggers muscle contraction is the action potential. Both skeletal and cardiac muscles undergo a reversal of the membrane potential from -60 mV to +30 mV.

The action potential is initiated from adjacent cells: in the case of skeletal muscle, the action potential comes from the axon of a motor neuron, which then reaches the synapsis of the neuron and the neurotransmitter acetylcholine gets released.

Acetylcholine binds to receptors in the membrane of the muscle fibres called sarcolemma, which cause an influx of Na^+ ions that depolarize the membrane potential of the sarcolemma.

In case of the cardiac muscle, the action potential initiates from the cardiac pacemaker, the sinoatrial (SA) node that is located at the top of the right atrium. The SA node initiates a membrane depolarization that spreads along the heart. The action potential passes from the atrial cells to the ventricular cells through the atrioventricular (AV) node that is located posterior of the atrial septum. Without the AV node the action potential cannot pass through to the ventricular myocytes. The AV node is responsible for the delay of the depolarization in the cardiac muscle, which allows the blood present in the atrial to have enough time to move into the ventricles. Connected to the AV node are fibres known as the bundle of His that pass through the septum into the ventricles. Purkinje fibres are located at the termini of the His bundles which are responsible for transmitting the wave of polarization to the ventricles (Levick, 2009). Figure 1-13 shows the action potential propagation in the heart.

Figure 1-13: The action potential propagation in the heart



This figure shows the action potential depolarization across the heart, starting from the SA node and reaching the ventricles (Cunningham, 2002).

1.3.3 The excitation-contraction coupling

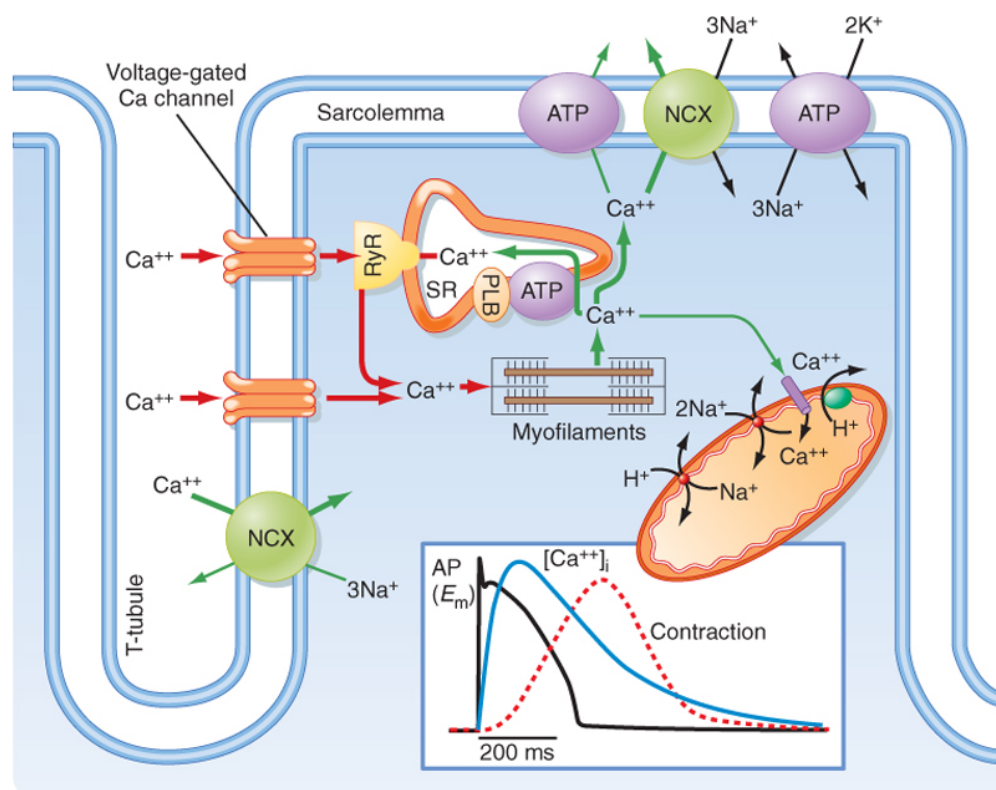
The action potential propagation is linked to muscle contraction through Ca^{2+} in a process called excitation-contraction coupling (EC coupling). The EC coupling has been shown to be very similar between skeletal and cardiac muscle, although the dynamics of the process has differences because of the different isoforms of the proteins involved in the process.

The arrival of the action potential causes a change in the Ca^{2+} concentration in the sarcoplasm from $0.1 \mu\text{M}$ to around $2 \mu\text{M}$. The rise in free Ca^{2+} concentration comes from the release of Ca^{2+} from the store of Ca^{2+} of the myocyte, the sarcoplasmic reticulum (SR) and from the plateau current, i_{Ca} . The action potential spreads over the sarcolemma membrane, and this induces L type Ca^{2+} channels which are located on the surface of the membrane to open and this cause an increase of the intracellular Ca^{2+} concentration, mainly close to the membrane. This is called the plateau current i_{Ca} . The slight increase in intracellular Ca^{2+} concentration induces the opening of

channels on the SR, called the ryanodine receptors to open by a process called calcium-induced calcium release (CICR). The Ca^{2+} concentration within the myocyte rises within 10 milliseconds. This sudden increase in the intracellular Ca^{2+} concentration triggers muscle contraction. L type channels are negatively regulated by Ca^{2+} by binding to calmodulin, which then binds to the L type channel inducing its closure. Ca^{2+} is actively pumped again back to the sarcoplasmic reticulum by an ATPase called SERCA (Sarco- Endoplasmic Reticulum Ca^{2+} ATPase). An additional 10 % of Ca^{2+} is exported out of the myocyte by sarcolemmal transporters such as NCX (Na^+ Ca^{2+} exchanger) (Bers, 2002) (Figure 1-14).

Ca^{2+} binds to troponin, the Ca^{2+} sensing component of the myofilament, which then regulates its contraction, making the Ca^{2+} important for the muscle function. Any change of the ability of troponin to sense Ca^{2+} , or Ca^{2+} irregularity in the myocyte is therefore important for the development of diseases.

Figure 1-14: Ca^{2+} cycle in the cardiomyocyte



The major components of the Ca^{2+} contraction system in the cardiomyocyte is shown. Ca^{2+} enters via the L type Ca^{2+} channels and via the Ryanodine Receptor (RyR) from the SR. It is uptaken by SERCA ATPase, which is regulated by phospholamban (PLB). NCX channel (Na^+ Ca^{2+} exchanger) removes the Ca^{2+} ions in exchange with Na^+ . The diagram also shows a time course of the EC coupling (Bers, 2002).

1.3.4 The sliding filament theory and the cross-bridge cycle

In order for the muscle to contract, thin and thick filaments make contact with each other and the thin filament slides along the thick filament. In the sarcomere thin and thick filaments are in parallel to each other and when they slide past each other, their length does not change but the length of the sarcomere in total changes. It has been proposed that the force of the myofibrils is proportional to the amount of filaments that overlap. This is because when thin and thick filaments overlap they form cross-bridges that are responsible for the production of force. These cross-bridges are in fact myosin heads that overhang from the thick filament.

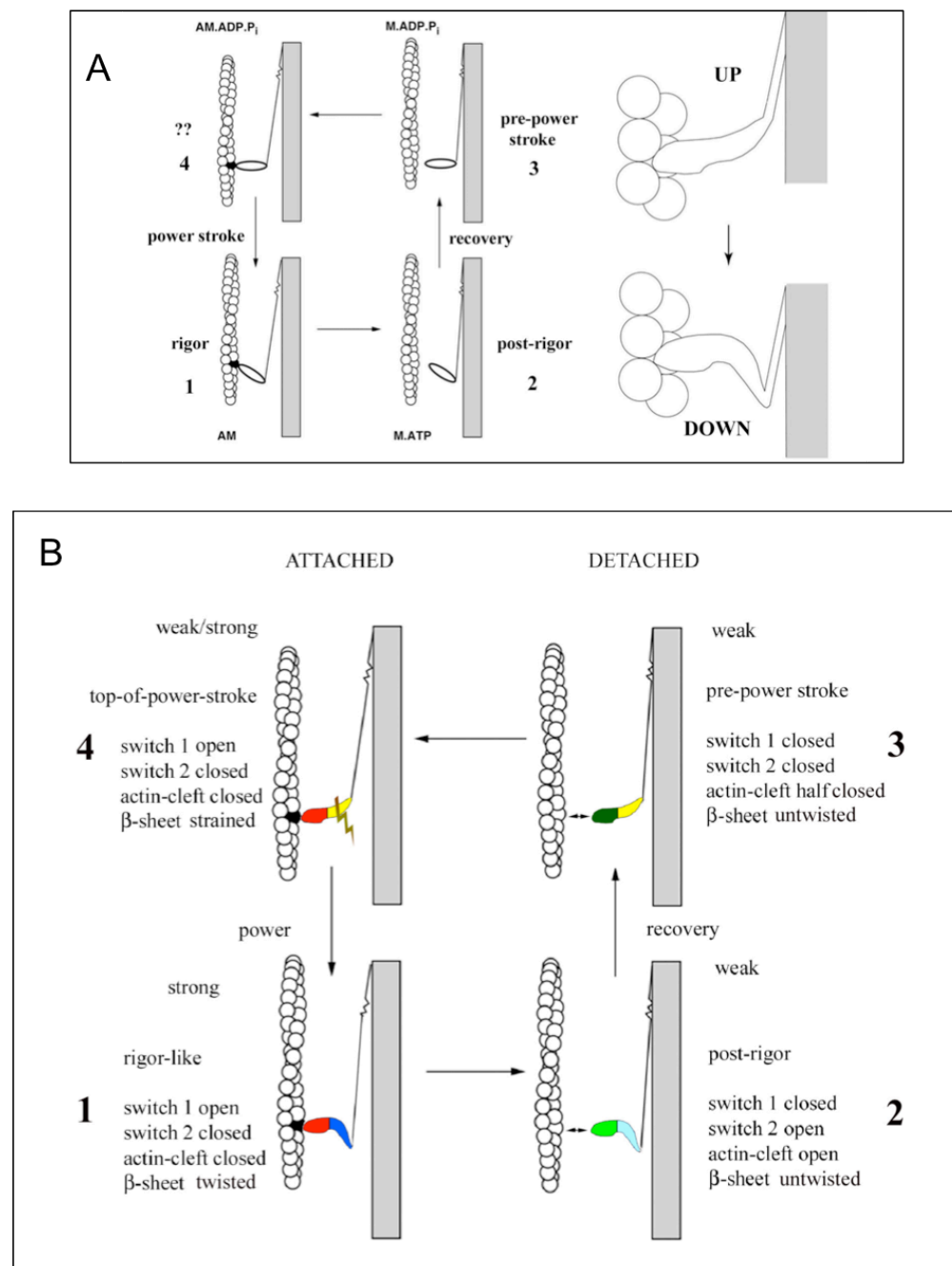
The detail of the cross-bridge mechanism derived mostly from X-ray crystallography. The theory of the cross-bridge cycle was developed from Huxley in 1957 and explains muscle contraction through the formation of these cross-bridges that generate tension (Huxley, 1957). The structure was linked to the enzymatic cycle by Lymn and Taylor who described how myosin also acts as an enzyme and uses ATP hydrolysis to drive the cross-bridge cycle (Lymn and Taylor, 1971). Myosin has ATPase activity, which provides the energy for contraction. The ATPase activity is activated by actin and inhibited by ADP and Pi. Through the cross-bridge cycle, ATP binds to the ATPase site on myosin, and gets hydrolysed to ADP and Pi. This energy is used to change the position of the myosin head to create power stroke.

The myosin head can exist in two different conformations, 90° (“up” position of the lever arm) and 45° (“down” position of the lever arm). In the beginning of the cycle, the myosin head is in the down position binding to actin. When ATP binds to the ATPase site, induces a dissociation of the actin molecule and also a conformational change, which changes the position of the myosin head from down to up. In the next step, ATP gets hydrolysed into ADP and Pi and myosin/ADP/Pi will rebind to actin. This rebinding of actin leads to the release of ADP and Pi from myosin. As the Pi is released the myosin head returns to its original down position, and this moves the actin along by ~10 nm. In the last step, ADP is also released and the cycle can start again (Spudich, 2001). Therefore, one cross-bridge cycle breaks down one molecule of ATP. The position of the myosin head depends on the existence of a kink on the relay helix which leads to a rotation of 60° and the structural changes in myosin in going through the different steps of the power stroke involve two switches (switch 1

and 2) and a β sheet (Geeves and Holmes, 2005). Figure 1-15 shows the cross-bridge cycle and also the structural changes that occur upon the cycle.

The total force of the muscle depends on the amount of cross-bridges formed in the sarcomere and their attached lifetimes.

Figure 1-15: The cross-bridge cycle



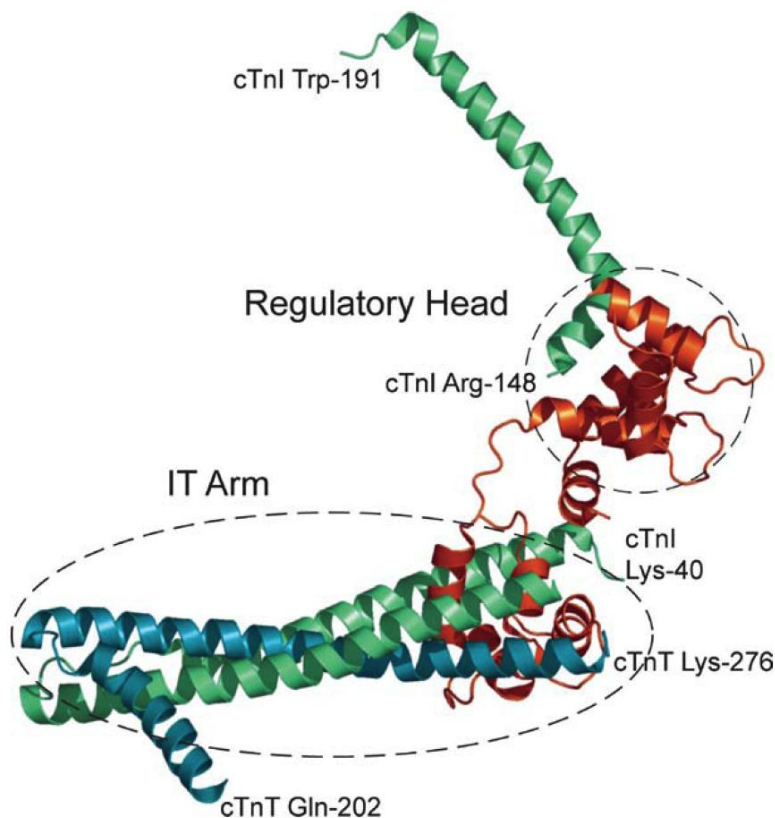
Schematic diagram showing the cross-bridge cycle. A) Different states of actin/myosin interactions are shown (weak/strong/unbound) and different nucleotide species (ATP, ADP and P_i) and the conformation of the myosin head during the different steps of the cross-bridge cycle. B) The structural changes that occur throughout the cross-bridge cycle (Geeves and Holmes, 2005).

1.3.5 Thin filament switch

The troponin complex has a specific 3D structure, which switches the filament between activated and inactivated states. The different components of troponin are responsible for different functions and can modulate muscle contraction. The functional properties of the switch are clear but the structural basis is not fully understood. In the last few decades, attempts have been made to build a model and the Takeda structure has helped to understand the mechanism of the filament switch to a large extent.

The troponin complex is divided into two regions: The troponin core domain and the extended structure of TnI (Figure 1-16). The core domain contains part of the TnC anchored to a W shaped TnT-TnI arm and the regulatory sequence of TnI that undergoes conformational changes according to Ca^{2+} . The TnI regulatory domain comprises of residues 137-210 (Takeda et al., 2003). The TnI/TnT arm make contact with tropomyosin through a tropomyosin binding site in TnT (Perry, 1998).

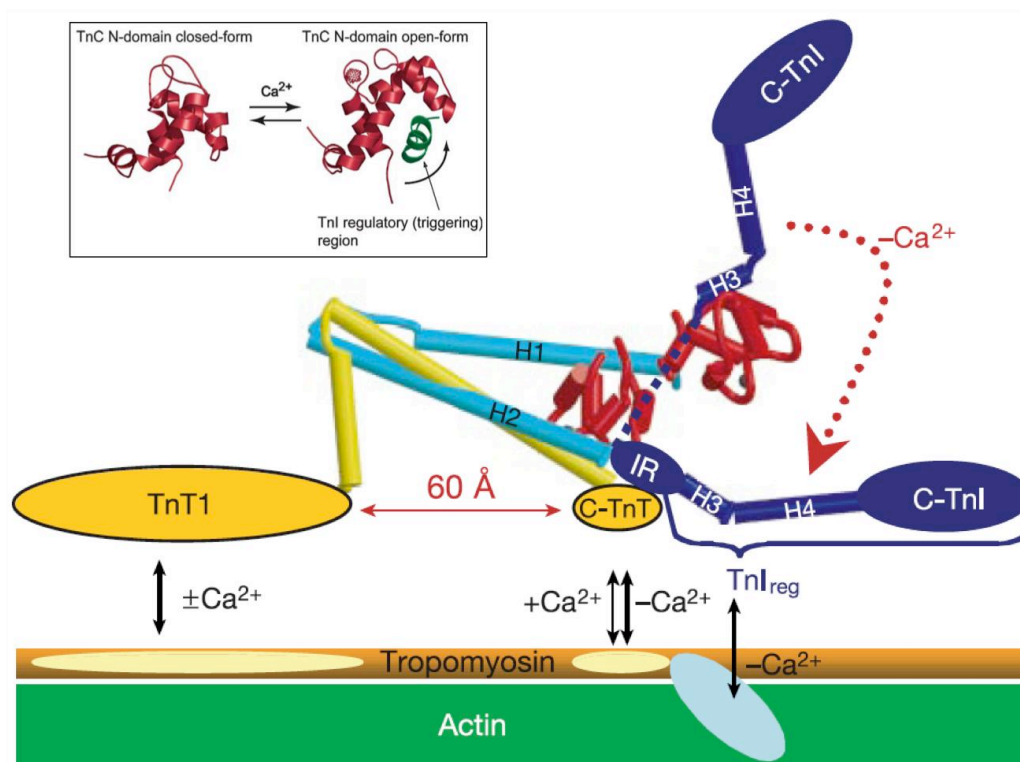
Figure 1-16: The structure of the core domain of the troponin complex



The core domain of the TnC when it is Ca^{2+} saturated. TnC is coloured red, TnT in blue and TnI in green. The regulatory head and the IT arm are shown (Kobayashi and Solaro, 2005).

In the absence of Ca^{2+} , the inhibitory region of TnI binds to actin, thus inhibiting its interaction with myosin and blocking contraction. The regulatory region consists of two actin binding sites (TnI₁₃₅₋₁₄₇ and TnI₁₆₀₋₂₀₉) and the “switch peptide” in the middle (TnI₁₄₈₋₁₅₈). The switch peptide is able to switch position when Ca^{2+} binds to the N terminal lobe of TnC and can bind to a hydrophobic pocket on TnC. This removes the actin blockage and allows it to interact with myosin. Furthermore, tropomyosin changes position relative to the actin groove and co-operatively releases the steric blockage allowing further cross-bridges to form. Figure 1-17 shows the structural changes that occur upon the thin filament switch. The thin filament switch model was first proposed by Malnic and the model states the two different interactions of troponin with actin/tropomyosin and how these interchange upon Ca^{2+} binding (Malnic et al., 1998). The first crystal structure of troponin by Takeda et al provided structural evidence for the Malnic model because it showed that the switch in the presence of Ca^{2+} is bound to TnC and this position inhibits contraction (Takeda et al., 2003).

Figure 1-17: The thin filament switch



This figure shows the thin filament switch in response to Ca^{2+} . TnI acts as the regulatory switch, where in the absence of Ca^{2+} interacts with actin preventing contraction. Ca^{2+} binds to a pocket in TnC and this causes a conformational change opening a hydrophobic pocket in TnC where the regulatory domain of TnI can bind, no longer inhibiting the actin/myosin interaction (Li et al., 2004)

1.3.6 The three state model

Muscle contraction can also be explained by the three state model of the troponin/tropomyosin/actin complex that oscillates between different states according to myosin and Ca^{2+} binding. The concept of the three state model was developed in the early 80s where it was proposed that the thin filaments can exist in two different conformations: a closed state and an open state.

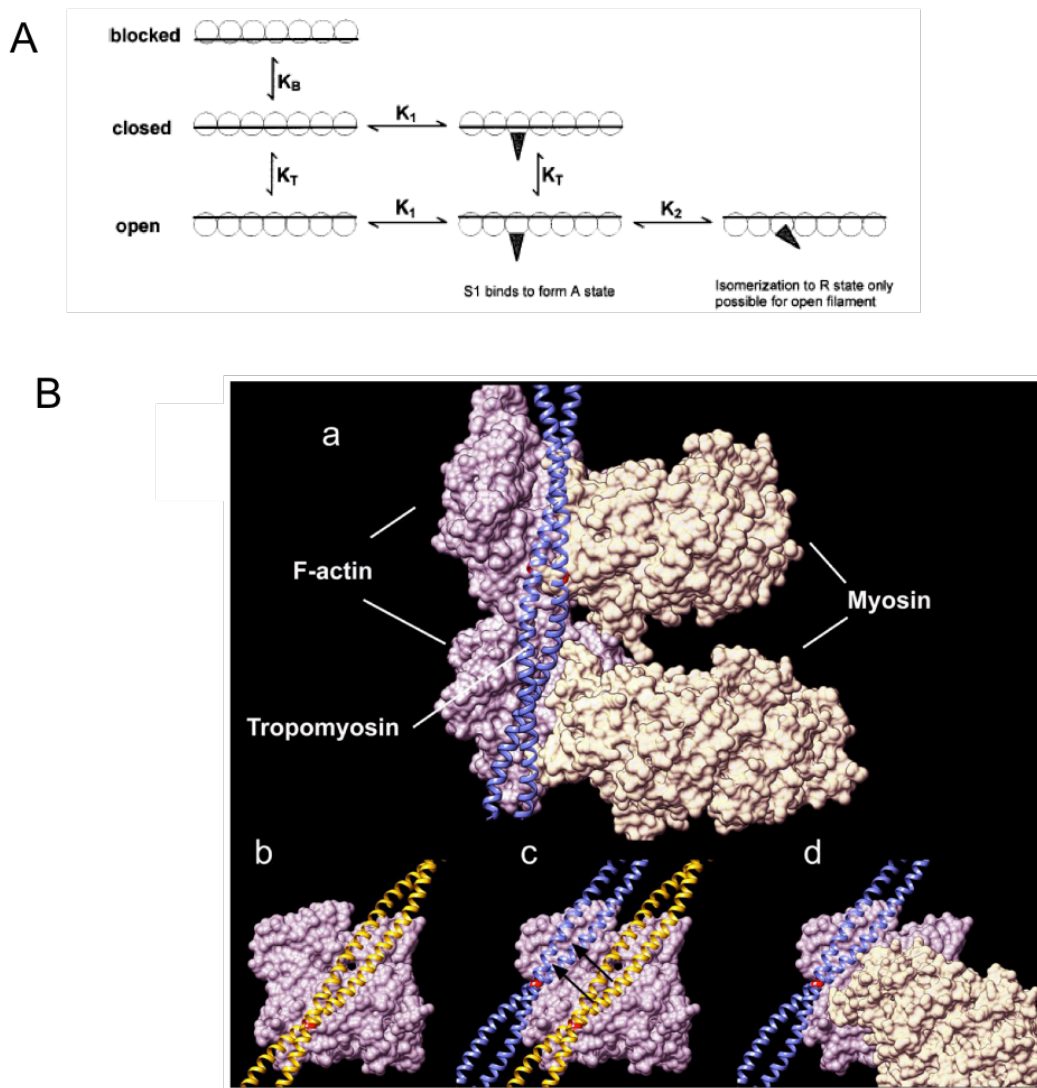
In the 90s the two state model was further developed according to new kinetic studies. Instead, the three state model was proposed, where thin filaments can be found in three conformational states, (“open”, “closed” and “blocked”) (McKillop and Geeves, 1993).

Actin on its own will always be active (in the “open” state), but in the presence of tropomyosin and troponin the three states would be possible. Actin and tropomyosin in the absence of troponin would only have two conformations, “open” and “closed”. Myosin S1 can bind to actin in two different ways, weak (attached or “A state”) and in this way the thin filament is found in the “closed” state, and strong (rigor or “R state”) where the thin filament is found in the “open” state. Only when S1 binds in the R position can there be ATPase activity and thus the cross-bridge cycle can start. In the presence of Ca^{2+} the equilibrium change between the “closed” and the “open” state and this is S1 dependent. In both states myosin binds first weakly (K_1), and this drives the equilibrium towards the “open” state (this step is regulated by K_T). Then thin filament is able to reach the R state (this step is regulated by K_2) and thus the cross-bridge cycle can complete. The third state is the “blocked” state, which only occurs when Ca^{2+} is not present. The equilibrium constant that drives the equilibrium from the “blocked” to the “closed” state is K_a and is Ca^{2+} dependent. Due to the inhibitory action of troponin, the tropomyosin and actin are found in a constrained position, being impossible for myosin to bind. The “blocked” and “closed” states are both called “B state” and the “open” state is called “M state”. Only in the “M state” can the cross-bridge cycle initiate. The three state model is illustrated in Figure 1-18 A.

Tropomyosin plays a central role in the blockage of contraction in absence of Ca^{2+} . In resting muscle tropomyosin is located in a position at the actin filament that would physically block the attachment of strong myosin cross-bridges to actin. When Ca^{2+}

binds to troponin, tropomyosin gets into the grooves between the actin strands and the steric block is removed (see Figure 1-18 B)

Figure 1-18: The three state model and the tropomyosin block



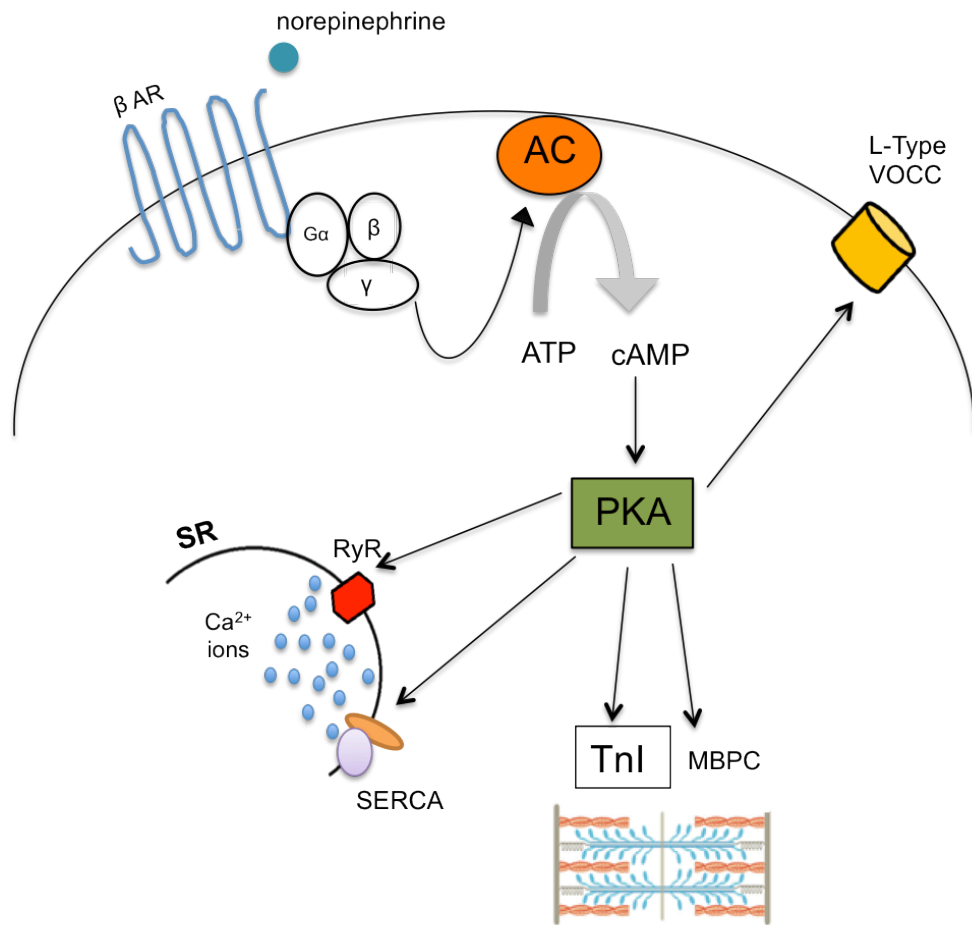
A) Diagram showing the three state model of thin filament activation. Actin is represented in circles, tropomyosin is represented by the solid line and myosin S1 is represented by the triangle. The equilibria between the states are also shown (Maytum et al., 1999). B) Model of regulation of muscle contraction showing the tropomyosin movement from B to M state. Actin is shown in light purple, myosin in beige, tropomyosin in the B state in yellow and in the M state in blue a) pseudo-atomic model of tropomyosin/actin complex showing tropomyosin in the M state b) tropomyosin in the B state c) the rotation of tropomyosin to reach the M state location d) tropomyosin is locked in the M state (Lehman et al., 2013)

1.3.7 The regulation of the cardiac muscle

The regulation of the skeletal and cardiac muscle is under the control of Ca^{2+} and it occurs through the thin filament switch, as described before. The cardiac muscle has an additional modulation in order to increase the cardiac output to meet the demands of the human body. The modulation of the cardiac muscle is under the sympathetic nervous system. The sympathetic nerves release the neurotransmitter adrenaline (epinephrine) or noradrenaline (norepinephrine) which have similar effects. These hormones lead to an increase in the heart rate (chronotropy), the force by which the cardiac muscle contracts (inotropy) and the rate of relaxation (lusitropy). Adrenaline and noradrenaline work by binding to β_1 adrenergic receptors (G coupled receptors), found on the plasma membrane of the cardiomyocytes. Binding of the agonists to the receptors stimulates the GS α -subunit of the receptor to activate adenylyl cyclase. Adenylyl cyclase is an enzyme that catalyses the conversion of ATP to cAMP. cAMP creates a signalling pathway which has different effects to regulate heart beating. The chronotropic effect occurs by an increase of the pacemaker potential rate taking less time to reach the threshold. This is due to the direct effect of the cAMP on the pacemaker current (i_F) and the calcium current (i_{Ca}). The inotropic effect arises from the action of Protein Kinase A (PKA), which is activated through cAMP. PKA phosphorylates many different targets for cardiac muscle contraction and relaxation (El-Armouche and Eschenhagen, 2009). Some of targets of PKA phosphorylation are proteins that control the E-C coupling, such as L-type Ca^{2+} channel, Ryanodine Receptors and Phospholamban (PLB). PLB is a protein that binds to SERCA and inhibits its action. When intracellular Ca^{2+} is increased, Ca^{2+} binds to SERCA and removes PLB from its binding site. However, if PLB is phosphorylated it forms polymers in its dissociated form and thus is no longer able to inhibit SERCA and Ca^{2+} ions will be moved out of the cytosol faster (MacLennan and Kranias, 2003). Another target of the PKA are the Ryanodine Receptors which is believed that their phosphorylation increases the probability of opening and releasing Ca^{2+} into the cytoplasm (Bers, 2002). PKA also phosphorylates MyBP-C that allows its integration into the cardiac cycle by dissociating it from the myosin head (Flashman et al., 2004). Finally, another important target of the PKA is TnI. The result of TnI phosphorylation is a decrease in the Ca^{2+} sensitivity of the myofilament and this is one of the most important aspects in this thesis. This is important because Ca^{2+} would dissociate faster

from the myofilament and the cardiac muscle can relax. TnI phosphorylation by the PKA thus leads to a positive lusitropic response (Solaro et al., 2008). The β adrenergic response and the proteins involved in the process are shown in Figure 1-19. In cardiomyopathies and heart failure, many of the downstream effectors of the β adrenergic response are compromised (El-Armouche and Eschenhagen, 2009). One example is the inability of Ca^{2+} sensitivity to change upon TnI phosphorylation, the phenomenon of which I will be studying extensively in this thesis and will be described in detail later on.

TnI gets phosphorylated by PKA upon β adrenergic stimulation (Solaro et al., 1976) and the phosphorylation of TnI happens in Serines 22 and 23 of the N terminal extension of cardiac TnI (Mittmann et al., 1990). Studies have demonstrated the effects of TnI phosphorylation and have shown that it reduces myofilament Ca^{2+} sensitivity and increase the cross-bridge cycle kinetics at high Ca^{2+} concentrations (Ray and England, 1976, Bailin, 1979, Mope et al., 1980). It has been suggested that TnI phosphorylation by PKA leads to a decrease of the Ca^{2+} sensitivity because of the higher dissociation rate of Ca^{2+} from TnC (Robertson et al., 1982, Zhang et al., 1995, Dong et al., 2007). In all cases the Ca^{2+} sensitivity change upon phosphorylation is 2-3 fold compared to the native. Ca^{2+} sensitivity change upon phosphorylation has been also observed in donor cardiomyocytes as shown in a study by Kooij et al, where the Ca^{2+} sensitivity of force development was reduced in cardiomyocytes treated with the catalytic subunit of PKA (Kooij et al., 2010). Similarly, Messer et al observed an increase in Ca^{2+} sensitivity of reconstituted donor thin filaments upon dephosphorylation of TnI using *in vitro* motility assay (Messer et al., 2007).

Figure 1-19: β adrenergic response of the cardiac muscle

The β adrenergic response of the cardiac muscle and the different targets of PKA phosphorylation upon β adrenergic stimulation are shown. PKA (protein kinase A) is shown in a green box and all its targets are indicated with arrows. β AR adrenergic receptor, which is a G protein coupled receptor, and in black circles its subunits. AC adenylyl cyclase, L-type VOCC L-type Ca^{2+} channel, SR sarcoplasmic reticulum, RyR ryanodine receptor, SERCA sarco/endoplasmic reticulum Ca^{2+} ATPase, TnI troponin I, MBPC myosin-binding protein C.

1.4 Primary cardiomyopathies

Cardiomyopathies can be subdivided into different types: Dilated Cardiomyopathy (DCM), Hypertrophic Cardiomyopathy (HCM), restrictive cardiomyopathy and Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC), all having different clinical characteristics. In this thesis I will investigate only two types of cardiomyopathies, DCM and HCM so they will be described in more detail.

Cardiomyopathies can be either primary or secondary. Primary cardiomyopathies occur when the heart is the primary organ affected and secondary cardiomyopathies occur as a result of other systemic disorders (Alves et al., 2010)

In this thesis I will concentrate on primary cardiomyopathies and more specifically genetic cardiomyopathies, whereby the cause of the disease is a genetic mutation.

The most described causes of cardiomyopathy are mutations in genes that express proteins of the cardiomyocyte. So far more than 900 different mutations of genes have been found expressed in the cardiomyocyte that lead to cardiomyopathies, more than 400 of them being in sarcomeric genes (including thin filament mutations which we study; actin, tropomyosin and troponin) (Harvey and Leinwand, 2011).

1.4.1 Dilated Cardiomyopathy (DCM)

DCM affects 36.5 in 100,000 individuals and is characterized by left ventricular dilation, enlargement of the ventricular chamber and thinning of the ventricular walls (Figure 1-20). This leads to systolic dysfunction with a reduced ejection fraction.

Usually DCM is prevalent in individuals around 30-60 years old, but can also occur in infants and young adults. DCM can lead to sudden death and 50% of patients die within five years after the symptoms begin to occur (Harvey and Leinwand, 2011).

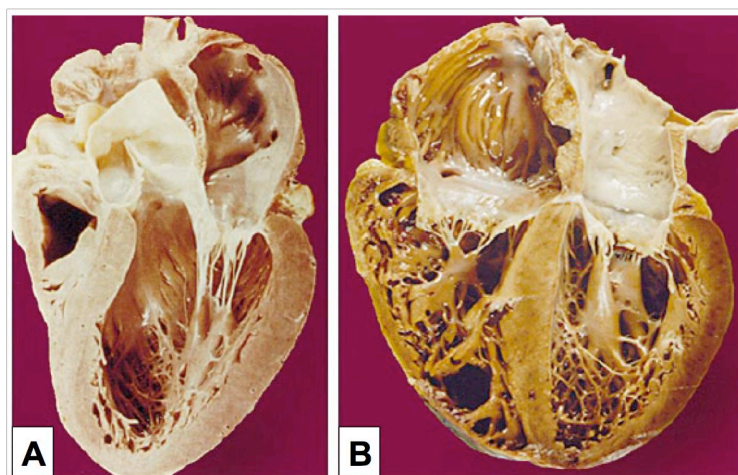
The most successful treatment so far is heart transplant.

Histological examination of DCM hearts has shown that the cardiomyocytes are increased in length and also become polyploid because of an increase in DNA synthesis. Moreover, the number of contractile myofibrils in the cardiomyocytes decreases so they appear empty and hollow in histological sections (Davies, 2000).

Death of cardiomyocytes also occurs through apoptosis and necrosis. This results in an increase in interstitial fibrosis at the space between the cardiomyocytes. Fibrosis attracts immune cells such as macrophages and T lymphocytes at the interstitial space

and it has been found a Th1 type of immune response in DCM patients. Th1 helper T cells play a major role in inflammation and healing process. (Efthimiadis et al., 2011).

Figure 1-20: Section of a DCM vs donor heart



A) Section of a healthy donor heart B) Section of a heart from a DCM patient (Seidman and Seidman, 2001)

DCM can have many causes. Many times DCM occurs as a secondary pathology of other diseases, such as coronary artery disease, thyroid disease, viral myocarditis, immunological diseases or alcohol. This is why the term DCM is imprecise, because it is based on the morphology of the left ventricle, regardless of the cause of the disease. Based on that, DCM can be divided into “ischemic” and “non-ischemic”. Most of the times, the primary cause remains unknown and the disease is called “idiopathic” and in that case DCM is not associated with another systemic cause. From the “idiopathic” DCM in about one third of the cases the disease is thought to be of genetic origin because it has been found in the family history. In that case it is called “familial DCM” (fDCM) (Hershberger et al., 2013).

About one third of the familial DCM patients have been associated with a specific mutation and mutations in more than 40 genes have been found. Most of the mutations are autosomal dominant but a few are found to be X linked and recessive (Hershberger and Siegfried, 2011).

These include genes that encode proteins in the sarcomere, the Z disk, ion channels, mitochondria, cytoskeleton or nuclear envelope. DCM mutations are usually

nonsynonymous missense (they create a protein with a different amino acid sequence than the wild type). However, deletions, nonsense and frameshift mutations have also been reported (Hershberger et al., 2013). The first gene mutation that was identified as cause of DCM was a mutation in dystrophin, which is a membrane-associated protein of the cytoskeleton (Muntoni et al., 1993) while the first mutation coding a sarcomeric protein was in *ACTC* encoding for actin (Olson et al., 1998). Recently titin truncating mutations have been found to be a major cause of fDCM (Herman et al., 2012). For the purpose of this thesis, I will focus on the mutations in the sarcomeric genes. In this thesis I will look at 5 DCM mutations in genes encoding proteins of the sarcomere: *ACTC* E361G, *TNNI* K36Q, *TNNC* G159D, and *TPMI* E54K and E40K based on the study by Memo et al (Memo et al., 2013). It is noted that mutations in thin and thick filament proteins that cause DCM are associated with DCM on its own, without additional phenotypes (Kamisago et al., 2000, Mogensen et al., 2004b).

1.4.2 Hypertrophic Cardiomyopathy (HCM)

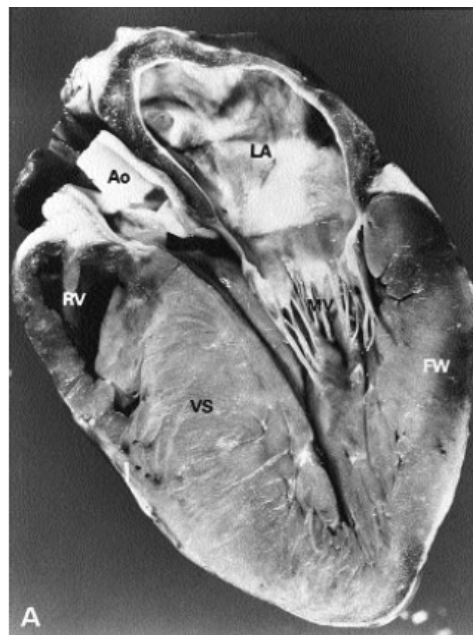
HCM is the most common inherited cardiac disease, with a prevalence of 1 in 500 individuals. HCM affects men more than women and has an age related phenotype; with symptoms occurring after adolescence and sometimes is asymptomatic (Maron et al., 1995, Maron et al., 1984). HCM is characterized by enlargement of the heart and asymmetric hypertrophy of the left or the right ventricle. Typically, the hypertrophy leads to a decrease in the ventricular volume.

Histologically HCM is characterized by cardiomyocyte hypertrophy and also myocyte disarray (disorientation of the enlarged cardiomyocytes). Cardiomyocytes of an HCM heart also undergo premature death that leads to increased interstitial fibrosis. The area affected by the disease and also the degree of myocyte hypertrophy varies among HCM patients (Wigle et al., 1985).

Patients with HCM can be asymptomatic while others might suffer shortness of breath and mild to moderate chest pain (Spirito et al., 1997). The disease can lead to heart failure or even sudden cardiac death (SCD). SCD is defined as “unexpected sudden collapse occurring <1 hour from the onset of symptoms in patients who had previously experienced a relatively stable and uneventful clinical course” (Maron et al., 2000) and occurs especially in athletes (Maron and Pelliccia, 2006). The prevalence of SCD is 1-2 % per year (Elliott and McKenna, 2004). 25% of the HCM

patients develop an obstruction to the ejection of blood from the left ventricle during systole, and these are called Hypertrophic Obstructive Cardiomyopathy (HOCM) patients. The obstruction is clinically characterized as Left Ventricular Outflow Tract Obstruction (LVOTO). The presence of LVOTO increases the probability of the patients developing heart failure and death (Maron et al., 2003).

Figure 1-21: Heart from an HCM patient



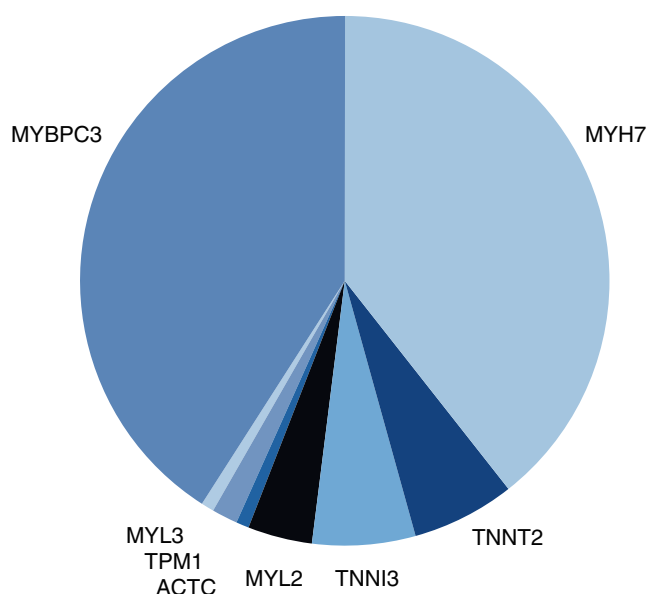
Heart from an HCM patient from a cross sectional plane. Ao represents the aorta, LA is the left atrium, RV is the right ventricle, FW is the left ventricular free wall and VS is the ventricular septum (Maron, 1997). It is clearly shown the enlargement of the heart and asymmetric hypertrophy.

HCM was the first cardiomyopathy that was linked to genetic mutations (Geisterfer-Lowrance et al., 1990) and about 50% of HCM cases are due to mutations. In contrast to DCM, the majority of the mutations are found mainly in genes that encode sarcomeric proteins. For this reason HCM is called “the disease of the sarcomere” (Thierfelder et al., 1994). Over 600 mutations in 12 different genes have been associated with the disease and HCM patients carrying a mutation in any of the sarcomeric genes show a more severe phenotype with greater hypertrophy than HCM patients with no known mutations (Van Driest et al., 2005). The HCM mutations are

usually autosomal dominant and a few patients carry more than one mutation (Ingles et al., 2005)

The first HCM mutation identified was β myosin heavy chain (*MYH7*) (Geisterfer-Lowrance, Kass et al. 1990) followed by mutations in myosin regulatory (*MYL2*) and essential light chain (*MYL3*), Myosin Binding Protein C (*MYBPC3*), cardiac Troponin T (*TNNT2*) and cardiac Troponin I (*TNNTI3*) (Redwood et al., 1999) but have received little mechanistic study to date. Mutations in cardiac actin (*ACTC*) and tropomyosin (*TPM1*) have also been found. Out of all the mutations causing HCM, the most frequent are mutations in genes encoding cardiac MyBP-C and β MHC (Xu et al., 2010). In this thesis three HCM mutations will be studied, E99K in *ACTC* (Olson et al.), E180G in *TPM1* (Thierfelder et al., 1994) and K280N in *TNNT2* (Bayliss, 2012, Ferrara et al., 2015).

Figure 1-22: The distribution of sarcomeric mutations in HCM



A pie chart showing the distribution of mutations in thick and thin filament proteins that cause HCM. *MYH7* β myosin heavy chain, *MYBPC3* myosin-binding protein C, *TNNT2* cardiac troponin T, *TNNI3* cardiac troponin I, *MYL2* myosin light chain 2, *ACTC* cardiac actin, *TPM1* Tpm1.1, *MYL3* ventricular myosin light chain 1. Taken from Marston, 2011.

1.4.3 Methodology to study familial cardiomyopathies

fDCM and fHCM are the two major familial cardiomyopathies in human, and thus there is a demand to study these diseases and the molecular mechanisms that are responsible for them. Different models in different levels have been developed in the recent years to study familial cardiomyopathies and all of them give us important insights about mechanisms of cardiomyopathies. In this section I will briefly describe some of these methodologies that others and us use and are relative to this thesis.

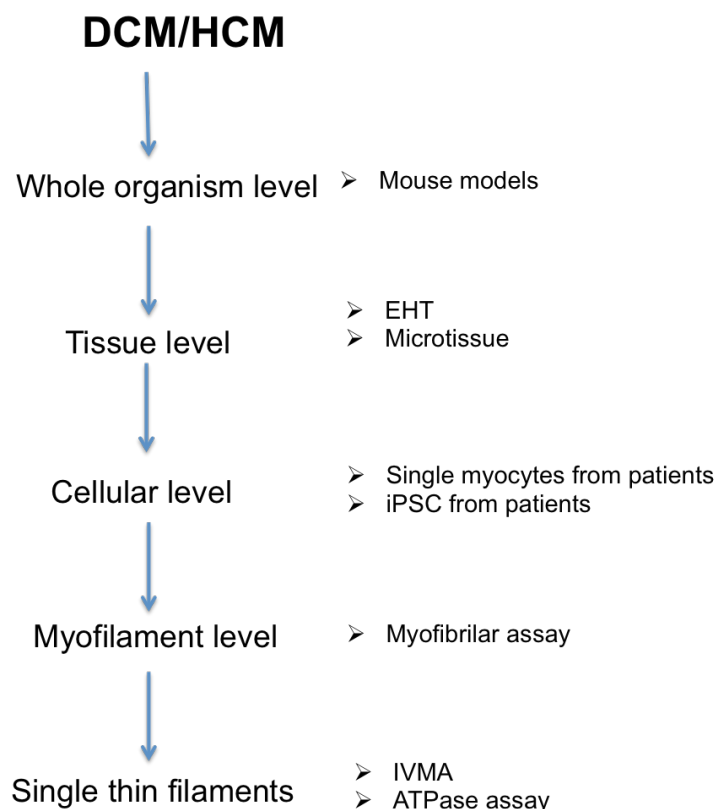
In order to study thin filament mutations (investigated in this thesis) that cause DCM or HCM in detail and be able to establish structure-function relationships, the *in vitro* motility assay (IVMA) is often used and is the main methodology used in this thesis. The IVMA was firstly developed in our lab by Dr Ian Frazer and Prof Steve Marston (Frazer and Marston, 1995b). In this technique, thin filaments can be reconstituted by proteins extracted from tissue (troponin, tropomyosin and actin) and slide along a bed of immobilised HMM in a Ca^{2+} dependent manner. In IVMA the movement of filaments can be studied, and the Ca^{2+} sensitivity of the thin filaments can be measured. Because the proteins can be extracted from different sources, different mutations in the thin filaments can be easily studied. Furthermore, the proteins can be easily manipulated in the post-translation level (such as phosphorylation) using biochemical methods (Messer et al., 2007, Bayliss et al., 2012, Memo et al., 2013), making IVMA a good model to study mutations in the filament level. Similarly to IVMA, the actomyosin S1 fragment ATPase activity can be measured using the ATPase assay to study the regulatory properties of thin filaments (Deng et al., 2001). However, an important limitation of the IVMA is the fact that the assay is unloaded and force cannot be measured. A complementary methodology to the IVMA to study mutations in the myofilament level is the myofibrillar assay. Such an assay was developed in our lab by Dr Petr Vikhorev. This apparatus can measure contractility of skinned myofibrils; force of contraction, kinetics of contraction and relaxation, and rates of Ca^{2+} activation (Vikhorev et al., 2014). While in IVMA thin filaments are reconstituted from proteins from different sources, in the myofibrillar assay all structural elements and contractile proteins are retained, representing better the physiological situation.

On the whole organism level, the most usual methodology to study DCM and HCM is through mouse models (Zaragoza et al., 2011). Over the last decades, several DCM

and HCM mouse models have been developed that incorporate mutations found in human and most of the times recapitulate many of the same characteristics of the human disease. A DCM mouse model has been developed in Steve Marston's lab with the E361G mutation in *ACTC* gene encoding for actin (Song et al., 2010, Wilkinson et al., 2015) and similarly an HCM mouse model was also developed with the *ACTC* E99K mutation (Song et al., 2011, Song et al., 2013). These two mouse models enabled us to study DCM and HCM and compare their mechanism of action in the molecular level, and will also be investigated in this thesis. Another mutation that is being studied in this thesis is the HCM-causing mutation E180G. A mouse model with this mutation has been developed by Alves et al (Alves et al., 2014) and showed many characteristics of HCM in human. Although mouse models can provide very insightful information about mechanisms of disease in the whole organism level, being able to link specific mutations with phenotype and disease progression, mouse models cannot represent the human situation 100%.

To overcome this problem, cardiovascular research recently has recently turned to the use of patient specific induced pluripotent stem cells (i-PSCs) derived cardiomyocytes for the study of DCM and HCM (Sun et al., 2012). These patient specific cells can be used study the diseases in the tissue level and not only the cellular, through Engineered Heart Tissue (EHT) or microtissue. EHT is a hydrogel based 3D muscle-like tissue whereby cells beat in synchrony and produce contractile force, a situation closely resembling that of intact heart tissue (Eschenhagen et al., 1997). Titin mutations causing DCM have been successfully modelled and studied using cardiac microtissue engineered from human iPSC (Hinson et al., 2015). In Figure 1-23 the different models and levels to study DCM and HCM are shown.

Figure 1-23: Methodology used to study HCM and DCM



Methodology used in research to study DCM and HCM. iPSC induced pluripotent stem cells, IVMA *in vitro* motility assay, EHT engineered heart tissue.

1.4.4 The molecular mechanisms of DCM and HCM mutations

It has been a mystery for many years how mutations in any of the sarcomeric proteins can lead to cardiomyopathies and still there has been a lot of research going on to elucidate the mechanisms behind the diseases.

It is important to establish that fDCM and familial HCM (fHCM) are two distinctive genetic diseases and do not overlap. For example, DCM can never develop to HCM but HCM can lead to heart failure after a long time and after the symptoms of HCM have been developed. fDCM and fHCM have also distinct molecular mechanisms. HCM has been associated with a hypercontractility demonstrated by an increased Ca^{2+} sensitivity. Initially, studies performed in *in vitro* models of HCM, mostly looking at mutations in the thin filament using recombinant proteins. These studies showed a higher Ca^{2+} sensitivity and increased Ca^{2+} affinity in mutant proteins, as

well as increased cross-bridge kinetics and incomplete relaxation (Marston, 2011). A study by Sequeira et al looking at human cardiomyocytes with HCM mutations in the thick and thin filament showed that in all cases HCM caused an increase of the myofilament Ca^{2+} sensitivity and impaired length-dependent activation (Sequeira et al., 2013).

It has been demonstrated that increased Ca^{2+} sensitivity is associated with higher risk of arrhythmias contributing to the phenotype of HCM. Increased Ca^{2+} sensitivity alone is sufficient to cause the HCM phenotype *ex vivo*, as it has been demonstrated by treating papillary muscles of wild type mice with the Ca^{2+} sensitizer EMD 57033 (Baudenbacher et al., 2008). However, the mechanisms by which increased Ca^{2+} sensitivity induces the histological changes observed in HCM are less clear. Sudden cardiac death correlates with increased Ca^{2+} sensitivity *in vitro* (Haim et al., 2007) but does not correlate with hypertrophy (Varnava et al., 2001), suggesting two separate mechanisms.

Early studies with recombinant proteins causing DCM have shown that DCM is linked to a decrease in Ca^{2+} sensitivity, decreased Ca^{2+} affinity for TnC and decreased cross-bridge cycle kinetics (Robinson et al., 2002, Mirza, 2005). However, studies from human and transgenic mice in Prof Steve Marston's laboratory and other studies have shown that DCM mutations can lead to either a decrease or an increased Ca^{2+} sensitivity (Marston, 2011, Messer and Marston, 2014). For example, *in vitro* motility assay studies with the DCM mutation E361G in actin showed similar Ca^{2+} sensitivity compared to normal. This led to the need to find a common mechanism for DCM mutations.

In a study by Memo et al, seven thin filament mutations leading to DCM were studied using IVMA. Phosphorylated and dephosphorylated thin filaments were compared and the Ca^{2+} sensitivity was independent of TnI phosphorylation (uncoupled) in all the DCM causing mutations, leading to the hypothesis that uncoupling is the common molecular mechanism of DCM (Memo et al., 2013).

Can uncoupling be a common molecular defect both HCM and DCM? The next section will cover the aspects of the uncoupling phenomenon and its importance in the field of cardiomyopathies.

1.5 The uncoupling of Ca^{2+} sensitivity to TnI phosphorylation and its importance

1.5.1 TnI phosphorylation by PKA

The phosphorylation of TnI and its effects have important implications in health and disease (Zhang et al., 2011). End-stage failing hearts have reduced TnI phosphorylation levels and this leads to an increase of the Ca^{2+} sensitivity seen in these hearts. (Wolff et al., 1995, Bodor et al., 1997, van der Velden et al., 2003). A study by Messer et al using IVMA has shown that phosphorylation changes in troponin alone can lead to contractile abnormalities in failing hearts (Messer et al., 2007). Failing hearts had a decreased level of TnI phosphorylation and this correlated with an increased Ca^{2+} sensitivity. When the TnI of the failing hearts was replaced with TnI treated with PKA the Ca^{2+} sensitivity was restored to the normal levels. In a study by Yasuda et al, phosphomimetic mutants S23/24D had no difference in isoproterenol stimulation relative to controls, whereas when cardiomyocytes were transfected with either non-phosphorylatable TnI or sTnI blunted the response to isoproterenol (Yasuda et al., 2007). It is noted that phosphomimetic mutants can represent TnI phosphorylation by PKA and the physiological implications. Transgenic (TG) mice with a mutated TnI lacking phosphorylation sites showed similar results, lacking lusitropic response to Isoproterenol (Pi et al., 2002). These studies highlight the importance of the TnI phosphorylation as well as the Ca^{2+} sensitivity in biology and disease.

1.5.2 The discovery of the uncoupling phenomenon

In the first investigations of the molecular mechanisms of cardiomyopathies, the effect of TnI phosphorylation was not considered. The first group to discover the uncoupling phenomenon was a study by Deng et al, whereby the Ca^{2+} sensitivity of the HCM causing mutation R145G in TnI was independent of phosphorylation (Deng et al., 2001). A few further independent studies have shown Ca^{2+} sensitivity uncoupled from TnI phosphorylation. For example, the mutation L29Q in TnC that causes HCM shows uncoupling, as measured by IVMA and also actomyosin ATPase activity (Schmidtman et al., 2005).

Dong et al looked at another mutation that also showed uncoupling, G159D. This mutation in TnC is a DCM causing mutation and is one of the best clinically characterised mutations (Mogensen et al., 2004a 2007, Kaski et al., 2007). For this reason, the G159D mutation has been intensively investigated in our lab and in others. Myofilaments containing the G159D mutation showed Ca^{2+} sensitivity independency to the change of TnI phosphorylation status (Biesiadecki et al., 2007). Dyer et al from Steve Marston's laboratory looked at Tn extracted from the human sample containing the G159D mutation and found that Ca^{2+} sensitivity of thin filaments reconstituted with the mutant troponin was not sensitive to TnI phosphorylation using IVMA (Dyer et al., 2009).

1.5.3 Uncoupling as a mechanism of cardiomyopathies with thin filament mutations

The first complete study of the uncoupling phenomenon was by Memo et al, where seven different mutations causing DCM were studied, and the mutations were not only limited to mutations in troponin. The mutations studied were E40K, E54K and D230N in Tpm1.1, R141W and Δ K210 in TnT, K36Q in TnI and E361G in actin. Using the IVMA in reconstituted thin filaments and comparing phosphorylated and dephosphorylated troponin it was clear that the Ca^{2+} sensitivity was independent from TnI phosphorylation (Memo et al., 2013). It is interesting to note that when the mutant thin filaments were compared to normal the Ca^{2+} sensitivity was increased, decreased or remained the same.

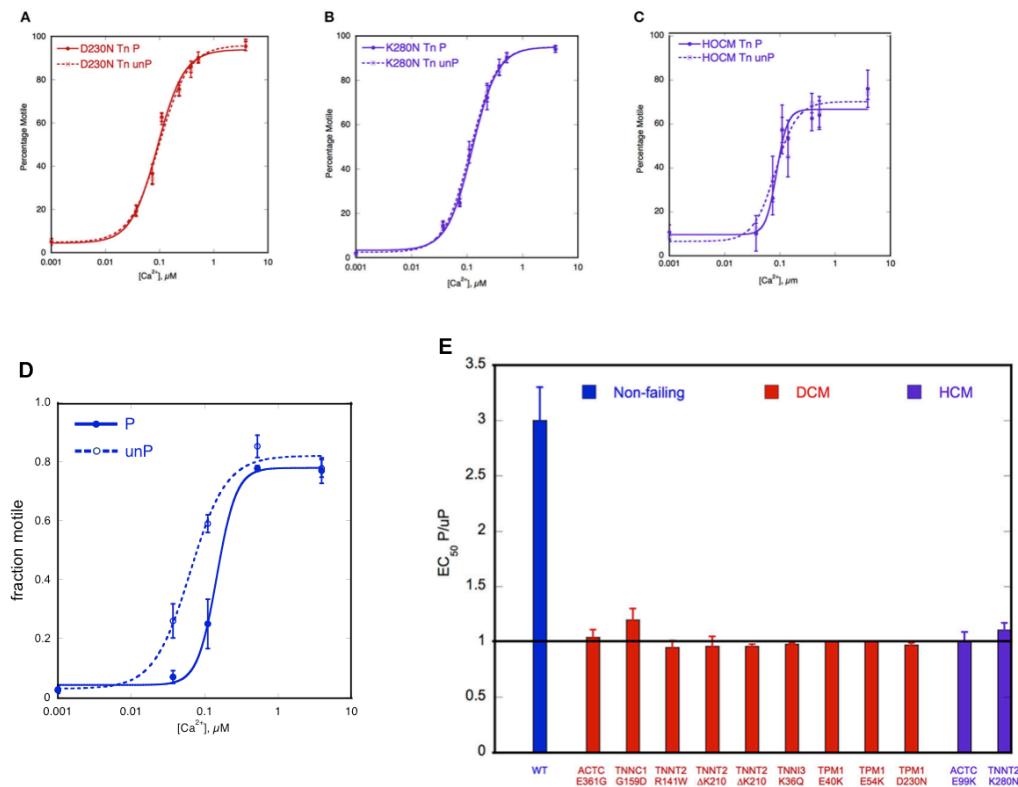
This led to the hypothesis that the molecular phenotype of DCM is uncoupling of Ca^{2+} sensitivity to TnI phosphorylation making the heart unresponsive to the β adrenergic response. The uncoupling phenomenon in DCM was confirmed by another study looking at rare TnC variants leading to DCM (Pinto et al., 2011).

The uncoupling phenomenon has also been demonstrated in some HCM mutations as well. For example, the Ca^{2+} sensitivity of mice carrying the HCM mutation R92W in TnT was uncoupled to TnI phosphorylation at 2 months of age (Guinto et al., 2009). Uncoupling has also been demonstrated in thin filaments reconstituted with the HCM mutation K280N in TnT (Messer et al., 2012) and also in thin filaments reconstituted with E99K mutant actin from transgenic mice (Song et al., 2011). However, the situation is less clear in permeabilised muscle. Sequeira et al found that some HCM

mutations uncouple and others partially uncouple Ca^{2+} sensitivity to TnI phosphorylation (Sequeira et al., 2013). DCM and HCM mutations where uncoupling has been observed are summarised in Table 1-1.

Unexpectedly, uncoupling of the Ca^{2+} sensitivity to TnI phosphorylation has also been observed as a secondary effect in myectomy samples independently of mutations. The purpose of myectomy operation is to relieve left ventricular outflow tract obstruction (LVOTO), which is causing pressure overload in HOCM patients. Several defects have been found in HOCM samples, including abnormal phosphorylation levels of TnI and MyBP-C (Messer et al., 2009) (Copeland et al., 2010) and actin isoform expression (Copeland et al., 2010). Myectomy samples with mutations in *MYBPC3*, *MYH7*, *TNNT2* and unknown mutations also showed uncoupling in IVMA when the phosphorylation levels were manipulated using standard biochemical methods (Bayliss et al., 2012b). Subunit exchange experiments were carried out to investigate which molecule was responsible for uncoupling and it was found to be in TnT. However, no post-translational modifications in TnT subunit were found to account for this abnormality. Figure 1-24 shows the uncoupling phenomenon in different situations measured by the IVMA by members of the Marston's laboratory.

Figure 1-24: The uncoupling phenomenon in DCM and HCM



Percentage motility of reconstituted thin filaments in response to different Ca^{2+} concentrations. Phosphorylated (solid lines) and dephosphorylated thin filaments (dotted lines) were compared. DCM mutations are represented in red, HCM mutations are represented in purple and non failing (donor) thin filaments are represented in blue. A) D230N tropomyosin. B) Thin filaments were reconstituted with human heart troponin with exchanged recombinant TnT K280N subunit C) Thin filaments were reconstituted from HOCM troponin or PKA treated HOCM troponin. D) Ca^{2+} regulation of phosphorylated or dephosphorylated donor thin filaments E) EC_{50} ratio of phosphorylated to dephosphorylated thin filaments containing different DCM (red) or HCM mutations (purple). Non failing (donor) thin filaments are represented in blue. Picture adapted from Messer and Marston, 2014.

Table 1-1: Examples of the uncoupling phenomenon in DCM and HCM

Mutation	Effect on Ca ²⁺ sensitivity	Measurement method	Publication
DCM			
ACTC E361G	No difference	IVMA	Song et al, 2010, Memo et al, 2013
TPM1 E54K	No difference	IVMA	Memo et al, 2013
TPM1 E40K	Decrease	IVMA	Memo et al, 2013
TPM1 D230N	Increase	IVMA	Memo et al, 2013
TNNC1 G159D	Increase	IVMA, Ca binding	Biesiadecki et al, 2007, Dong et al, 2008, Dyer et al, 2009, Memo et al, 2013
TNNC1 Y5H	Decrease	Skinned fiber	Pinto et al, 2011
TNNC1 M103I	Decrease	Skinned fiber	Pinto et al, 2011
TNNC1 I148V	Decrease	Skinned fiber	Pinto et al, 2011
TNNT2 ΔK210	Decrease	IVMA, Skinned fiber	Du et al, 2007, Memo et al, 2013
TNNT2 R141W	Decrease	IVMA	Memo et al, 2013
TNNI3 K36Q	Decrease	IVMA, ATPase	Carballo et al, 2009, Memo et al, 2013
HCM			
ACTC E99K	Increase	IVMA	Song et al, 2011
TNNC1 L29Q	Increase	Ca binding, ATPase	Schmidtman et al, 2005, Dong et al, 2008, Li et al, 2013
TNNT2 K280N	Increase	IVMA	Bayliss et al, 2012
TNNI3 R145G	Increase	IVMA, ATPase	Deng et al, 2001
TNNI3 R21C	Increase	Skinned fiber	Gomes et al, 2005, Wang et al, 2011
TNNI3 G203S	Increase	IVMA, ATPase	Deng et al, 2013
TNNI3 K206Q	Increase	IVMA, ATPase	Deng et al, 2013
TNNT2 R92W	Increase	Cardiac myocytes	Guinto et al, 2009

Adapted from Messer and Marston, 2014

1.5.4 The physiological importance of the uncoupling phenomenon

The implications of uncoupling of the Ca²⁺ sensitivity to the TnI phosphorylation in diseased heart is that even when TnI gets phosphorylated the Ca²⁺ sensitivity would not decrease, making the diseased heart unresponsive to part of the β adrenergic response. This would lead to a blunting of the lusitropic response, predisposing the heart to failure.

The physiological relevance of uncoupling has been demonstrated with DCM transgenic animals carrying the E361G mutation in *ACTC*. This mouse showed blunting of the lusitropic and inotropic response to adrenaline *in vivo* and *ex vivo*, as a result of uncoupling the Ca²⁺ sensitivity to TnI phosphorylation (Wilkinson et al., 2015). Furthermore, the HCM E99K transgenic mice showed blunted response to dobutamine (a drug which stimulates β1 adrenergic receptors), which would occur as

a result of uncoupling (Song et al., 2013). TnI G203S transgenic mice did not respond to chronic swimming exercise, which correlates with lack of response to β adrenergic stimulation. It is noteworthy that loss of one part of the system seems to lead to a loss of the whole. For example, PLB KO mice showed a blunted lusitropic and inotropic response to Isoproterenol, analogous to TG mice with the *ACTC* mutation E361G that uncouples TnI phosphorylation to Ca^{2+} sensitivity showing blunted response to dobutamine (Li et al., 2000a).

E361G TG mice did not show any phenotype of the disease unless under stress. This has been observed in other DCM mouse models such as TnT Δ K210, MyBP-C knockout and titin knock-in (Messer and Marston, 2014). This is also well-matched with the lack of response to adrenergic stimulation which would not be observable at rest. Targeting the uncoupling phenomenon pharmacologically might be therefore a way to treat cardiomyopathies.

The role of uncoupling in HCM is less clear. In all cases of HCM there is increased Ca^{2+} sensitivity and this might be the primary effect that causes the HCM phenotype and it dominates over the uncoupling phenomenon. The recent view for the case of HCM is that the common mechanism is increased force, and not increased Ca^{2+} sensitivity (Spudich, 2014). In any case, it will be interesting to investigate whether increasing Ca^{2+} sensitivity would also lead to uncoupling or Ca^{2+} sensitivity and uncoupling act via separate mechanisms. In this thesis this will be investigated, as well as the structural mechanisms that lead to uncoupling.

1.6 Aims

The uncoupling phenomenon has been shown to play a very important role in the development of cardiomyopathies. In this thesis I will investigate some the aspects of the uncoupling phenomenon, its molecular mechanism, its correlation to Ca^{2+} sensitivity, and the potential to induce it or reverse it using pharmacological agents. The main method used in the thesis was the *in vitro* motility assay (IVMA). The advantage of this technique is that is a simple technique to screen functional properties of the contractile proteins *in vitro*. Subunits of the thin filaments can be purified separately and from different sources, the Ca^{2+} sensitivity can be easily measured as a function of movement of the filaments in relationship to Ca^{2+} and the phosphorylation of the different proteins can be manipulated easily by biochemical

methods. Thus this method is very appropriate for the extensive study of the uncoupling phenomenon.

In order to familiarise myself with the IVMA, I also got involved in a small project concerning skeletal muscle myopathies. I studied 7 mutations in Tpm2.2 or Tpm3.12 that lead to various skeletal muscle diseases and evaluated whether there is a common molecular behaviour in terms of the location of the mutation. The aim of this study was to elucidate a structure-function mechanism for tropomyosin mutations that would lead to future predictions. For the purpose of the thesis, this project is included as an entire different chapter, with its own introduction and discussion separate from the rest of the thesis, which investigates the uncoupling phenomenon in cardiomyopathies.

The study by Memo et al showed that uncoupling of TnI phosphorylation to Ca^{2+} sensitivity is the molecular mechanism behind DCM. In this study mutations in all the components of the thin filament were studied (actin, troponin, tropomyosin) and in all cases TnI phosphorylation was uncoupled to Ca^{2+} sensitivity. How is it possible for a mutation in any component of the thin filament to cause perturbations in troponin and thus cause uncoupling?

As suggested by recent studies, troponin can have two conformations depending on the phosphorylation level and there is a specific interaction between TnC and TnI that occurs when TnI is phosphorylated and accounts for the change in Ca^{2+} sensitivity that makes the myofilament respond to β adrenergic response.

We propose that this interaction between TnC and TnI is destabilised or change if there is a perturbation (for example a mutation) in any of the components of the thin filament that causes the uncoupling phenomenon to occur. To test this hypothesis, I used small molecules, which increase or decrease the Ca^{2+} sensitivity by binding to TnC and are thought to affect this interaction. The hypothesis is that the small molecules will cause perturbations in the TnC-TnI interaction and might induce uncoupling. This will give insights about the mechanisms of the uncoupling phenomenon. The most surprising and important finding results I got was that the Ca^{2+} desensitiser EGCG could reverse the uncoupling in mutant thin filaments.

As part of my PhD I initiated a collaboration with Dr Ian Gould from the Department of Chemistry and he performed molecular dynamics (MD) simulations in troponin to observe the structural differences upon phosphorylation.

2 MATERIALS AND METHODS

2.1 Sources of sample proteins

2.1.1 Sources of human heart samples

2.1.1.1 Sources of donor heart samples

All donor heart samples were provided by Prof Cristobal Dos Remedios, University of Sydney, Sydney, Australia. Ethical approval for usage of the samples was obtained from the Brompton, Harefield & NHLI, London and from St Vincent's Hospital, Sydney. Non-failing adult myocardium was obtained from patients who were first assigned as donors but no suitable recipient was found or the heart was considered unusable for other reasons. The donors had no history of cardiac disease, had a normal cardiac examination, a normal admission ECG and normal ventricular function on transthoracic echocardiography as performed within 24 hours of heart explantation. Immediately after explantation of the heart, the ventricular wall was cut into 1g pieces and stored in liquid nitrogen. The clinical details of the donors are shown in Table 2-1.

2.1.1.2 Sources of Dilated Cardiomyopathy

Two samples were obtained from patients diagnosed with DCM undergoing heart transplant. An explanted heart sample with the missense mutation Lys36Gly (K36Q) in Troponin I (*TNNI*) that causes DCM was used (Carballo et al., 2009). The heart sample carrying this mutation was kindly provided by Prof Cristobal Dos Remedios from University of Sydney, Sydney, Australia. The other sample of DCM mutation used in this study was from a patient carrying the missense mutation Gly159Asp (G159D) in Troponin C (*TNNC*). A sample from a transplanted heart from the patient was collected by Dr Adam Jacques from the Great Ormond Street Hospital, London (Dyer et al., 2009). Ethical approval for usage of the samples was obtained from the Brompton, Harefield & NHLI, London and from St Vincent's Hospital, Sydney. Both samples were stored in liquid nitrogen at all times. The clinical characteristics of both DCM mutations are summarised in Table 2-1.

2.1.1.3 Sources of Hypertrophic Cardiomyopathy

For this study, a sample from a patient diagnosed with HCM and carrying the missense mutation Lys280Asp (K280N) on Troponin T (*TNNT2*) was donated by Prof Cristobal Dos Remedios, University of Sydney, Sydney, Australia. Ethical approval for usage of the sample was obtained from the Brompton, Harefield & NHLI, London and from St Vincent's Hospital, Sydney. The mutation was identified by Prof Van der Velden (Bayliss et al., 2012a).

Table 2-1: Table of human heart samples used in this study

Sample		Diagnosis	Gender	Age	LVEF on ECO
Donor	NH	SAH	Female	48	
	NJ	SAH	Female	55	
	NK	Cardioplegia	Male	45	
	NL	SAH/Aneurism	Female	42	65%
	NN	Right MCA infarct	Male	40	
	NS	Family of hypertension	Female	55	
DCM	G159D	DCM		3	20%
	K36Q	DCM	Male	15	15-20%
HCM	K280N	HCM	Male	26	

Hypertrophic Cardiomyopathy (HCM), Dilated Cardiomyopathy (DCM), Subarachnoid Haemorrhage (SAH), Middle Cerebral Artery (MCA), Left Ventricular Ejection Fraction (LVEF), Echocardiography (ECO)

2.1.2 Sources of mouse heart samples

2.1.2.1 The Dilated Cardiomyopathy mouse model

A DCM mouse model was developed in our lab by Dr Weihua Song carrying the mutation Glu361Gly (E361G) on actin (*ACTC*) which is correlated with DCM (Song et al., 2010, Wilkinson et al., 2015). Whole hearts from the transgenic mice were extracted and frozen in liquid nitrogen.

2.1.2.2 The Hypertrophic Cardiomyopathy mouse model

An HCM mouse model overexpressing the mutation Glu99Lys (E99K) on actin (*ACTC*) was developed in our laboratory by Dr Weihua Song (Song et al., 2011). Whole hearts from the transgenic mice were extracted from these mice and frozen in liquid nitrogen.

2.1.3 Tropomyosin samples

2.1.3.1 *TPM1* mutants causing cardiomyopathy

Two mutations in a tropomyosin that cause DCM have been used in this study: Glu40Lys (E40K) and Glu54Lys (E54K), both in *TPM1*. These two mutations were identified as a cause of DCM by a study on two families by Olson et al (Olson et al., 2001). Purified recombinant AS α tropomyosins E40K and E54K and wild type (WT) were provided by Dr Charles Redwood, University of Oxford, Oxford, UK. Furthermore, a tropomyosin mutation that causes HCM and has been characterised in this lab (Bing et al., 2000) is a mutation in *TPM1* Glu180Gly (E180G). Purified recombinant AS α tropomyosin E180G was provided by Dr Charles Redwood, University of Oxford, Oxford, UK. The recombinant proteins were expressed in baculovirus/*sf9* insect cells.

2.1.3.2 *TPM2* mutants causing skeletal muscle myopathy

Mutations in *TPM2* that cause skeletal muscle diseases were studied in this thesis. Briefly, mutations were introduced into *TPM2* cDNA by site-directed mutagenesis. The wild type and also the mutant tropomyosins were cloned into *sf9* insect cells and recombinant proteins were isolated using the protocol of Akkari et al (Akkari et al., 2002). These cells are able to keep the post-translational modifications of the eukaryotic system including the N terminal acetylation. Purified WT tropomyosin was also provided. Mutant Tpm2.2 proteins included in this study are: a deletion in Lys49 (Δ K49), which is associated with Cap disease (Ohlsson et al., 2008), Arg91Gly (R91G), associated with distal arthrogryposis (Sung et al., 2003) and a deletion in Glu139 (Δ E139) found in a patient with Cap disease (Clarke et al., 2009). Baculovirus/*sf9* pellets with the mutations Δ K49 and R91G Tpm2.2 mutants

were supplied by Dr Kristen Nowak and Dr Elyshia McNamara. Δ E139 mutant pellets were provided by Mintu Martilla, University of Helsinki, Helsinki, Finland. WT purified Tpm2.2 was provided by Dr Mohammed El-Mezgueldi, University of Leicester, Leicester, UK.

2.1.3.3 *TPM3* mutants that cause skeletal muscle myopathy

Baculovirus/*sf9* pellets from recombinant Tpm3.12 mutants Lys168Glu (K168E), deletion in E139 (Δ E139), Arg167His (R167H) and Arg244Gly (R244G) were provided by Dr Kristen Nowak, Dr Elyshia McNamara and Royston Ong, University of Western Australia, Nedlands, Australia. The skeletal muscle diseases associated with these mutations can be found in Table 2.2.

Tpm3.12 218del (Δ E218) and 224del (Δ E224) were provided by Dr Kristen Nowak, Dr Elyshia McNamara and Royston Ong already purified from baculovirus/*sf9* (see Section 2.1.3.2) in a lyophilised form. A summary of all the tropomyosin mutations used in this study can be found in Table 2-2. WT Tpm3.12 was supplied by Dr Mohamed El-Mezgueldi, University of Leicester, UK and Dr Kristen Nowak, University of Western Australia, Nedlands, Australia.

Table 2-2: Summary of the Tropomyosin mutations used in this study

Mutation	Disease	Gene	Isoform
E40K	DCM	<i>TPM1</i>	Tpm1.1
E54K	DCM	<i>TPM1</i>	Tpm1.1
E180G	HCM	<i>TPM1</i>	Tpm1.1
Δ K49	Cap disease	<i>TPM2</i>	Tpm2.2
R91G	Distal arthrogryposis	<i>TPM2</i>	Tpm2.2
Δ E139	Cap disease	<i>TPM2</i>	Tpm2.2
K168E	CFTD	<i>TPM3</i>	Tpm3.12
R167H	Congenital myopathy	<i>TPM3</i>	Tpm3.12
R244G	CFTD	<i>TPM3</i>	Tpm3.12
Δ E218	Severe stiff myopathy	<i>TPM3</i>	Tpm3.12
Δ E224	Severe stiff myopathy	<i>TPM3</i>	Tpm3.12

Dilated Cardiomyopathy (DCM), Hypertrophic cardiomyopathy (HCM), Congenital Fibre type Disproportion (CTFD). Isoform description according to Geeves et al, 2014

2.2 Protein preparation

2.2.1 Purification of myosin from rabbit muscle

Heavy Meromyosin (HMM) is a crucial motor protein for the IVMA. HMM is the soluble part of myosin thus myosin was firstly isolated from rabbit by Mr O'Neal Copeland as by the method of Margossian and Lowey (Margossian and Lowey, 1982).

Immediately after the rabbit was sacrificed, the back muscle was taken and cooled down on ice for one hour. The muscle was then skinned and diced before being minced two times in a pre-cooled meat grinder. The minced muscle was then weighed, on average around 300g of minced muscle was obtained from one rabbit. Myosin was extracted from the minced muscle in three volumes of myosin extraction buffer (300 mM KCl, 150 mM potassium phosphate (48 mM K_2HPO_4 / 102 mM KH_2PO_4), 20 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP and 5 mM DTT pH 6.5 and protease inhibitors: E-64, chymostatin, leupeptin & pepstatin A) for exactly 10 minutes with continual stirring. The extract was centrifuged at 25,000 x g (13,000 rpm, Sorvall Evolution RC, SLA-1500 rotor) for 5 minutes. The pellet was saved for F-actin preparation (see section 2.2.3). The supernatant was filtered through glass wool and diluted gradually with 12 volumes of cold distilled H_2O . Myosin was allowed to precipitate overnight with no agitation. The next day, myosin was pelleted by centrifugation at 13,000 rpm (Sorvall Evolution RC, SLA-3000 rotor) for 6 minutes. The resulting pellet was resuspended with 3 M KCl at the ratio of 220 ml per kg of starting muscle. Once dissolved, the myosin solution was dialysed overnight (600 mM KCl, 25 mM potassium phosphate (8 mM K_2HPO_4 / 17 mM KH_2PO_4), 10 mM EDTA and 2 mM DTT, pH 6.5 and protease inhibitors: E-64, chymostatin, leupeptin & pepstatin A).

The dialysate was gradually diluted two-fold with cold distilled H_2O , stirred for 30 minutes and then centrifuged at 50,000 x g (20,000 rpm Sorvall Evolution RC SS-34 rotor) for 1 hour. This step was designed to remove any actomyosin still present. The resulting supernatant was diluted eight-fold with cold distilled H_2O and left for 1 hour to allow the myosin to precipitate. The myosin was then pelleted by centrifugation at 25,000 x g (13,000 rpm, Sorvall Evolution RC, SLA-1500 rotor) for 10 minutes. This pellet was resuspended in 1/4 volume of 4 M KCl. Finally the dissolved myosin was

centrifuged at 45,000 rpm (Sorvall Discovery 90SE, T-865 rotor) for 1 hour. After which the final product was preserved by dropping 100 μ l aliquots directly into liquid nitrogen to form myosin pellets, which were then stored in liquid nitrogen for future use. The concentration of the myosin was determined by Lowry assay (see Section 2.3.1.2) this was usually in the range of 30- 60 mg/ml.

2.2.2 Preparation of Heavy meromyosin (HMM)

HMM was prepared from the myosin pellets stored in liquid nitrogen. The preparation of myosin is based on the protocol of Okamoto (Okamoto and Sekine, 1985, Frazer and Marston, 1995). Two pellets of myosin per HMM sample were removed from liquid nitrogen and were put in an Eppendorf tube to thaw on ice. This was then diluted in HA buffer (0.1 mM NaCO₃, 0.1 mM EGTA, 2 mM DTT, pH 7.4) to a final concentration of 1.9 mg/ml, mixed thoroughly and left on ice for 20 minutes. The myosin was centrifuged for ten minutes at 21,100 x g (14,800 rpm, Thermo Scientific Heraeus Fresco 21) at 4°C. The supernatant was discarded and the pellet was carefully mixed with 100 μ l Buffer HB (20 mM imidazole, 1 M KCl, 4 mM MgCl₂, 2 mM DTT, pH 7 measured at 25°C) and left on ice for 10 minutes. The total volume was then made up to 200 μ l with Buffer HA and incubated at 25°C for 10 minutes in an Eppendorf thermomixer. The myosin was then digested with 7.4 μ l of 4 mg/ml TLCK α chymotrypsin (Sigma type VII, C-3142, UK) and incubated for 8 minutes at 25°C. The digestion was stopped by adding the myosin to STOP solution (27000 μ l Buffer HA with 3 mM MgCl₂ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and leaving it on ice for one hour. It was then centrifuged at 33,7000 x g (100,000 rpm, Sorvall MC-120 SE, S100-AT6 rotor) for 20 minutes at 4°C. The supernatant contained pure HMM, the concentration of which was determined by the Bradford assay (see Section 2.3.1.1). The usual concentration range of HMM obtained was 0.3-0.7 mg/ml. HMM was stored at 4°C to be used for the IVMA and remained viable for 5 days.

2.2.3 Preparation of skeletal F-actin.

2.2.3.1 Acetone powder preparation from skeletal muscle

The preparation of F-actin from acetone powder is based on the method of Straub (Straub, 1942). The acetone powder preparation has the same starting material as the

myosin preparation from the rabbit muscle (see Section 2.2.1). Acetone powder was made by washing the myosin pellet obtained from the first centrifugation step of the myosin preparation (see Section 2.2.1) before being dried to form the acetone powder. The preparation of the acetone powder was carried out by Mr O'Neal Copeland. In each step, the muscle residue was dispersed by manual homogenising and then filtered through a nylon mesh and squeezed to drain liquid. The pellet was weighed and washed as follows:

1. 3 washes with 10 volumes of cold dH₂O for 5 minutes at 4°C
2. 1 wash with 4 volumes of 0.4 NaHCO₃ for 45 minutes at room temperature (stirring)
3. 3 washes with 2 volumes of 1 M Tris-HCl pH 8.5 for 5 minutes at 4°C
4. 5 washes with 4 volumes of acetone for 10 minutes at 4°C

After the final acetone wash, the pellet was left to dry to form powder in the fume hood overnight. The following day it was stored at -20°C for future use.

2.2.3.2 Preparation of F-actin from acetone powder

F-actin was prepared from the acetone powder based on the method of Spudich and Watt (Spudich and Watt, 1971). A sample of around 2.5 g of acetone powder (see Section 2.2.3.1) was removed from -20°C storage and was left to thaw on ice. The acetone powder was dissolved in 50 ml of G-actin Buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.1 mM CaCl₂, 1 mM NaN₃, 500 nM DTT, pH 8.0) and stirred for 30 minutes at 4°C. The solution was centrifuged at 15,000 x g (18,000 rpm, Sorvall Evolution RC, SS-34 rotor) for 20 minutes at 4°C. The pellet was resuspended in an additional 15 ml of G-actin Buffer and was centrifuged as before. The supernatants were combined and filtered through glass wool into a measuring cylinder. G-actin polymerization was initiated by the addition of 0.1 volume of 10 x KME buffer, (0.5 M KCl, 100 mM Tris-HCl, 25 mM MgCl₂, 10 mM EGTA, 10 mM NaN₃, pH 8.0). The solution was gently mixed by sealing the measuring cylinder with parafilmTM and inverted slowly several times. It was then incubated in a water bath at 30°C for 45 minutes. The KCl concentration was adjusted to 0.8 M by the addition of 0.34 volumes of 3 M KCl. This solution was mixed at room temperature for 20 minutes by intermittent inversion of the resealed measuring cylinder. The F-actin was pelleted by centrifugation at

146,000 x g (45,000 rpm Sorvall Discovery 90SE, T-865 rotor) for 1.5 hours at 4°C. The supernatant was discarded, the pellets rinsed in distilled H₂O and then dissolved in 12-13 ml of G-actin buffer. The dissolved actin was then resuspended before being dialysed against G-actin buffer for 48 hours. The actin was depolymerized due to the reduction in the concentration of KCl. The solution was centrifuged at 146,000 x g (45,000 rpm, Sorvall Discovery 90SE, T-865 rotor) for 1.5 hours at 4°C.

The G-actin supernatant was filtered through glass wool and loaded onto a 2.5 x 90cm Sephacryl S300 gel filtration column, which had previously been equilibrated with 1.5 L of G-actin buffer. It was run overnight at a flow rate of 50 ml/hour and fractions were collected every 6 minutes in a fraction collector (Frac 920, GE Healthcare, UK). A single peak was recorded on Pharmacia U.V. monitor II system. Fractions producing the peak were checked by measuring their optical density (absorbance) at 290 nm in a quartz cuvette. Any fractions that gave reading of higher than 0.35 were pooled together.

The G-actin was polymerized for the final time by the addition of 0.1 volumes of 10xKME buffer at 30°C for 45 minutes and centrifuged at 146,000 x g (45,000 rpm, Sorvall Discovery 90SE, T-865 rotor) for 1.5 hours at 4°C as before. The supernatant was discarded and the F-actin pellets were dissolved in ATPase buffer (100 mM KCl, 5 mM PIPES-K₂, 2.5 mM MgCl₂, 1 mM DTT, 10 mM NaN₃, pH 7.3) at around 10 mg/ml. This was stored for up to one month at 4°C for experimental use. For later experiments, F-actin was stored as 100 µl aliquots to avoid cross-contamination.

2.2.3.3 F-actin preparation from mouse heart

F-actin was isolated from WT, E361G or E99K transgenic mice at 2-6 months of age (See section 2.1.2). The method of purification of actin from the mouse hearts was developed in the lab by Dr Emma Dyer (Song et al., 2010). One heart (50-100 mg) was pulverised and homogenised with 10 x volume of wash buffer (3.9 mM NaH₂PO₄, 0.1 mM Na₂HPO₄, 0.1 M NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 5 mM DTT and 2 mg/ml protease inhibitors chymostatin, leupeptin and pepstatin, pH 7.0) in an electric homogeniser. The samples were spun at 16,662 x g (13,400 rpm, Thermo Scientific Heraeus Fresco 21) for 3 minutes at 4°C. The wash step was repeated four times; 2 times with Triton x 100 (Sigma), 2 without. The supernatant was discarded, the pellet was extracted twice in 1.5 volume of thin filament extraction buffer (5 mM

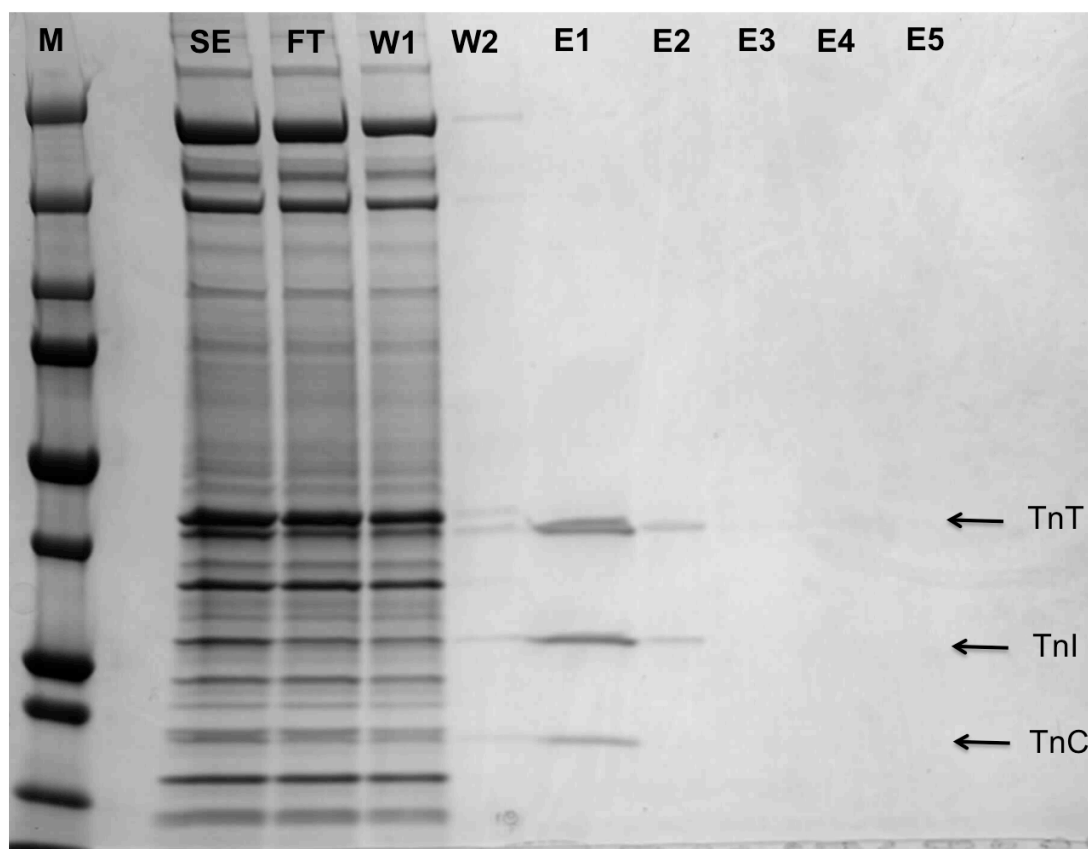
ATP and 1.5 mM blebbistatin in wash buffer) and spun at 21,000 x g (13,400 rpm, Thermo Scientific Heraeus Fresco 21). The supernatants were combined and spun at 446,800 x g (100,000 rpm, Sorvall RCM-120 SE, RCM-120 rotor) for 25 minutes at 4°C. Thin filaments are sedimented and the pellets containing thin filaments were resuspended 3 times in 50 µl “actin resuspension buffer” (2.6 mM NaH₂PO₄, 17.4 mM Na₂HPO₄, 0.1 M NaCl, 5 mM MgCl₂, 5 mM DTT, 1 mM ATP, 3 mM blebbistatin, pH 6) and dialysed in the same buffer for 2 hours at 4°C. The samples were then spun at 16,662 x g (13,000 rpm, Thermo Scientific Heraeus Fresco) at 4°C for 2 minutes. 3 M KCl was added to the supernatant to a concentration of 0.8 M to dissociate actin from troponin-tropomyosin. 150 nM TRITC-phalloidin (Sigma, UK) was added to keep actin polymerised and stable. The actin was then spun at 446,800 x g (100,000 rpm, Sorvall MC-120 SE, S100-AT6 rotor) for 25 minutes at 25°C and the pellet containing the F-actin was resuspended in 50 µl of ACEX buffer (2 mM Tris-HCl, 0.2 mM CaCl₂, 0.2 mM ATP, 1 mM DTT, pH 8.0) overnight at 4°C. The actin was then labelled with TRITC-phalloidin and remained functional for IVMA for 10-15 days. (See Section 2.5.2).

2.2.4 Purification of the troponin complex

Troponin was purified from human and mouse heart (See section 2.1.1 and 2.1.2) by a method developed by Messer (Messer et al., 2007). The sample was removed from liquid nitrogen. One mouse heart (50-100 mg) or 100-200 mg of human heart was pulverised in a percussion motor, which was cooled with liquid nitrogen. All steps of troponin preparation were performed at 4°C to ensure stability of the protein. The tissue sample was washed by homogenisation with 1.5 ml of wash buffer (5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1 M NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 0.1% Triton X-100, 5 mM DTT, 2 mg/ml of the protease inhibitors chymostatin, leupeptin and pepstatin A, pH 7.0) using a micropestle. The sample was then centrifuged at 21,000 x g (14,800 rpm Thermo Scientific Heraeus Fresco 21) for 3 minutes at 4° C. The supernatant was discarded and the pellet was further washed three more times with the same procedure. After the last washing step, the supernatant was discarded and the pellet was homogenized with 5 x volume of extraction buffer (0.7 M LiCl, 25 mM Tris, 5 mM EGTA, 0.1 mM CaCl₂, 5 mM DTT, 2 mg/ml of each protease inhibitor, pH 7.5) to extract the myofilament proteins from the tissue. The sample was then

mixed in a rotating wheel for 25 minutes at 4° C and afterwards was spun at 21,000 x g (14,800 rpm, Thermo Scientific Heraeus Fresco 21) for three minutes at 4°C. The supernatant was kept at 4°C and the pellet underwent three more extractions steps for 20, 15 and 10 minutes respectively. All the supernatants from the extraction steps were kept and pooled together and centrifuged at 337,000 x g (100,000 rpm, Sorvall RCM-120 SE, S100-AT6 rotor) for 20 minutes at 4°C. The supernatant (spun extract) contained soluble myofilament proteins was kept at 4°C. Troponin was purified from the spun extract using affinity column chromatography consisting of 0.6 ml of CNBr Sepharose CL-4B conjugated with a monoclonal anti-troponin I antibody (14G5) gift from N. Gusev and A. Katrukha (Katrukha et al., 1995). The matrix was equilibrated in 4.5 ml of troponin extraction buffer prior to the addition of spun extract. The spun extract was added to the sepharose column and incubated for 15 minutes at 4° C on the rotating wheel to ensure that the troponin is bound to the antibody of the column. Eight 2 ml tubes were setup and labelled: 1 flow through (FT), 2 wash (W1 and W2), and 5 containing the elution fractions (E1-E5). After incubation, the column containing the spun extract was placed in the FT tube, and the soluble proteins such as actin, tropomyosin and myosin were run through. The column was then washed two times with 0.8 ml extraction buffer (tubes W1 and W2). Finally the troponin was eluted with 0.6 ml elution buffer (0.1 M glycine, 5 mM DTT and 2 mg/ml protease inhibitors, pH 2) in each elution tube (E1-E5). To neutralize the low pH of the elution buffer 100 µl of 1 M MOPS, pH 8 was added in each of the elution fractions during preparation of the tubes. A sample was taken from each step and run on an SDS PAGE gel (see 2.3.2.) to determine in which fraction the troponin was eluted (see Figure 2-1). The troponin was usually eluted in fraction E1 and some times in fraction E2. The fraction in which troponin was eluted was therefore dialysed in small dialysis tubing (BioDesignDilaysis Tubing D001, Fisher Scientific, UK) against dialysis buffer (1 M KCl, 10 mM MES, 0.1 mM CaCl₂, 5 mM DTT, 2 mg/ml protease inhibitors, pH 6) overnight at 4°C with gentle stirring. The next day, troponin was centrifuged at 21,100 x g (18,400 rpm Thermo Scientific Heraeus Fresco 21) for 5 minutes at 4°C to remove any insoluble material. The supernatant was kept and stored at 4°C for use with the IVMA. The final concentration was measured using the Lowry assay (see Section 2.3.1.2). The usual concentration obtained was 4-10 µM and the troponin remained stable for three days after extraction.

Figure 2-1: Troponin extraction steps



Different troponin extraction steps are represented in each lane. M molecular weight marker, SE spun extract, FT flow through, W1 wash 1, W2 wash 2, E1 elution 1, E2 elution 2, E3 elution 3, E4 elution 4, E5 elution 5. TnI troponin I, TnT troponin T, TnC troponin C.

2.2.5 Further manipulation of the troponin complex

2.2.5.1 Phosphorylation of Human Cardiac Troponin

For the purpose of the thesis, it was desirable to increase the phosphorylation levels of the mutant K280N and K36Q produced by the exchange method (see Section 2.2.5.3). Mutant troponin was taken after column elution (see Section 2.2.4) and was dialysed against “phosphorylation buffer P” (0.5 M KCl, 50 mM MOPS, 20 mM NaF, 1 mM DTT, 10 mM MgCl₂, pH 7.0) for four hours at 4°C. The PKA catalytic subunit from bovine pancreas (Sigma, UK, P2645) was prepared to 200 units/ml solution in “phosphorylation buffer A” (same as “phosphorylation buffer P” without KCl). 300 µl of troponin (4-10 µM) was mixed together with 6.75 µl of Mg²⁺-ATP (100 mM), 24 µl of Protein Kinase A (PKA) (200 units/ml) and 6.75 µl of “phosphorylation buffer

P” in a 1.5 ml Eppendorf tube. The mixture was left to incubate overnight at 4°C on the rotating wheel. The next day, it was centrifuged at 21,000 x g (14,800 rpm, Thermo Scientific Heraeus Fresco 21) for 5 minutes at 4°C to remove any insoluble material. The supernatant contained phosphorylated troponin and was stored at 4°C.

2.2.5.2 Dephosphorylation of Human Cardiac Troponin

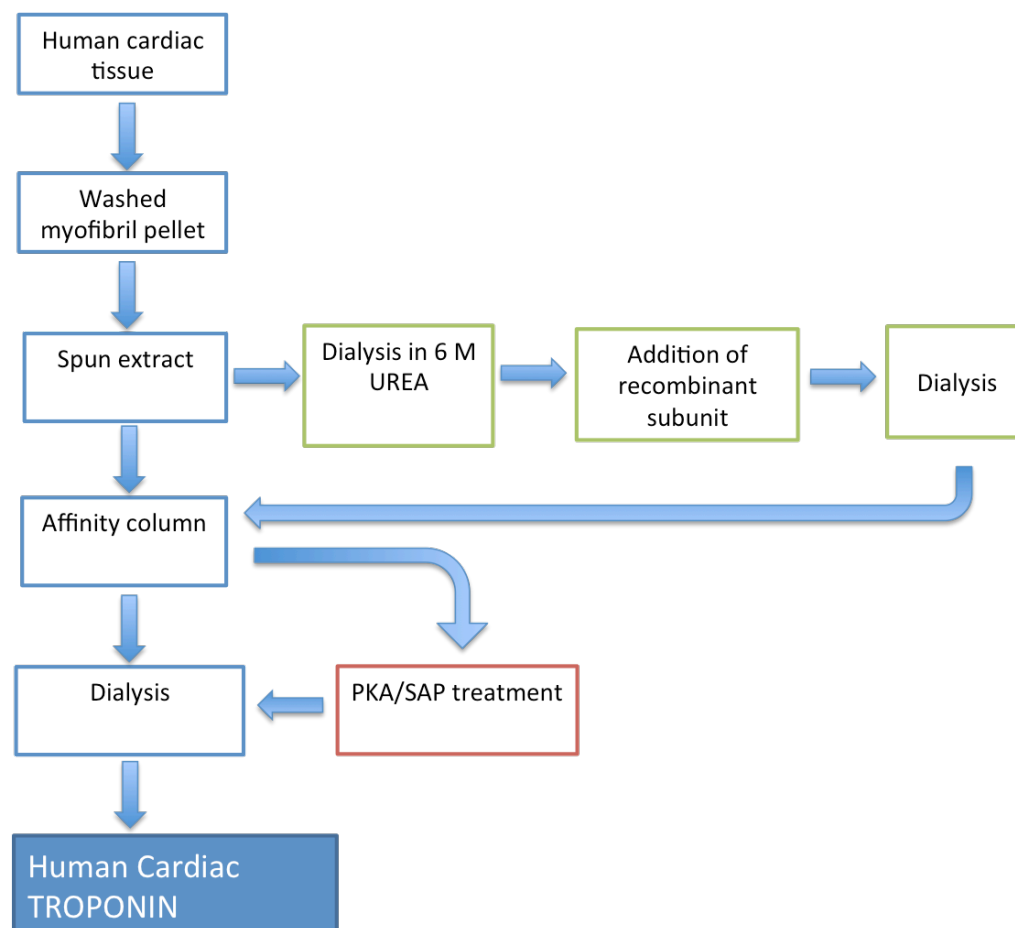
All the donor samples have been shown to have high phosphorylation levels so for the purpose of this study it was needed to completely dephosphorylate the troponin complex. TnI was dephosphorylated by incubating troponin with 90 U/ml Shrimp Alkaline Phosphatase (SAP) (Sigma, P9088, UK) after the column elution (see Section 2.2.4). The reaction mixture was then dialysed overnight with dialysis buffer at 4°C. The next day the sample was centrifuged at 21,000 x g (14,800 rpm, Thermo Scientific Heraeus Fresco 21) for 5 minutes at 4°C.

2.2.5.3 Exchange of native troponin subunits with recombinant TnI or TnT

Mutant troponin K820N and K36Q were produced by exchanging the native TnI from donor hearts with mutant recombinant TnI subunit (in the case of K36Q) or TnT with mutant recombinant TnT (in the case of K280N). All the recombinant subunits were provided by Dr Charles Redwood, University of Oxford, Oxford, UK. The exchange process was based on the troponin extraction protocol (see Section 2.2.4) with a further step as described by Bayliss et al (Bayliss et al., 2012b). After obtaining the “spun extract” from high speed centrifugation, the “spun extract” was dialysed in “denaturing buffer” (6 M Urea, 1 M KCl, 10 mM MES, 0.1 mM CaCl₂, 5 mM DTT, 2 mg/ml protease inhibitors, pH 6) for 6 hours at 4°C to denature the protein complexes instead of loading it onto the affinity column. A 10 fold excess of the mutant recombinant TnT or TnI subunit was added to the sample and the mix then was dialysed overnight at 4°C in dialysis buffer. The next day, the sample was centrifuged at 21,000 x g (14,800, Thermo Scientific Heraeus Fresco 21) for 20 minutes at 4°C. The samples were loaded into the affinity column and troponin was eluted. Different antibody columns were used dependent on the subunit exchanged. For example, when TnI was exchanged, an anti-TnT column was used (7F4, sepharose column donated by N. Gusev) whereas when TnT was exchanged an anti-TnI column was used (14G5). The fraction that contained troponin was dialysed in dialysis buffer at 4°C for

at least 4 hours. Finally the sample was centrifuged at $21,000 \times g$ (14,800 rpm, Thermo Scientific Heraeus Fresco 21) for 5 minutes at 4°C . These exchanged troponins had a relatively short functional life and they remained viable for 2 days. A diagram for preparation of troponin (exchanged and native) is shown in Figure 2-2.

Figure 2-2: Troponin extraction steps



A diagram of the basic troponin extraction steps is shown in blue. The method for the subunit exchange is shown in green and the phosphorylation/dephosphorylation in red.

2.3 Analysis of proteins

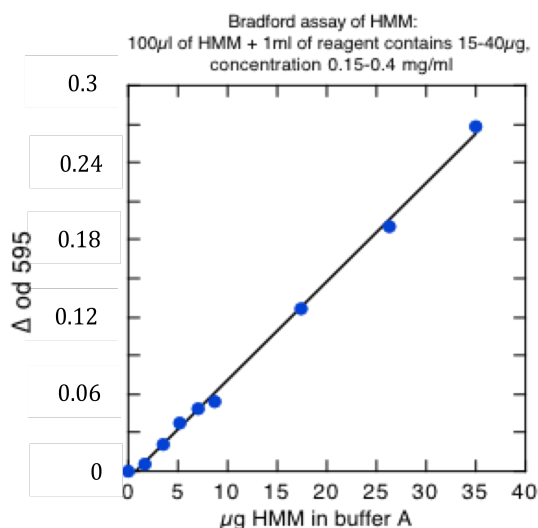
2.3.1 Determination of protein concentration

2.3.1.1 Bradford assay

The Bradford assay was used to determine the concentration of the HMM preparation (Bradford, 1976). 100 μl of HMM was mixed thoroughly with 1 ml Bradford reagent (Sigma, B6916, UK) and was left to incubate at room temperature for 5 minutes. The absorbance of the solution was measured at 595 nm (Ultrospec III, Pharmacia LKB).

The concentration of each HMM sample was calculated using a previously established calibration curve (see Figure 2-3).

Figure 2-3: The calibration curve for the Bradford assay



The calibration curve for Bradford assay that was calculated using skeletal HMM.

2.3.1.2 The Lowry assay

In order to determine the concentration of actin, tropomyosin and troponin the Lowry assay was used (Lowry et al., 1951). Lowry solution A was prepared which consisted of 48 parts of 2% Na_2CO_3 , 1 part of 0.5 CuSO_4 , 1 part of 1% NaK titrate. 5, 10 and 15 ml of the protein were made up to 500 μ l of NaOH, to which 5 ml of solution A was added. The mixture was vortexed and incubated at room temperature for 10 minutes. Then, 500 μ l of Lowry solution B (1 part Folin's solution reagent (VWR) to 1 part of water) was added. The solution was vortexed thoroughly and was left to incubate for 30 minutes at room temperature. Finally the absorbance was measured at 595 nm and the protein concentration was calculated using a previously established calibration curve.

2.3.2 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.3.2.1 Sample preparation

Protein solutions were diluted 1:1 (v/v) with 2 x SDS sample buffer (125 mM Tris-HCl, 10% SDS, 130 mM DTT, 0.05 bromophenol blue, pH 6.8). The samples were heated at 98°C for five minutes.

2.3.2.2 Criterion TGX gels

Two types of precast SDS gels were used in this study: Criterion TGX gel and Mini Protean TGX gels. Gradient Criterion TGX 4-15% (Biorad Laboratories, US) was run at 200 V for 40 minutes in standard Laemmli running buffer (25 mM Tris-Base, 192 mM Glycine, 0.1% SDS) (Laemmli, 1970)

2.3.2.3 Mini-Protean TGX gels

Mini-Protean TGX 4-15% (Biorad Laboratories, US) were run at 200 V for 30 minutes in standard Laemmli running buffer (25 mM Tris-Base, 192 mM Glycine, 0.1% SDS) (Laemmli, 1970).

2.3.2.4 Phosphate affinity SDS-PAGE

Phosphate affinity SDS-PAGE is a technique used to separate proteins according to their phosphorylation status (Kinoshita et al., 2006). This technique has been used to investigate the phosphorylation of cardiac TnI in human heart by Messer et al (Messer et al., 2009) . 50 μ M of Phos-tag conjugated moieties (NARD Institute, Hyogo, Japan) were added into the resolving gel of the polyacrylamide gel. The polyacrylamide gel consisted of 10% resolving (including Phos-tag) and 4% stacker polyacrylamide gel. The gel was run in 100 V for 90 minutes and then a Western blot was performed (See section 2.3.4)

2.3.3 Protein staining

2.3.3.1 Protein staining with Coomassie blue

Coomassie blue staining solution (800 ml) was made up by Mr O'Neal Copeland from 2 PhastGelTMBLue R tablets (GE Healthcare, 17-0518-01) dissolved in 60% methanol for 30 minutes and then diluted 1:1 with 20% acetic acid. Upon completion of electrophoresis the gel was immersed in fixing solution (50% methanol, 10% acetic acid) for 5 minutes with agitation at room temperature. The gel was then immersed in ~100 ml Coomassie Blue staining solution for 15 minutes with agitation at room temperature. The gel was then destained by immersion in 'destain solution' (25% methanol, 8% acetic acid) with agitation for 30 minutes or until satisfactorily destained at room temperature.

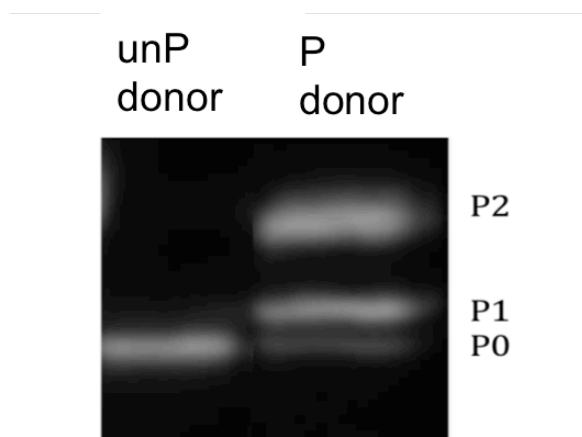
2.3.4 Western Blot

Western blot was performed to measure the phosphorylation status of TnI by blotting the Phosphate affinity SDS-PAGE (See section 2.3.2.4) and incubating it using a primary antibody against TnI. Proteins were separated using Phosphate affinity SDS PAGE (see Section 2.3.2.4) and then they were transferred to a Nitrocellulose Transfer membrane (Trans-Blot, 0.2 μ m Nitrocellulose, Bio-Rad, UK) using the Trans-Blot Turbo (Bio-Rad, UK) system for 7 minutes at 25 V, 1.3 mA. The membrane was then blocked in 10 ml of 20% Sea-block blocking buffer (Thermo Scientific, UK) in PBS-Tween (PBS, 0.1% Tween-20) for one hour at room temperature with agitation to prevent non-specific binding of the antibody to the membrane. It was then probed with the primary antibody, a cardiac TnI specific antibody (P4-14G5, Abcam, UK) that was diluted in 10 ml 20% Sea-block in PBS-Tween (1:1000). The membrane was incubated with the antibody overnight at 4° C with agitation.

The following day the membrane was briefly rinsed 3 times and then washed 3 times for 5 minutes agitating with PBS-Tween. The secondary antibody (anti- mouse IgG, horseradish peroxidase linked whole antibody, GE Healthcare, UK) was diluted in 10 ml 20% Sea-block in PBS-Tween (1:10,000). The membrane was incubated with the secondary antibody for one hour at room temperature with agitation. It was then rinsed 3 times and then washed 3 times for 5 minutes agitating with PBS-Tween. The

proteins were visualised using ECL Prime Western Blotting Detection reagent (GE Healthcare, UK) and the chemiluminescence was detected by a camera based gel imager (G:BOX Chemi XT16, Syngene) (Figure 2-4).

Figure 2-4: Phosphate affinity SDS PAGE showing phosphorylated and dephosphorylated troponin

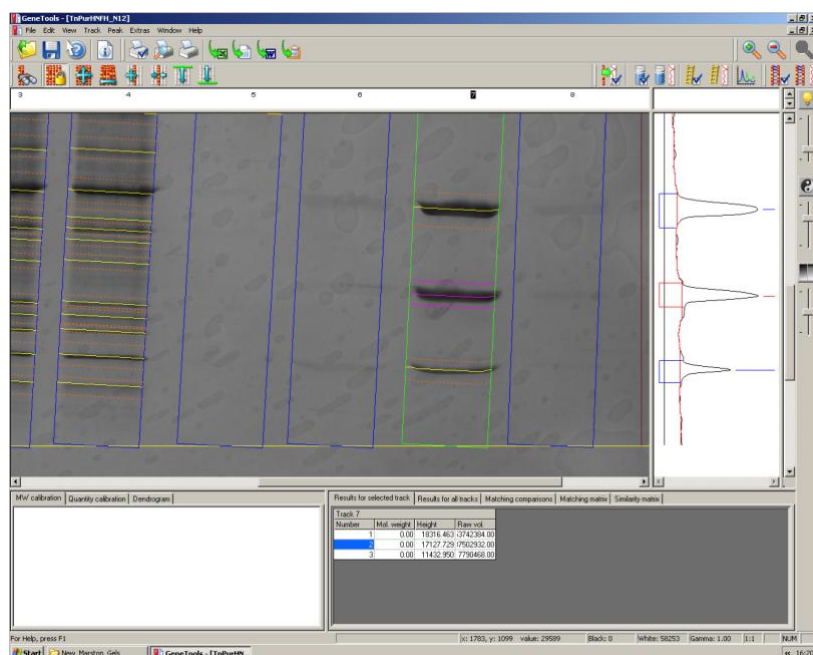


Representative western blot of phosphate affinity SDS-PAGE showing phosphorylated and dephosphorylated troponin extracted from donor sample. On the left the dephosphorylated troponin is shown (treated with shrimp alkaline phosphatase) and phosphorylated troponin is shown on the right with the three different phosphorylation levels (P2, P1 and P0).

2.3.4.1 Densitometric Analysis

Digital images of gels and Western blots were visualised with a CCD camera based gel imager (G:BOX Chemi XT16, Syngene) and GeneSnap (version 7.05, Synoptics Ltd) software. Exposure time for gels stained with Coomassie Blue was 80 milliseconds and for Western blots it was 10 and 30 seconds, then 1, 2 and 5 minutes. Protein bands were analysed by scanning densitometry using GeneTools software (version 4.00, Synoptics Ltd, Figure 2-5) (Messer et al., 2009, Copeland et al., 2010).

Figure 2-5: Densitometric analysis



Analysis of the troponin complex stained in Coomassie blue is shown. The analysis of the three protein bands is shown on the bottom.

2.4 Sources of drugs and compounds

2.4.1 Ca^{2+} sensitisers

EMD 57033 was kindly donated by E Merck KGaA, Darmstadt. Bepridil was purchased from Sigma (Bepridil Hydrochloride, Sigma B5016, UK). Both were diluted in motility buffer A, and for later experiments in DMSO (3 mM stock for EMD57033, 10 mM stock for Bepridil) and stored at -20°C .

2.4.2 Ca^{2+} desensitisers

Epigallocatechin-3-gallate (EGCG) (Minimum 95%, E4143, Sigma, UK) and Silybin (Minimum 98%, S0417, Sigma, UK) were dissolved in DMSO (10 mM) and stored at -20°C .

2.4.3 EGCG analogue compounds

EGCG and Silybin-like compounds originally developed as HSP90 inhibitors were kindly donated by Dr Brian Blagg, University of Kansas (Khandelwal et al., 2013a,

Zhao et al., 2011). Their details are summarised in Table 2-3. The compounds were diluted in DMSO to make 10 mM stock and then stored in -20° C for use.

Table 2-3: EGCG and Silybin analogues

Vial No	Molecular weight	Quantity
1	482.49	7.9 mg
2	406.43	4.0 mg
3	314.34	4.8 mg
4	300.31	6.5 mg
5	394.42	3.0 mg
6	270.33	15.1 mg
7	344.36	5.7 mg
8	440.49	4.4 mg
9	300.31	2.8 mg
10	510.54	1.1 mg
11	420.46	4.7 mg
12	302.28	1.4 mg
13	328.37	5.6 mg
14	480.56	5.6 mg
15	606.67	12.1 mg
16	358.39	3.1 mg
17	316.31	2.6 mg
19	374.39	4.7 mg
20	393.39	7.1 mg
21	498.53	1.2 mg
22	367.41	3.9 mg
23	286.28	1.7 mg
24	304.34	4.5 mg
26	304.34	2.1 mg
27	515.61	13.7 mg
28	420.46	2.0 mg
S-1	480.43	N/A
S-2	482.44	5.7 mg

Table of the EGCG or Silybin analogue compounds donated by Dr Brian Blagg, showing their molecular weight and the quantity given. N/A: Not applicable (not given). Compounds 1-28 are EGCG analogues. S1 and S2 are Silybin analogues.

2.5 The *in vitro* motility assay (IVMA)

2.5.1 Buffers for IVMA

The main buffer for the *in vitro* motility assay (IVMA) is motility buffer A (50 mM KCl, 25 mM Imidazole, 4 mM MgCl₂, 1 mM EDTA, 5 mM DTT, pH 7.4). Apart from motility buffer A, motility buffer B, C and D are required for IVMA. Motility buffer B consists of 4 ml of motility buffer A and 12 µl of 22% BSA (Albumin from bovine serum, Ultra pure, Sigma, A3675, UK). Buffer C consisted of 850 µl of cold dH₂O, 52 µl catalase (26 mg/ml stock, Sigma, C3515, UK), 52 µl glucose oxidase (100 mg/ml stock, Sigma, G6641, UK), 40 µl glucose (stock 100 mg/ml), 6 µl of 22% BSA and 1 ml of 1% methylcellulose dissolved in 2 x motility buffer A. Buffer D was identical to buffer C with an additional 40 µl of 100 nM Mg²⁺-ATP.

2.5.2 Fluorescent labelling of F-actin

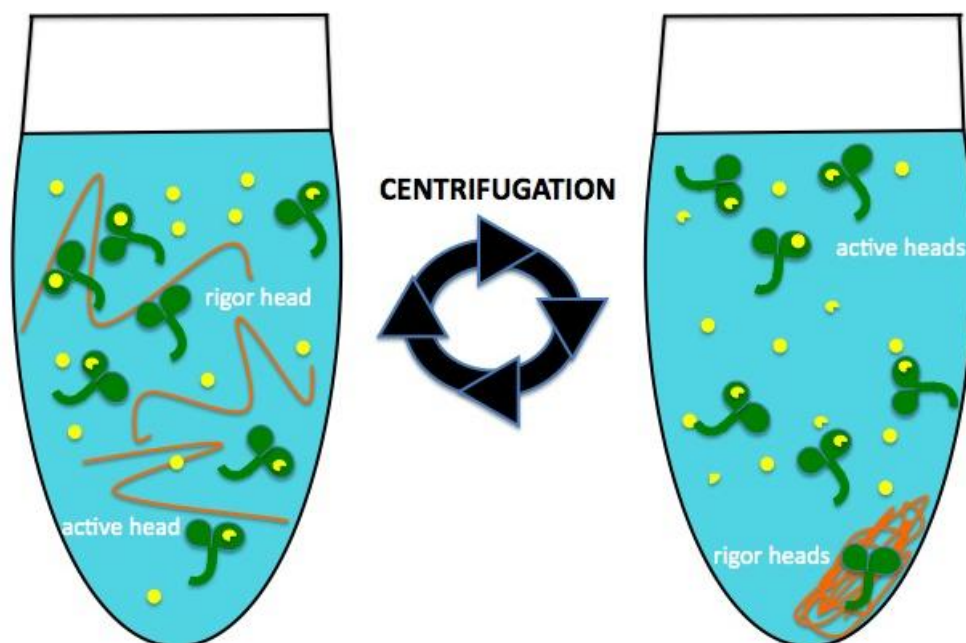
F-actin was labelled with TRITC-phalloidin to visualise the filaments. 36 µl of 33 mM TRITC-phalloidin was added in 438 µl of motility buffer A and mixed by vortexing. 20 µl of 1 mg/ml of F-actin was then carefully added to the solution, and was gently mixed. The tube was then wrapped in aluminium foil and incubated overnight at 4°C. Labelled actin (φ actin) was always stored at 4°C and was stable for 4 weeks.

2.5.3 Affinity precipitation of HMM

Affinity precipitation of HMM is required for the IVMA, in order to remove the “rigor heads” (Fraser and Marston, 1995a). These bind irreversibly to the myosin even in the absence of ATP and hence lead to a low fraction of filaments moving. To minimise the presence of “rigor heads”, HMM obtained from rabbit myosin (see Section 2.2.2) was treated with F-actin in the presence of ATP (Fraser and Marston, 1995). 200 mg/ml of HMM were mixed with 150 mg/ml of F-actin and 1 mM Mg²⁺-ATP in the presence of motility buffer A, total of 1,500 µl. The sample was incubated on ice for 10 minutes and then it was centrifuged at 337,000 x g (100,000 rpm, Sorvall MC-120SE, S100- AT6 rotor) for 20 minutes at 4°C to pellet F-actin with the associated rigor heads (Figure 2-6). The supernatant which represents the functional

HMM was collected and was stored at 4°C. The functional HMM was used within 6 hours.

Figure 2-6: Affinity precipitation of HMM



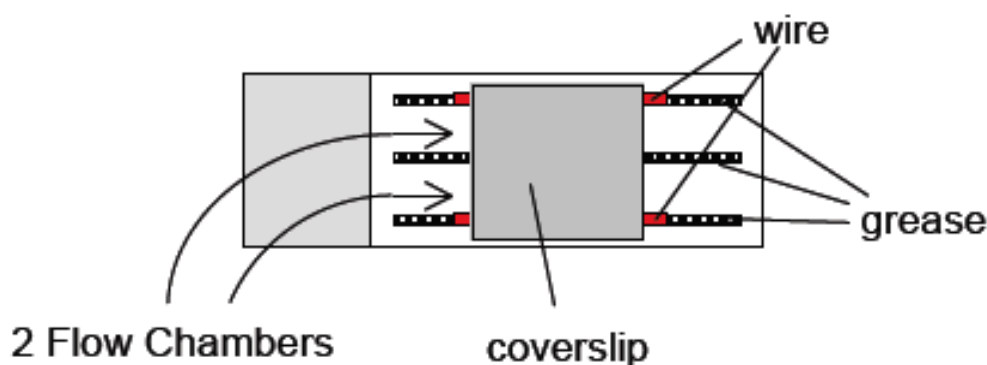
Here is shown the affinity precipitation of HMM. Full yellow dots represent ATP. $\frac{3}{4}$ of a yellow dot represent ADP+Pi. The orange lines represent the actin and myosin is shown in green.

2.5.4 Preparation of the flow cells

Flow cells were constructed using microscope slides (Sigma, S8400, UK) and coverslips (VWR, 630125, UK) that were put together using silicon grease (BDH, 6380 2B, UK). Each motility flow cell consisted of two flow chambers. Prior to the formation of the motility chambers, the coverslips were first siliconised by immersing for 30 seconds with agitation in a solution containing 40 ml of chloroform (Sigma, 496189) and 0.2 % dichloromethylsilane (Fluka, 39909, UK). The purpose of this process was to create clean coverslips with a hydrophobic surface to which HMM could adhere. Following the coverslip preparation, the microscope slides were placed with the frosted side up and were layered with three parallel longitudinal rows of silicon grease at the top, middle and bottom of the slide. 2-3 cm of copper wire was then placed on the top and bottom layer of grease. Finally, the siliconised coverslip

was placed on top of the slide, covering all the layers of the grease (Figure 2-7). The motility chambers were prepared at least one day before performing the IVMA experiment. All solutions and motility chambers were kept dry by storage in a desiccator cabinet (Nalgene, 5317-0120, UK).

Figure 2-7: Construction of the flow cells



The construction of a flow motility cell, consisting of two flow motility chambers.

2.5.5 Reconstitution of the thin filaments

Stock solutions of 2 μM of troponin and tropomyosin were made in motility buffer A and kept on ice. Thin filaments were constructed by adding together all the components (troponin, tropomyosin and actin) at 10 fold of the required concentration. Drugs dissolved in motility buffer A or DMSO (See Section 2.4) were also added in the mix at the appropriate concentration (Table 2-4). The ϕ actin was added lastly, and the solution was carefully mixed. The proteins ($\pm$ drug) were incubated at 4°C for 15 minutes before 10 fold dilution with motility buffer A and addition to the flow motility cell. Actin and actin/tropomyosin were also analysed by this method.

Table 2-4: 10 x thin filament preparation for the IVMA

	Volume/ μ l						
10 x Protein preparation Final Tn concentration 60 nM	Actin Only	Actin + Tm	Actin + Tm + Tn	Drug 25 μ M	Drug 50 μ M	Drug 100 μ M	Drug 200 μ M
1 μ M Actin (10 nM)	10	10	10	10	10	5	5
2 μ M Tropomyosin (60 nM)	0	30	30	30	30	15	15
2 μ M Troponin (60 nM)	0	0	30	30	30	15	15
Drug (stock at 10 μ M DMSO)	0	0	0	2.5	5	5	10
Buffer A	90	60	30	27.5	25	10	5

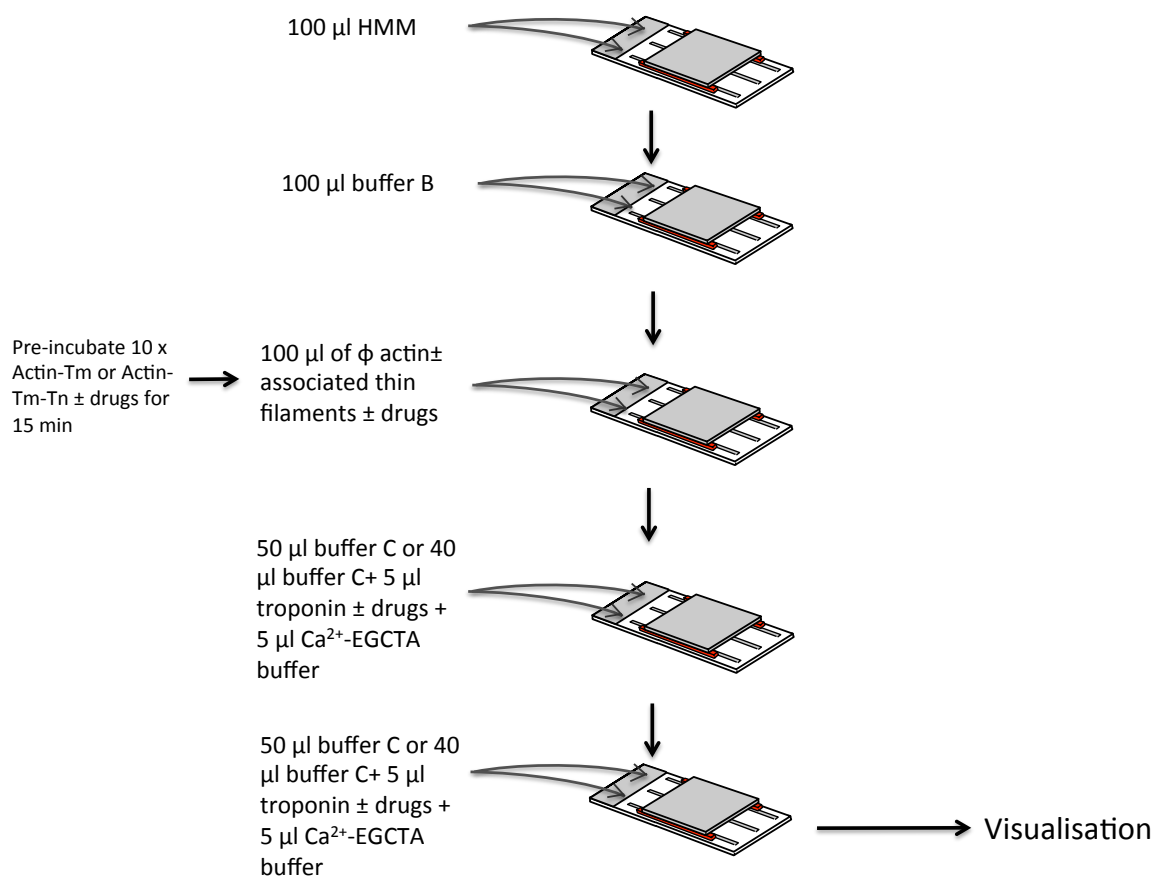
2.5.6 Loading of the flow motility cell

The flow cell was placed at an angle of 30° with the frosted side of the microscope slide facing upwards. Each of the components was added separately, in the order indicated, to the flow cell with a 30 second gap (see Figure 2-8). In each chamber, the same components were added apart from the parameter examined. Firstly, 100 μ l of the affinity precipitated HMM was added followed by 100 μ l of motility buffer B. 100 μ l of the reconstituted thin filaments were added (See section 2.5.5) and lastly 50 μ l of buffers C and D were loaded.

Buffers C and D contained the free radical scavenging system to prevent photobleaching and also methylcellulose to keep the filaments attached to the flow cell. BSA also was contained in buffers C and D to remove and minimise any non-specific binding of the proteins. If complete thin filaments were being examined then buffers C and D also contained Ca^{2+} -EGTA buffer (see Section 2.5.7) and troponin ($\pm$ drugs). The troponin and drug concentrations added in buffers C and D were the same as the concentration of 10 fold thin filament incubation (60 nM troponin for all the experimental setups, see Table 2-5). The incubation was diluted 10 fold by adding 5

μl of the incubation to 40 μl of buffers C or D. 5 μl of the EGTA- Ca^{2+} buffers were also added (see Table 2-6).

Figure 2-8: Flow motility assay protocol



This figure shows all the steps of the IVMA protocol including a small description of each step.

Table 2-5: 10 x troponin preparation for Buffers C and D

10 x Tn 60 nM Buffer for C and D Buffer Preparation	Volume/ μl				
	No Drug	Drug 25 μM	Drug 50 μM	Drug 100 μM	Drug 200 μM
1 μM Actin (10 nM)	15	15	15	15	15
Drug (stock at 10 μM DMSO)	0	1.25	2.5	5	10
Buffer A	35	32.5	25	30	25

2.5.7 Preparation of Ca^{2+} - EGTA buffers

Different Ca^{2+} concentrations were incorporated into the IVMA by the addition of Ca^{2+} -EGTA buffers into IVMA buffers C & D as previously stated. Ca^{2+} -EGTA buffers were prepared as described in the method of McGuigan et al (McGuigan et al., 1991). Buffers of different Ca^{2+} concentrations were made by mixing an EGTA buffer (50 mM KCl, 25 mM Imidazole pH 7.4, 50 mM EGTA, 1 mM EDTA, 4 mM MgCl_2 , 95 mM KOH, 1.43 mM HCl, 8mM NaCl, pH adjusted using concentrated HCl) and a Ca^{2+} -EGTA buffer (50 mM KCl, 25 mM Imidazole pH 7.4, 50 mM EGTA, 50 mM CaCl_2 , 1 mM EDTA, 4 mM MgCl_2 , 8 mM NaCl, pH adjusted using 1M KOH) at appropriate ratios to obtain Ca^{2+} -EGTA buffers ranging from 1 nM to 3.9 μM . The free Ca^{2+} concentration values were determined using the Maxchelator computer programme.

Table 2-6: The Ca^{2+} - EGTA Buffers

Solution Name	Volume of 50mM EGTA buffer added (ml)	Volume of 50mM Ca/EGTA buffer added (ml)	Ratio	pCa	Free [Ca^{2+}]
A	47	0.5	94/1	9.00	1.00×10^{-9}
C	40	4	10/1	8.14	7.24×10^{-9}
D	40	10	4/1	7.74	1.82×10^{-8}
E	30	15	2/1	7.43	3.72×10^{-8}
F	25	17.5	1/0.7	7.24	5.75×10^{-8}
G	25	25	1/1	7.13	7.41×10^{-8}
H	20	30	1/1.5	6.95	1.12×10^{-7}
I	15	30	1/2	6.84	1.45×10^{-7}
J	10	30	1/3	6.64	2.29×10^{-7}
K	10	40	1/4	6.52	3.02×10^{-7}
L	8	40	1/5	6.42	3.80×10^{-7}
M	7	42	1/6	6.34	4.57×10^{-7}
N	6	42	1/7	6.28	5.25×10^{-7}
P	5	40	1/8	6.23	5.89×10^{-7}
Q	5	45	1/9	6.18	6.61×10^{-7}
R	2	40	1/20	5.88	1.32×10^{-6}
S	1.5	45	1/30	5.75	1.79×10^{-6}
T	0	50	-1	5.43	3.9×10^{-6}

2.5.8 Experimental strategy

The IVMA was used to investigate the effects of different compounds on the Ca^{2+} regulation of donor and mutant reconstituted thin filaments. As mentioned previously, thin filaments were engineered using proteins of different origin.

The first step of the IVMA was to make sure that the assay was functioning properly. For this reason, plain actin was first loaded into the flow cell to confirm the presence of thin filaments and also their motility. The experiments were automatically tracked (see Section 2.5.10.1) and the motility parameters were analysed. The normal sliding speed of the filaments was 2.8–3 $\mu\text{m}/\text{sec}$ and the fraction motile was 70-90%. If these criteria were met, then we could proceed to the next step. On the occasion that this was not the case, this could have been because either the HMM, the flow cells, the ϕ actin or the buffers were faulty. To identify the faulty reagent, every component was replaced in turn. The next control experiment was to test actin with tropomyosin (A-Tm), using 60 nM as tropomyosin concentration. Usually, one motility chamber consisted of plain actin and the other one with actin-tropomyosin. A slight increase in both sliding speed and percentage motility was observed with the addition of tropomyosin compared with plain actin, possibly due to better co-operativity of the system. In the case of skeletal mutant tropomyosin experiments, the second flow cell consisted of actin with wild type tropomyosin (A-TmWT) on one motility chamber and actin with mutant tropomyosin (A-TmMut) on the other motility chamber. Actin and tropomyosin are not Ca^{2+} regulated proteins, thus 50 μl of buffer C and D (without troponin/ Ca^{2+} -EGTA buffers) was added as the last component on the flow cell.

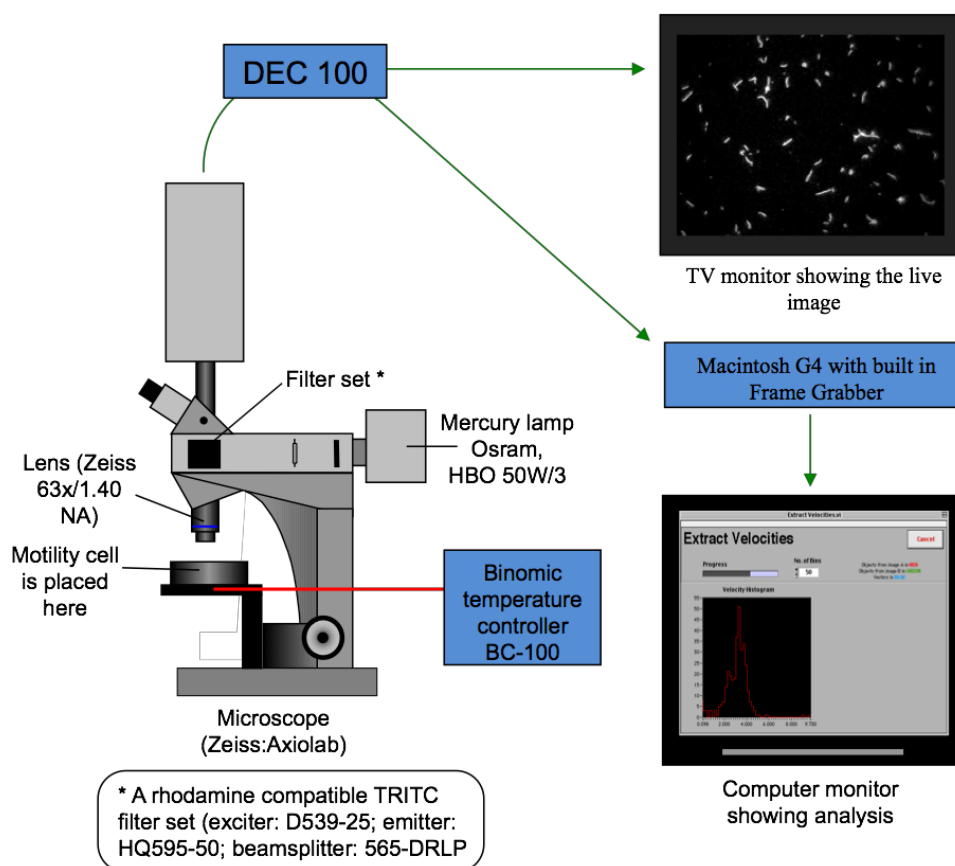
After all the controls were performed ensuring that the IVMA was functioning adequately, reconstituted thin filaments with actin, tropomyosin and troponin mixtures were used for the completion of the experiment. The troponin concentration used for the study was 60 nM. Two experimental designs were carried out in this study: constructions of Ca^{2+} activation curves, where filaments were compared over a range of Ca^{2+} concentrations, and dose response curves, where different concentrations of the drug were compared using a single Ca^{2+} concentration. In the case of Ca^{2+} activation curves, 4-8 points were selected between 1 nM- 3.9 μM Ca^{2+} and a Ca^{2+} concentration curve could then be plotted by fitting the Hill equation on the data obtained (see Section 2.5.10.2). For the dose response curves, a single Ca^{2+}

concentration was chosen according to the concentration that gave the biggest difference in the motile parameters on the Ca^{2+} curve. That was usually $0.0037 \mu\text{M}$ Ca^{2+} (Ca^{2+} - EGTA buffer E) or $0.0074 \mu\text{M}$ Ca^{2+} (Ca^{2+} - EGTA buffer G). More details about the dose response curves can be found in the appropriate chapters. The Hill equation was fitted to the data obtained. From the Hill equations, two important parameters can be obtained: The EC_{50} value, which is the value at which 50 % of the filaments were activated, and the Hill coefficient, which relates to the steepness of the fitted curve.

2.5.9 Recording Apparatus

The movement of thin filament over the immobilized bed of HMM was visualised by translocation of the fluorescently labelled actin. The microscope and recording apparatus was setup as illustrated in Figure 2-9.

Figure 2-9: Recording apparatus



Recording apparatus of the IVMA showing the microscope, the thin filaments on the TV monitor from the live image and the computer monitor showing the analysis of the motility of the filaments.

The flow cell was placed in the metal temperature control chamber on the stage of the Zeiss Axiolab microscope, with a Zeiss 63x / 1.40 NA Plan Apochromat objective lens (44 07 60- 03) fitted with an epifluorescence illuminator (Zeiss, 39 26 42-9901) containing a 50 W mercury high pressure arc lamp (Osram, HBO 50W/3) and a rhodamine compatible filter set (Chrome technology, emission: HQ 595-50, excitation: D539-25, dichroic: 565-DRLP special). The microscope was connected through a C-mount (45 61 05), to a highly sensitive video camera (Hamamatsu C2400), which in turn was linked through a Time Base Corrector (DEC100) to both a television monitor and a Macintosh G4. A drop of immersion oil, type A (Cargille laboratories, 16482) was placed on the coverslip of the flow cell and the image of the fluorescently labelled actin filaments were brought into focus on the television screen.

2.5.9.1 Temperature Control of the IVMA

The temperature was controlled using a Bionomic Controller BC-100 (20/20 Technology Inc.) This controller sets and maintains the temperature of a metal heating platform onto which a flow cell is placed. All IVMA experiments were completed at 28°C. To obtain this temperature in the motility chamber, the temperature was set to 30°C.

2.5.9.2 Analysis of Filament Movement

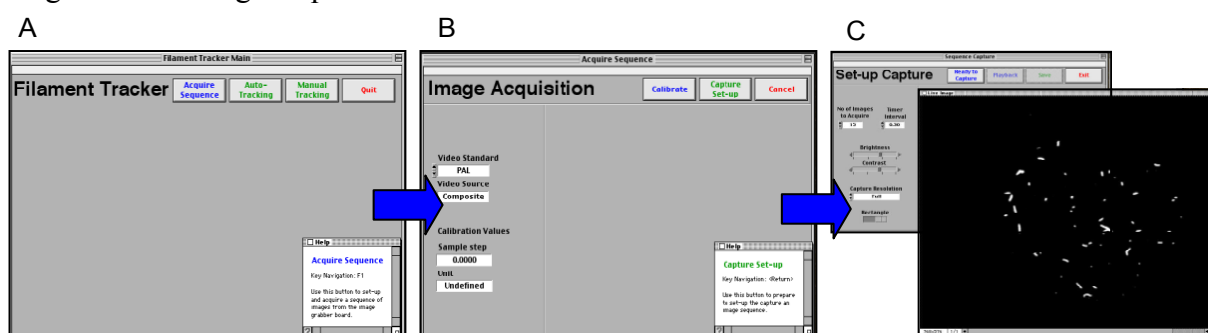
To complete quantitative analysis of filament movement, bespoke computer software had been designed and provided by ME Electronics (Reading, UK) for use on a Macintosh computer with Operating System 9 (Marston et al., 1996). The name of the software package was 'Filament Tracker 2.0.3'. This was run using LabVIEW-3.1.1 run-Time image analysis application (National Instruments Corp.). The software completes two tasks: the first was to digitise a series of filament images; the second was the image analysis function. This determines the movement of each filament by comparing sequential images.

2.5.9.3 Acquisition of a Sequence of Images

To record images using Filament Tracker 2.0.3 the following command sequence was followed (see Figure 2-10). The first step was to select the 'Acquire Sequence' in the tracking panel (Figure 2-10 A). Next the 'Capture Setup' was selected (Figure 2-10

B). This would open the ‘Capture Setup’ screen where the live image from the microscope could be viewed in the image window. Also displayed were four parameters that could be adjusted for optimal image acquisition, including ‘contrast’ and ‘brightness’, which along could be adjusted to improve the quality of the image. The number of images acquired and the time interval between image acquisitions could also be adjusted. The setting for the number of images acquired depended on the density of the filaments in the microscope field. The aim was to record 250-500 filament vectors, which usually meant 10-16 images were acquired (Marston et al., 1996)

Figure 2-10: Image acquisition



The process of how to acquire the IVMA data is shown, with the image of the filaments as a final step.

The time interval was dependent on filament sliding speed, and was set to allow filaments to move 1.6-1.8 μm between image acquisitions. The distance had previously been determined to facilitate the most accurate results from the tracker software (Marston et al., 1996). A calibration curve for filament velocity and frame interval is shown below (Figure 2-11). Once set, time intervals were kept constant during a set of measurements.

2.5.10 Analysis of Filament Movement

2.5.10.1 Automatic Filament Tracking

Image files of filament movement were analysed using the automatic-tracking segment of the tracker program. Auto tracking was selected from the main ‘Filament tracker’ panel, from there the desired set of images were loaded. In the ‘Pre-process’

panel filaments that fulfilled the set criteria were displayed and highlighted as green objects on the original black and white image. Selection of the 'Change Settings' command allowed the adjustment of the movement analysis parameters. The maximum velocity cut-off was set at 8 $\mu\text{m/s}$, which is 2.5x mean velocity based on prior experiments in this configuration (Bayliss, 2012). Finally, the selection sensitivity was set to discriminate objects from noise. When these settings had been optimised, the 'Extract Velocities' command was selected (Figure 2-11 A)

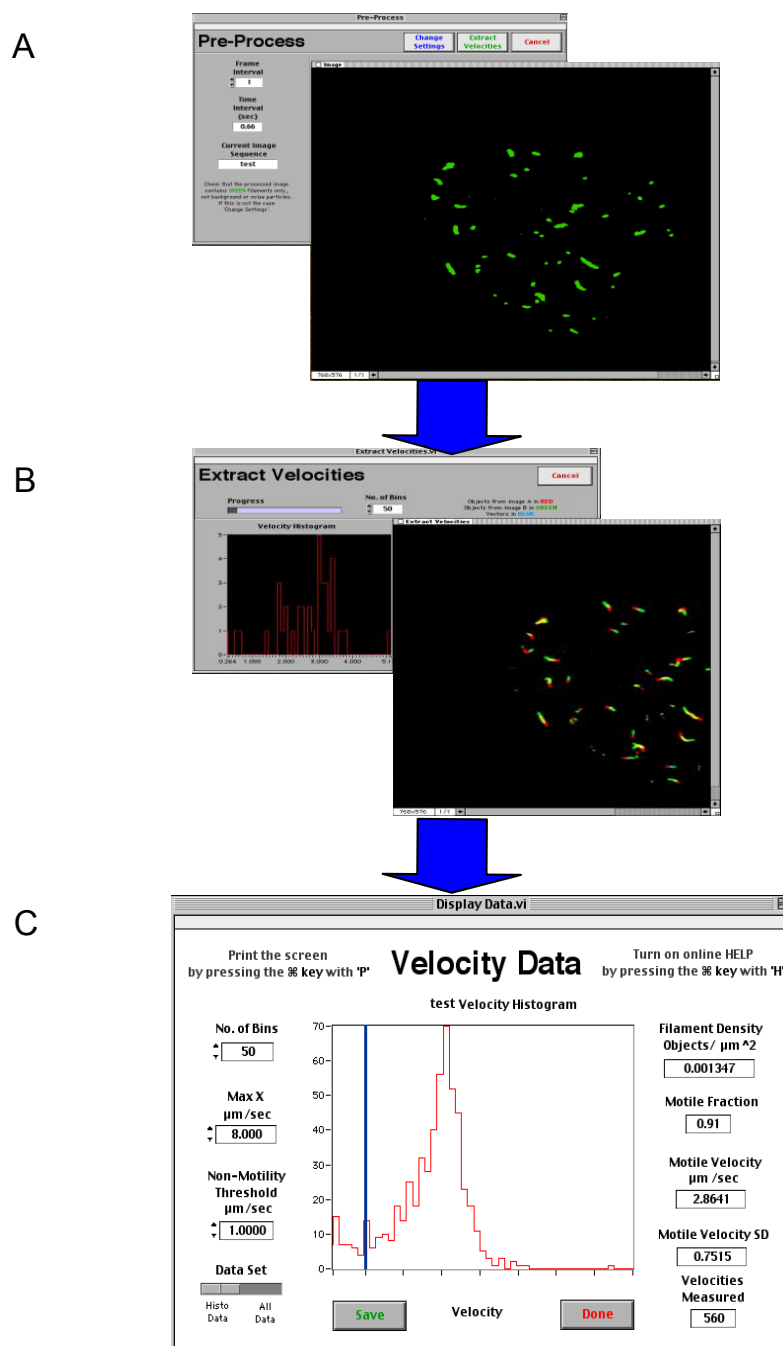
As the tracking progresses two images are superimposed over each other on a screen similar to the live image screen. In the first image the filaments are coloured green and in the second the filaments are coloured red (Figure 2-11 B). Therefore any filaments present in the same position in both images will appear yellow. The program searches for the centroid in the second image nearest the centroid in the first image for each object. We select short filaments and use low filament density to avoid tracking mistakes. White lines connecting two filaments represent the vectors of filament movement.

After sequential tracking of all of the images is complete, a histogram will appear (Figure 2-11 C). On the left hand side three parameters to further optimise tracking can be set: the number of bins (e.g. size of velocity channels), maximum velocity and the velocity, which defines the threshold between non-motile and motile filaments. This velocity is a visually selected value deemed to separate motile and non-motile fractions, since quantitative analysis has shown that curve-fitting produces the same result as direct observation (Marston et al., 1996). Even at low Ca^{2+} or ATP concentrations, not all the filaments will remain static. One weakness of the tracker program is that it cannot distinguish between linear movement and oscillatory movement around a fixed point. Some filaments may move with a Brownian motion and other filaments may 'wobble' if only in the 'weak' binding state with myosin. By reviewing the results from a set of thin filaments in a high Ca^{2+} concentration in the presence of ATP, a value for this threshold is selected. For consistency, this value must remain constant for the entire data set. This was normally set at 1 $\mu\text{m/s}$.

The calculated parameters: (filament density, motile fraction, motile velocity, standard deviation of velocities and velocities measures) are displayed on the right hand side. These values are manually recorded and linked to the experiment they represent. It was assumed the sliding speeds were distributed as a single Gaussian distribution, based on the initial setup of this system (Marston et al., 1996). Thus

mean values and standard deviation were calculated by the software. If the standard deviation was $> 0.4 \times$ mean sliding speed this data was discarded.

Figure 2-11: Automatic filament tracking



This figure shows the automatic tracking of the actin filaments. In A) filaments are shown in green. In B) filaments from the first image acquisition (green) are overlaid with filaments from the second image acquisition image acquisition (red); therefore, filaments that have not moved are yellow. In C) the histogram resulted from all the images is shown.

2.5.10.2 Construction of Ca^{2+} curves

To produce a Ca^{2+} titration curve, measurements were completed on the same incubation of thin filaments (actin-Tm-Tn) over a range of concentrations from 1 nM to 3.9 μM . For the dose response, a single Ca^{2+} concentration was used with different incubations of thin filaments (actin-Tm-Tn $\pm$ drugs).

In the case of Ca^{2+} titration curve, histograms from measurements at the different Ca^{2+} concentration were taken. As an example, in Figure 2-12 histograms taken in 8 different Ca^{2+} concentrations is shown. This demonstrates how the fraction of filaments moving and the sliding speed increases with the increase in Ca^{2+} concentration and how this is measured by the tracker software. In the case of the dose response curve, histograms were taken from measurements where the same Ca^{2+} concentration was used, but different incubations (concentration of the drug was the variable).

It should be noted that for the experiments in this thesis four measurements were completed at each point (at each Ca^{2+} or drug concentration); therefore there are four histograms per point from areas of the flow cell chosen at random. In order to eliminate the effect of time in the motility of the filaments, two images were recorded first from one chamber of the motility flow motility cell, then all four images were recorded from the second motility chamber and lastly the remaining two images were recorded from the first chamber, so that four images per chamber are recorded. Data from obviously defective areas (low or zero motility, usually occurred when bubbles were incorporated into the flow motility cell) were discarded. The mean fraction motile values from these measurements were then plotted and fitted with the 4-parameter Hill equation using KaleidaGraph (Synergy Software, version 4.5). In some cases m_1 and/or m_2 were fixed at reasonable values to obtain a 3 or 2-parameter fit. A sample of a Ca^{2+} activation curve and its fitted equation are shown in Figure 2-13. Two parameters were obtained by fitting the Hill equation to the data: the EC_{50} that was used as a measure of thin filament Ca^{2+} -sensitivity and the Hill co-efficient (n_H), which is an indicator of co-operativity.

Hill equation:

$$y = \frac{m1 + (m2 \times m0^{m3})}{m4^{m3} + m0^{m3}}$$

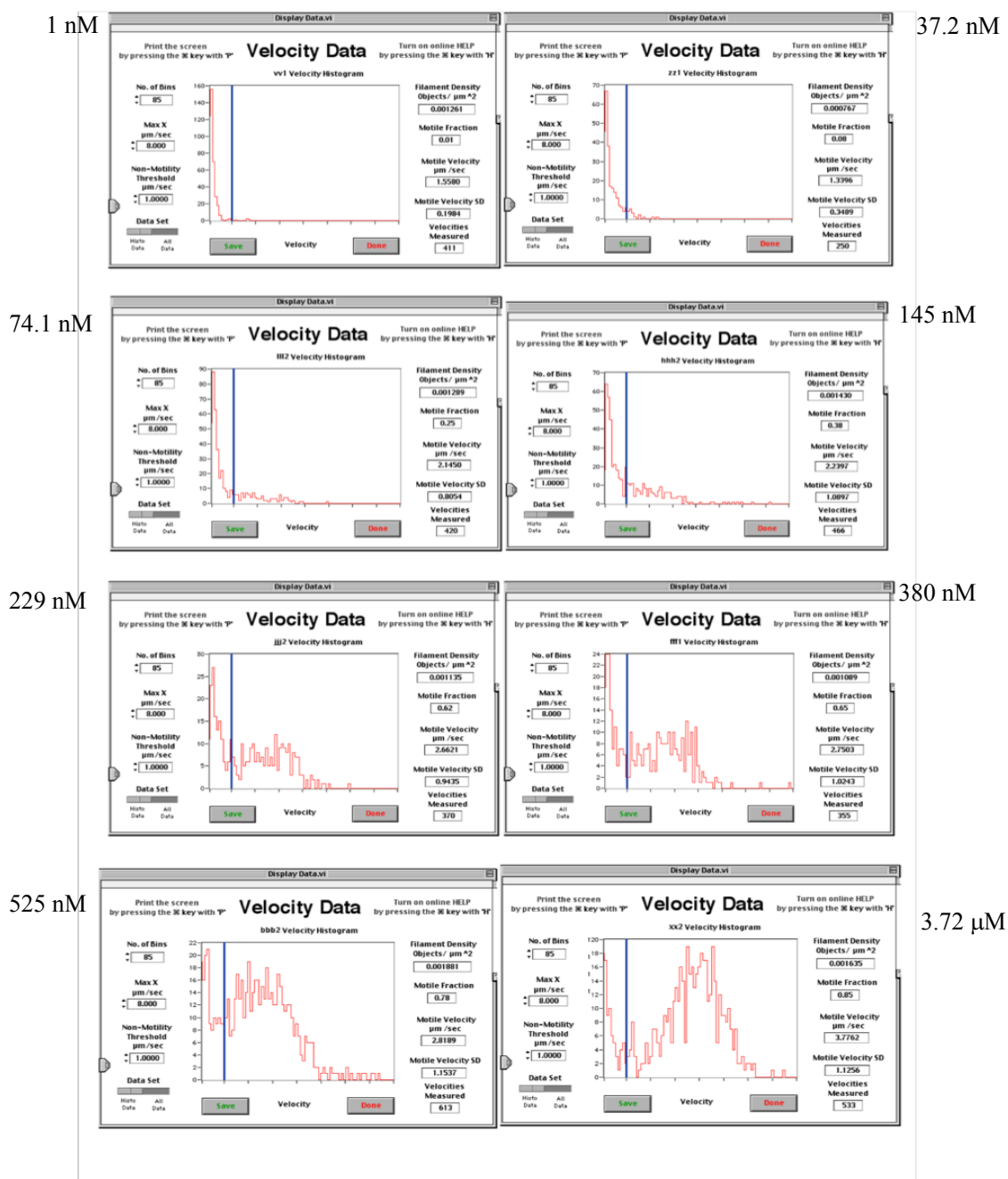
m1 = min value

m2 = max - min value

m3 = n_H

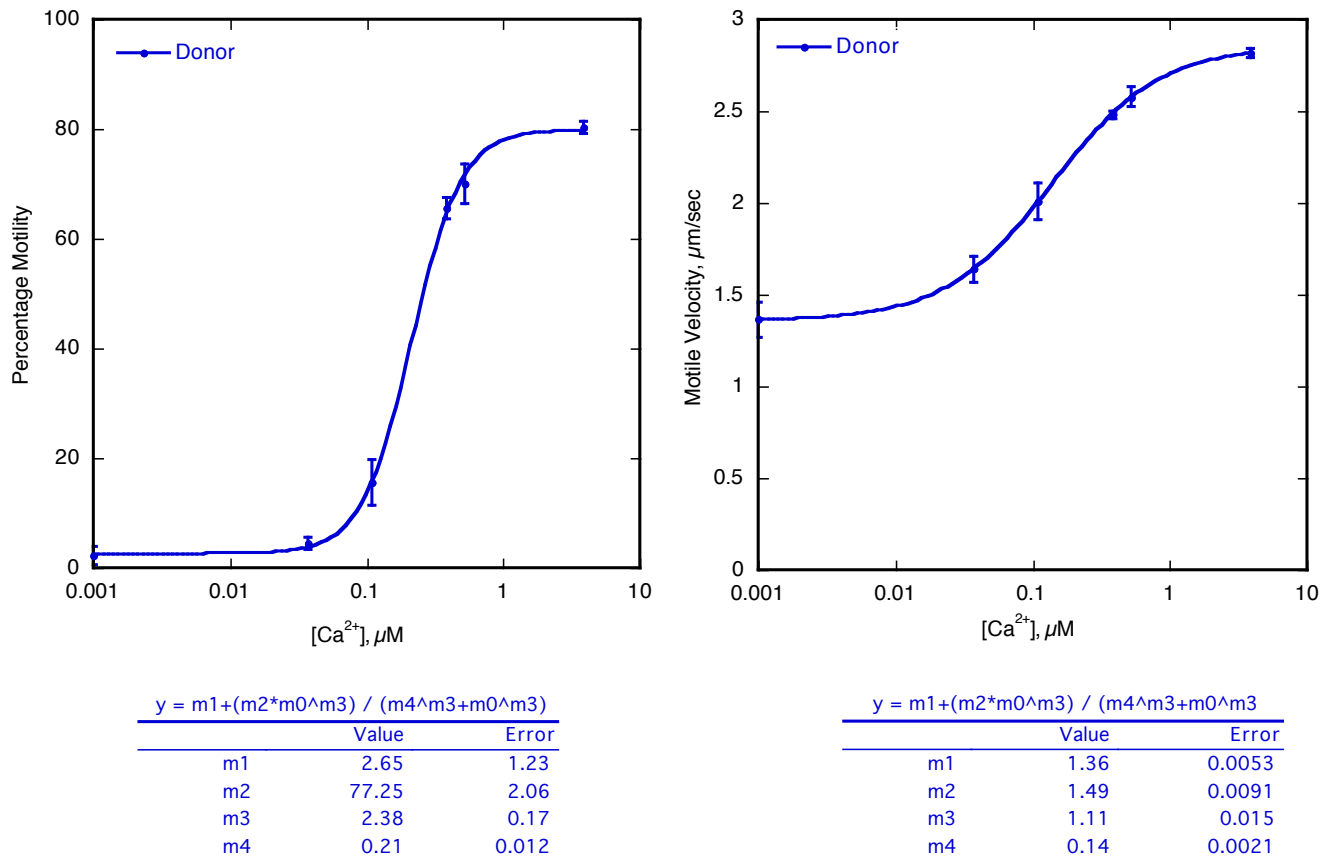
m4 = EC_{50}

Figure 2-12: Histograms from the tracking software to construct the Ca^{2+} activation curves



Histogram produced at different Ca^{2+} concentrations (1 nM, 74.1 nM, 229 nM and 525 nM) from the tracker program is shown. Using these data, a Ca^{2+} titration curve can be produced (see Figure 2-13).

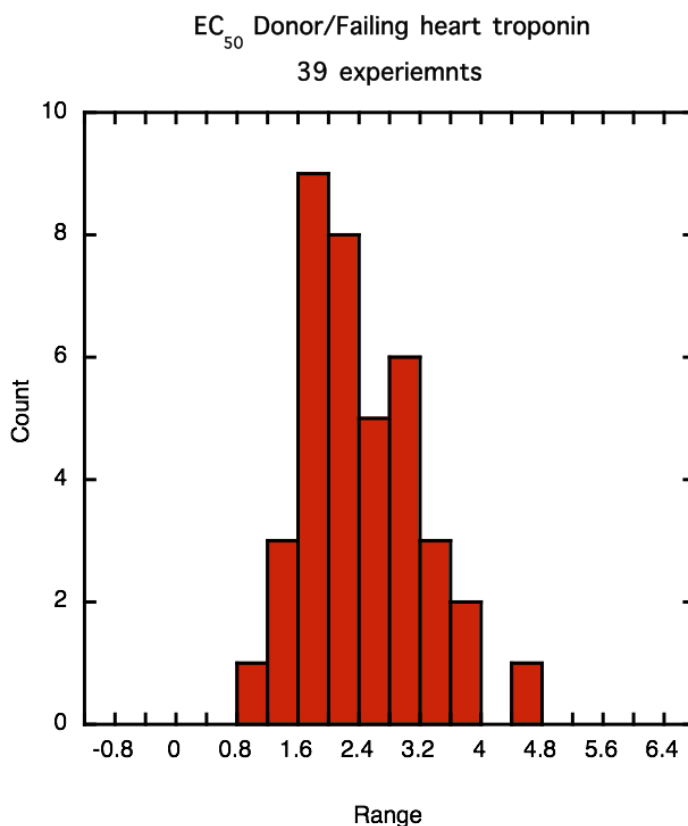
Figure 2-13: Ca^{2+} activation curves of thin filaments reconstituted from proteins extracted from donor samples



Donor thin filaments were reconstituted with troponin extracted from donor hearts, recombinant WT AS α cardiac tropomyosin and rabbit skeletal actin. Percentage motility (left) and motile velocity (right) of the thin filaments was measured in the different Ca^{2+} concentrations and the data were plotted. Error bars represent SEM of four measurements in the same motility chamber. The Hill equation for percentage motility and motile velocity was fitted to the data and is shown under the Ca^{2+} activation curves. m1 represents the min value, m2 represents the max-min value, m3 represents the Hill coefficient (n_H) and m4 the EC_{50} .

2.5.11 Statistical analysis

Mean data values are shown alongside standard deviation of the mean unless otherwise stated. Extensive measurements by Dr Messer have established that this is true for the IVMA system used here (Messer et al., 2007). Considering 39 paired measurements of EC_{50} in donor and failing hearts using the same methods as in this thesis, it was shown that the distribution of the data produced was normal, see Figure 2-14.

Figure 2-14: Distribution of the EC₅₀ ratios Donor/Failing troponin

Distribution of EC₅₀ donor/failing ratio shows that data are normally distributed.

For this reason, data was compared for statistical significance using the Student t-test where $p < 0.05$ was classed as significant. The Student t-test is the best method for significance tests on small populations, such as in this thesis (< 10 replicates) provided the distribution of the EC₅₀ parameter is normal (Messer, 2007).

Furthermore, 2- way ANOVA (Turkey correction) was also performed to see the combined phosphorylation status and response to treatment. 2-way ANOVA required equal numbers of samples for each treatment and this has meant some data is excluded from the analysis where the n value of some datasets was not equal.

3 Mutations in tropomyosin that cause gain of function in skeletal muscle myopathy patients

3.1 Introduction

As an introduction to the *in vitro* motility assay (IVMA) and to the proteins of the human myocyte, firstly I looked at tropomyosin mutations that lead to skeletal muscle myopathies. This helped me understand about the molecular mechanism of myopathies and structure-function relationships of protein mutations that lead to diseases, building on previous studies in the Marston lab. Tropomyosin mutations are commonly found in patients with congenital myopathies (Memo and Marston, 2013). There are many types of skeletal muscle myopathies, such as Nemaline myopathy, Cap disease and fibre type disproportion. Nemaline myopathy is the most widely studied of the skeletal myopathies and is characterized by nemaline (rod) bodies in the myocytes. Congenital myopathies can also be found in the form of cap-like structures at the periphery of the muscle (Cap disease) and congenital fibre-type disproportion (CFTD) with a pronounced hypertrophy of Type 1 skeletal muscle fibres. Histology of the different congenital myopathies can be found more detailed in APPENDIX. The diseases have different clinical and histological characteristics but they all result in muscle weakness and are caused by mutations in skeletal muscle proteins. For example, mutations in these diseases are found in *TPM2*, *TPM3* and *ACTC*. Mutations in different positions in *ACTA1* have been extensively studied in order to establish a mechanism in the single myofilament level that would be responsible for the diseases, however it was not possible to find such mechanism (Feng and Marston, 2009).

On the other hand, looking at mutations in specific positions helped us elucidate a common mechanism for mutations found in these positions. More specifically, mutations at the interface where tropomyosin interacts with actin are of particular importance. Starting with actin, the mutation K326N in *ACTA1* found in a patient with stiff muscle showed an increase Ca^{2+} sensitivity which was associated with a hypercontractile phenotype in the patient (Jain et al., 2012). K326N mutation in actin is found close to one site of interaction with tropomyosin (Li et al., 2011). Studying tropomyosin mutations is easier due to tropomyosin's simple structure.

Thus, mutations in tropomyosin could potentially help us identify a mechanism at the molecular level that is responsible for the onset of skeletal myopathies.

So far, 27 mutations have been found in *TPM2* gene and 22 mutations have been found in *TPM3* gene (Marston et al., 2013, Marttila et al., 2014).

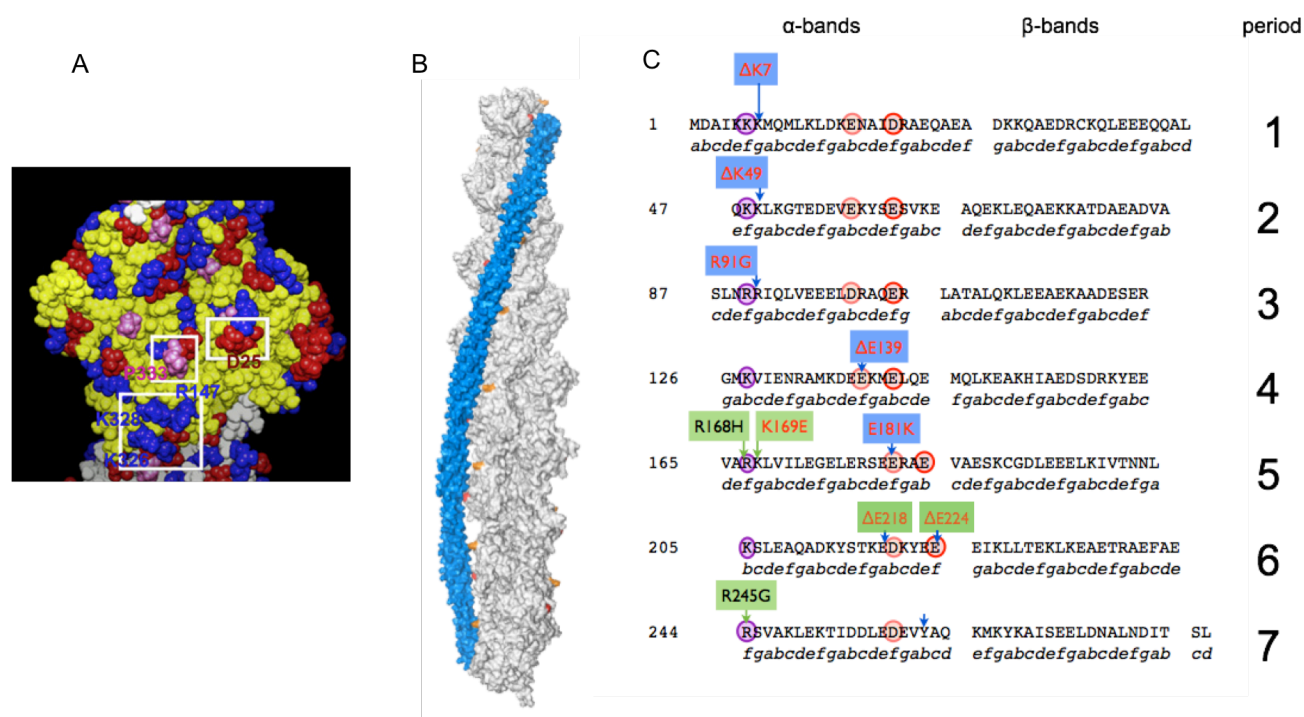
The first tropomyosin mutation studied at the molecular level was *TPM2* R91G and showed a hypercontractile function (Robinson et al., 2007). Another mutation that results in hypercontractile phenotype is a deletion on K7 residue in Tpm2.2 (Δ K7), which is a common mutation in patients with skeletal muscle myopathies (Davidson et al., 2013). This mutation has been found in nine families and clinical investigations found the presence of nemaline bodies as a common feature of patients with this mutation. Studies from skinned muscle isolated from patient biopsies and also *in vitro* motility studies showed that the mutation leads to an increased Ca^{2+} sensitivity, cross-bridge kinetics and force, which is linked with the presence of a hypercontractile phenotype of the patients (Mokbel et al., 2013).

Interestingly, this mutation is found near another site where tropomyosin interacts with actin. A high resolution structure of Tpm1.1 interacting with actin has been published, showing the interactions between tropomyosin and actin (Li et al., 2011). Li et al defined the interfaces between actin and tropomyosin using a combination of cryo-electron microscopy (cryo-EM) and computational simulations. The actin residues that appear to make contacts with tropomyosin include the basic amino acids Lys326, Lys328 and Arg147, the acidic amino acids Asp25 and Arg28 and the hydrophobic Pro333 (Figure 3-1A). Tropomyosin itself has a repeating amino acid motif in the 7 positions where it interacts with the actin molecules. These conserved seven amino acids are K6, K48, R90, K128, R167, K205 and R244 and interact with Asp25 on actin. Four of these share the same motif, with two basic amino acids next to each other: K6- K7, K48-K49, R90-R91 and R167-R168.

Figure 3-1 shows the structural motif of tropomyosin and its interaction with actin. This new structure could provide a molecular explanation of how mutations in tropomyosin would lead to diseases. Mutations in the second amino acid of all the four motifs have been reported in skeletal myopathies (Marston et al., 2013) and Δ K7 and R91G are already known to be hypercontractile. In this chapter I will look at the mutations found in the actin-tropomyosin interface and propose a mechanism of how these mutations lead to diseases. The starting point of this study is that the loss of K7 residue that, as explained, leads to a gain of function and is positioned adjacent to the

K6 residue, which interacts with Asp25 on actin. We therefore hypothesize that the mutations might destabilize the inhibitory action of tropomyosin to actin (the “off” state) leading to hypercontractility. Taking advantage of tropomyosin’s conserved structural motif at the sites of interaction with actin it would be interesting to see if other mutations found at these sites give a similar phenotype. I therefore predict that the mutations that are found in one of these conserved amino acids in Tpm2.2 and Tpm3.12 that interact with Asp25 would cause a gain of function like $\Delta K7$. Mutations in acidic residues of tropomyosin where it interacts with Lys326 of actin have also been found ($\Delta E139$, E181K, $\Delta E218$, $\Delta E224$), so we predict mutations found in these amino acids would also lead to gain of function. It was discovered that some of the mutations that lead to skeletal myopathies are associated with a hypercontractile phenotype, thus being “gain of function” mutations. The hypercontractile phenotype indicates that mutations cause abnormalities in the contractile apparatus of the myocyte that influence the force production of the muscle.

Figure 3-1: Tropomyosin- actin interactions



A) Actin subunit showing the residues whereby it interacts with tropomyosin. Basic amino acids are coloured blue, acidic amino acids are coloured red and proline is pink (Li et al., 2011). B) Surface model of tropomyosin (blue) interacting with actin (grey). Actin Asp25 is red and Lys326 is orange. C) Sequence Tpm2.2 divided in 7 periods. The purple circles represent the residues interacting with Asp25. The orange and red circles represent residues interacting with actin R147, K326 and K328. The blue boxes represent β tropomyosin mutations and green boxes represent Tpm3.12 mutations. The mutations predicted to be gain of function are written in red and those predicted to be loss of function in black (Marston et al., 2013)

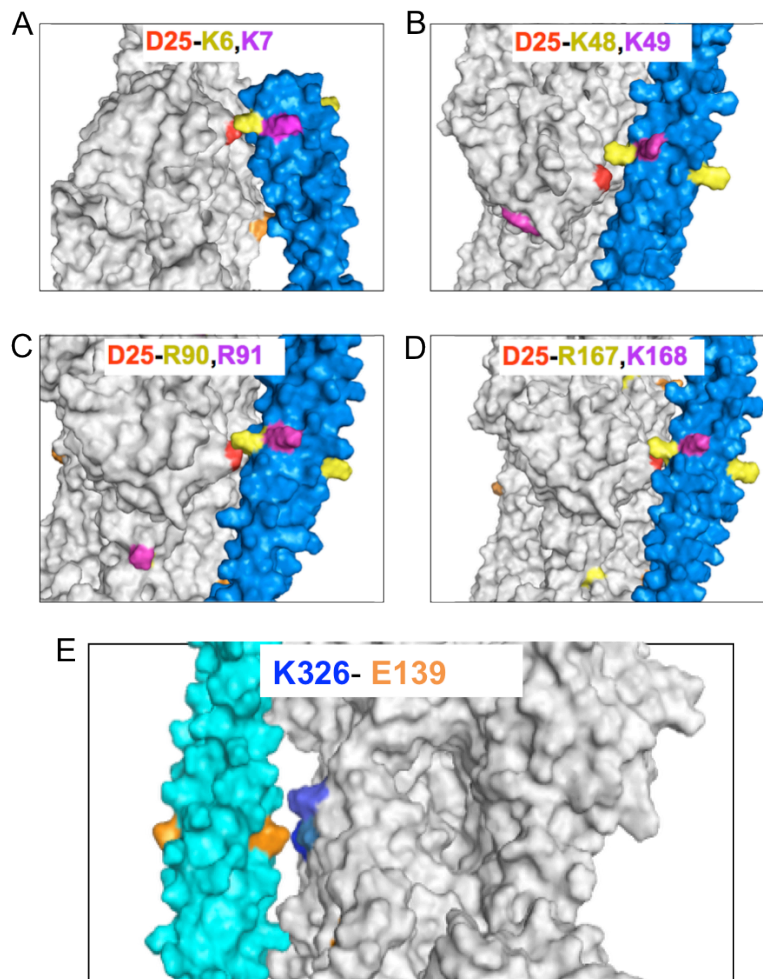
3.2 Tropomyosin mutations in *TPM2* and *TPM3* that cause gain of function

In this chapter, four mutations Tpm2.2 or Tpm3.12 predicted to cause gain of function based on their position relative to actin were studied. The mutations are the following: Δ K49, Δ E139, R91G in Tpm2.2 and K168E in Tpm3.12. These mutations are found in the second amino acid of the motif where tropomyosin binds to actin and as Δ K7 was found to be gain of function mutation, they are predicted to be gain of function too.

Δ K49 is associated with cap disease and has been found in one patient who had muscle weakness at the age of 8 with healthy relatives. Biopsies taken from the patient showed cap-like structures (Ohlsson et al., 2008). Δ E139 is also associated with cap disease and it was actually the first mutation in *TPM3* known to be causative for the disease. The mutation was found on a patient diagnosed at age 11 with muscle weakness, hypotonia and who presented cap-like structures in Type 1 and Type 2 fibres (Lehtokari et al., 2007). R91G is known to cause distal arthrogryposis and *in vitro* investigation showed that this mutation increased ATPase activity and caused incomplete relaxation (Robinson et al., 2007).

TPM3 mutation K168E is linked to CFTD and was found in one family whose members were affected by this disease by Clarke et al (Clarke et al., 2008). The patient carrying the mutation was diagnosed at 4 years of age. All of these mutations are, like Δ K7, closely positioned to the place where tropomyosin interacts with actin and the location of these mutations can be found in Figure 3-2.

Mutations in basic or acidic pairs in Tpm2.2 and Tpm3.12 lead to a charge reversal or loss placed one amino acid downstream of the site of interaction with Asp25 or K326 on actin. In this chapter, the molecular mechanism of these mutations using the IVMA will be investigated, to elucidate whether all the mutations lead to a gain of function, as the structural model predict.

Figure 3-2: Location of *TPM2* and *TPM3* mutations

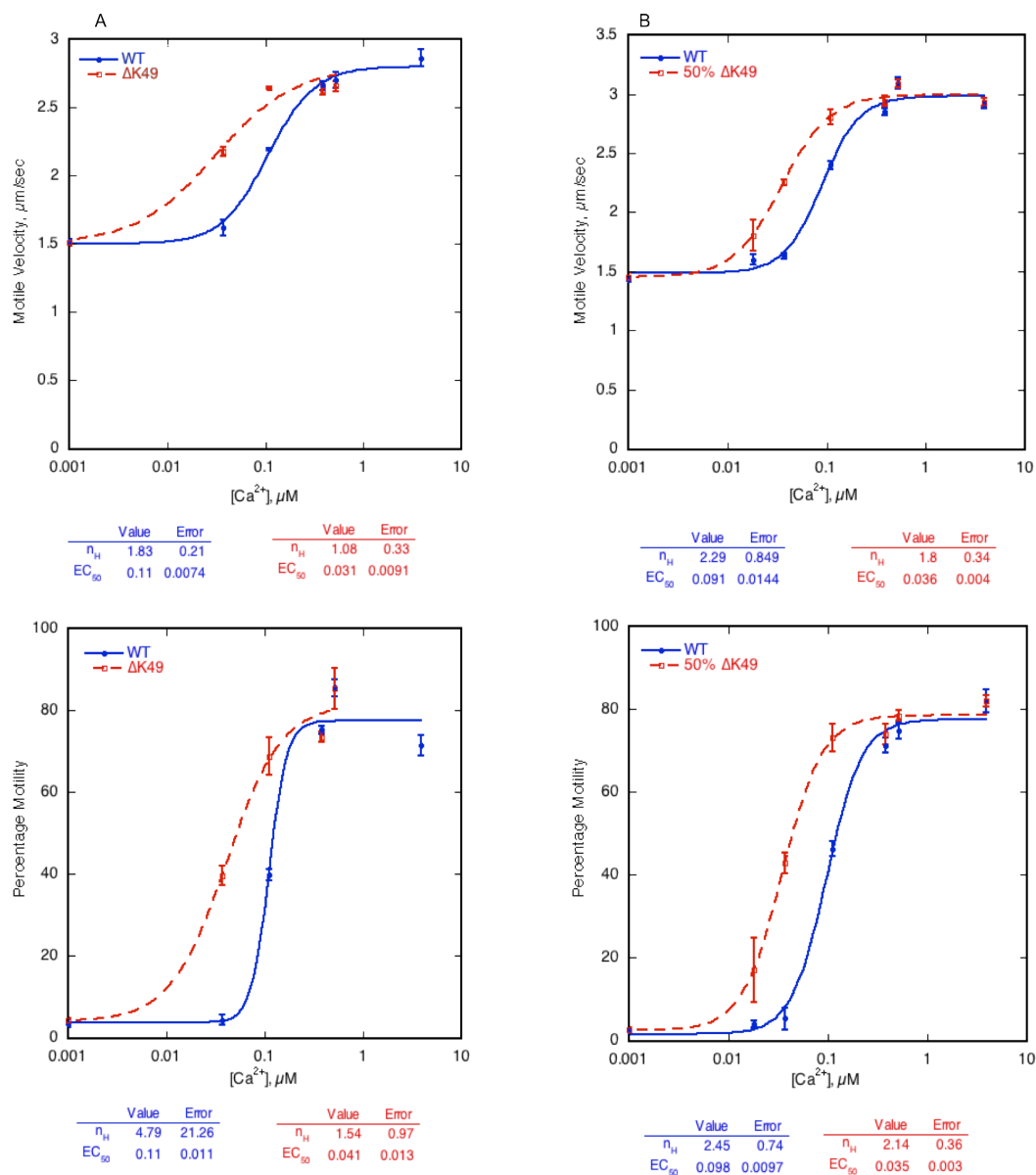
The locations of the predicted gain of function mutations in relationship to Asp25 (red) or Lys326 (blue) on actin. Actin is shown in shown in grey and tropomyosin is shown in blue or light blue. The residues in tropomyosin that interact with Asp25 on actin are indicated in purple and yellow and the residues interacting with Lys326 are indicated in orange. A) Tm K6-K7 B) Tm K48-K49 C) Tm R90-R91 D) Tm R167-K168 E) Tm E139 with K326 on actin.

3.2.1 In vitro motility analysis of *TPM2* and *TPM3* mutations

Thin filaments were reconstituted using 60 nM skeletal troponin, 60 nM wild type (WT) or mutant tropomyosin that was produced by the providers using baculovirus/*sf9* cells with native post translational modifications including N terminal acetylation and 10 nM rabbit skeletal actin. Each mutation was tested for both 100% and 50% reconstitution. Because the mutations are not expressed 100 % *in vivo*, thin

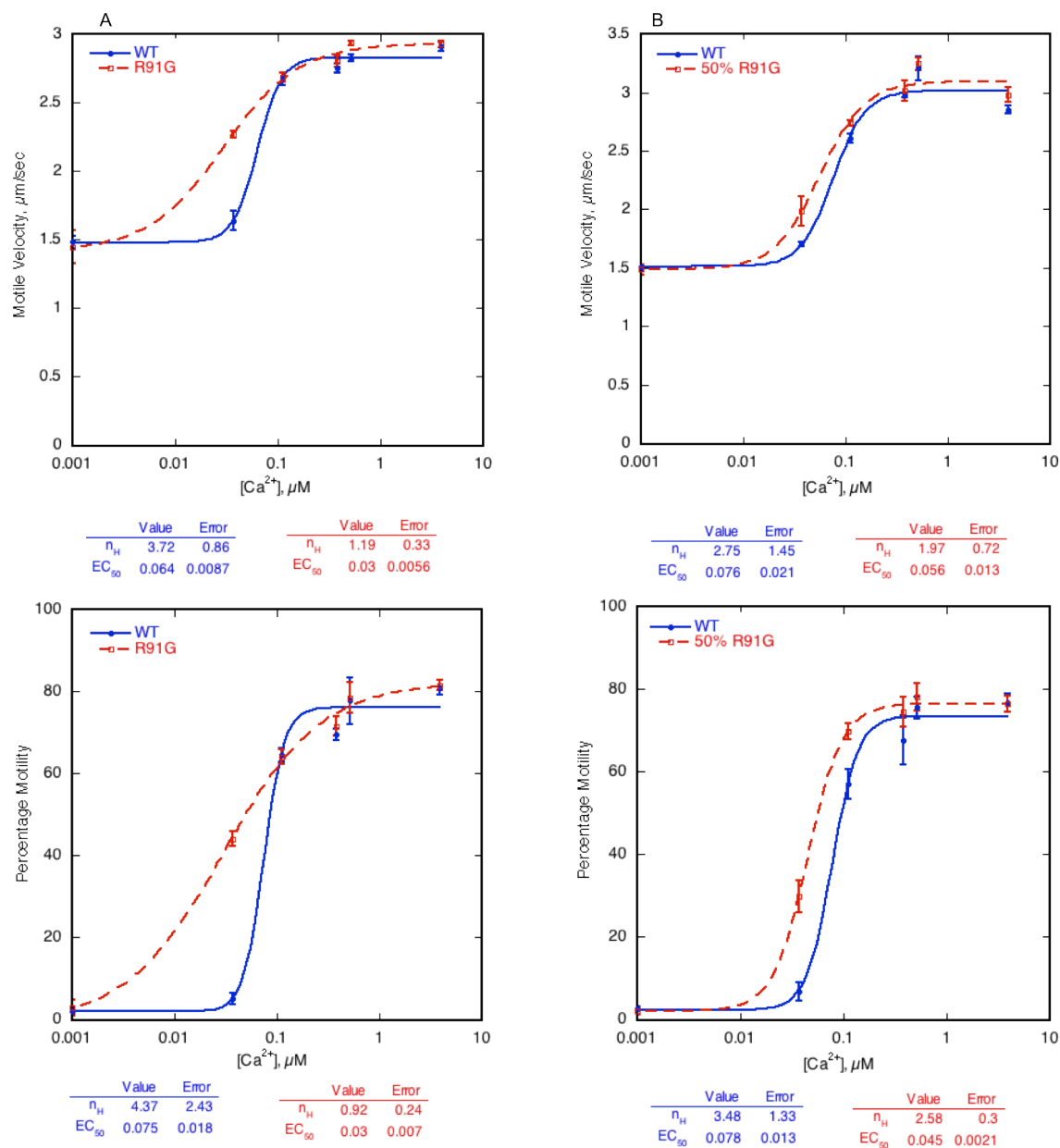
filaments reconstituted with 50% of the mutation might represent the physiological situation better. Three Ca^{2+} activation curves were performed for the 50% of each of the mutations and one for the 100% for the mutations. Figure 3-3, Figure 3-4, Figure 3-5 and Figure 3-6 show representative Ca^{2+} activation curves for $\Delta\text{K49 TPM2}$, R91G *TPM2*, $\Delta\text{E139 TPM2}$ and K168E *TPM3* mutations respectively. Data were combined with previous measurements from Dr Massimiliano Memo and Dr Andrew Messer and are summarised in Table 3-1. Interestingly, all the mutations showed an increase in Ca^{2+} sensitivity, as shown by the decrease in EC_{50} , which is in line with the “gain of function” prediction. The motile velocities were not different between wild type (WT) and mutant thin filaments and there was no difference in the results between 50% and 100% mutant thin filaments. Thus even the partial replacement of tropomyosin as occurs *in vivo* is likely to provoke the maximal abnormality in function.

Figure 3-3: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with ΔK49 mutant tropomyosin vs WT



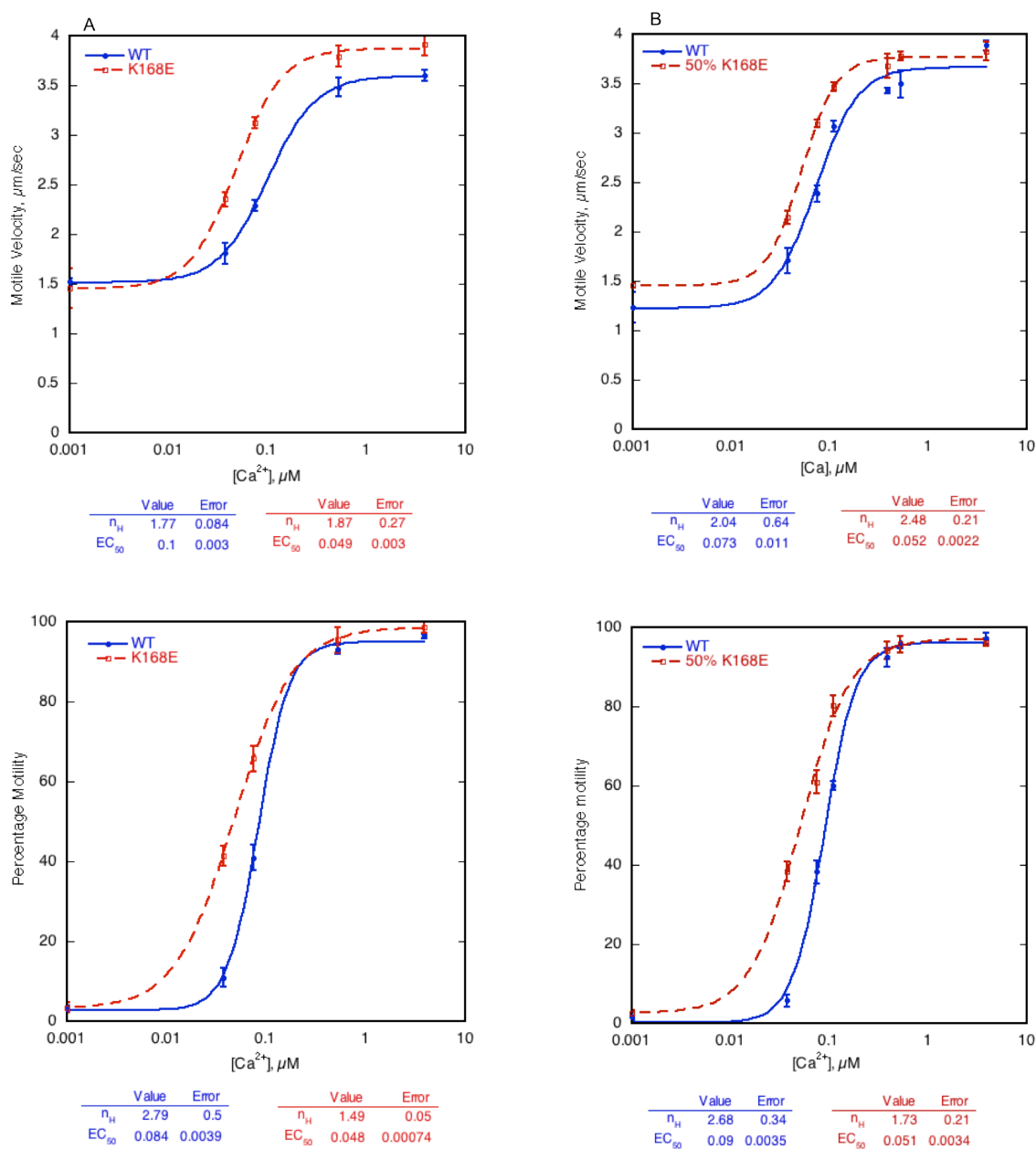
Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % ΔK49 tropomyosin vs WT tropomyosin and B) 50% ΔK49 tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Figure 3-4: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with R91G mutant tropomyosin vs WT



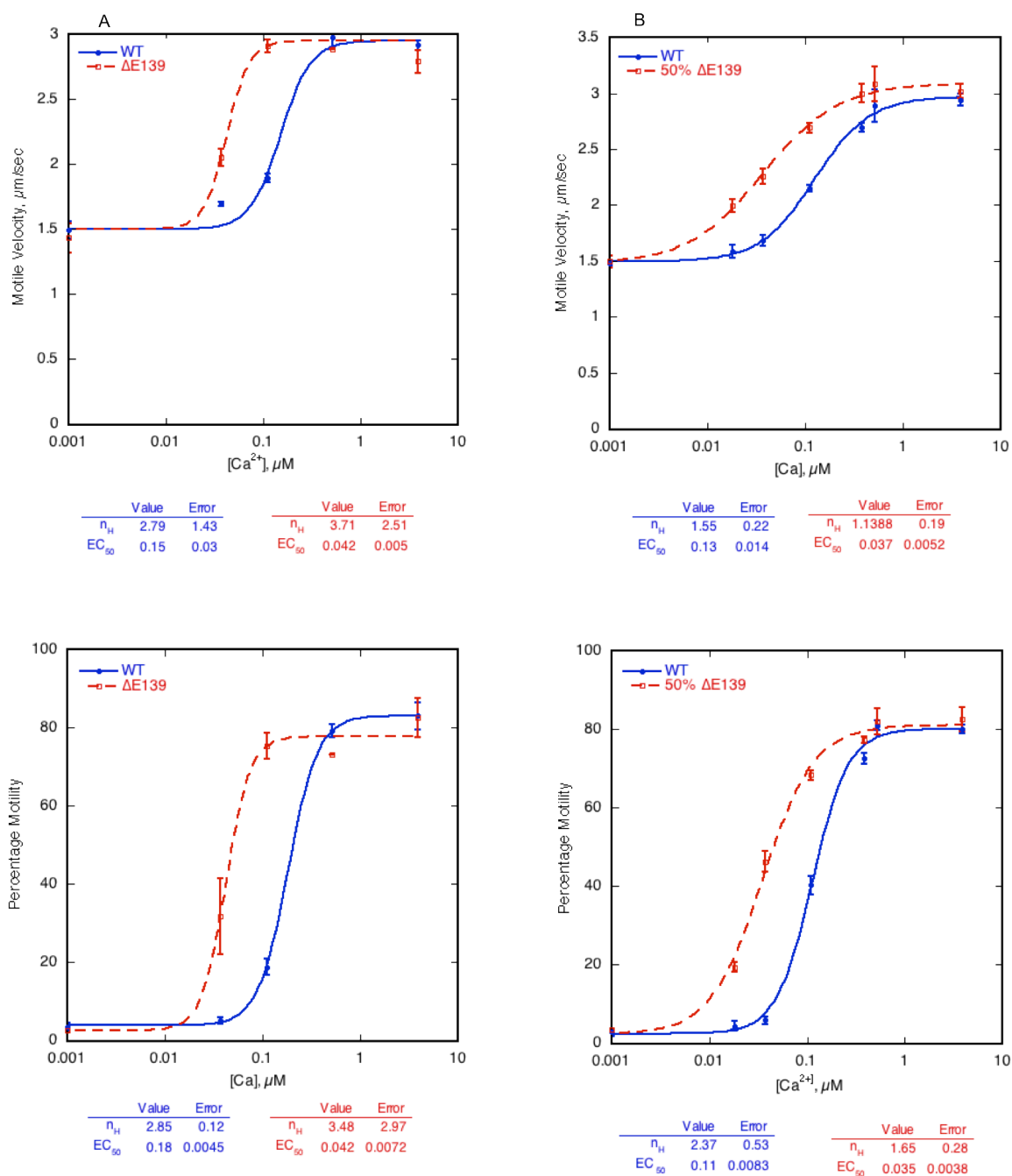
Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % R91G tropomyosin v WT tropomyosin and B) 50% R91G tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Figure 3-5: Ca^{2+} activation curves for motile velocity and percentage motility of thin filaments reconstituted with K168E mutant tropomyosin vs WT



Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % K168E tropomyosin v WT tropomyosin and B) 50% K168E tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Figure 3-6: Ca^{2+} activation curves for motile velocity and percentage motility of thin filaments reconstituted with ΔE139 mutant tropomyosin vs WT



Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % ΔE139 tropomyosin vs WT tropomyosin and B) 50% ΔE139 tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Table 3-1: Summary of tropomyosin mutations in *TPM2* and *TPM3* which give gain of function

Mutation	EC ₅₀ Mut, μ M	EC ₅₀ WT, μ M	Ratio EC ₅₀ WT/Mut	Max sliding speed relative to WT, %
ΔK49 100%	0.04 $\pm$ 0.002 (6)	0.08 $\pm$ 0.01 (6)	2.42 $\pm$ 0.25 (6)**	98.1 $\pm$ 2.7 (3)
50%	0.03 $\pm$ 0.002 (6)	0.09 $\pm$ 0.003(6)	2.71 $\pm$ 0.06 (6)***	101 $\pm$ 0.6 (3)
R91G 100%	0.05 $\pm$ 0.01 (6)	0.09 $\pm$ 0.01 (6)	1.83 $\pm$ 0.18 (6)**	102.2 $\pm$ 1 (3)
50%	0.06 $\pm$ 0.0 (6)	0.09 $\pm$ 0.004(6)	1.55 $\pm$ 0.05 (6) **	100.2 $\pm$ 0.6 (3)
ΔE139 100%	0.03 $\pm$ 0.004 (6)	0.12 $\pm$ 0.03 (6)	2.95 $\pm$ 0.43 (6) **	101.1 $\pm$ 2.4 (3)
50%	0.03 $\pm$ 0.004 (6)	0.11 $\pm$ 0.01 (6)	2.58 $\pm$ 0.49 (6) **	104.2 $\pm$ 1.1 (3)
K168E 100%	0.05 $\pm$ 0.01 (6)	0.08 $\pm$ 0.01 (8)	2.17 $\pm$ 0.32 (8) **	107.2 $\pm$ 1.6 (4)
50%	0.05 $\pm$ 0.003 (12)	0.09 $\pm$ 0.001 (12)	1.84 $\pm$ 0.07 (12) ***	105.4 $\pm$ 2.7 (6)

Data were combined from experiments performed by Dr Massimiliano Memo, Dr Andrew Messer and myself. EC₅₀ values were combined for percentage motility and motile velocity Ca²⁺ activation curves. **p<0.01, ***p<0.001 for single value t test compared to one. In brackets the n number.

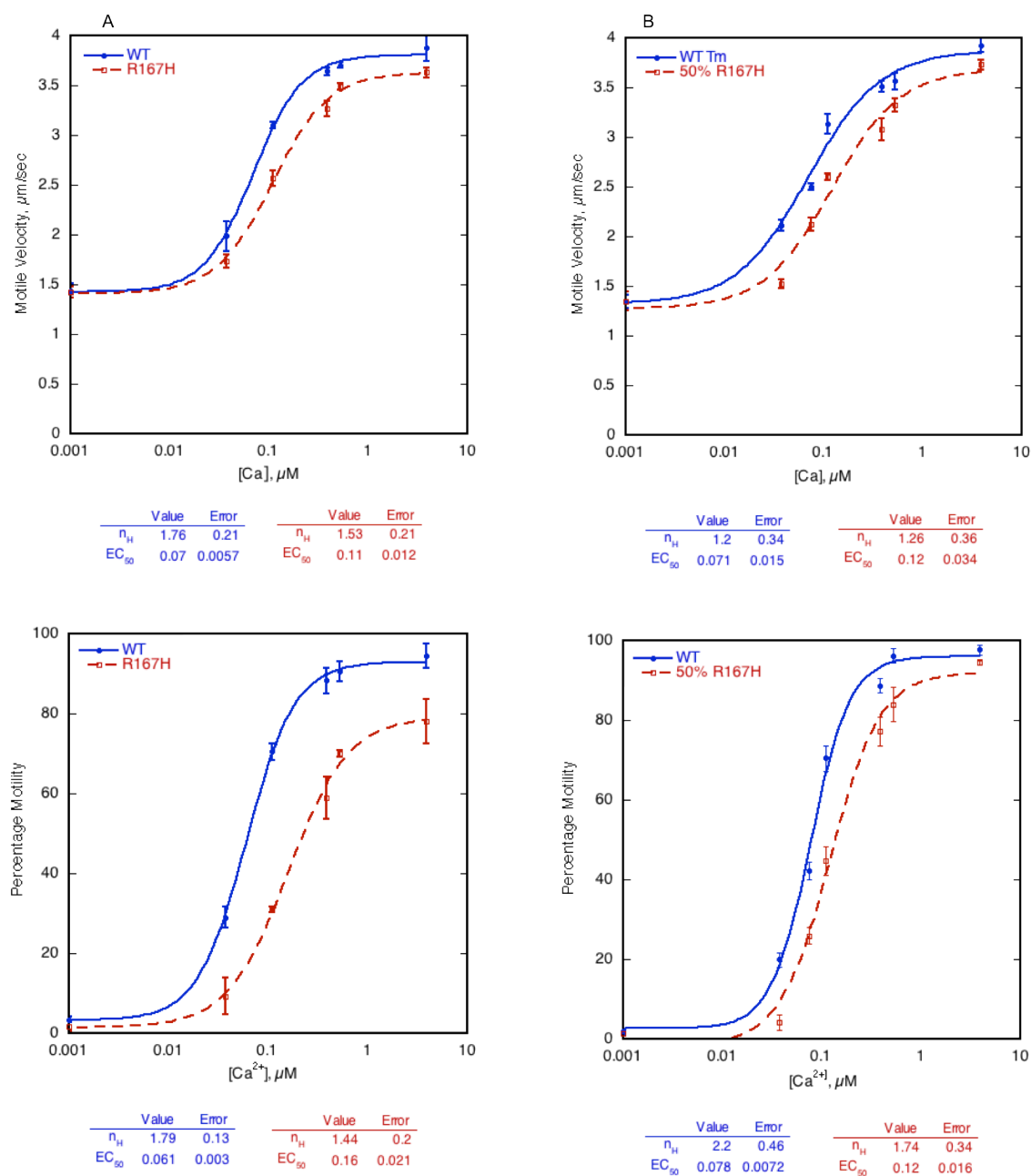
3.3 Tropomyosin mutations in *TPM3* that cause a loss of function

Two tropomyosin mutations that are in the exact amino acid where tropomyosin interacts with actin rather than one amino acid downstream were examined. R167H and R244G in Tpm3.12 are found in the amino acid directly interacting with Asp25. R167H has been identified in a large study by Clarke et al, where they screened 23 unrelated individuals affected with CFTD (Clarke et al, 2008). Position R167 in *TPM3* is a hotspot for mutations, as multiple mutations in this position linked with skeletal muscle myopathies have been found, such as R167C linked to CFTD and Cap disease (Ohlsson et al., 2009) and R167H. R244G in *TPM3* was another mutation identified in the study of Clarke et al and has been one of the mutations that cause CFTD. R244G is found in Tpm3.12 at the position where tropomyosin directly interacts with actin. It is noted that Tpm3.12 numbering is based on the native protein and is one less than the numbering based on the DNA sequence.

3.3.1 *In vitro* motility assay analysis of R167H and R244G

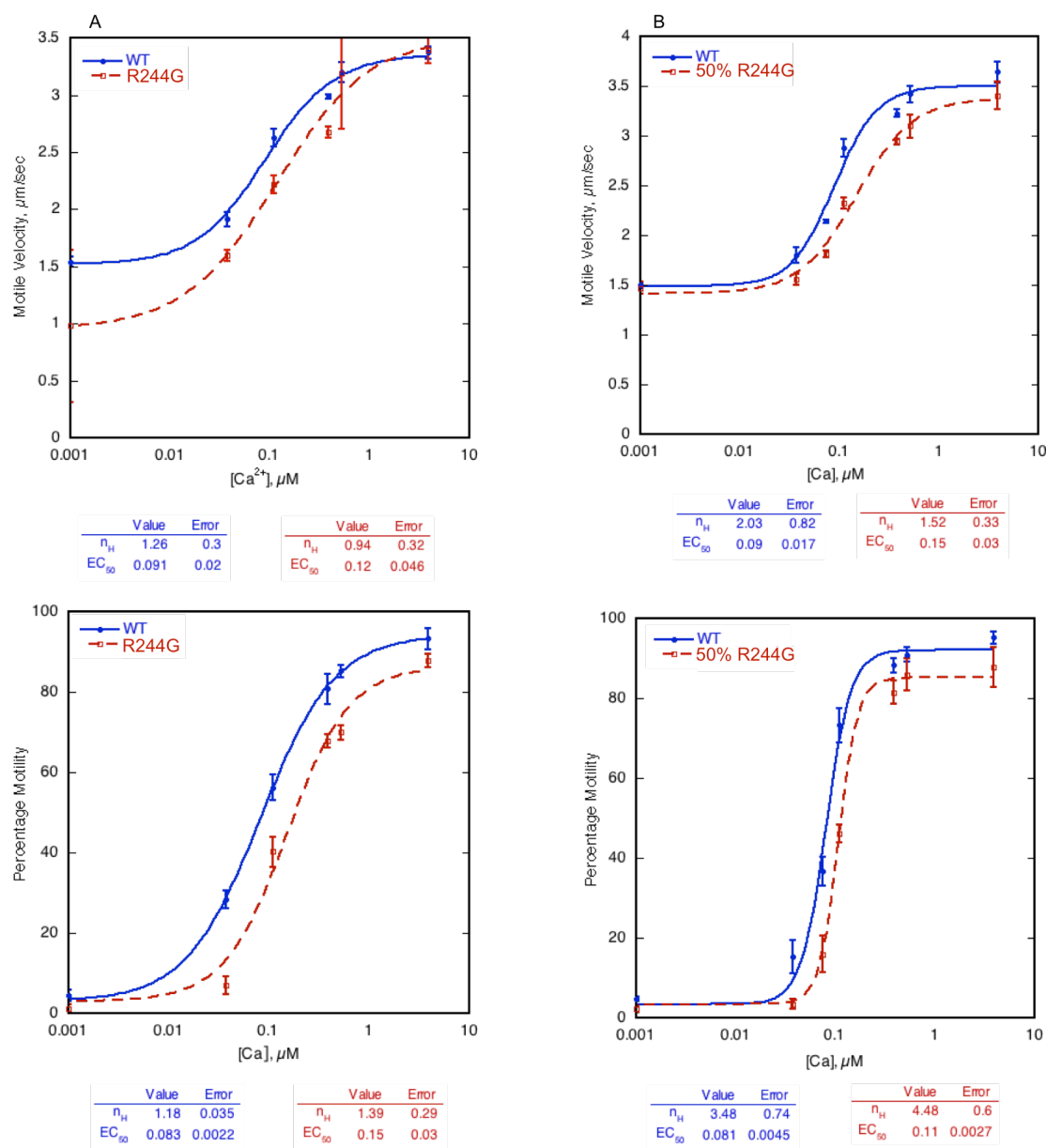
Thin filaments were reconstituted using 60 nM skeletal troponin, 60 nM wild type or mutant tropomyosin that was produced by the providers using baculovirus/*sf9* cells and 10 nM rabbit skeletal actin. Ca^{2+} activation curves were constructed for the percentage motility and motile velocity of thin filaments reconstituted with WT or mutant tropomyosin. Again, 100 % and 50% of the mutation was used for the production of the thin filaments. Three Ca^{2+} activation curves were produced for 100% R167H and one for 50% R167H mutation versus WT, examples of which are shown in Figure 3-7. Three Ca^{2+} activation curves were produced for 100% R244G mutation and one for 50% R244G mutation versus WT, examples of which are shown in Figure 3-8. The data are summarized in Table 3-2. As we observe from the Ca^{2+} activation curves and also from the summary tables, both of these mutations showed a decreased in Ca^{2+} sensitivity or loss of function (EC_{50} WT/ R167H 100% = 0.59 ± 0.08 , $n = 6$, $p = 0.04$ and EC_{50} WT/ R244G 100% = 0.46 ± 0.08 , $n = 6$, $p = 0.001$). Interestingly, 100% and 50% of the mutation gave similar changes to Ca^{2+} sensitivity for all the mutations.

Figure 3-7: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with mutant R167H tropomyosin vs WT



Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % R167H tropomyosin vs WT tropomyosin and B) 50% R167H tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Figure 3-8: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with R244G mutant tropomyosin vs WT



Representative Ca^{2+} titration curves for motile velocity and percentage motility for thin filaments reconstituted with A) 100 % R244G tropomyosin vs WT tropomyosin and B) 50% R244G tropomyosin vs WT tropomyosin. WT tropomyosin in blue and mutant tropomyosin in red.

Table 3-2: Summary of the mutations in *TPM3* that cause loss of function

Mutation	EC ₅₀ Mut, μ M	EC ₅₀ WT, μ M	Ratio EC ₅₀ WT/Mut	Max sliding speed relative to WT, %
R167H 100%	0.16 $\pm$ 0.02 (6)	0.07 $\pm$ 0.01 (6)	0.59 $\pm$ 0.08 (6) *	98.1 $\pm$ 2.7 (3)
50%	0.13 $\pm$ 0.002 (2)	0.08 $\pm$ 0.01 (2)	0.59 $\pm$ 0.01 (2)	95 (1)
R245G 100%	0.12 $\pm$ 0.02 (6)	0.05 $\pm$ 0.02 (6)	0.46 $\pm$ 0.08 (6)*	97.1 $\pm$ 4.7 (3)
50%	0.11 $\pm$ 0.003 (2)	0.08 $\pm$ 0.01(2)	0.67 $\pm$ 0.07 (2)	93.6 (1)

EC₅₀ values were combined for percentage motility and motile velocity Ca²⁺ activation curves. **p<0.01 for single value t test compared to one. In brackets the number of experiments.

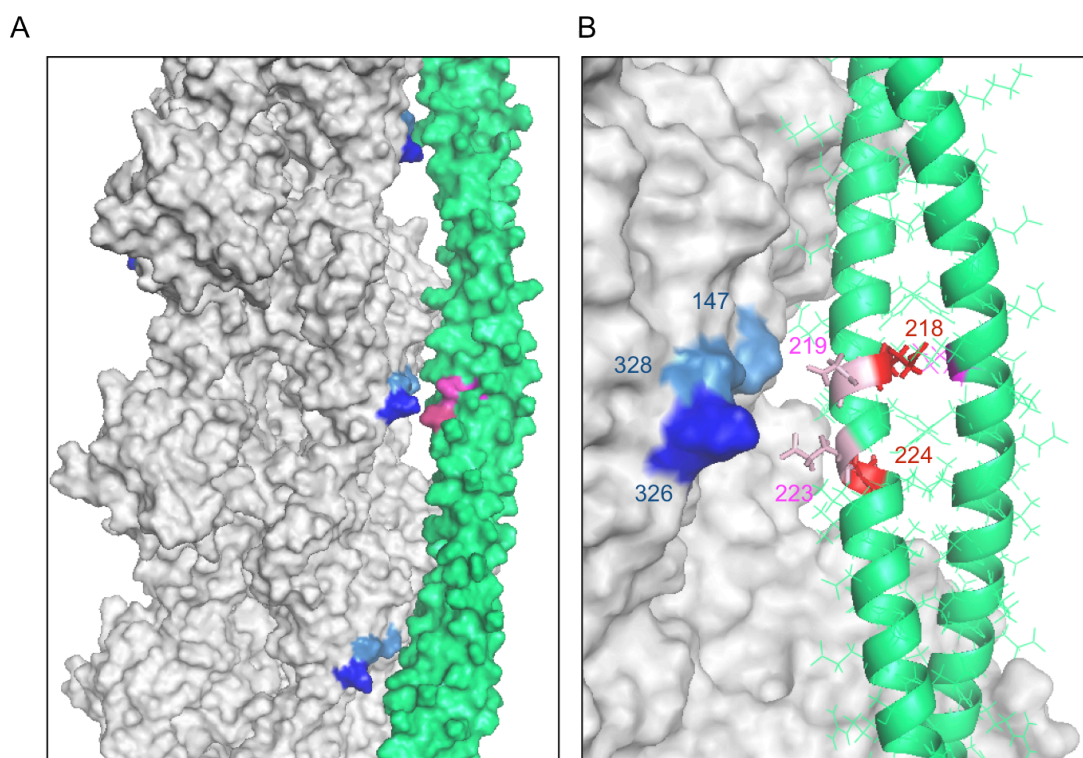
3.4 Two novel *TPM3* deletions also cause a hypercontractile phenotype with gain of function.

Two novel mutations in *TPM3* in positions very close to each other have been recently identified in two unrelated stiff patients. These mutations were a deletion in Glutamine 224 ($\Delta E224$) and the other one in Glutamine 218 ($\Delta E218$) (Donkervoort et al., 2015). Gain of function associated with a stiff patient phenotype is rare. *ACTA1* K236N has been the only mutation so far that has been previously identified in a muscle protein.

The proband carrying the $\Delta E224$ mutation was 5 years old and was diagnosed with generalized muscle stiffness. The patient had a history of overheating and inappropriate sweating. He had problems of moving and breathing and he uses a Cough Assist device to help him with ventilation. The relatives of the individual were unaffected.

The patient carrying the $\Delta E218$ had a more severe phenotype. She was three years old when she was diagnosed with the disease but she presented severe muscle stiffness even from birth. The respiratory muscles were affected by the myopathy and the patient underwent three episodes of apnoea. She still remains fully respirator dependent at three years of age.

The two mutations are found in the second acidic amino acid in the motif of where Tpm3.12 interacts with a basic cluster consisting of K326, K328 and R147 on actin (see Figure 3-9). It is predicted that these two mutations would also be gain of function mutations similar to the mutations in mutations described in the previous section.

Figure 3-9: Location of the novel mutations in *TPM3*

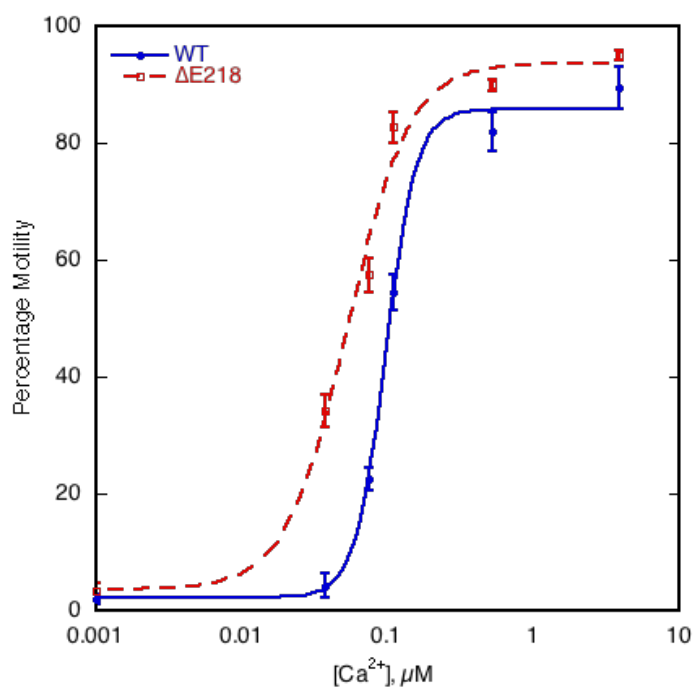
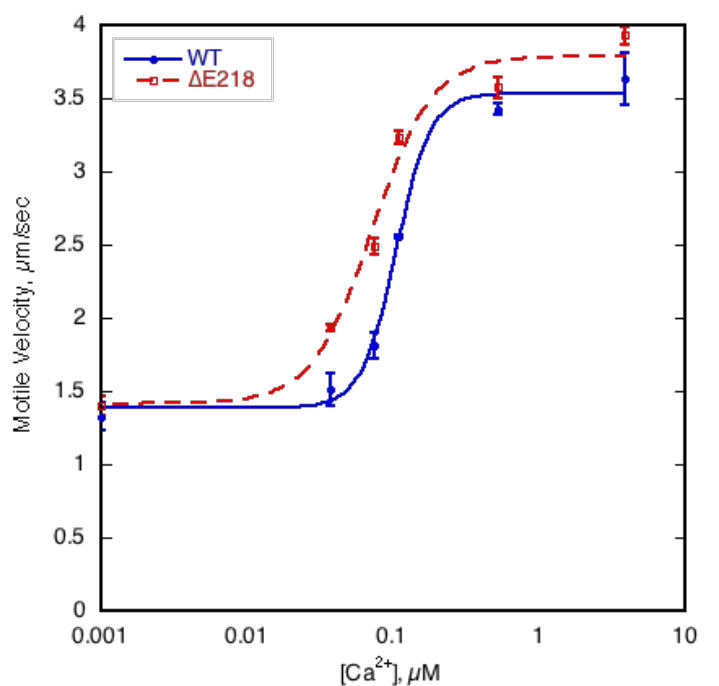
Location of the novel mutations in Tpm3.12 Δ E224 and Δ E218 and their interaction with actin. A) the interface of tropomyosin (green) period 6 with actin (grey). K326, K328 and R147 in actin are shown in different shades of blue, E218 and E224 are shown in red and E219 and E223 are shown in pink. B) A closer view of the site of interaction of actin K326 with the two tropomyosin mutations.

3.4.1 *In vitro* motility analysis of Δ E224 and Δ E218 *TPM3* mutations

Thin filaments were reconstituted using 60 nM WT or Δ E224 and Δ E218 recombinant mutant Tpm3.12 provided by Dr Elyshia McNamara, 60 nM skeletal troponin and 10 nM rabbit skeletal actin. Four Ca^{2+} activation curves were performed for each of the mutation comparing mutant or wild type thin filaments. Representative Ca^{2+} activation curves for percentage motility and motile velocity of the thin filaments are shown in Figure 3-10 and Figure 3-11 and data for percentage motility and motile velocity of thin filaments for each of the mutations are summarized in Table 3-3, Table 3-4, Table 3-5 and Table 3-6. Δ E218 increased the Ca^{2+} sensitivity by 2.5 fold for the percentage motility and by 2 fold for the motile velocity of the thin filaments. Δ E224 also led to an increase in Ca^{2+} sensitivity in both motile parameters, by 2.2

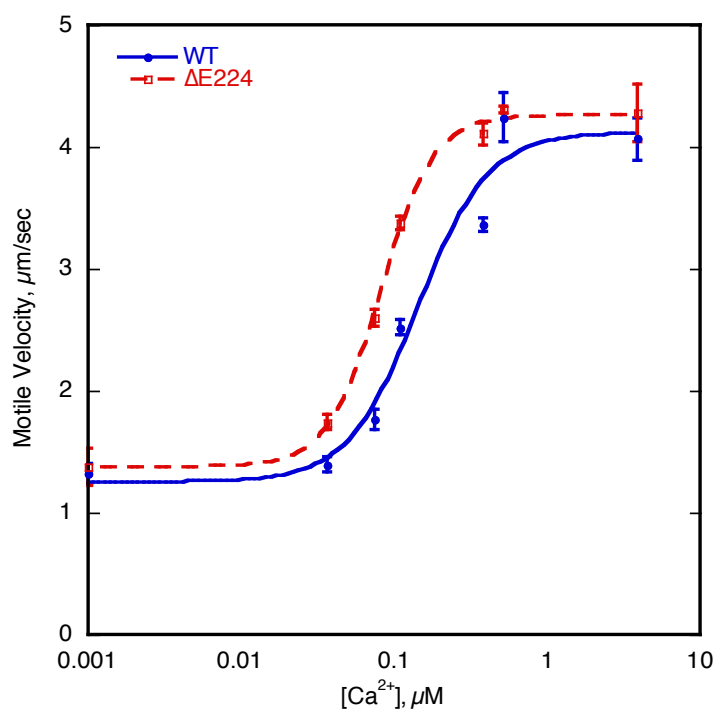
fold and 3 fold respectively ($EC_{50} \Delta E224/WT = 0.46 \pm 0.09$, $p = 0.01$, $n = 4$ for percentage motility and $EC_{50} \Delta E224/WT = 0.33 \pm 0.08$, $p = 0.004$, $n = 4$ for sliding speed). These results indicate that both of these mutations also cause a gain of function. There was no significant difference in maximum sliding speed of the filaments, or the Hill coefficient between either of the mutations and wild type thin filament

Figure 3-10: Ca^{2+} activation curve for percentage motility and motile velocity of thin filaments reconstituted with ΔE218 mutant tropomyosin vs WT



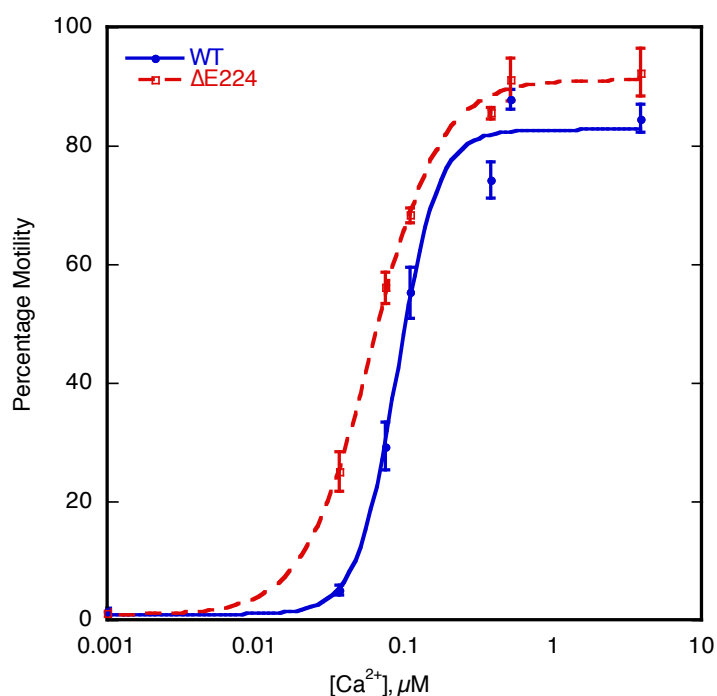
Representative Ca^{2+} titration curves for motile velocity (top) and percentage motility (bottom) for thin filaments reconstituted with 100 % ΔE218 tropomyosin (red) vs WT tropomyosin (blue).

Figure 3-11: Ca^{2+} activation curves for percentage motility and motile velocity of thin filaments reconstituted with ΔE224 mutant tropomyosin vs WT



	Value	Error
n_H	1.89	0.81
EC_{50}	0.14	0.043

	Value	Error
n_H	2.53	0.3
EC_{50}	0.082	0.0035



	Value	Error
n_H	3.18	1.01
EC_{50}	0.09	0.0076

	Value	Error
n_H	1.91	0.19
EC_{50}	0.061	0.0033

Representative Ca^{2+} titration curves for motile velocity (top) and percentage motility (bottom) for thin filaments reconstituted with 100 % ΔE224 tropomyosin (red) vs WT tropomyosin (blue).

Table 3-3: Summary of motility data for motile velocity of thin filaments reconstituted with $\Delta E218$ mutant tropomyosin vs WT

EXP N	EC ₅₀ Motile velocity, μ M WT	EC ₅₀ Motile velocity, μ M $\Delta E218$	EC ₅₀ Ratio Mut/WT	η_H Motile velocity WT	η_H Motile velocity $\Delta E218$	Max Sliding speed WT	Max Sliding speed $\Delta E218$
1	0.11 $\pm$ 0.0073	0.07 $\pm$ 0.01	0.69	3.66 $\pm$ 1.17	2.11 $\pm$ 0.77	2.15 $\pm$ 0.14	2.37 $\pm$ 0.28
2	0.12 $\pm$ 0.003	0.072 $\pm$ 0.0094	0.62	3.69 $\pm$ 0.46	2.25 $\pm$ 0.48	2.48 $\pm$ 0.54	2.91 $\pm$ 0.20
3	0.46 $\pm$ 0.031	0.06	0.13	0.99 $\pm$ 0.06	1.94	2.74 $\pm$ 0.06	2.6
4	0.22 $\pm$ 0.063	0.11 $\pm$ 0.058	0.69	0.98 $\pm$ 0.16	0.88 $\pm$ 0.22	2.92 $\pm$ 0.22	3.11 $\pm$ 0.33
Mean $\pm$ SE	0.22 $\pm$ 0.08	0.08 $\pm$ 0.01	0.48 $\pm$ 0.12*	2.33 $\pm$ 0.77	1.72 $\pm$ 0.38	2.57 $\pm$ 0.16	2.75 $\pm$ 0.16

Table 3-4: Summary of motility data for percentage motility of thin filaments reconstituted with $\Delta E218$ mutant tropomyosin vs WT

EXP N	EC ₅₀ % Motility, μ M WT	EC ₅₀ % Motility, μ M $\Delta E218$	EC ₅₀ Ratio Mut/WT	η_H % Motility WT	η_H % Motility $\Delta E218$	Max % Motile WT	Max % Motile $\Delta E218$
1	0.10 $\pm$ 0.004	0.05 $\pm$ 0.01	0.55	4.12 $\pm$ 0.84	2.06 $\pm$ 0.53	89.5 $\pm$ 3.9	95 $\pm$ 0.82
2	0.099 $\pm$ 0.0002	0.05 $\pm$ 0.004	0.5	4.87 $\pm$ 0.072	1.87 $\pm$ 0.3	86.5 $\pm$ 3.11	94.25 $\pm$ 1.71
3	0.36 $\pm$ 0.078	0.12 $\pm$ 0.003	0.33	0.97 $\pm$ 0.19	0.99 $\pm$ 0.02	74.5 $\pm$ 6.36	84.5 $\pm$ 3.05
4	0.25 $\pm$ 0.049	0.06 $\pm$ 0.096	0.24	1 $\pm$ 0.16	0.88 $\pm$ 0.16	78 $\pm$ 16.25	84.67 $\pm$ 4.73
Mean $\pm$ SE	0.20 $\pm$ 0.06	0.07 $\pm$ 0.02	0.4 $\pm$ 0.07*	2.74 $\pm$ 1.02	1.45 $\pm$ 0.3	82.13 $\pm$ 3.52	89.6 $\pm$ 2.90

* $p < 0.05$ for single value t test compared to one.

Table 3-5: Summary of motility data for motile velocity of thin filaments reconstituted with $\Delta E224$ mutant tropomyosin vs WT

EXP N	EC ₅₀ Motile velocity, μM WT	EC ₅₀ Motile velocity, μM $\Delta E224$	EC ₅₀ Ratio Mut/WT	n _H Motile velocity WT	n _H Motile velocity $\Delta E224$	Max Sliding speed WT	Max Sliding speed $\Delta E224$
1	0.14 $\pm$ 0.044	0.08	0.57	1.89 $\pm$ 0.81	2.53 $\pm$ 0.29	2.86 $\pm$ 0.46	2.88 $\pm$ 0.1
2	0.27 $\pm$ 0.18	0.078 $\pm$ 0.0063	0.29	0.99 $\pm$ 0.5	2.65 $\pm$ 0.57	2.82 $\pm$ 0.71	2.58 $\pm$ 0.16
3	0.52 $\pm$ 0.0024	0.015 $\pm$ 0.08	0.30	1.18 $\pm$ 0.01	0.65 $\pm$ 0.15	2.73	2.97 $\pm$ 0.08
4	0.29 $\pm$ 0.4	0.05 $\pm$ 0.034	0.17	0.7 $\pm$ 0.56	0.61 $\pm$ 0.44	3.16 $\pm$ 0.46	2.66 $\pm$ 0.96
Mean $\pm$ SE	0.3 $\pm$ 0.08	0.09 $\pm$ 0.02	0.33 $\pm$ 0.08*	1.19 $\pm$ 0.25	1.61 $\pm$ 0.56	2.89 $\pm$ 0.09	2.77 $\pm$ 0.09

Table 3-6: Summary of motility data for percentage motility of thin filaments reconstituted with $\Delta E224$ mutant tropomyosin vs WT

EXP N	EC ₅₀ % Motility, μM WT	EC ₅₀ % Motility, μM $\Delta E224$	EC ₅₀ Ratio Mut/WT	n _H % Motility WT	n _H % Motility $\Delta E224$	Max % Motile WT	Max % Motile $\Delta E224$
1	0.09 $\pm$ 0.01	0.06 $\pm$ 0.03	0.68	3.18 $\pm$ 1.01	1.91 $\pm$ 0.19	84.5 $\pm$ 2.38	92.33 $\pm$ 4.4
2	0.11 $\pm$ 0.12	0.06	0.54	2.98 $\pm$ 1.06	2 $\pm$ 0.26	94.75 $\pm$ 6.18	96.25 $\pm$ 4.35
3	0.31 $\pm$ 0.003	0.08 $\pm$ 0.013	0.25	1.96 $\pm$ 0.05	1.03 $\pm$ 0.15	61 $\pm$ 2.24	61.5 $\pm$ 3.11
4	0.22 $\pm$ 0.011	0.09 $\pm$ 0.001	0.39	1.65 $\pm$ 0.02	1.33 $\pm$ 0.03	82.5 $\pm$ 16.25	84.67 $\pm$ 4.73
Mean $\pm$ SE	0.19 $\pm$ 0.05	0.07 $\pm$ 0.01	0.46 $\pm$ 0.09*	2.44 $\pm$ 0.37	1.57 $\pm$ 0.23	79.81 $\pm$ 7.06	83.15 $\pm$ 7.77

* p<0.05 for single value t test compared to one.

3.5 Discussion

3.5.1 IVMA Advantages and Limitations

The main technique used for the experiments in this thesis was the *in vitro* motility assay (IVMA). This assay relies on the ability of thin filaments to move over a bed of immobilised myosin. A silane-coated glass was the substrate for attaching the HMM, and this replaced the more widely adopted nitrocellulose surface (Marston, 2003). In the case of a nitrocellulose surface, thin filaments are completely stalled at low Ca^{2+} concentrations, so with increasing Ca^{2+} concentrations the filaments start moving at increasing speed, until the point where the Ca^{2+} saturation is reached. On the other hand, on the silane-coated surface there are two populations of filaments, one of which will be moving and the other one not. The moving population will have a motile velocity even at low Ca^{2+} concentration, which most of the times exceeds $2 \mu\text{m}/\text{sec}$. At high Ca^{2+} concentration, the population of moving filaments will be larger, so in this context the percentage motile represents the fragment of thin filaments that are in the “moving” or activated population. The Hill equation can be fitted for both percentage motility and motile velocity data and the EC_{50} can be calculated, which represents the Ca^{2+} sensitivity of the filaments, and also the n_H , which represents the co-operativity of the system. It is noted that when tropomyosin and troponin are added to the system, the percentage motility and the motile velocity increase slightly compared to plain actin, suggesting that the co-operativity of the system has increased. The Ca^{2+} activation curves for both parameters, percentage motility and motile velocity gave very similar results, showing that the IVMA is a reliable assay for the experimental questions. Motile velocity also is dependent of the cross-bridge cycle rate turnover and thus can also give information about the cross-bridge cycle kinetics.

The IVMA relies in a large extent to the purity of the reconstituted proteins, and also in their half-life. For example, both cardiac troponin and actin are unstable (viable for 3 days and four weeks respectively), which can be a limitation to the IVMA experiments. An advantage of the IVMA is that the filaments can be reconstituted using sarcomeric proteins from different species (mouse, human, rabbit or recombinant proteins), which makes IVMA a good model to study the response of different proteins to different Ca^{2+} concentration. Therefore, the IVMA is an

appropriate technique for this thesis, looking at both skeletal muscle diseases and cardiomyopathies. Reconstituting the thin filaments with either skeletal or cardiac troponin means that both of these types of diseases can be efficiently studied. As the rest of the proteins used in IVMA are the same, the effect of the change in one of the proteins or the addition of a drug can be observed. Before each experiment the different components are tested individually, making sure that the assay works correctly prior to performing the experiment and incubating all the proteins together. The different repeats gave consistent results, even though the proteins were not necessarily of the same isolation. This assay has been used in our lab and others for many years and is thus a well-established technique. The assay uses very small amounts of sample and small differences between samples can be detected. However, the fact that that proteins from different species are used for the assay, for example rabbit myosin and actin are used make the IVMA not really representative of the human condition. It would be more appropriate to model human disease if human cardiac myosin and actin could be used. Furthermore, the IVMA only represents individual reconstituted filaments and not a higher-order system. In reality, the filaments all come together to form myofilaments and in their turn they form myocytes that interact with each other so there are more components in the physiological condition. IVMA can be a useful tool to study the molecular mechanism of myopathies but should be combined with other studies to provide a more complete result.

3.5.2 Structure of the tropomyosin-actin interface predicts mutations that cause gain of function in skeletal muscle diseases

Here I show for the first time that it is possible to predict the function of mutations in tropomyosin based on their location. In total six of the motifs where tropomyosin interacts with actin and has a structural motif of two basic or acidic amino acids next to each other were investigated. It was found that all of the mutations lead to a “gain of function” as demonstrated by an increase in Ca^{2+} sensitivity.

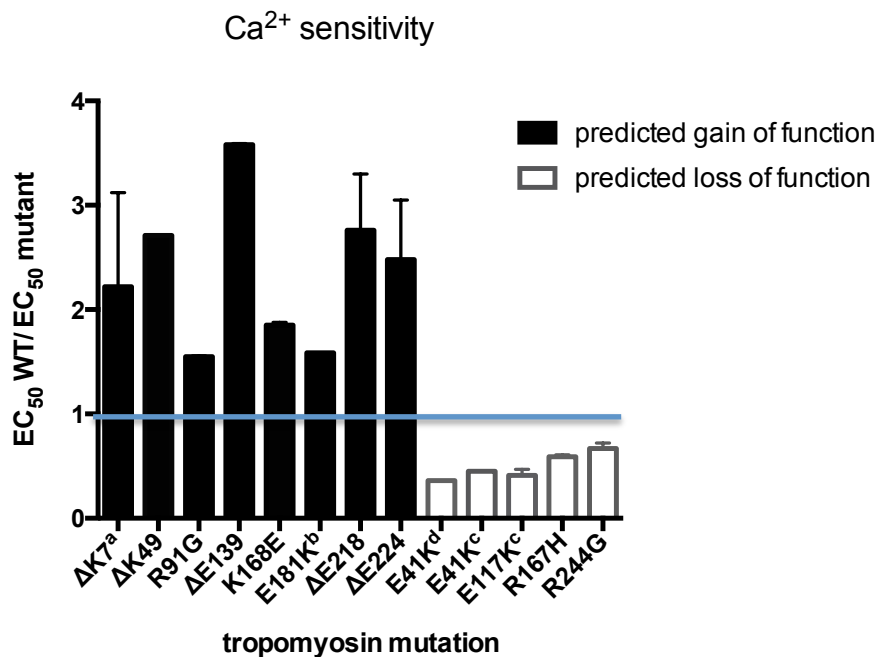
The mutations were ΔK49 , R91G in *TPM2* and K168E , ΔE224 and ΔE218 in *TPM 3* that interact with Asp25 on actin. ΔE139 mutation in *TPM2* interacts with actin on K326 and was also found to be a gain of function mutation. This investigation was inspired by the observation that a mutation in K326 of actin to Asparagine was found in a child with stiff muscles (Jain et al., 2012). The next observation was that the

TPM2 Δ K7 mutation that interacts with Asp25 on actin also leads to a gain of function. Thus, with these two studies, the key initial results were already known when we started. Li et al have calculated the electrostatic interactions of tropomyosin and F-actin, identifying protein complexes that make contacts in both molecules (Li et al., 2011). This enable us to predict similar motifs on tropomyosin interacting with Asp25 and Lys326 in which mutations would also cause gain of function.

Initially four motifs were identified; Δ K49, Δ E139, R91G and K168E. Δ K218 and Δ K224 are novel and were recently identified in two unrelated patients. All of the mutations involve a change in a motif of two basic or acidic amino acids next to each other. There are nine such motifs in all tropomyosin isoforms. Together with other groups, we have identified gain of function mutations in eight of these motifs, and Δ E224 and Δ E218 were the last mutations in such domains to be identified.

The structure-function relationship described here is further supported by the fact that the two mutations *TPM3* R167H and R245G in skeletal muscle myopathy patients that are found adjacent to the predicted gain of function sites produced the exact opposite effect and lead to a loss of function, demonstrated by a decrease in Ca^{2+} sensitivity. Figure 3-12 summarises the predicted gain of function mutations versus loss of function mutations found by us and others predicted by the molecular model by Li et al.

Figure 3-12: Synopsis of the effect of skeletal muscle myopathy mutations on Ca^{2+} sensitivity



Ca^{2+} sensitivity effect on mutations that are predicted to cause gain (black bars) or loss of function (white bars) based on the structure by Li et al, found by us and others. a) From Mokbell et al (Mokbell et al, 2007) b) From Ochala et al (Ochala et al., 2012) c) From Martilla et al (Martilla et al., 2012) d) From Ochala et al (Ochala et al., 2008)

3.5.3 Linkage between the Ca^{2+} sensitivity caused by skeletal muscle myopathy and patient phenotype

Historically, skeletal muscle myopathy patients are characterized by the histopathology of the muscle. In our case, there is no correlation between the disease classification and the gain of function phenotype. Among all the mutations studied by us and others, patients were diagnosed with a range of skeletal muscle myopathies, such as Cap disease, Nemaline Myopathy, CFTD, rod-core disease, distal arthrogryposis and trismus-pseudocamptodactyly. Actually, different diseases can sometimes be found within patients carrying the same mutations. For example, the single mutation ΔK7 studied in 10 families included diagnosis of rod-core disease, CFTD and trismus-pseudocamptodactyly (Davidson et al., 2013, Mokbell et al., 2013). Similarly, as mentioned before, mutations in R167 also give diverse

phenotypes, where patients with Nemaline myopathy, cap disease and mixed pathologies carry mutation in this amino acid.

It was very interesting that in 8 out of 9 sites of tropomyosin/actin interactions with the same motif mutations causing congenital skeletal muscle diseases have been found and all of them are associated with a gain of function at the molecular level. The gain of function observed in the Tpm2.2 and Tpm3.12 mutations described in this study, are sometimes associated with hypercontractile phenotype in patients but not always (Table 3-7). For example, distal arthrogryposis that was found in patients with E181K, R91G and Δ K7 is linked to hypercontractility. It is noted that this study led to the re-assessment of the diagnosis. However, hypercontractility was not found in patients with all the mutations. Patients with three gain of function mutations, Δ K49, Δ E139 and K168E were not diagnosed with a hypercontractile phenotype, but in these cases muscle weakness is not congenital. The reason of these discrepancies might be the progress of the disease and the secondary effects to the muscle. It is noted that these patients were not examined from birth, and a hypocontractile phenotype from birth would clearly exclude a gain of function type of mutation. For example, the patient carrying the Δ E139 mutation was only diagnosed at age 11.

Table 3-7: Summary of tropomyosin mutations causing gain of function

Actin/ Tm interface		Tm Mutation	My contribution
Asp 25	K6/K7	Δ K7 #	
	K48/K49	Δ K49	✓
	R90/R91	R91G #	✓
	K167/K168	K168E	✓
K326	E138/E139	Δ E139	✓
K328	E180/E181	E181K #	
K147	E218/D219	Δ E218 #	✓
	E223/E224	Δ E224 #	✓
	E257/E258	N/A	

Mutations in 8 out of 9 sites of tropomyosin/ actin interaction with the same motif have been found and are all gain of function at the molecular level. # indicates mutations associated with a gain of function (hypercontractile) phenotype in patients. N/A, not applicable.

In that respect, the situation can be compared to Hypertrophic Cardiomyopathy (HCM), which is also associated with increased Ca^{2+} sensitivity. In HCM, symptoms develop later on in life. In HCM a hypercontractile phenotype which is demonstrated by an increase of Ca^{2+} sensitivity and hypertrophy may later on cause hypocontractility as shown in biopsies of interventricular septum of these patients (Elliott and McKenna, 2004). It is possible that this is the case also in patients with gain of function mutations, where the hypercontractile phenotype is masked by the secondary effects of the skeletal muscle disease later on.

A special case has been made for ΔE224 and ΔE218 patients. These two patients showed a severe phenotype of skeletal muscle diseases from a young age. Both patients showed a marked hypercontractile phenotype with muscle stiffness and no indication of muscle weakness. The hypercontractility had major implications in patients respiratory system, especially for the more severe patient. In the case of these two mutations, the IVMA results completely support the clinical characteristics of the patients. Furthermore, an increase in the Ca^{2+} sensitivity was reported in skinned fibres from the ΔE224 patient.

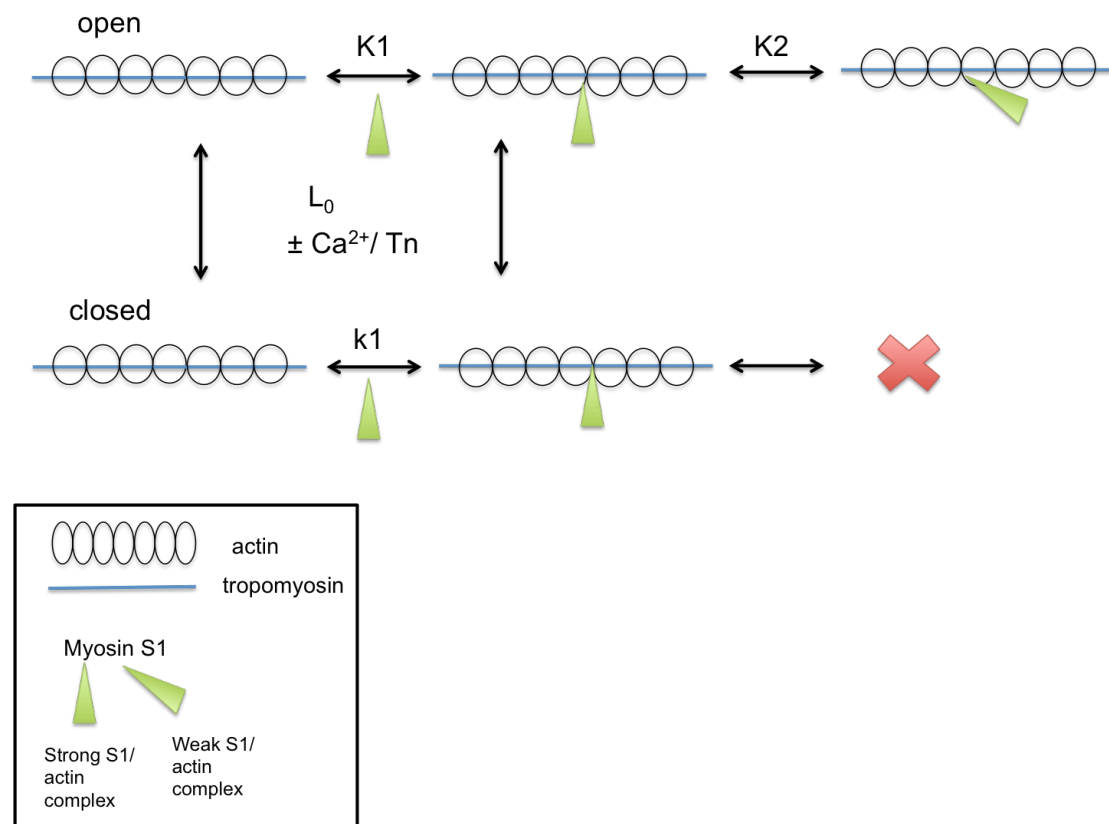
Cross-bridge cycle kinetics was shown to be reduced in ΔE224 skinned fibres. However, the IVMA results suggest that the mutations do not alter the cross-bridge cycle kinetics, as the maximum sliding speed was not affected by the mutations. Unfortunately cross-bridge cycle kinetics could not be studied from the ΔE218 patients but in both patients there was a reduced force generation in skinned fibres (Donkervoort et al., 2015). Histopathologically the two patients could not be assigned to conventional myopathies, as characteristics from different types of myopathies were found in biopsies of both patients, such as nemaline rods, CFTD or cap-like structures.

Based on these results, the interesting question why these patients have a stiff phenotype from birth arises. This might be because the mutations are in *TPM3*, the slow skeletal isoform. *TPM2* is expressed in both fast and slow twitch fibres. Patients with distal arthrogryposis, which is associated with increased Ca^{2+} sensitivity and muscle hypercontractility, have mutations in genes which are encoded specifically to fast switch fibres (Robinson et al, 2007). Fast switch fibres typically develop prenatally and do not change over development. It is possible that Tpm3.12 in which the mutations ΔE224 and ΔE218 mutations are found is predominantly expressed in slow fibres, such as diaphragm and may thus have particular effects on respiration.

3.5.4 Structural analysis of tropomyosin-actin regulation.

The regulation of muscle occurs through interactions between proteins of the thick and thin filaments, myosin, actin, troponin and tropomyosin. A review by Lehrer and Geeves relates the actin/tropomyosin/troponin complex to the haemoglobin/ aspartate transcarbamylase complex, where troponin and Ca^{2+} coordinate transitions between “on” and “off” states of the system. Ca^{2+} sensitivity is therefore a way to identify the position of the equilibrium between the system states (Lehrer and Geeves, 1998). Figure 3-13 illustrates the “on” and “off” states of actin/tropomyosin complex. Li et al have resolved a structure of skeletal muscle tropomyosin on actin and have shown that when actin is free from inhibition by the troponin molecule, tropomyosin localizes to a favourable position on F-actin (Li et al, 2011). The position of tropomyosin to actin is calculated to be at the “closed” or “off” state (Lehman et al., 2009). Energy landscape calculations have shown that tropomyosin is located in a shallow energy well on actin (Lehman et al., 2013). Structural studies have shown that the “open” or “on” state is energetically unfavourable and it becomes favourable in the presence of myosin binding to actin (Behrmann et al., 2012).

Figure 3-13: Tropomyosin and actin complex open and closed states



This picture shows the two states of the tropomyosin/actin complex, open and closed state. $K1$ is the initial actin/binding constant which is independent of myosin, Ca^{2+} and troponin. $K2$ is the isomerization constant, which allows myosin to bind weakly to actin and requires the actin/tropomyosin complex to be in “open” state. Ca^{2+} and troponin additionally affect the L_0 equilibrium constant from “closed” to “open” state.

The mutations in all the cases are found in a location critical to the interaction between actin and tropomyosin in the “off” state according to these structures. We hypothesize that all the gain of function mutations destabilize the “off” state of the system, driving the equilibrium towards the “on” or “open” state of the system. In that way, the protein complex has more probability to be “active” and so a gain of function is observed. The increase of the Ca^{2+} sensitivity as measured by the IVMA can be an indicator of the change between states. In four of the gain of function mutations reported in this study, $\Delta K7$, $\Delta K49$, $R91G$ and $\Delta E139$ it was found to be decreased affinity of tropomyosin and actin (Marttila et al., 2012) (Mokbel et al.,

2013) (Ochala et al., 2008) supporting our hypothesis. Furthermore, energy landscape calculations have shown that in the gain of function mutation in actin K326N, which was the first gain of function mutation reported, the most energetically favourable position of tropomyosin is in the “open” state (Orzechowski et al., 2013).

It is very peculiar that two mutations found just next to the amino acid mutation that causes the gain of function phenotype, are loss of function mutations. Although this result provided the negative control to our study, it is difficult to explain these results. This might have to do with the nature of the interactions between tropomyosin and actin. It would be interesting to look at mutations that occur at the site where tropomyosin interacts with the hydrophobic Pro333. Another unexplained phenomenon is the fact that most of the gain of function mutations are found in tropomyosin and not in actin. This might be related to the fact that only four amino acids in actin interact with tropomyosin, opposed to 18 amino acids in tropomyosin interacting with actin thus there are more probabilities of having a mutation in tropomyosin. Furthermore, most actin mutations do not affect actin-tropomyosin interactions at all (Feng and Marston, 2009).

In summary, these results suggest that it is possible to predict the effect of mutations in tropomyosin in terms of muscle function based on the structure. This is partly because tropomyosin has a repeating like structure with conserved motifs. This study has also important implications for therapy, as it would be possible to treat gain of function mutations with agents that desensitise the thin filaments to Ca^{2+} , such as EGCG, Silybin and their analogues. These drugs and their action on the cardiac muscle proteins will be described in detail in Chapter 5 and 6.

4 Investigation of the effect of Ca^{2+} sensitisers on reconstituted human heart thin filaments

4.1 General Introduction

In the previous chapter I looked at the molecular mechanism of mutations in tropomyosin that cause skeletal muscle myopathies. In the next chapters of the thesis I will investigate the molecular mechanisms of mutations in the cardiac thin filament proteins that cause cardiomyopathies, giving particular importance to the “uncoupling phenomenon”. Dilated Cardiomyopathy (DCM) and Hypertrophic Cardiomyopathy (HCM) are inherited diseases that can arise in the case of mutations on the genes that encode proteins of the contractile apparatus of the myocardium (Kamisago et al., 2000) (Elliott and McKenna, 2004). Some of these mutations that cause HCM or DCM are found in the proteins of the thin filament (troponin, tropomyosin or actin) and investigations of these mutations have shown abnormalities in the Ca^{2+} regulatory system of the thin filament that have been proposed to be the cause of the diseases (Marston, 2011).

HCM mutations lead to an increased Ca^{2+} sensitivity of the myofilament. However, the situation in DCM seems to be more complex because DCM mutations have been found to increase or decrease the Ca^{2+} sensitivity of the myofilament. Recent data have indicated that the common molecular mechanism of DCM is the absence of the response in the Ca^{2+} sensitivity upon troponin I (TnI) phosphorylation (uncoupling). TnI is phosphorylated upon the adrenergic response and this leads to a decrease of the Ca^{2+} sensitivity of the cardiac muscle, so that the muscle is able to relax. In DCM, the change of the Ca^{2+} sensitivity is uncoupled to the TnI phosphorylation (Memo et al., 2013) blunting the response of the diseased heart to adrenaline. The uncoupling phenomenon seems to be also present in some HCM-causing mutations in the thin filament (Messer and Marston, 2014).

The uncoupling phenomenon may thus be a common feature of thin filament cardiomyopathies (see Section 1.5). We hypothesise that there is a specific interaction between the TnI and troponin C (TnC) upon phosphorylation, which transduces the signal from the TnI to the TnC and thus changing the Ca^{2+} dissociation rate, causing the Ca^{2+} sensitivity to decrease. Therefore, we predict that in case of a mutation in the

thin filament, this interaction is abolished and the Ca^{2+} sensitivity cannot change upon phosphorylation, giving rise to the uncoupling phenomenon. The objective of this thesis is to test this hypothesis, and to discover the molecular mechanism that is responsible for the uncoupling. This can be done with the aid of different agents that can bind to troponin and therefore could affect the TnC-TnI interaction in a similar fashion to mutations.

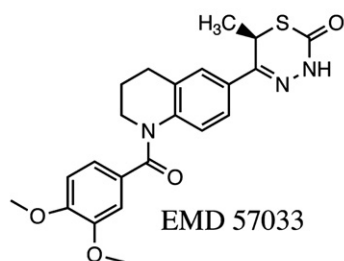
Ca^{2+} sensitisers alter the Ca^{2+} sensitivity of the myofilament by binding to TnC but their effect on coupling of the Ca^{2+} sensitivity with TnI phosphorylation has never been considered. The purpose of this study is to investigate the effects of these molecules that increase the Ca^{2+} sensitivity of the thin filament in relationship with the phosphorylation of TnI, which is a downstream effector of the adrenergic response. The two Ca^{2+} sensitisers used in this study are EMD 57033 and Bepridil. The method by which this question will be investigated is the well-established technique of *in vitro* motility assay (IVMA). As it was demonstrated in the previous chapter, in this method, actin thin filaments slide over a bed of immobilised myosin in response to Ca^{2+} . The two parameters measured is the percentage motility of the sliding actin thin filaments and also the sliding speed, which are indicators of thin filament switching, co-operativity and cross-bridge cycling. The IVMA allows for direct comparison between samples, because they have equal experimental factors during the assay, apart from one factor, so that any differences due to this particular factor (for example presence of mutant protein, phosphorylation status or effect of a drug) can be detected. This method has been extensively used by the Marston's lab to study the uncoupling phenomenon because direct phosphorylation of troponin can be manipulated in donor and mutant thin filaments (Memo et al., 2013, Bayliss et al., 2012b). Phosphorylation studies in the heart samples have been done in the lab by Dr Andrew Messer and thus the phosphorylation status of all the samples is known (Messer, 2007). Moreover, the effect of mutations can be investigated because mutant proteins can be incorporated in the assay instead of native.

4.2 Investigation of the effects of EMD 57033 on human heart thin filaments

4.2.1 Introduction

The first sensitiser studied was EMD 57033 which is a small molecule found to increase the Ca^{2+} sensitivity of the cardiac muscle (Baudenbacher et al., 2008). EMD 57033 is one of the enantiomers of thiadiazinone EMD 53998, a sensitiser drug originally developed by E. Merck (Darmstadt). The other enantiomer of EMD 53998 is EMD 57439 which is a phosphodiesterase inhibitor, making EMD 53998 both a Ca^{2+} sensitiser through the action of EMD 57033 and a phosphodiesterase inhibitor through the action of EMD 57439 (Solaro et al., 1993). The clinical development of EMD 57033 was stopped because of lack of bioavailability of the drug (Kass and Solaro, 2006). However, EMD 57033 is a useful tool to study thin filament properties since it increases the Ca^{2+} sensitivity of the myofilament through interaction with the sarcomeric proteins. The structure of EMD 57033 is shown in Figure 4-1.

Figure 4-1: Chemical structure of EMD 57033



Structure of EMD 57033.

EMD 57033 increases the myofilament sensitivity to Ca^{2+} by binding to the hydrophobic cleft of the cardiac TnC (Wang et al., 2001). This region is close to the N terminal peptide of the TnI where the phosphorylation of Serine 22 and 23 take place, making this region important for the transduction of the Ca^{2+} binding signal to the N-lobe. The fact that EMD 57033 binds close to this TnC-TnI interaction led to the hypothesis that EMD 57033 would have an effect on the coupling between TnI phosphorylation and Ca^{2+} sensitivity. In this section I will describe the effects of

EMD 57033 on phosphorylated, dephosphorylated donor and mutant reconstituted human heart thin filaments.

The mutation studied in this chapter is *TPMI* Glu54Lys (E54K), a mutation that causes DCM in human. This mutation was first identified by Olson et al, in a study screening a big cohort of DCM patients for mutations in the *TPMI* gene. One patient was identified with the E54K mutation and although the patient had a normal echocardiogram at the age of 17, he was diagnosed with DCM at the age of 26 after presenting shortness of breath, oedema, and non-sustained ventricular tachycardia. The patient died at the age of 27, and his father and uncle also died from heart failure at ages of 27 and 49 respectively (Olson et al., 2001). The mutation results in a charge reversal from acidic to basic in a conserved region of the tropomyosin molecule and it is proposed that this disrupts the structural integrity of the sarcomere that would lead to defected cardiomyocyte contraction and myocyte death under stress. A transgenic mouse model carrying the mutation E54K was developed by Rajan et al that developed DCM, further linking the mutation to the disease (Rajan et al., 2007). This mutation was studied in depth in the Marston lab by Dr Mahmooda Mirza and Dr Massimiliano Memo, and it was found that mutant reconstituted thin filaments were insensitive to Ca^{2+} sensitivity change upon TnI phosphorylation (uncoupling) (Memo et al., 2013, Mirza et al., 2005).

4.2.2 *In vitro* motility analysis on EMD 57033 on donor thin filaments

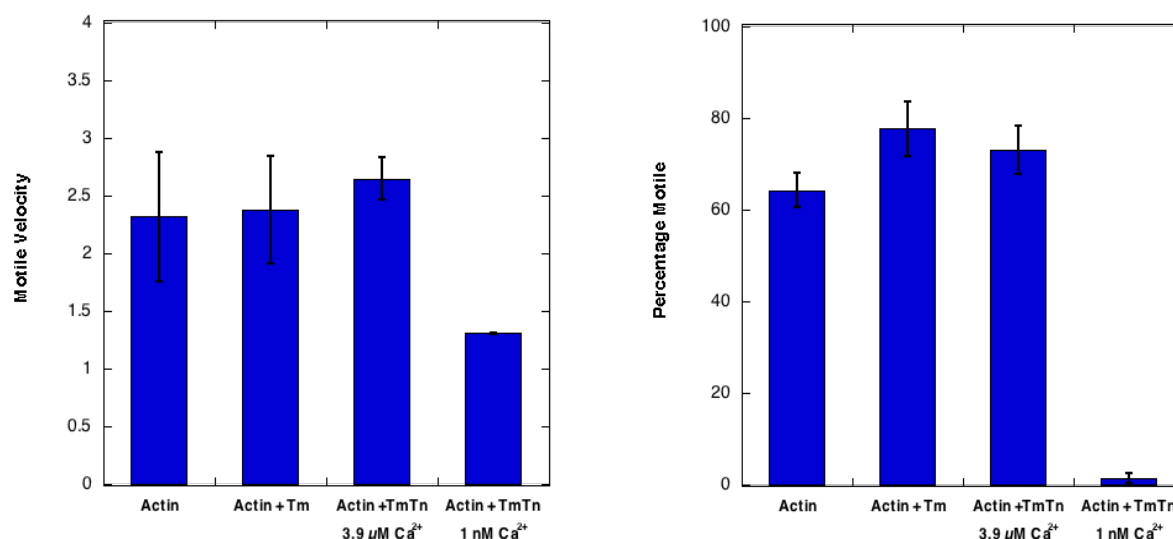
4.2.2.1 Ca^{2+} titration curves using different concentrations of EMD 57033 on donor reconstituted thin filaments.

Thin filaments were reconstituted using troponin from donor heart, wild type Tpm1.1 produced from baculovirus/*sf9* cells and ϕ actin from rabbit skeletal muscle. Full Ca^{2+} activation curves were constructed using donor thin filaments or donor thin filaments in the presence of different concentrations of EMD 57033. The phosphorylation studies performed previously by Dr Andrew Messer have shown that the donor hearts were highly phosphorylated, the level of TnI phosphorylation being 1.62 ± 0.02 mols Pi/mols TnI (Messer et al., 2009).

To construct the filaments 10 nM actin was used together with 60 nM tropomyosin and 60 nM troponin. A titration of troponin from 30 nM- 60 nM has been performed previously by Dr Andrew Messer and Dr Massimiliano Memo and it was found that

60 nM of troponin gives the best switch (Memo, 2012). Rabbit skeletal actin alone gave a motility of 66.9 ± 3.7 % at a speed of 2.33 ± 0.56 $\mu\text{m}/\text{sec}$. With the addition of 60 nM tropomyosin the motility of the filaments increased to 78 ± 6 % at a speed of 2.38 ± 0.47 $\mu\text{m}/\text{sec}$. Upon the addition of cardiac troponin at high Ca^{2+} ($3.9 \mu\text{M} \text{Ca}^{2+}$) thin filaments were motile at a percentage of 73 ± 5 % and velocity of 2.65 ± 0.18 $\mu\text{m}/\text{sec}$. In low Ca^{2+} concentration (1 nM) the thin filaments were switched off with the percentage motile falling to 1.38 ± 1.19 % and the velocity falling to 1.31 ± 0.006 $\mu\text{m}/\text{sec}$. These data are graphically presented in Figure 4-2.

Figure 4-2: *In vitro* motility measurements comparing the addition of troponin and tropomyosin to actin filaments



Means and SE were made from the first two experiments comparing Actin, Actin-Tropomyosin (Tm) and Actin-Tropomyosin-Troponin (Tn). Percentage motility and motile velocity is of donor thin filaments is shown and error bars represent SEM. In the presence of troponin, filaments were Ca^{2+} regulated.

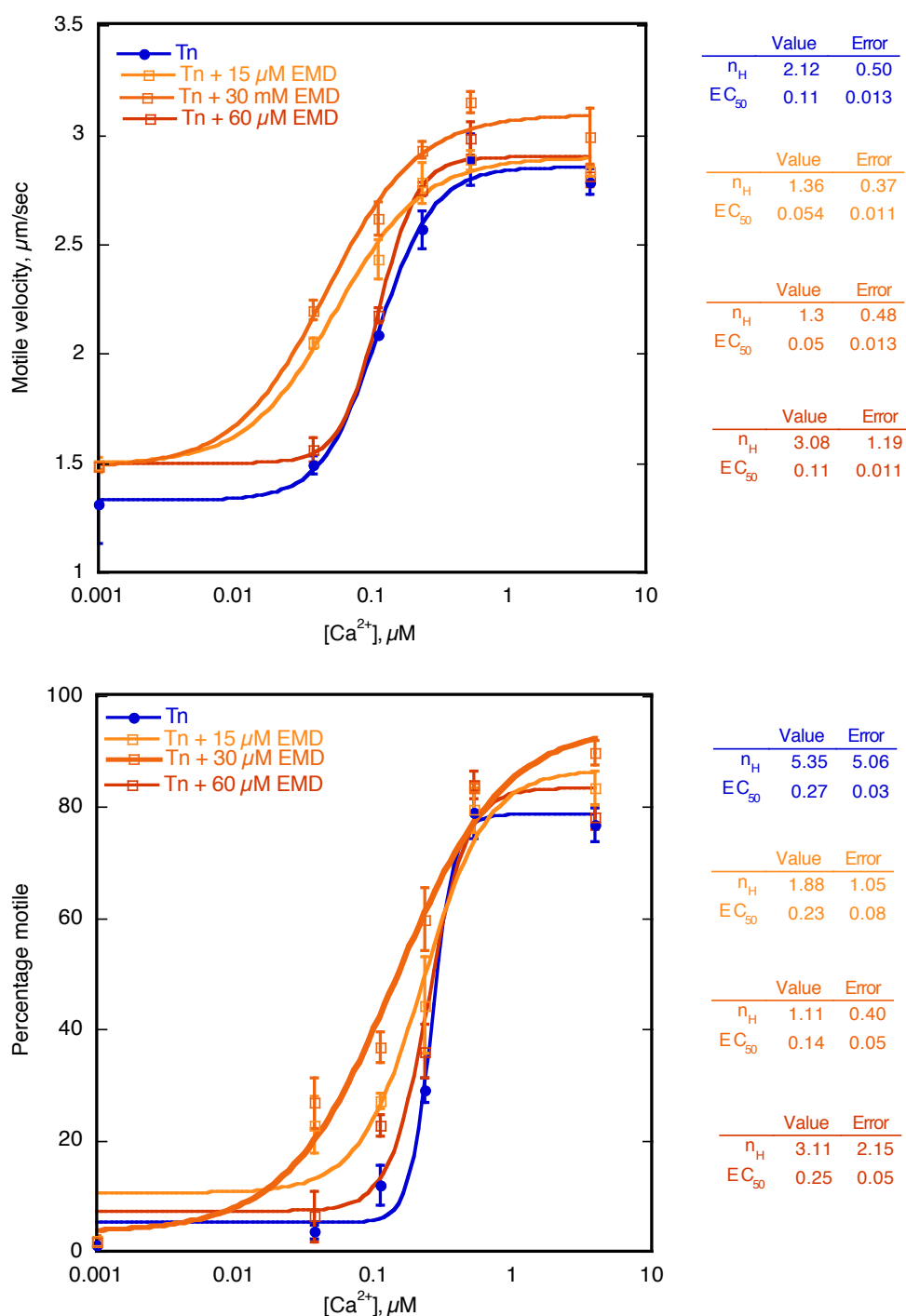
Dr Weihua Song has performed experiments examining the action of EMD 57033 on isolated papillary muscle of wild type and E99K transgenic mice and found a working optimal concentration of $30 \mu\text{M}$ (Song, personal communication). For this reason, Ca^{2+} sensitivity curves were produced using 15 - $60 \mu\text{M}$ EMD 57033 to find the optimal EMD 57033 concentration for the IVMA. EMD 57033 was pre-incubated in different concentrations (15 , 30 and $60 \mu\text{M}$) along with actin (10 nM), tropomyosin (60 nM) and troponin (60 nM). In the same flow cell, donor troponin or donor

troponin + EMD 57033 were added in the two different motility chambers so direct comparison for the presence or absence of drug could be performed for each Ca^{2+} point. Two complete Ca^{2+} titrations were performed comparing thin filaments reconstituted with donor troponin and different concentrations of EMD 57033 (shown in Figure 4-3). Summary of the experiments can be found in APPENDIX.

From the Ca^{2+} curve we can observe that EMD 57033 increases the Ca^{2+} sensitivity of the thin filaments. The maximal calcium sensitivity was seen with 30 μM EMD 57033 ($\text{EC}_{50} = 0.12 \pm 0.02$, $n = 2$ compared to donor troponin with $\text{EC}_{50} = 0.22 \pm 0.04$, $n = 2$) whereas when the EMD 57033 concentration was increased to 60 μM the movement of the filaments was inhibited ($\text{EC}_{50} = 0.19 \pm 0.03$, $n = 2$). EMD 57033 had a positive effect also on the maximum sliding speed of the filaments. 30 μM of EMD 57033 increased the maximum sliding speed from $2.65 \pm 0.13 \mu\text{m/sec}$, $n = 2$ to $2.97 \pm 0.02 \mu\text{m/sec}$, $n = 2$.

However, performing full Ca^{2+} activation curves is very time-consuming. To save time, the drug dose response was detected by selecting the Ca^{2+} concentration that gave the biggest motility difference between the donor thin filaments and thin filaments treated with EMD 57033. The corresponding Ca^{2+} concentration was 0.037 μM Ca^{2+} for donor thin filaments treated with EMD 57033. Different concentrations of EMD 57033 were tested in thin filaments at 0.037 μM Ca^{2+} to create a dose response curve.

Figure 4-3: The effect of EMD 57033 on thin filaments containing donor troponin



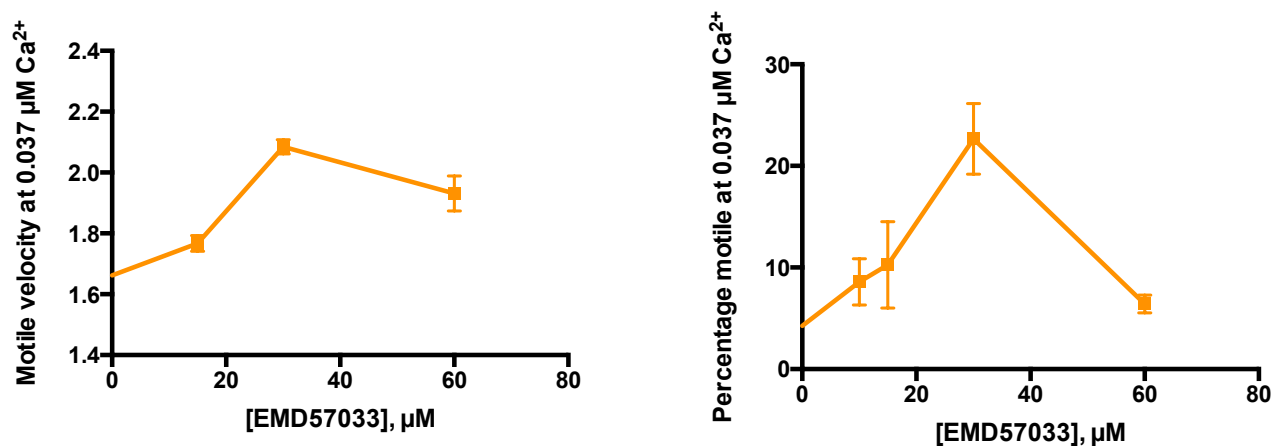
Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native donor troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Blue solid lines represent donor thin filaments and different shades of orange represent thin filaments treated with 15 μM EMD 57033 (light orange) 30 μM EMD 57033 (orange), or 60 μM EMD 57033 (dark orange).

4.2.2.2 Dose response curve of EMD 57033 on donor thin filaments

One Ca^{2+} concentration was used for the dose response curve, $0.037 \mu\text{M}$ Ca^{2+} . For this Ca^{2+} point, different EMD 57033 concentrations were tried out. This specific Ca^{2+} point was also included in the Ca^{2+} titration curves.

Figure 4-4 shows the dose response curve for EMD 57033 for motile velocity and percentage motility of the filaments treated with EMD 57033. We can see from the dose response curves that the maximal EMD 57033 concentration was $30 \mu\text{M}$, which increased the percentage motility of the filaments from $4.25 \pm 0.56 \%$ ($n = 6$) to $22.6 \pm 3.49 \%$ ($n = 5$, $p < 0.05$) and the sliding speed of the filaments from 1.66 ± 0.05 ($n = 7$) to 2.08 ± 0.05 ($n = 6$, $p < 0.05$). When the EMD 57033 concentration was increased to $60 \mu\text{M}$, the percentage motility dropped to $6.42 \pm 0.87 \%$ ($n = 3$) and the motile velocity to 1.93 ± 0.13 ($n = 5$) $\mu\text{m}/\text{sec}$. For the rest of the experiments $30 \mu\text{M}$ EMD 57033 was used, which was the optimal concentration.

Figure 4-4: Dose response curve for EMD 57033 on donor thin filaments



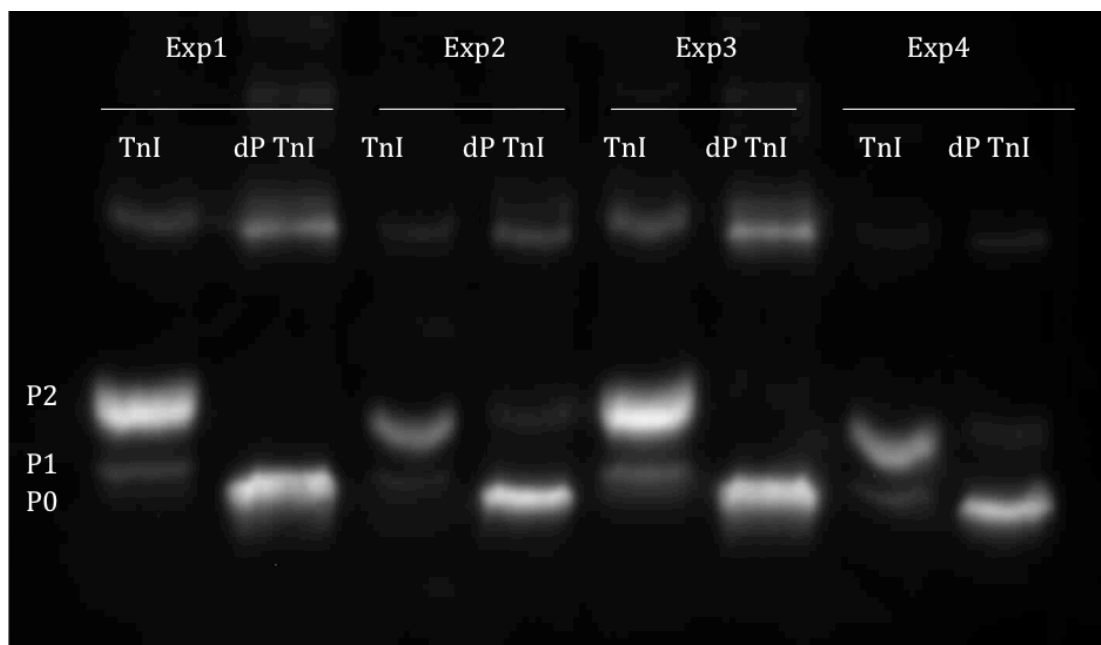
Motile velocity (left) and percentage motility (right) and of thin filaments reconstituted with native donor troponin treated with 0-60 μM EMD 57033 was measured at $0.037 \mu\text{M}$ Ca^{2+} to create a dose response curve. A single EMD 57033 concentration was tested in one motility flow chamber.

4.2.3 The effect of EMD 57033 on the coupling between TnI phosphorylation and Ca^{2+} sensitivity on donor thin filaments.

The effect of EMD 57033 on the relationship between Ca^{2+} sensitivity and TnI phosphorylation was then studied. In order to do so, extracted troponin was dephosphorylated using Shrimp Alkaline Phosphatase (SAP), see Section 2.2.5.2. Then Ca^{2+} sensitivity of the dephosphorylated troponin was tested against donor samples using IVMA.

To make sure that all the proteins used for the experiments had the correct phosphorylation status, a Phos-tag gel was run and a western blot was performed in all the troponin samples. The donor troponin had a high phosphorylation status (1.9 mol Pi/mol TnI) and the dephosphorylated troponin had very low phosphorylation levels (0.08 mol Pi/mol TnI). (Figure 4-5)

Figure 4-5: TnI Western blot of Phos-tag gels



Troponin samples and dephosphorylated troponin samples used for the IVMA for four experiments performed (Exp 1-4) were run on Phos-tag gel, transferred to a membrane and incubated with anti-TnI antibody. This blot shows the different phosphorylation levels of cardiac TnI. TnI can be separated in three phosphorylation states: P0; which indicates dephosphorylated TnI; P1 which indicates the first phosphorylated state of the protein and P2; which indicates fully phosphorylated protein. TnI from Donor troponin was found mostly in the P2 state, whereas TnI from dephosphorylated troponin was found mostly in the P0 state.

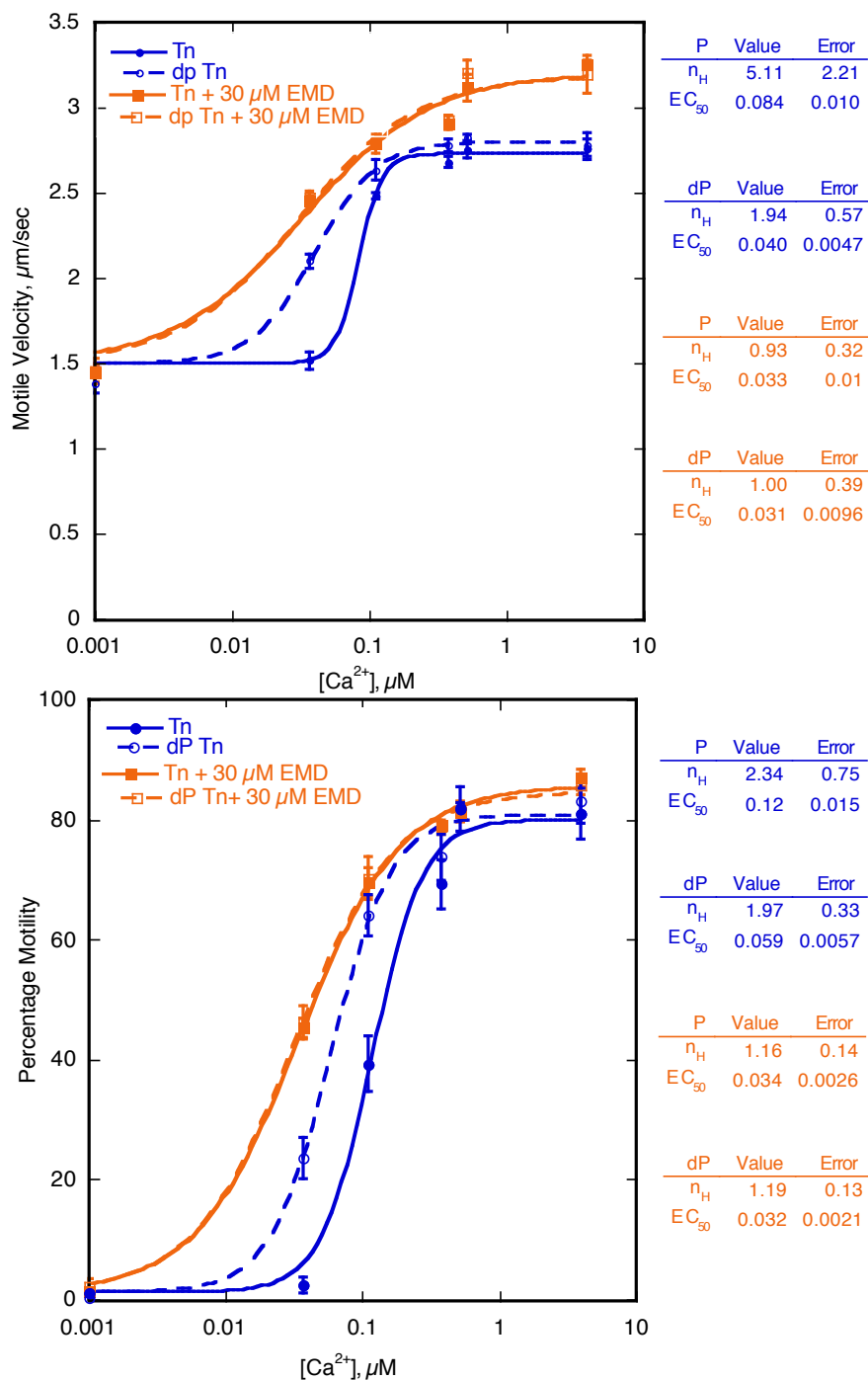
Four Ca^{2+} titration curves were performed with phosphorylated or dephosphorylated donor troponin treated with 30 μM EMD 57033. An example of such a Ca^{2+} titration curve can be found in Figure 4-6. All the titration curves gave similar EC_{50} and n_H values and the data for both percentage motility and motile velocity of the thin filaments are summarized in Table 4-1 and Table 4-2. The raw data for all the experiments are shown in APPENDIX. The EC_{50} and n_H values were also similar between Ca^{2+} curves of percentage motility and motile velocity. The Ca^{2+} sensitivity of dephosphorylated thin filaments was significantly decreased compared to phosphorylated thin filaments (mean ratio $\text{EC}_{50}\text{P}/\text{EC}_{50}\text{dP} = 1.77 \pm 0.13$, $p < 0.01$, $n = 4$ for percentage motility and 1.87 ± 0.12 , $p < 0.01$, $n = 4$ for motile velocity) in accordance with previous measurements (Messer et al., 2007).

As expected, 30 μM EMD 57033 increased the Ca^{2+} sensitivity of phosphorylated thin filaments as shown previously, from 0.16 ± 0.03 to $0.057 \pm 0.01 \mu\text{M}$ ($p < 0.05$, $n = 4$) for percentage motility and from 0.14 ± 0.049 to $0.038 \pm 0.006 \mu\text{M}$ ($p < 0.01$, $n = 4$) for motile velocity.

Interestingly, we observe that 30 μM of EMD 57033 increased the Ca^{2+} sensitivity of phosphorylated thin filaments more than this of dephosphorylated troponin (mean ratio $\text{EC}_{50} \text{P}/\text{EC}_{50} \text{P} + \text{EMD 57033} = 3.07 \pm 0.71$, $p < 0.001$, $n = 4$ in phosphorylated compared with a mean ratio of $\text{EC}_{50} \text{dP}/\text{EC}_{50} \text{dP} + \text{EMD 57033} = 1.83 \pm 0.36$, $p = 0.07$, $n = 4$ in dephosphorylated thin filaments for percentage motility). Consequently, in the presence of 30 μM EMD 57033 the Ca^{2+} sensitivity of the filaments became uncoupled from the TnI phosphorylation (mean ratio $\text{EC}_{50} \text{P} + \text{EMD 57033}/\text{EC}_{50} \text{dP} + \text{EMD 57033} = 1.08 \pm 0.03$, $p = 0.06$, $n = 4$ for percentage motility and $\text{EC}_{50} \text{P} + \text{EMD 57033}/\text{EC}_{50} \text{dP} + \text{EMD 57033} = 1.07 \pm 0.038$, $p = 0.06$, $n = 4$ for motile velocity).

Treatment with 30 μM EMD 57033 also decreased the n_H of phosphorylated thin filaments. EMD 57033 increased sliding speed slightly at saturating Ca^{2+} concentration in phosphorylated thin filaments and in dephosphorylated thin filaments by $18.4 \pm 4.1\%$ ($p = 0.02$, $n = 4$).

Figure 4-6: Ca^{2+} activation curves comparing donor thin filaments containing phosphorylated or dephosphorylated troponin treated with 30 μM EMD 57033



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Blue lines represent donor thin filaments and orange lines represent thin filaments treated with 30 μM EMD 57033.

Table 4-1: Ca^{2+} sensitivity data for motile velocity of phosphorylated vs dephosphorylated thin filaments treated with 30 μM EMD 57033

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
Donor	P	0.14 ± 0.049 (4)	1.87 ± 0.12 (4) $\dagger\dagger$	3.42 ± 0.91 (4)	2.93 ± 0.06 (4)
Donor	dP	0.08 ± 0.04 (4)		1.42 ± 0.32 (4)	2.82 ± 0.05 (4)
EMD 57033	P	0.038 ± 0.006 (4)	1.07 ± 0.038 (5)	0.87 ± 0.027 (4) $**$	3.068 ± 0.082 (4)
EMD 57033	dP	0.035 ± 0.0051 (4)		0.94 ± 0.028 (4)	3.06 ± 0.065 (4)

Table 4-2: Ca^{2+} sensitivity data for percentage motility of phosphorylated vs dephosphorylated thin filaments treated with 30 μM EMD 57033

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
Donor	P	0.16 ± 0.03 (4)	1.77 ± 0.13 (4) $\dagger\dagger$	2.08 ± 0.14 (4)	79.69 ± 1.06 (4)
Donor	dP	0.091 ± 0.019 (4)		1.51 ± 0.25 (4)	79.38 ± 2.05 (4)
EMD 57033	P	0.057 ± 0.01 (4)	1.08 ± 0.026 (5)	1.26 ± 0.07 (4) $*$	82.56 ± 2.07 (4)
EMD 57033	dP	0.053 ± 0.01 (4) $\dagger$		1.34 ± 0.14 (4)	81.81 ± 2.14 (4)

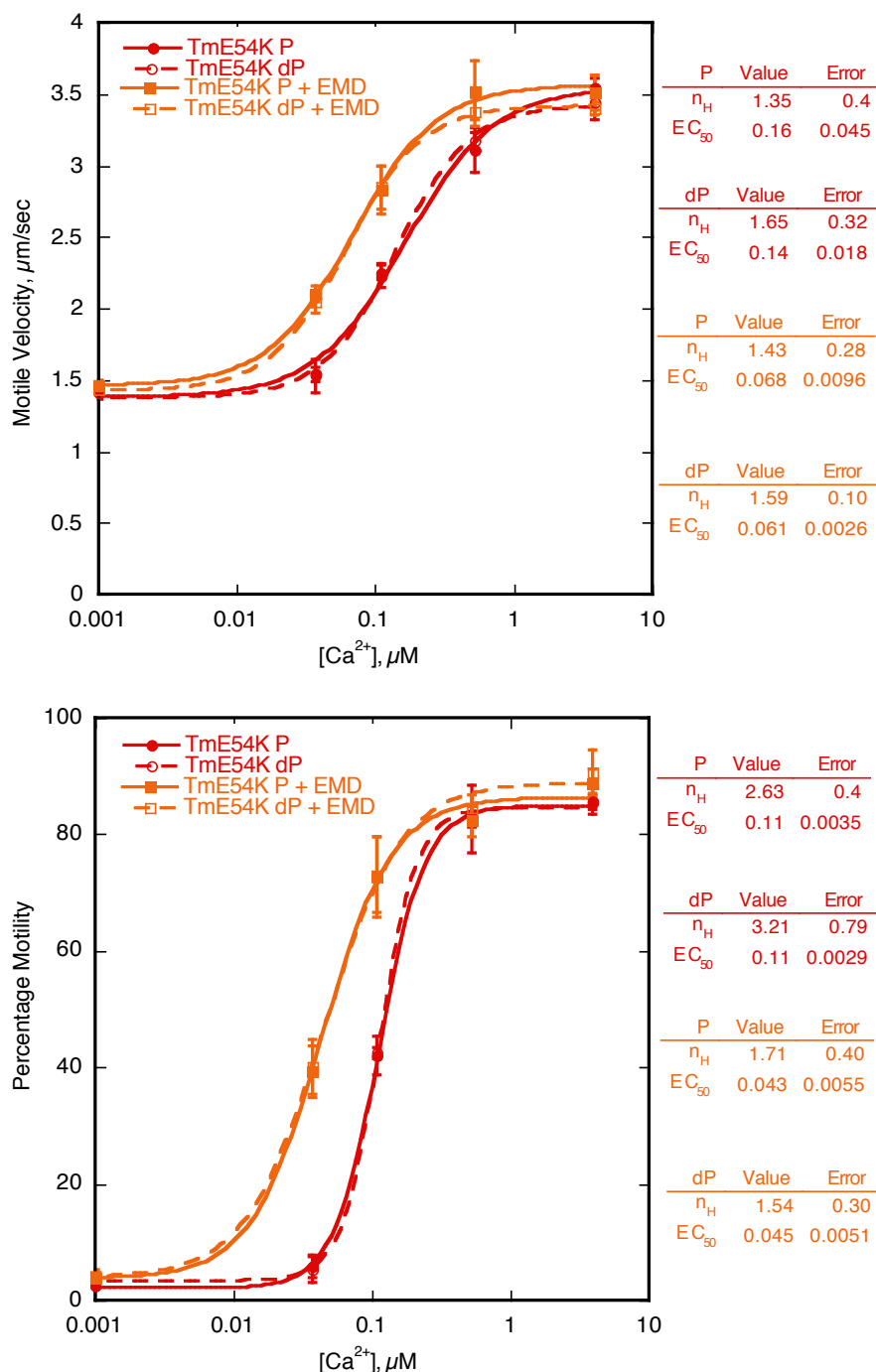
$**$ $p < 0.01$; for presence and absent of reagent. $\dagger$ $p < 0.05$, $\dagger\dagger$ $p < 0.01$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the number of experiments.

4.2.4 Investigation of the effects of EMD 57033 on DCM E54K mutant human thin filaments.

Next, the effect of EMD 57033 on *TPMI* E54K mutant phosphorylated and dephosphorylated thin filaments was studied. Previously, Dr Massimiliano Memo has found that in E54K mutant filaments the Ca^{2+} sensitivity is uncoupled from TnI phosphorylation (Memo et al., 2013). Thin filaments were reconstituted with rabbit skeletal muscle actin (10 nM), human cardiac troponin (60 nM) and 60 nM E54K tropomyosin purified from baculovirus/*sf9* pellets. Three experiments were performed comparing phosphorylated or dephosphorylated E54K reconstituted thin filaments treated with 30 μM EMD 57033. An example of such an experiment is shown in Figure 4-7. Summary of the data for the percentage motility of the thin filaments and the motile velocity can be found in Table 4-3 and Table 4-4.

30 μM EMD 57033 increased the Ca^{2+} sensitivity of phosphorylated E54K thin filaments by 3.07 ± 0.35 fold for percentage motility ($p < 0.01$, $n = 3$) and by 3.41 ± 0.6 fold for motile velocity ($n = 3$). The Ca^{2+} sensitivity was independent of TnI phosphorylation in the E54K thin filaments and remained independent after the treatment with 30 μM EMD 57033 (mean ratio $\text{EC}_{50}\text{P} + \text{EMD 57033} / \text{EC}_{50}\text{dP} + \text{EMD 57033} = 1.00 \pm 0.013$, $p = 0.38$, $n = 3$ for percentage motility, mean ratio $\text{EC}_{50}\text{P} + \text{EMD 57033} / \text{EC}_{50}\text{dP} + \text{EMD 57033} = 1.06 \pm 0.05$, $p = 0.94$, $n = 3$ for motile velocity)

Figure 4-7: Ca^{2+} activation curves comparing thin filaments containing phosphorylated or dephosphorylated troponin and E54K mutant tropomyosin treated with EMD 57033



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin and mutant E54K tropomyosin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent E54K thin filaments and orange lines represent thin filaments treated with 30 μM EMD 57033.

Table 4-3: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E54K mutant thin filaments treated with EMD 57033

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E54K	P	0.12 ± 0.024 (3)	1.03 ± 0.075 (3)	2.39 ± 0.63 (3)	3.20 ± 0.23 (3)
E54	dP	0.11 ± 0.015 (3)		2.24 ± 0.66 (3)	3.21 ± 0.21 (3)
EMD 57033	P	0.053 ± 0.008 (3)	1.06 ± 0.05 (3)	2.07 ± 0.035 (3)	3.24 ± 0.17 (3)
EMD 57033	dP	0.051 ± 0.0056 (4)		2.21 ± 0.39 (3)	3.2 ± 0.11 (3)

Table 4-4: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E54K mutant thin filaments treated with $30 \mu\text{M}$ EMD 57033

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E54K	P	0.13 ± 0.014 (3)	1.08 ± 0.06 (3) $\dagger\dagger$	2.33 ± 0.14 (3)	85.58 ± 3.89 (3)
E54K	dP	0.11 ± 0.008 (3)		2.85 ± 0.56 (3)	84.83 ± 3.19 (3)
EMD 57033	P	0.041 ± 0.0018 (3) **	1 ± 0.013 (3)	2.38 ± 0.46 (3)	85.33 ± 3.8 (3)
EMD 57033	dP	0.04 ± 0.002 (3) **		2.07 ± 0.31 (3)	86 ± 3.68 (3)

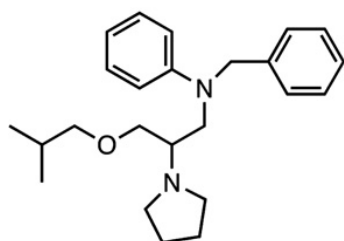
****** $p < 0.01$ for presence and absent of reagent. $\dagger\dagger$ $p < 0.01$ for the effect of phosphorylation. p values were calculated using one- way ANOVA. p values for the ratios were calculated using value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the number of experiments.

4.3 The effect of Bepridil on human heart thin filaments

4.3.1 Introduction

The other Ca^{2+} sensitiser used in this study is Bepridil (trade name Vascor) that acts as a Ca^{2+} channel blocker and calmodulin antagonist and was used for the treatment of hypertension and chronic stable angina. It is no longer for sale in the US, as it caused ventricular arrhythmias as a side effect (Drugbank). Bepridil has the ability to penetrate the cardiomyocytes and bind to the thin filament of the sarcomere. The chemical structure of Bepridil is shown in Figure 4-8. Early studies have found that it binds to the N-terminal region of the TnC with a K_D of around 10 μM and as a consequence promotes the Ca^{2+} release from the N terminal domain of the cTnC (MacLachlan et al., 1990). Later studies have found that Bepridil has actually three binding sites on TnC (Kleerekoper et al., 1998, Abusamhadneh et al., 2001) but it is possible that TnI binding can inhibit two of the sites, because an NMR study by Abusamhadneh et al found only one Bepridil binding site in the cTnC N terminal region (Abusamhadneh, Abbott et al. 2001). The region where Bepridil binds is important because it is at the site where TnI₁₄₇₋₁₆₃ switch region acts, that is responsible for promoting the tropomyosin-actin interaction and consequently the muscle contraction in the presence of Ca^{2+} . Similarly to EMD 57033, the effect of Bepridil on the coupling between the phosphorylation of TnI peptide and Ca^{2+} release has never been studied before. In this section I will describe the effect of Bepridil in terms of Ca^{2+} sensitivity and TnI phosphorylation in donor and E54K mutant thin filaments.

Figure 4-8: Chemical structure of Bepridil

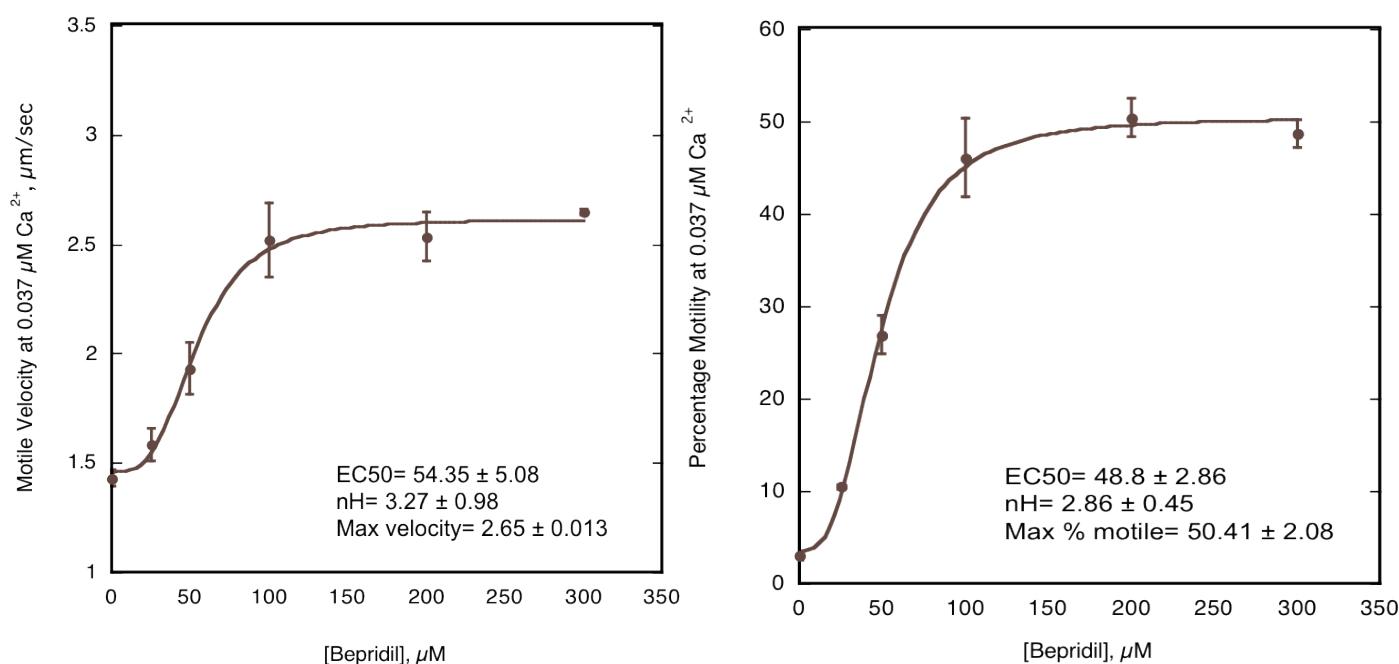


Structure and chemical groups of Bepridil.

4.3.2 Dose response curve of Bepridil on donor human heart thin filaments

In the previous section I described an assay in the *in vitro* motility system for performing dose responses for the drugs, using a single Ca^{2+} concentration. The effect of Bepridil was tested by this method in reconstituted thin filaments with 60 nM donor troponin, 60 nM human cardiac tropomyosin and 10 nM rabbit skeletal actin. 0, 25, 50, 100, 200 and 300 μM of Bepridil were screened, at the same Ca^{2+} concentration as used for the dose response of EMD 57033, 0.037 μM Ca^{2+} and the data are shown in Figure 4-9. The resulting dose response curve was co-operative and thus the Hill equation was fitted to the data to determine the EC_{50} and the Hill coefficient. The optimal Bepridil concentration is 100 μM and the EC_{50} of the dose response curve is 48.8 ± 2.86 μM for the percentage motility and 54.35 ± 5.08 μM for the motile velocity of the filaments.

Figure 4-9: Bepridil dose response curve on reconstituted donor thin filaments

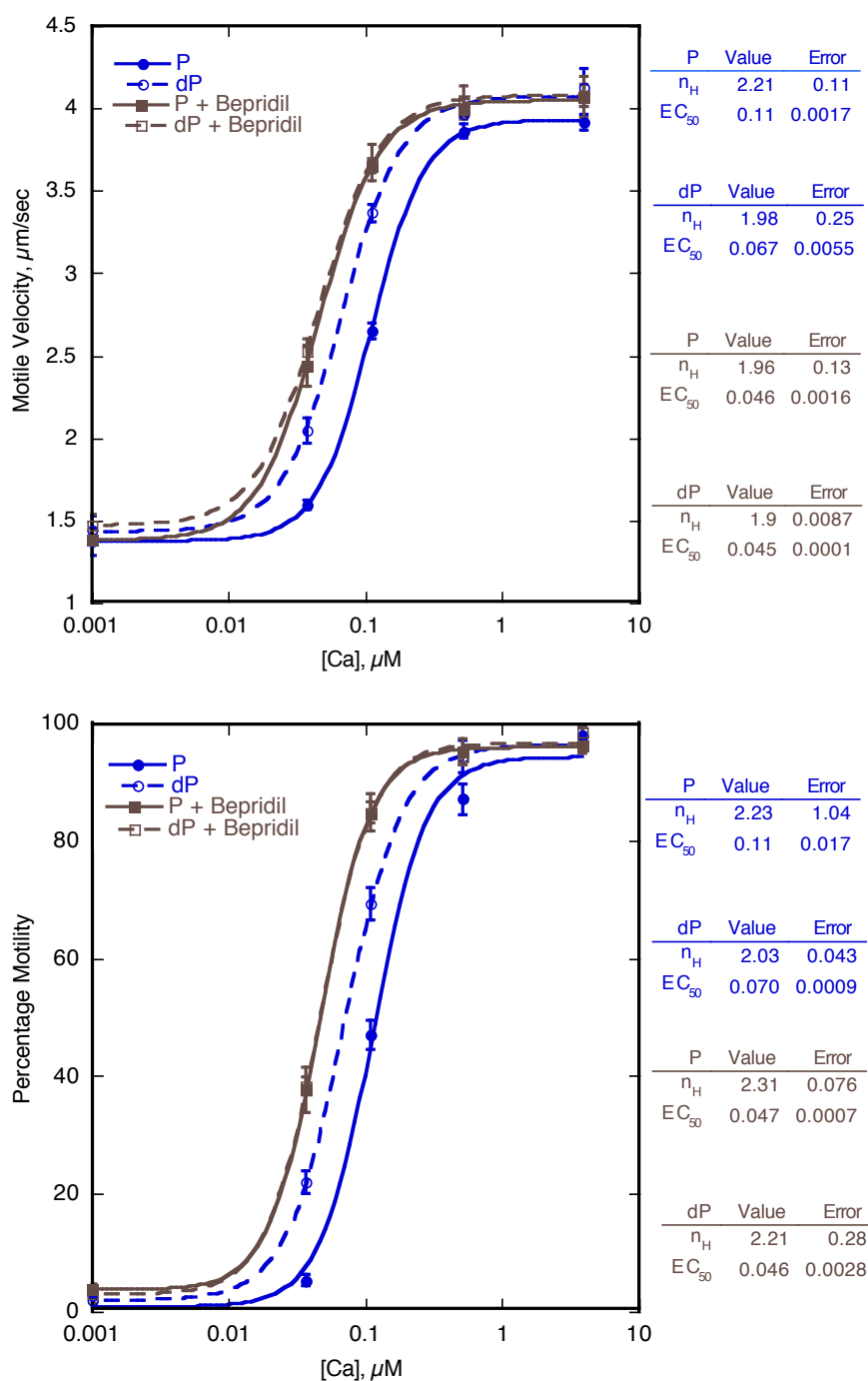


Motile velocity (left) and percentage motility (right) and of thin filaments reconstituted with native donor troponin treated with 0-300 μM Bepridil was measured at 0.037 μM Ca^{2+} to create a dose response curve. A single Bepridil concentration was tested in one motility flow chamber. The Hill equation was fitted to the data to calculate the EC_{50} and n_H of the dose response curve.

4.3.3 The effect of Bepridil on the coupling between phosphorylated and dephosphorylated donor thin filaments

Ca^{2+} activation curves were then performed using phosphorylated or dephosphorylated donor thin filaments to observe the effect of Bepridil on the relationship between phosphorylation and Ca^{2+} sensitivity. Since the optimal Bepridil concentration was 100 μM , this concentration was used for the rest of the experiments. Thin filaments were reconstituted with 60 μM phosphorylated or dephosphorylated troponin extracted from donor heart, 60 μM human cardiac tropomyosin and 10 μM rabbit skeletal actin. Six Ca^{2+} activation curves were performed with phosphorylated and dephosphorylated thin filaments treated or not with Bepridil. An example of such is shown in Figure 4-10. A summary of all the experiments can be found in Table 4-5 and Table 4-6. The raw data for all the experiments are shown in APPENDIX.

Figure 4-10: The effect of Bepridil on phosphorylated and dephosphorylated donor thin filaments



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Blue lines represent donor thin filaments and brown lines represent thin filaments treated with 100 μM Bepridil.

Table 4-5: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated donor thin filaments treated with 100 μM Bepridil

Treatment		EC_{50} , μM	Ratio EC_{50} P/dP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
Donor	P	0.13 ± 0.011 (6)	1.67 ± 0.12 †	2.1 ± 0.24	2.05 ± 0.18 (6)
Donor	dP	0.077 ± 0.0056 (6) †		1.33 ± 0.15	2.13 ± 0.20 (6)
Bepridil	P	0.046 ± 0.007 (6) **	0.98 ± 0.11	1.36 ± 0.14	2.35 ± 0.20 (6)*
Bepridil	dP	0.047 ± 0.0037 (6) **		1.43 ± 0.16	2.24 ± 0.16 (6)

Table 4-6: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated donor thin filaments treated with 100 μM Bepridil

Treatment		EC_{50} , μM	Ratio EC_{50} P/dP	n_H	Max % motile
Donor	P	0.13 ± 0.005 (6)	1.73 ± 0.03 ††	2.51 ± 0.28 (6)	93 ± 1.93 (6)
Donor	dP	0.072 ± 0.002 (6)††		1.96 ± 0.08 (6)	95.13 ± 1 (6)
Bepridil	P	0.046 ± 0.001 (6)**	1.03 ± 0.01	1.97 ± 0.10 (6)	95.6 ± 0.83 (6)
Bepridil	dP	0.045 ± 0.001 (6)**		2.03 ± 0.13 (6)	96.46 ± 0.83 (6)

* $p < 0.05$, ** $p < 0.01$; for presence and absent of reagent. † $p < 0.05$, †† $p < 0.01$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the number of experiments.

From the Ca^{2+} sensitivity curves it can be observed that there is a decrease in Ca^{2+} sensitivity upon phosphorylation of TnI in the donor troponin, as expected. The Ca^{2+} sensitivity change was from $\text{EC}_{50} = 0.13 \pm 0.005$ for phosphorylated to $\text{EC}_{50} = 0.072 \pm 0.002$ for dephosphorylated donor thin filaments for percentage motility ($n = 6$, $p < 0.01$) and from $\text{EC}_{50} = 0.13 \pm 0.011$ for phosphorylated to $\text{EC}_{50} = 0.072 \pm 0.002$ for dephosphorylated donor thin filaments for motile velocity ($n = 6$, $p < 0.05$). It is noted that the EC_{50} and n_H values were very similar for the percentage motility Ca^{2+} activation curves and the motile velocity curves. 100 μM of Bepridil increased the Ca^{2+} sensitivity of phosphorylated thin filaments by 2.8 ± 0.14 fold ($p < 0.0001$, $n = 6$) and of dephosphorylated donor thin filaments by 1.6 ± 0.06 fold ($p < 0.01$, $n = 6$) for percentage motility.

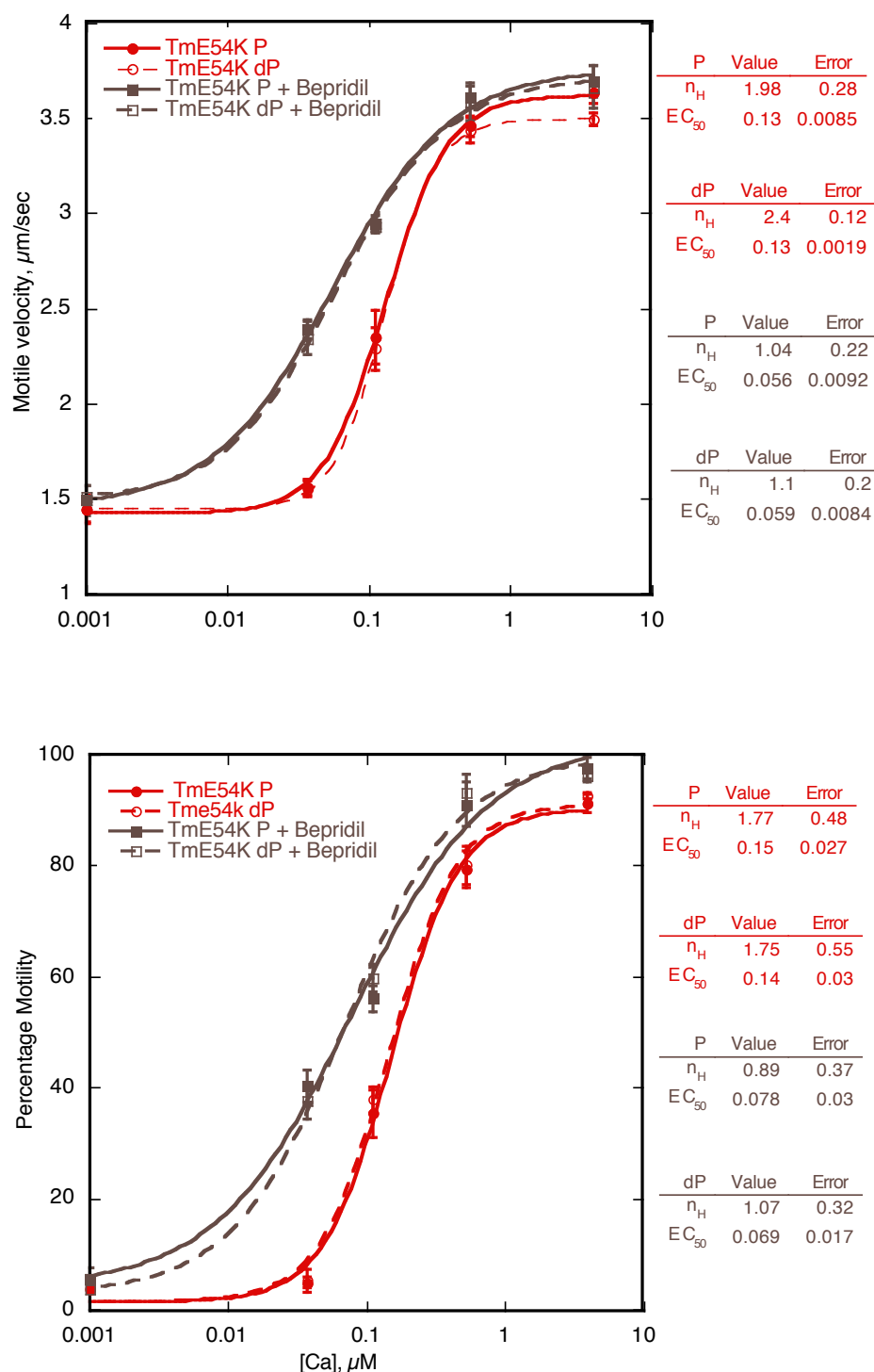
Ca^{2+} sensitivity curves that are different in phosphorylated and dephosphorylated donor troponin were found to be the same in phosphorylated and dephosphorylated donor thin filaments treated with Bepridil. Thus, the Ca^{2+} sensitivity is uncoupled from the TnI phosphorylation in thin filaments treated with 100 μM Bepridil (mean ratio $\text{EC}_{50} \text{ P} + \text{Bepridil} / \text{EC}_{50} \text{ unP} + \text{Bepridil} = 1.08 \pm 0.03$, $p = 0.04$, $n = 6$ for percentage motility and $\text{EC}_{50} \text{ P} + \text{Bepridil} / \text{EC}_{50} \text{ unP} + \text{Bepridil} = 0.98 \pm 0.11$, $p = 0.84$, $n = 6$ for motile velocity). Bepridil had no effect on the n_H for either phosphorylated or dephosphorylated donor thin filaments. Finally, Bepridil increased the motile velocity of the phosphorylated donor thin filaments at saturating Ca^{2+} concentrations significantly (by $14.8 \pm 3.2\%$, $p = 0.006$, $n = 6$) but not in dephosphorylated thin filaments (velocity only increased by $7.2 \pm 4.6\%$, $p = 0.18$, $n = 6$). Therefore, similar to EMD 57033, Bepridil increased the Ca^{2+} sensitivity of donor thin filaments and also caused uncoupling of the Ca^{2+} sensitivity from TnI phosphorylation.

4.3.4 The effect of Bepridil on DCM E54K mutant thin filaments

In this section the effect of Bepridil on DCM mutant thin filaments will be investigated. Thin filaments were reconstituted with 60 nM troponin extracted from donor heart, 60 nM Tpm1.1 E54K from baculovirus/*sf9* and 10 μM actin. 2 Ca^{2+} activation curves were performed using 100 μM Bepridil on phosphorylated and dephosphorylated DCM E54K mutant thin filaments. An example of such curve is shown in Figure 4-11. Table 4-7 and Table 4-8 show the summary of the two

experiments performed. As we can see from these results, Bepridil increased the Ca^{2+} sensitivity of phosphorylated and dephosphorylated thin filaments equally (from $\text{EC}_{50} = 0.14 \pm 0.008$ to $\text{EC}_{50} = 0.063 \pm 0.001$, $n = 2$ for phosphorylated and from $\text{EC}_{50} = 0.14 \pm 0.014$ to $\text{EC}_{50} = 0.061 \pm 0.008$, $n = 2$ for dephosphorylated thin filaments, for percentage motility parameter). The Ca^{2+} sensitivity was uncoupled from TnI phosphorylation in E54K mutant thin filaments and remained uncoupled for the E54K mutant thin filaments treated with Bepridil (mean ratio $\text{EC}_{50} \text{ P/dP} = 1.05 \pm 0.02$, $n = 2$, $\text{EC}_{50} \text{ P} + \text{Bepridil} / \text{dP} + \text{Bepridil} = 1.01 \pm 0.12$, $n = 2$ for percentage motility and mean ratio $\text{EC}_{50} \text{ P/dP} = 1.062 \pm 0.038$, $n = 2$, $\text{EC}_{50} \text{ P} + \text{Bepridil} / \text{EC}_{50} \text{ dP} + \text{Bepridil} = 1.057 \pm 0.11$ for motile velocity). In conclusion, Bepridil showed the same effects as EMD 57033 in both donor and E54K mutant thin filaments.

Figure 4-11: Ca^{2+} activation curve of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 μM Bepridil



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin and mutant E54K tropomyosin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent E54K thin filaments and brown lines represent E54K thin filaments treated with Bepridil.

Table 4-7: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 μM Bepridil

Treatment	EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity
E54K P	0.14 ± 0.06 (2)	1.062 ± 0.038 (2)	1.72 ± 0.26 (2)	3.54 ± 0.01 (2)
E54K dP	0.13 ± 0.0013 (2)		1.95 ± 0.45 (2)	3.45 ± 0.045 (2)
Bepridil P	0.071 ± 0.015 (2)	1.057 ± 0.11 (2)	0.85 ± 0.2 (2)	3.71 ± 0.095 (2)
Bepridil dP	0.067 ± 0.0071 (2)		1.01 ± 0.09 (2)	3.67 ± 0.01 (2)

Table 4-8: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E54K mutant thin filaments treated with 100 μM Bepridil

Treatment	EC_{50} , μM	Ratio EC_{50} P/dP	n_H	Max % motile
E54K P	0.14 ± 0.008 (2)	1.05 ± 0.02 (2)	1.93 ± 0.16 (2)	93.13 ± 1.85 (2)
E54K dP	0.14 ± 0.014 (2)		2.24 ± 0.49 (2)	92.38 ± 0.13 (2)
Bepridil P	0.063 ± 0.001 (2)	1.01 ± 0.12 (2)	1.45 ± 0.56 (2)	95.5 ± 2 (2)
Bepridil dP	0.061 ± 0.008 (2)		1.3 ± 0.23 (2)	95.38 ± 0.88 (2)

Values for Ca^{2+} sensitivity data for motile velocity and percentage motility in reconstituted E54K thin filaments. EC_{50} was rounded to two significant figures. In brackets the n number. Note that statistical analysis could not be performed due to lack of experimental repeats.

4.4 Discussion

4.4.1 Studying the mechanism of the uncoupling phenomenon using pharmacological agents

The objective of this chapter was to investigate the effects of two known Ca^{2+} sensitisers that bind to TnC on human muscle heart thin filaments in relationship to TnI phosphorylation. This contributes to the overall objective of my PhD thesis to investigate the molecular mechanism of the uncoupling phenomenon. Is the TnC-TnI N terminal extension interaction responsible for the uncoupling phenomenon seen in HCM and DCM mutations? Can agents that bind to TnC and affect the TnC-TnI interaction have an effect on the coupling between TnI phosphorylation and Ca^{2+} sensitivity? As we observed from the previous chapter, the IVMA is a very reliable assay for this investigation because the Ca^{2+} sensitivity of reconstituted thin filaments can be accurately measured and thus the molecular mechanism can be established. Functional cardiac troponin can be successfully extracted from samples of human heart and the phosphorylation status can also be manipulated using phosphatases or kinases (see Section 2.2.5). The uncoupling phenomenon was itself discovered using this assay (Memo et al, 2013, Bayliss et al, 2012) and the effect on the Ca^{2+} regulation of a single parameter (for example a mutation, effect of the phosphorylation status or addition of a drug) could be directly observed.

4.4.2 Ca^{2+} sensitisers mimic the effects of HCM mutations

The first agents that bind to TnC that I looked at were two Ca^{2+} sensitisers, EMD 57033 and Bepridil. The effect of EMD 57033 and Bepridil was first tested in thin filaments reconstituted with donor troponin. EMD 57033 increased the Ca^{2+} sensitivity of the thin filaments, in agreement with previous measurements showing the action of EMD 57033 as a Ca^{2+} sensitiser on myofilaments in mice (Baudenbacher et al., 2008). Bepridil also increased the Ca^{2+} sensitivity of the native donor thin filaments by 3 fold. In addition, here for the first time I showed that the Ca^{2+} sensitisers EMD 57033 and Bepridil are able to uncouple the Ca^{2+} sensitivity response to the TnI phosphorylation by increasing the Ca^{2+} sensitivity of phosphorylated thin filaments more than the Ca^{2+} sensitivity of the dephosphorylated thin filaments. The uncoupling phenomenon has been proposed to be causative of DCM and has also

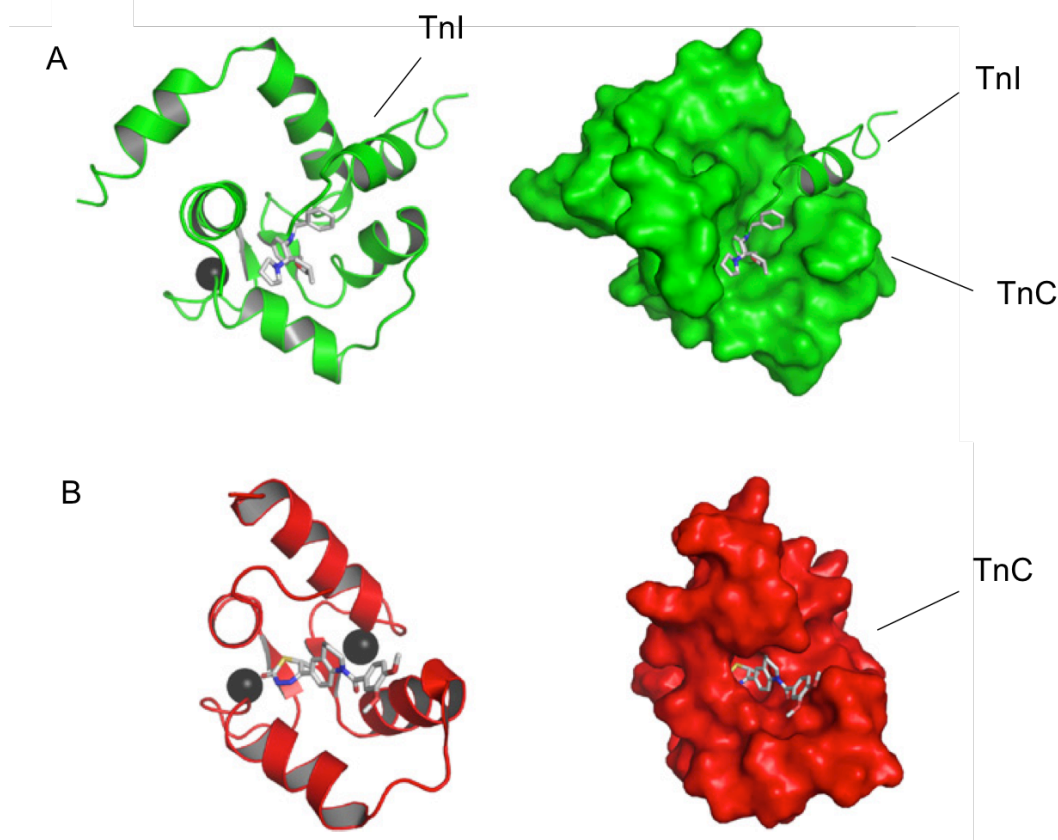
been found in some HCM mutations at the single filament level (reviewed by Messer and Marston, 2014). Thus the effect of the Ca^{2+} sensitisers on thin filaments is parallel to the effect of these HCM mutations, since it increases the Ca^{2+} sensitivity of the thin filaments and also uncouples the Ca^{2+} sensitivity from TnI phosphorylation.

4.4.3 Mechanism of action of the Ca^{2+} sensitisers

As we can observe from the IVMA experiments, EMD 57033 and Bepridil affected the EC_{50} of the Ca^{2+} activation curves rather than the maximum velocity, which suggest that it is likely that the Ca^{2+} sensitisers act directly on troponin to modulate the Ca^{2+} sensitivity rather than the cross-bridge cycle of the system. In fact, it has been shown that EMD 57033 binds to the C terminal domain of cTnC, using direct binding studies (Pan and Johnson, 1996) but also NMR studies (Maclachlan et al., 1990). The binding of Bepridil to the hydrophobic pocket of TnC has been shown by the crystal structure of cTNC-3 Ca^{2+} -3 Bepridil (Li et al., 2000b).

NMR studies on the binding of the small molecules to the troponin complex can give insights about the mechanism by which the molecules alter the Ca^{2+} sensitivity of the filaments. The sensitising action of EMD 57033 is considered to be indirect, by modifying the interaction of TnC with the TnI inhibitory peptide, thus promoting the “open” structure of troponin. EMD 57033 is shown to compete with TnI₃₄₋₇₁ peptide for cTnC, but not with TnI₁₂₈₋₁₄₇ (the inhibitory peptide). It is thought that EMD 57033 disrupts the interaction of TnC-TnI₃₄₋₇₁ and thus promoting the binding of the switch region of TnI to TnC (the troponin “open state”) (Wang et al., 2001). On the other hand, Bepridil is thought to also promote the troponin “open” state directly. Bepridil binds to the centre of the hydrophobic pocket of the TnC, at the same position where the switch peptide TnI₁₄₇₋₁₆₃ binds. Bepridil is able to bind to the TnC-TnI₁₄₇₋₁₆₃ complex, but with negative co-operativity. NMR studies of the TnC/TnI₁₄₇₋₁₆₃/Bepridil complex have shown that Bepridil can decrease the binding affinity of TnI₁₄₇₋₁₆₃ for the hydrophobic pocket of TnC by 3.5 fold and vice versa (Wang et al., 2002). Binding of Bepridil promotes the opening of the complex, inhibiting the release of Ca^{2+} and thereby enhancing the Ca^{2+} sensitivity of the myofilament (Li et al., 2008). Figure 4-12 shows the binding of EMD 57033 and Bepridil to TnC.

Figure 4-12: Binding of the Ca^{2+} sensitisers to TnC



A) NMR structure of Bepridil /cTnC/TnI₁₄₇₋₁₆₃/Ca²⁺ complex in green. On the left, TnI is represented as helix and on the right, TnC is represented as surface and TnI₁₄₇₋₁₆₃ is represented as a helix. B) NMR structure of EMD 57033/ cTnC/2Ca²⁺ complex in red, as helical representation on the left and as a surface on the right. The black sphere represents the Ca²⁺. Adapted from Li et al (Li et al., 2008)

The Ca²⁺ sensitivity of the myofilament is modulated by the phosphorylation of Serine 22 and 23 on the N terminal peptide of TnI, TnI₁₋₃₀. This peptide interacts with the N terminal domain of the cTnC, close to the Ca²⁺ binding site and when the two Serines get phosphorylated, this interaction is changed. I propose that the Ca²⁺ sensitisers can induce uncoupling because they disrupt this interaction of the N terminal region of TnI with TnC allosterically. As mentioned earlier, EMD 57033 is able to displace TnI₃₁₋₇₀. Bepridil binding is more complicated because not many recent studies have looked at this interaction directly. A study by Abusamhadneh et al, has shown through NMR that Bepridil and the N terminal extension of TnI binding give similar chemical shifts to the regulatory domain of cTnC (Abusamhadneh et al., 2001). It is possible that both Ca²⁺ sensitisers interfere with the modulation of Ca²⁺

sensitivity upon phosphorylation by displacing the TnI₁₋₃₀ helix. TnI₁₋₃₀ is intrinsically disordered therefore its structural location cannot be defined by structural strategies such as NMR or X-ray crystallography. One possible way to determine the exact mechanism by which EMD 57033 and Bepridil induce uncoupling is via molecular dynamics (MD) simulation of the whole troponin molecule. The effect of Ca²⁺ sensitisers that are found to uncouple the Ca²⁺ sensitivity from the TnI phosphorylation highlights the important role of the TnI N terminal peptide and its interaction with TnC. As mentioned earlier, DCM or HCM mutations found in any component of the thin filament also have the capability of uncoupling, and they might also do so by destabilising this interaction allosterically although its actual structure remains unknown.

Interestingly, in experiments measuring isometric force in cardiac muscle myofibrils, EMD 57033 increased Ca²⁺ sensitivity but only caused uncoupling at short sarcomere lengths (Vikhorev et al., 2014). Thus uncoupling may not be an all-or-none phenomenon. One possibility is that uncoupling may be related to Ca²⁺ sensitivity if the Ca²⁺ sensitiser shifts the conformational equilibrium so far towards the N terminal bound conformation that it cannot be significantly influenced by phosphorylation of the N terminal peptide of TnI.

EMD 57033 had a maximal effect at 30 µM in IVMA, which is in accordance with the *in vivo* experiments that also showed the same optimum concentration for enhancing contractility (Song, unpublished data). Furthermore, EMD 57033 had a biphasic dose response curve, compared with Bepridil that reached a plateau at 100 µM. EMD 57033 concentrations higher than 30 µM inhibited the motility of thin filaments. It has been proposed that EMD 57033 also acts upon myosin apart from troponin and prevents it from denaturation (Radke et al., 2014). It might be possible that EMD 57033 at high concentration acts upon myosin and this leads to the inactivation of the filaments. However, there has not been much evidence in the recent years about the action of EMD 57033 on myosin, thus a conclusion on that cannot be reached. Another possibility is that high concentrations of EMD 57033 inhibit troponin activation resulting in the promotion of the “off” state.

In conclusion, the major finding was that the two small molecules that act as Ca²⁺ sensitisers, EMD 57033 and Bepridil, increased the Ca²⁺ sensitivity of actin thin filaments *in vitro* by 2.8 and 3.5 fold respectively. Furthermore, both small molecules uncoupled the Ca²⁺ sensitivity from TnI phosphorylation, mimicking the effect of

HCM mutations on the thin filaments. I propose that small molecules can induce uncoupling by altering the interaction of the phosphorylatable peptide in TnI with TnC.

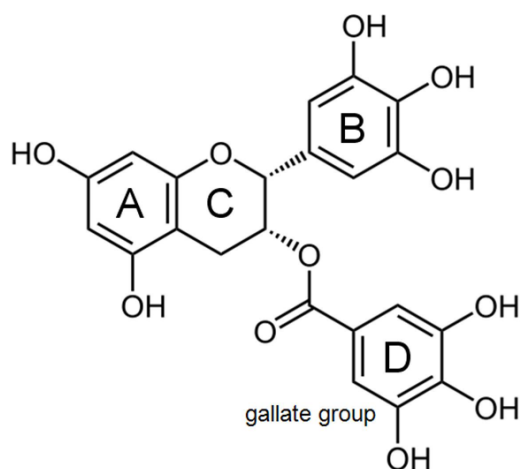
5 Investigation of the effect of EGCG on reconstituted human thin filaments

5.1 Introduction

In the previous chapter I examined the effect of two Ca^{2+} sensitisers in terms of the relationship between the Ca^{2+} sensitivity and troponin I (TnI) phosphorylation in mutant and donor thin filaments. In this chapter I will examine the effect of a Ca^{2+} desensitiser for the same property.

The Ca^{2+} desensitiser used in this chapter is Epigallocatechin-3-gallate (EGCG) (Tadano et al., 2010). EGCG is the most abundant polyphenol of green tea. The structure of EGCG can be found in Figure 5-1. Its benefits have been linked to many diseases, such as cancer (Chen et al., 2011), Alzheimer's disease (Walker et al., 2015) and diabetes (Ahmad et al., 2015). EGCG also has antioxidant properties and it can affect many pathways such as growth factor-mediated pathways, the mitogen-activated protein kinase-dependent pathway and the ubiquitin/proteasome degradation pathway (Mereles and Hunstein, 2011) Recently it has also been found to be cardioprotective, mainly ascribed to its antioxidant and anti-inflammatory properties. For example, EGCG protected the cardiovascular system of rats that were on a high fat diet (Zhong et al., 2015)

Figure 5-1: Chemical structure of EGCG



Structure of Epigallocatechin-3-gallate (EGCG).

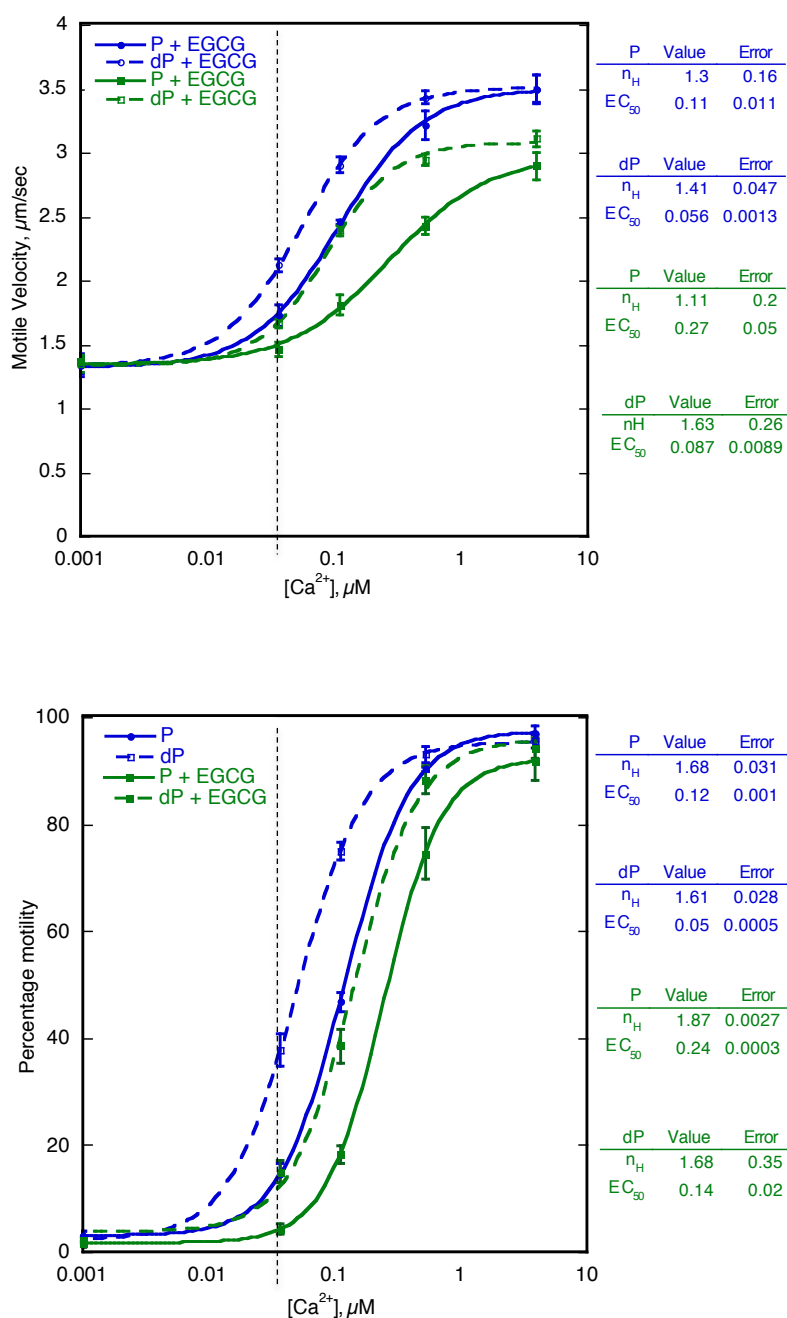
EGCG has been found also to interact with the myofilament, and more specifically with cardiac troponin C (cTnC) (Tadano et al., 2010, Robertson et al., 2009, Fuchs and Grabarek, 2013). EGCG has been found to decrease the Ca^{2+} sensitivity of skinned cardiac muscle fibres (Tadano et al., 2010). It also decreased slightly the sensitivity of skeletal fibres, but the effect was much weaker. Using NMR approaches it was then proposed that EGCG decreases the affinity of Ca^{2+} to the cTnC by binding to cTnC. EGCG binds to the C terminus of the cTnC, in the presence of cTnI, as opposed to other cardiotonic molecules such as EMD 57033 (Botten et al., 2013). EGCG is another molecule that could affect the TnC-TnI interaction that we hypothesise being crucial for the uncoupling phenomenon found in cardiomyopathies of the thin filament. The aim of this chapter is to observe the effect of EGCG on the coupling between the TnI phosphorylation and the Ca^{2+} sensitivity in wild type (WT) and Dilated Cardiomyopathy (DCM) or Hypertrophic Cardiomyopathy (HCM) mutant thin filaments using the *in vitro* motility assay (IVMA).

5.2 Investigation of the effect of EGCG on donor thin filaments

5.2.1 *In vitro* motility assay results of donor phosphorylated and dephosphorylated thin filaments treated with EGCG.

Based on the results of Tadano et al (Tadano et al., 2010) 100 μM EGCG was tested on donor thin filaments. Two Ca^{2+} curves were determined using phosphorylated and dephosphorylated thin filaments. Thin filaments were reconstituted using 60 nM human cardiac tropomyosin, 60 nM troponin extracted from donor heart and 10 nM rabbit skeletal actin. Six experiments were performed with phosphorylated and dephosphorylated thin filaments treated with EGCG. An example of a Ca^{2+} curve is shown in Figure 5-2 and a summary of the experiments can be found in Figure 5-2. Full tables for all the data can be found in APPENDIX.

Figure 5-2: Ca^{2+} activation curve of phosphorylated or dephosphorylated donor thin filaments treated with 100 μM EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Blue lines represent donor thin filaments and green lines represent donor thin filaments treated with 100 μM EGCG. The black dashed line represents 0.074 μM Ca^{2+} concentration.

Table 5-1: Ca^{2+} sensitivity data for motile velocity of donor thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/ unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
Donor	P	0.11 ± 0.082 (6)	2.11 ± 0.25 (6) $\dagger\dagger$	1.73 ± 0.34 (6)	3.31 ± 0.15 (6)
Donor	dP	0.053 ± 0.014 (6)		1.63 ± 0.2 (6)	3.38 ± 0.11 (6)
EGCG	P	0.3 ± 0.059 (6)	2.91 ± 0.44 (6) $\dagger\dagger$	1.42 ± 0.23 (6)	2.79 ± 0.099 (6)*
EGCG	dP	0.1 ± 0.01 (6)		1.54 ± 0.18 (6)	2.94 ± 0.12 (6) *

Table 5-2: Ca^{2+} sensitivity data for percentage motility of donor thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
Donor	P	0.14 ± 0.03 (6)	2.24 ± 0.1 (6) $\dagger\dagger$	1.67 ± 0.22 (6)	88.17 ± 4.45 (6)
Donor	dP	0.059 ± 0.01 (6)		1.5 ± 0.21 (6)	88.42 ± 4.81 (6)
EGCG	P	0.25 ± 0.029 (6)	1.73 ± 0.16 (6) $\dagger\dagger$	2.04 ± 0.27 (6)	79.83 ± 4.81 (6)
EGCG	dP	0.15 ± 0.02 (6) **		1.78 ± 0.3 (6)	83 ± 4.99 (6)

* $p < 0.05$, ** $p < 0.01$; for presence and absent of reagent. $\dagger\dagger$ $p < 0.01$; for the effect of phosphorylation. p values were calculated using one- way ANOVA. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

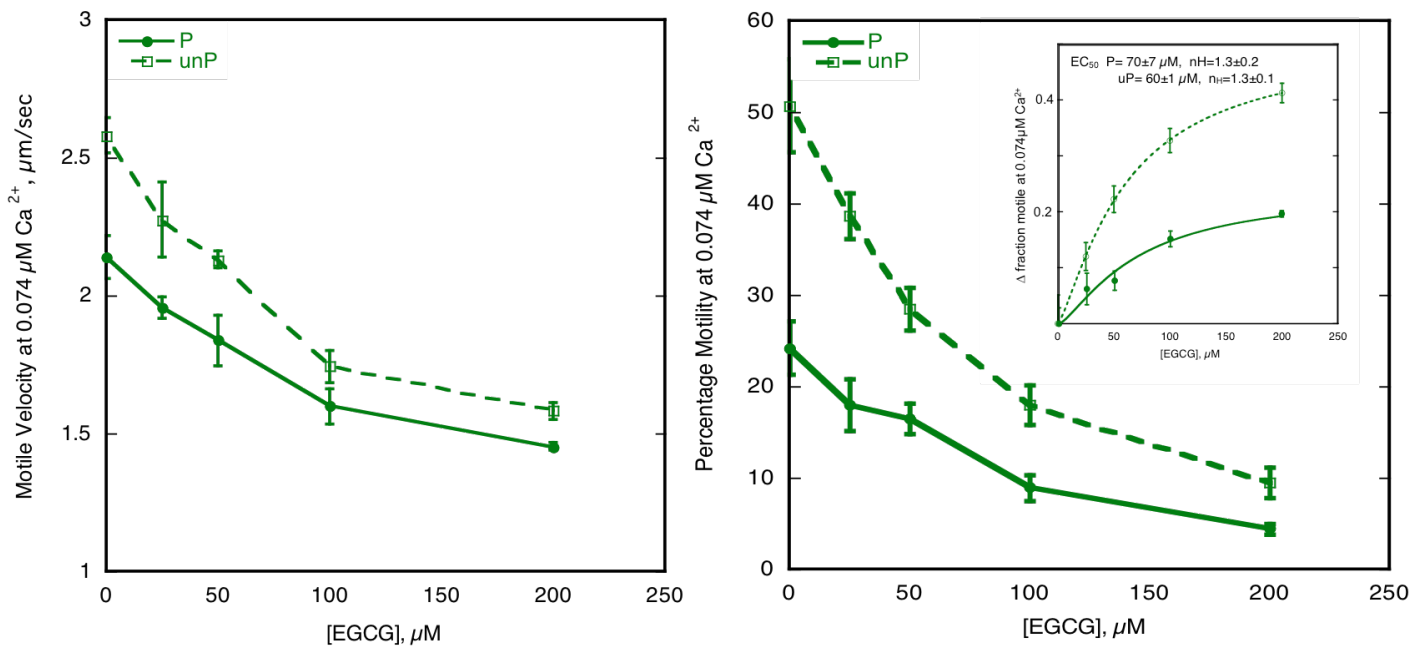
We can see from the Ca^{2+} sensitivity curves that 100 μM EGCG decreased the Ca^{2+} sensitivity of both phosphorylated and dephosphorylated thin filaments. The Ca^{2+} sensitivity decreased for the phosphorylated thin filaments by 2.15 ± 0.45 fold and by 2.80 ± 0.48 fold for the dephosphorylated thin filaments, as measured by the mean ratio of $\text{EC}_{50} \text{ P} + \text{EGCG} / \text{EC}_{50} \text{ P}$ for percentage motility of the filaments. The motile velocity gave similar results, with a mean ratio of $\text{EC}_{50} \text{ P} + \text{EGCG} / \text{EC}_{50} \text{ P} = 2.74 \pm 0.48$ for phosphorylated and a mean ratio of $\text{EC}_{50} \text{ dP} + \text{EGCG} / \text{EC}_{50} \text{ dP} = 2.07 \pm 0.31$ for dephosphorylated thin filaments. From these results, we can observe that EGCG does not have the same effect as EMD 57033 and Bepridil, as it retains the coupling between Ca^{2+} sensitivity and TnI phosphorylation on donor thin filaments. Furthermore, EGCG had an effect on the maximum motile velocity of the filaments. EGCG decreased the maximum velocity of the filaments (from 3.38 ± 0.17 to 2.79 ± 0.099 $\mu\text{m}/\text{sec}$ for phosphorylated thin filaments, $p = 0.03$, $n = 6$ and from 3.38 ± 0.17 to 2.94 ± 0.12 $\mu\text{m}/\text{sec}$ for dephosphorylated thin filaments, $p = 0.01$, $n = 6$). EGCG had no effect on n_{H} .

5.2.2 Dose response curve of EGCG on donor thin filaments

The next step was to determine a dose response curve for EGCG on donor thin filaments using the established technique of the IVMA and testing different concentrations of EGCG at a single Ca^{2+} concentration. Thin filaments were reconstituted using 60 nM phosphorylated or dephosphorylated troponin extracted from donor hearts, 60 nM human cardiac tropomyosin and 10 nM rabbit skeletal actin. Because EGCG decreased the Ca^{2+} sensitivity of the filaments, a slightly higher Ca^{2+} concentration was chosen compared to this one used for EMD 57033 and Bepridil. The chosen Ca^{2+} concentration was 0.074 μM Ca^{2+} . A dose response curve for phosphorylated and dephosphorylated donor thin filaments was created. One experiment was performed with 0, 25, 50, 100 and 200 μM EGCG in filaments containing phosphorylated and dephosphorylated troponin extracted from donor heart, shown in Figure 5-3. From the dose response curve for the percentage motility and motile velocity we can observe that EGCG inhibited the movement of the filaments. EGCG decreased the percentage motility of phosphorylated thin filaments from 24.25 ± 2.87 to $4.5 \pm 0.58\%$ (with 200 μM) and the motile velocity from 2.14 ± 0.07 to 1.45 ± 0.01 (with 200 μM). The percentage motility of dephosphorylated thin filaments

was decreased from 50.75 ± 5.12 to 9.5 ± 1.73 (200 μM) and the motile velocity from 2.58 ± 0.064 to 1.58 ± 0.03 (200 μM). EGCG does not saturate and the dose response curve is not co-operative. The change of fraction motile was plotted for phosphorylated and dephosphorylated filaments treated with different concentrations of EGCG and the Hill equation was fitted to the data. The dose response in both cases reached a plateau at concentration of 100 μM , with an EC_{50} of 70 ± 7 μM and n_H of 1.3 ± 0.2 for the phosphorylated and EC_{50} of 60 ± 1 μM and n_H 1.3 ± 0.1 for the dephosphorylated thin filaments.

Figure 5-3: EGCG dose response curve for phosphorylated and dephosphorylated thin filaments

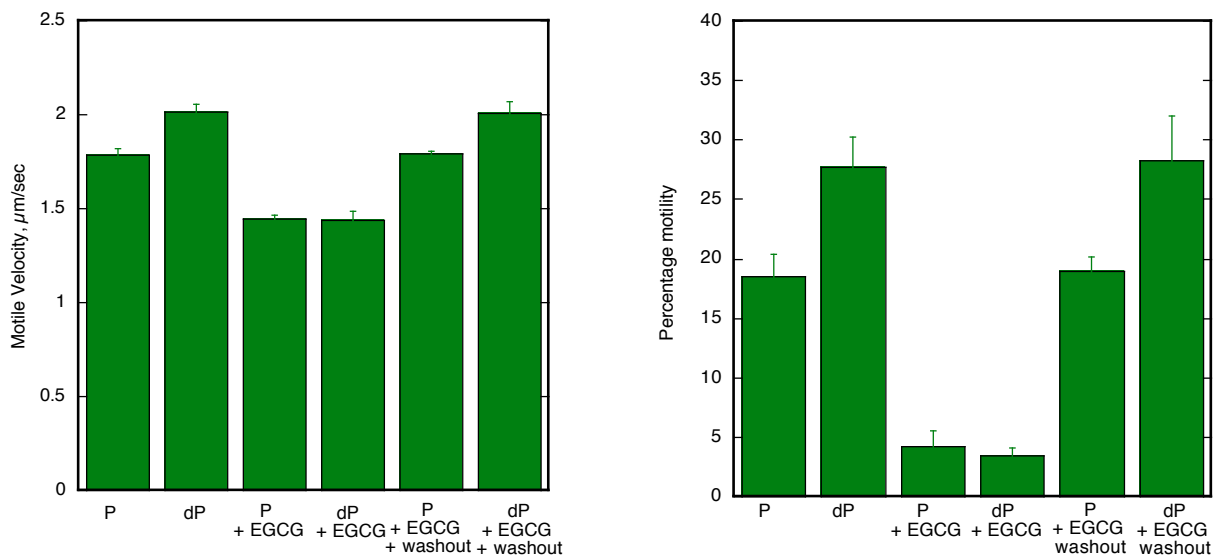


Motile velocity (left) and percentage motility (right) and of thin filaments reconstituted with phosphorylated (solid lines) or dephosphorylated (dotted lines) donor troponin treated with 0-200 μM EGCG was measured at 0.074 $\mu\text{M Ca}^{2+}$ to create a dose response curve. A single EGCG concentration was tested in one motility flow chamber. The inset plots the change in fraction motility at 0.074 Ca^{2+} of phosphorylated (solid lines) and dephosphorylated (dotted lines) thin filaments treated with increased EGCG concentrations. The Hill curve was fitted to the data to calculate the EC_{50} and n_H .

5.2.3 Reversibility of the action of EGCG

The reversibility of EGCG effect was tested in reconstituted thin filaments from donor proteins. Thin filaments were reconstituted by incubating these components: 60 nM phosphorylated or dephosphorylated troponin extracted from donor heart, 60 nM human cardiac tropomyosin, 10 nM rabbit skeletal actin with or without 100 μ M EGCG, in a similar fashion to all the experiments carried out in this chapter. Thin filaments were then added to the flow cell chambers. Subsequently, Buffers C and D containing 0.074 μ M Ca^{2+} and 100 μ M EGCG were added to the flow cell in the same manner as for the dose response curves, and images were recorded. To wash out the drug, buffers C and D containing 0.074 μ M Ca^{2+} without EGCG were added in the same flow cell and images were recorded. Results of the reversibility experiment is shown in Figure 5-4. It is clear from the graph that after the EGCG was washed out, the percentage motility and the motile velocity at 0.074 μ M Ca^{2+} were returned to the initial values without EGCG, making EGCG fully reversible.

Figure 5-4: Reversibility of EGCG



Motile velocity (left) and percentage motility (right) of thin filaments treated with 100 μ M EGCG at 0.074 μ M Ca^{2+} before and after drug washout. 4 measurements of motility of phosphorylated or dephosphorylated donor thin filaments were taken. Phosphorylated thin filaments were added in one motility chamber and dephosphorylated thin filaments in the other motility chamber of the same motility cell. For the reversibility experiment, 4 measurements were taken of thin filaments treated with 100 μ M (C and D buffers containing 100 μ M EGCG) and then the same motility chamber was washed out with 50 μ l of buffers C and D without EGCG and 4 measurements were taken. Bar charts represent the average of the 4 motility measurements for both percentage motility parameter and motile velocity with SEM.

5.3 The effect of EGCG on *TPM1* mutations that cause DCM or HCM

5.3.1 The effect of EGCG on *TPM1* E54K DCM mutation mutant thin filaments

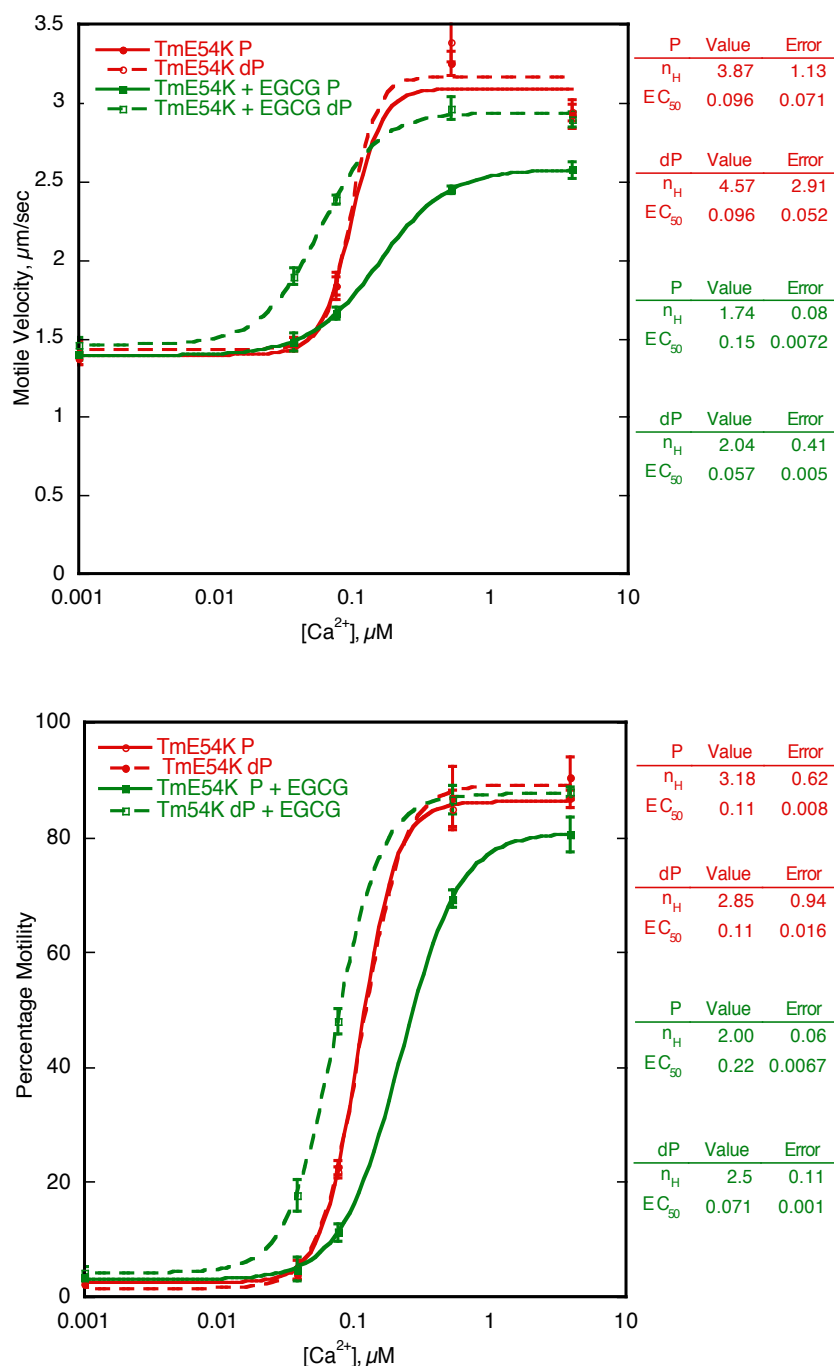
After the effect of EGCG on donor thin filaments was tested, the effect of EGCG on mutant thin filaments was tested. The first mutation that was examined was the DCM mutations *TPM1* E54K. The tropomyosin was purified from baculovirus/*sf9* cells so they are easy to study by the IVMA since proteins do not need to be extracted from tissue.

Every experiment described in this section was performed using thin filaments reconstituted using 60 nM phosphorylated or dephosphorylated troponin extracted from cardiac tissue, 60 nM recombinant Tpm1.1 E54K and 10 nM rabbit skeletal actin.

E54K thin filaments were reconstituted with phosphorylated or dephosphorylated troponin and were treated with 100 μ M EGCG similar to the donor thin filaments. Five experiments were performed comparing phosphorylated and dephosphorylated mutant thin filaments treated with 100 μ M EGCG and representative Ca^{2+} curves for percentage motility and motile velocity are shown in Figure 5-5. A summary of the experiments can be found in Table 5-3 and Table 5-4. We can see from the Ca^{2+} curves, the Ca^{2+} sensitivity of phosphorylated and dephosphorylated E54K mutant thin filaments is very similar. This is also shown from the calculated EC_{50} of the Ca^{2+} activation curves (mean ratio of $\text{EC}_{50}\text{P/dP} = 0.97 \pm 0.041$ for motile velocity and mean ratio $\text{EC}_{50}\text{P/dP} = 0.95 \pm 0.0035$ for percentage motility). This confirms the experiments with E54K thin filaments described in Section 4.2.4 and Section 4.3.4 and also the results found by Dr Massimiliano Memo. 100 μ M EGCG decreased the Ca^{2+} sensitivity of phosphorylated thin filaments by 2.33 ± 0.53 , $n = 5$ for percentage motility and 1.45 ± 0.43 , $n = 5$ as measured by the mean ratio $\text{EC}_{50}\text{P} + \text{EGCG}/\text{EC}_{50}\text{P}$. However, 100 μ M increased the Ca^{2+} sensitivity slightly for dephosphorylated thin filaments (mean ratio $\text{dP EC}_{50} + \text{EGCG}/\text{EC}_{50}\text{dP} = 0.68 \pm 0.1$ for percentage motility and 0.47 ± 0.072 for motile velocity). This had as a result to re-couple the Ca^{2+} sensitivity to TnI phosphorylation (mean ratio $\text{EC}_{50}\text{P/unP} = 3.17 \pm 0.37$, $p = 0.008$, n

= 5 for percentage motility and mean ratio $EC_{50}P/dP = 2.75 \pm 0.44$, $p = 0.016$, $n = 5$ for motile velocity).

Figure 5-5: Ca^{2+} activation curve comparing phosphorylated or dephosphorylated E54K mutant thin filaments treated with 100 μM EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin and mutant E54K tropomyosin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent E54K thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-3: Ca^{2+} sensitivity data for motile velocity of phosphorylated or dephosphorylated E54K DCM mutant reconstituted thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E54K	P	0.15 ± 0.043 (5)	0.97 ± 0.0413 (5)	2.73 ± 0.55 (5)	3.27 ± 0.29 (5)
E54K	dP	0.16 ± 0.043 (5)		2.8 ± 0.63 (5)	3.26 ± 0.24 (5)
EGCG	P	0.17 ± 0.021 (5)	2.75 ± 0.44 (5) †	2.18 ± 0.59 (5)	2.64 ± 0.024 (5)
EGCG	dP	0.062 ± 0.0058 (5) †		3.33 ± 1.15 (5)	2.9 ± 0.11 (5)

Table 5-4: Ca^{2+} sensitivity data for percentage motility of phosphorylated or dephosphorylated E54K DCM mutant reconstituted thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E54K	P	0.11 ± 0.013 (5)	0.95 ± 0.0033 (5)	3.4 ± 0.39 (5)	89.98 ± 0.48 (5)
E54K	dP	0.11 ± 0.019 (5)		3.84 ± 0.48 (5)	90.45 ± 3.53 (5)
EGCG	P	0.27 ± 0.034 (5)	3.17 ± 0.37 (5) ††	3.68 ± 0.78 (5)	84.3 ± 3.37 (5)
EGCG	dP	0.071 ± 0.0046 (5) †		3.25 ± 0.34 (5)	88.55 ± 3.31 (5)

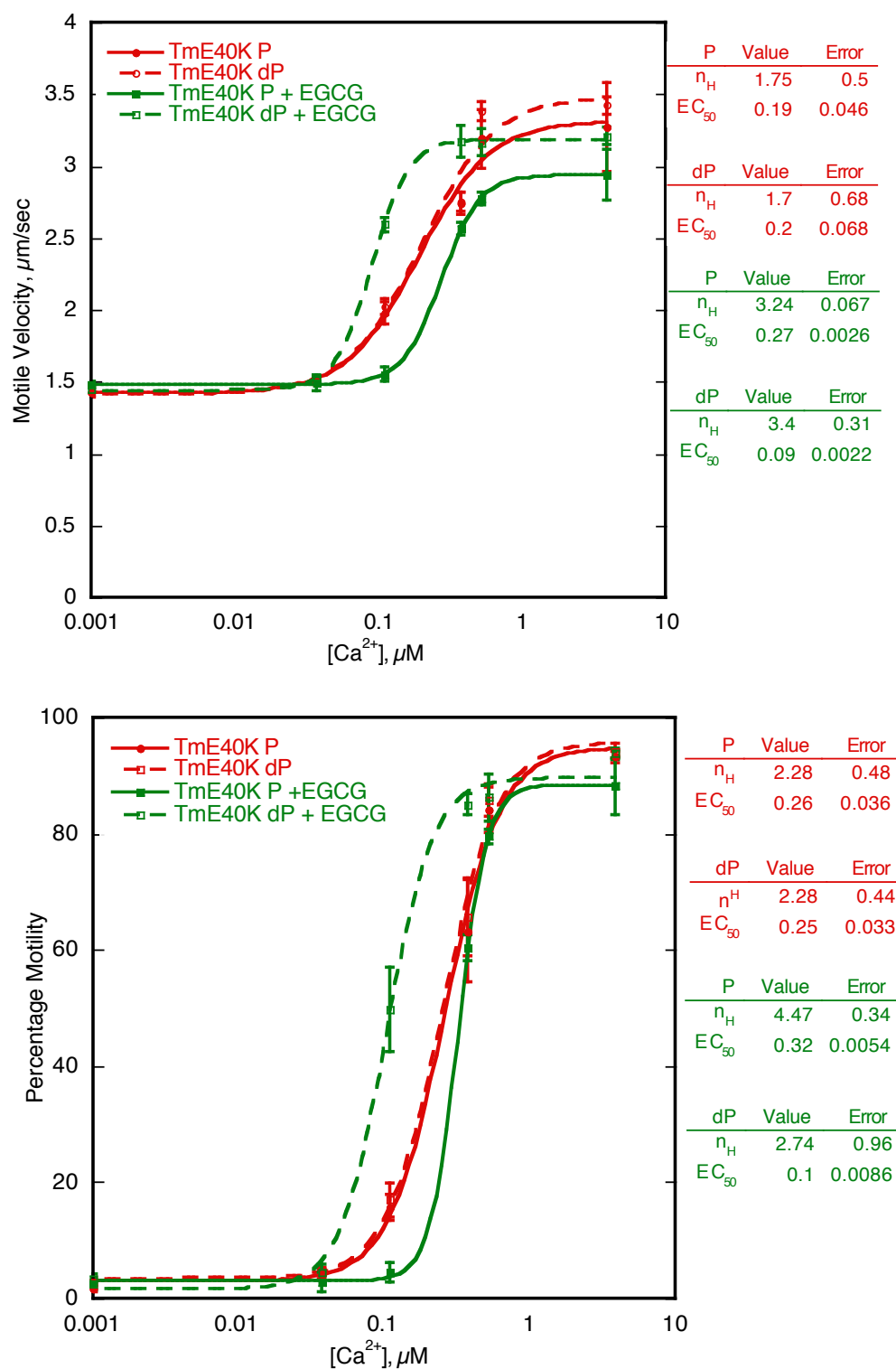
† $p < 0.05$, †† $p < 0.01$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.3.2 The effect of EGCG on *TPM1* E40K DCM mutant thin filaments

TPM1 E40K was identified in the same study as E54K by Olson et al as a DCM causing mutation (Olson et al., 2001). This mutation has been also extensively studied by the Marston group. E40K mutation causes a decrease in the Ca^{2+} sensitivity as shown by IVMA experiments with reconstituted thin filaments but also using ATPase assay (Mirza et al., 2005, Mirza et al., 2007)). Memo et al confirmed these findings and furthermore showed that this mutation causes uncoupling in reconstituted thin filaments (Memo et al, 2013).

E40K thin filaments reconstituted with phosphorylated or dephosphorylated troponin and recombinant E40K Tpm1.1 were treated with 100 μM EGCG similar to the donor thin filaments. Three Ca^{2+} curves were constructed comparing phosphorylated or dephosphorylated E40K mutant thin filaments treated or untreated with 100 μM EGCG, and a representative Ca^{2+} curve for percentage motility and motile velocity is shown in Figure 5-6. Data for phosphorylated and dephosphorylated E54K thin filaments were combined with data from Dr Massimiliano Memo's thesis. Two Ca^{2+} activation curves were performed; only comparing treated phosphorylated and dephosphorylated thin filaments, to increase the n numbers for the treatment group. It is noted that paired one-way ANOVA could not be performed using all the data because it requires data to be available for all the four groups. For this reason, the data for E40K were analysed using paired t test. Summary of all the data can be found in Table 5-5 and Table 5-6. Thin filaments reconstituted with both E40K and E54K showed a similar trend. Phosphorylated and dephosphorylated E40K thin filaments had analogous Ca^{2+} sensitivities, as shown from the Ca^{2+} curves and the $\text{EC}_{50}\text{P/dP}$ ratios ($\text{EC}_{50}\text{P/dP}$ ratio for motile velocity = 1.02 ± 0.055 , $n = 6$ and $\text{EC}_{50}\text{P/dP}$ ratio for percentage motility = 1.02 ± 0.0069 , $n = 8$). 100 μM EGCG decreased the Ca^{2+} sensitivity of phosphorylated thin filaments by 1.7 ± 0.43 fold, $n = 3$ for percentage motility and 1.56 ± 0.13 fold, $n = 3$ for motile velocity. On the other hand, EGCG increased the Ca^{2+} sensitivity of dephosphorylated thin filaments from $\text{EC}_{50} = 0.11 \pm 0.022$ ($n = 6$) to $\text{EC}_{50} = 0.062 \pm 0.0085$ ($n = 5$) for motile velocity and $\text{EC}_{50} = 0.17 \pm 0.03$ ($n = 8$) to $\text{EC}_{50} = 0.052 \pm 0.013$ ($n = 5$, $p < 0.05$) for percentage motility. This resulted in Ca^{2+} sensitivity restoration upon TnI phosphorylation, shown by the EC_{50} ratios ($\text{EC}_{50}\text{P/dP} + \text{EGCG} = 3.05 \pm 0.56$, $n = 5$, $p = 0.02$ for motile velocity and $\text{EC}_{50}\text{P/dP} + \text{EGCG} = 4.63 \pm 0.95$, $n = 5$, $p = 0.02$ for percentage motility).

Figure 5-6: Ca^{2+} activation curve comparing phosphorylated or dephosphorylated E40K mutant thin filaments treated with 100 μM EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin and mutant E40K tropomyosin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent E40K thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-5: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E40K thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E40K	P	0.11 ± 0.019 (6)	1.02 ± 0.055 (6)	2.56 ± 0.55 (6)	3.02 ± 0.17 (6)
E40K	dP	0.11 ± 0.022 (6)		2.74 ± 0.85 (6)	3.13 ± 0.2 (6)
EGCG	P	0.18 ± 0.033 (5) *	3.05 ± 0.56 (5) †	2 ± 0.39 (5)	2.86 ± 0.047 (5)
EGCG	dP	0.062 ± 0.0085 (5) †		1.84 ± 0.4 (5)	3.15 ± 0.11 (5)

Table 5-6: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E40K mutant thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E40K	P	0.18 ± 0.029 (8)	1.02 ± 0.0069 (8)	2.04 ± 0.33 (8)	90.74 ± 1.37 (8)
E40K	dP	0.17 ± 0.03 (8)		2.03 ± 0.27 (8)	91.85 ± 1.48 (8)
EGCG	P	0.22 ± 0.038 (5)	4.63 ± 0.95 (5) †	2.82 ± 0.48 (5)	89.3 ± 2.76 (5)
EGCG	dP	0.052 ± 0.013 (5) *†		1.46 ± 0.36 (5)	91.85 ± 1.78 (5)

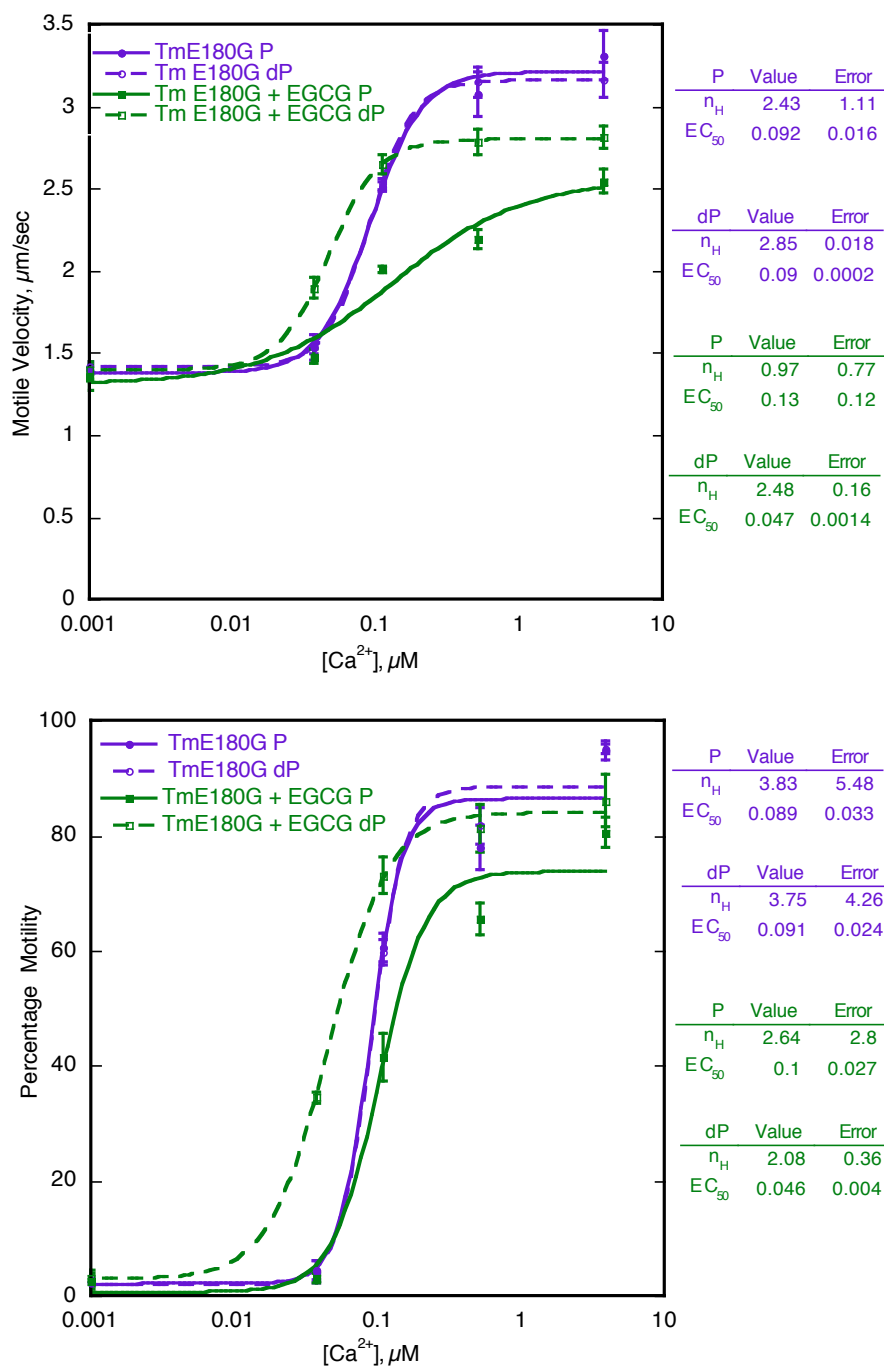
* $p < 0.05$; for presence and absent of reagent. † $p < 0.05$; for the effect of phosphorylation. p values were calculated using multiple t tests. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.3.3 The effect of EGCG on *TPM1* E180G HCM mutant thin filaments

TPM1 E180G mutation was first identified in a study by Thierfelder et al, where a family affected by fHCM was screened for mutations in the α tropomyosin gene (Thierfelder et al., 1994). The affected individuals were heterozygous for the mutation at nucleotide position 595 at exon 5 of the *TPM1* gene, which encodes for an exchange of a glutamic acid residue to a glycine residue in position 180. A previous study by the Marston lab has shown that reconstituted thin filaments with recombinant AS α tropomyosin E180G had an increased Ca^{2+} sensitivity (Bing et al., 1997), consistent with the hypothesis that the molecular mechanism of HCM is increased Ca^{2+} sensitivity. A transgenic (TG) mouse model expressing Tpm1.1 E180G has been developed that showed signs of hypertrophy: increased body weight ratios, fibrosis and increased sensitivity to Ca^{2+} of the myofilaments (Prabhakar et al., 2001). Since the two DCM mutations in Tpm1.1 uncouple the Ca^{2+} sensitivity to TnI phosphorylation and EGCG can reverse this, we asked whether the same applied to HCM. There are no data on the coupling between TnI phosphorylation and Ca^{2+} sensitivity in the E180G mutation on the thin filaments.

Experiments with E180G mutant thin filaments were performed using the same protocols as previously described. A representative Ca^{2+} titration curve is shown in Figure 5-7 and the results are summarized in Table 5-7 and Table 5-8. Three Ca^{2+} activation curves were performed with phosphorylated or dephosphorylated E180G mutant tropomyosin treated or untreated with 100 μM EGCG. Two additional Ca^{2+} activation curves were performed, only comparing phosphorylated and dephosphorylated E180G mutant thin filaments treated with 100 μM EGCG. In order to be able to include all the data in the analysis, a t test was performed. For the first time it was demonstrated that E180G mutant thin filaments were uncoupled, with $\text{EC}_{50} = 0.086 \pm 0.013 \mu\text{M}$ for the percentage motility of phosphorylated thin filaments and $\text{EC}_{50} = 0.087 \pm 0.011 \mu\text{M}$ for dephosphorylated thin filaments, giving a ratio of 0.98 ± 0.022 ($n = 3$). However, in the presence of 100 μM EGCG, the EC_{50} was $0.11 \pm 0.011 \mu\text{M}$ for phosphorylated and $0.048 \pm 0.0033 \mu\text{M}$ for dephosphorylated thin filaments, giving a ratio of 2.41 ± 0.32 ($p = 0.01$, $n = 5$). The motile velocities data gave very similar results. EGCG also had an effect on the maximum velocities of the filaments, where there was a decrease of V_{max} from $3.1 \pm 0.17 \mu\text{m/sec}$ to $2.7 \pm 0.094 \mu\text{m/sec}$ ($n = 5$, $p = 0.03$). These results also show that EGCG restored the Ca^{2+} sensitivity response to TnI phosphorylation.

Figure 5-7: Ca^{2+} activation curve comparing phosphorylated and dephosphorylated E180G mutant thin filaments treated with 100 μM EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) donor troponin and mutant E180G tropomyosin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Purple lines represent E180G thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-7: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E180G mutant thin filaments treated with EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/ unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E180G	P	0.089 ± 0.0019 (3)	0.86 ± 0.13 (3)	2.66 ± 0.13 (3)	3.1 ± 0.17 (3)
E180G	dP	0.11 ± 0.019 (3)		3.74 ± 0.61 (3)	3.04 ± 0.13 (3)
EGCG	P	0.11 ± 0.0076 (5)	2.71 ± 0.78 (5) †	2.87 ± 0.88 (5)	2.7 ± 0.094 (5)
EGCG	dP	0.058 ± 0.0045 (5) *†		2.7 ± 0.094 (5)	2.87 ± 0.057 †(5)

Table 5-8: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E180G mutant thin filaments treated with EGCG

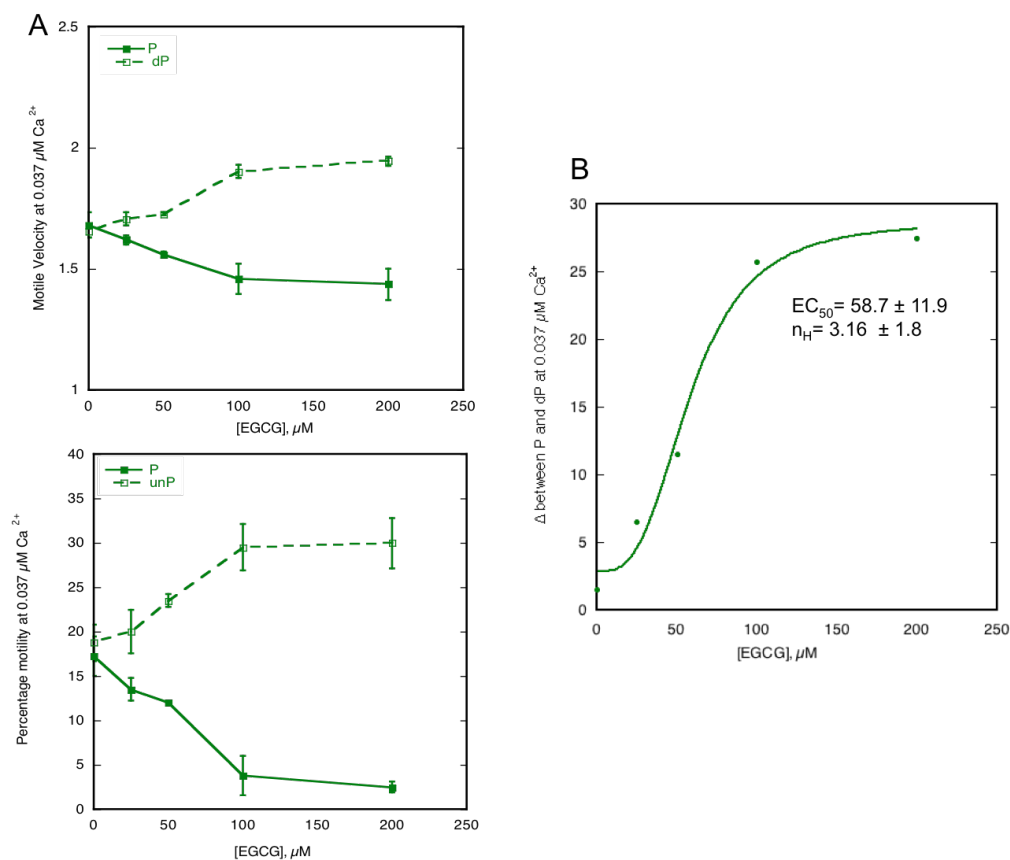
Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E180G	P	0.086 ± 0.013 (3)	0.98 ± 0.022 (3)	5.38 ± 1.8 (3)	93.91 ± 1.23 (3)
E180G	dP	0.087 ± 0.011 (3)		4.38 ± 0.98 (3)	92.92 ± 1.06 (3)
EGCG	P	0.11 ± 0.011 (5)	2.41 ± 0.32 (5) †	3.3 ± 0.69 (5)	82.77 ± 2.2 (5)
EGCG	dP	0.048 ± 0.0033 (5) *†		2.68 ± 0.84 (5)	90.4 ± 2.43 (5)

* $p < 0.05$; for presence and absent of reagent. † $p < 0.05$; for the effect of phosphorylation. p values were calculated using multiple t tests. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.3.4 Dose response curve of re-coupling by EGCG

Similarly to the dose response of EGCG in phosphorylated and dephosphorylated donor thin filaments (see Section 5.2.2), a dose response curve was constructed using Tpm1.1 E180G thin filaments. 25-200 μM EGCG were tested in a single Ca^{2+} concentration, $0.037 \mu\text{M}$ Ca^{2+} . The dose response curve for percentage motility and motile velocity of the filaments is shown in Figure 5-8. Initially motility is the same but with increasing concentrations of EGCG, motility of phosphorylated thin filaments was reduced and motility of dephosphorylated thin filaments was increased at $0.037 \mu\text{M}$ Ca^{2+} , indicating that EGCG re-coupled the Ca^{2+} change due to the phosphorylation. The Ca^{2+} curves reached a plateau at $100 \mu\text{M}$. The difference in percentage motility between phosphorylated and dephosphorylated mutant filaments was plotted and Hill equation was fitted to the data. The calculated EC_{50} was $58.7 \pm 11.9 \mu\text{M}$ and the n_H was 3.16 ± 1.8 .

Figure 5-8: Dose response curve for EGCG on E180G mutant thin filaments



A) Motile velocity (top) and percentage motility (bottom) of phosphorylated (solid lines) or dephosphorylated (dotted lines) E180G mutant thin filaments treated with 0-200 μM EGCG was measured at $0.037 \mu\text{M}$ Ca^{2+} . B) Percentage motility difference between phosphorylated and dephosphorylated E180G mutant filaments treated with increased EGCG concentrations at $0.037 \mu\text{M}$ Ca^{2+} . The Hill curve was fitted to the data to calculate the EC_{50} and n_H .

5.4 The effect of EGCG on three further DCM mutations

5.4.1 The effect of EGCG on *TNNC2* G159D DCM mutant thin filaments

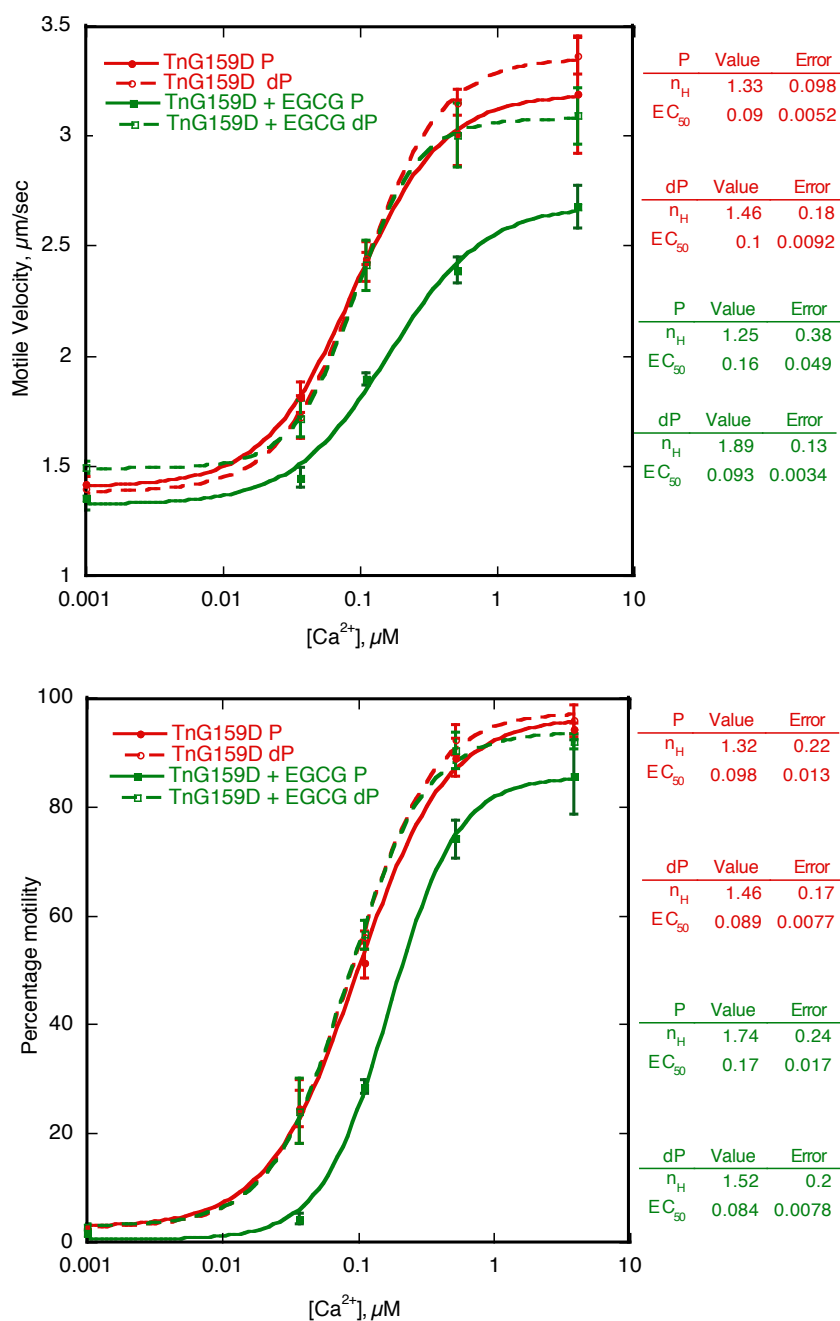
In the initial studies tropomyosin mutation were investigated because native mutant tropomyosin can be produced by baculovirus/*sf9* cells and tested against human cardiac troponin. DCM and some HCM mutations in troponin and actin are also able to uncouple the Ca^{2+} sensitivity from the TnI phosphorylation. In order to determine whether re-coupling by EGCG is a general feature of DCM and HCM more mutants were examined. First of all, the effect of EGCG was tested in the TnC mutation G159D. The missense mutation in TnC (*TNNC2*) Gly159Asp (G159D) was first discovered by Mogensen et al, in a study screening 235 patients with idiopathic DCM (Mogensen et al., 2004b). Steve Marston's lab obtained a cardiac muscle sample from an affected member of the original family from this study who has had a heart transplant.

Structural studies have shown that the position of the mutation is close to the N terminal regulatory peptide of TnI, which is important for the phosphorylation dependent modulation of Ca^{2+} sensitivity. Dr Massimiliano Memo has performed IVMA experiments with this DCM mutation and has found that there is uncoupling of the Ca^{2+} sensitivity from the TnI phosphorylation (Memo et al., 2013) For this reason, this mutation was selected to observe the action of EGCG on the coupling between Ca^{2+} sensitivity and TnI phosphorylation.

Thin filaments were reconstituted using 60 nM human cardiac tropomyosin, 60 nM troponin extracted from human heart sample containing the G159D mutation and 10 nM rabbit skeletal actin. To investigate the effect of EGCG on the Ca^{2+} sensitivity in relationship with TnI phosphorylation, the G159D extracted troponin was divided into two samples. One sample was kept in the native form and the other one was thoroughly dephosphorylated (see section 2.2.6.2). The original phosphorylation level of G159D sample was 1.47 molsPi/mol TnI and after dephosphorylation was 0.2 molsPi/mol TnI (see APPENDIX). Five Ca^{2+} activation curves were constructed comparing phosphorylated and dephosphorylated G159D mutant thin filaments with and without 100 μM EGCG. A representative Ca^{2+} curve for percentage motility and motile velocity is shown in Figure 5-9. The data are summarised in Table 5-9 and Table 5-10. We can see from the Ca^{2+} sensitivity curves, that in G159D mutant thin

filaments the Ca^{2+} sensitivity is uncoupled to the TnI phosphorylation ($\text{EC}_{50} \text{P/dP} = 0.97 \pm 0.035$ for percentage motility and 0.92 ± 0.0031 for motile velocity, $n = 5$), supporting the results of Dr Massimiliano Memo and Dr Emma Dyer. In the presence of $100 \mu\text{M}$ EGCG, the Ca^{2+} sensitivity was re-coupled to TnI phosphorylation ($\text{EC}_{50}\text{P/dP} + \text{EGCG} = 2.25 \pm 0.41$, $p = 0.04$, $n = 5$ for percentage motility and $\text{EC}_{50}\text{P/dP} + \text{EGCG} = 2.29 \pm 0.46$, $p = 0.048$, $n = 5$ for motile velocity). EGCG also had an effect on the motile velocity of the G159D reconstituted thin filaments, decreasing the motile velocity of phosphorylated thin filaments from $3.65 \pm 0.15 \mu\text{M}$ to $2.47 \pm 0.13 \mu\text{M}$, $p < 0.05$, $n = 5$.

Figure 5-9: Ca^{2+} activation curves for phosphorylated and dephosphorylated G159D mutant thin filaments treated with EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with native (solid lines) or dephosphorylated (dotted lines) troponin from G159D sample was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent G159D thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-9: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated G159D DCM mutant thin filaments treated with 100 μM EGCG

Treatment	EC_{50} , μM	Ratio EC_{50} P/ unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
G159D P	0.091 ± 0.0062 (5)	0.92 ± 0.031 (5)	2.33 ± 0.77 (5)	3.65 ± 0.15 (3)
G159D dP	0.1 ± 0.009 (5)		2.65 ± 1.12 (3)	3.56 ± 0.15 (3)
EGCG P	0.17 ± 0.037 (5)	2.29 ± 0.46 (5) †	1.73 ± 0.4 (5)	2.47 ± 0.13 (5) *
EGCG dP	0.074 ± 0.011 (5) *		2.53 ± 1.51 (5)	2.83 ± 0.19 †(5)

Table 5-10: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated G159D mutant thin filaments treated with 100 μM EGCG

Treatment	EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
G159G P	0.092 ± 0.0041 (5)	0.97 ± 0.035 (5)	2.79 ± 0.93 (5)	94.5 ± 0.54 (5)
G159D dP	0.095 ± 0.0054 (5)		2.52 ± 0.79 (5)	95.65 ± 0.48 (5)
EGCG P	0.19 ± 0.029 (5)	2.25 ± 0.41 (5) †	2.26 ± 0.48 (5)	89.1 ± 2.89 (5)
EGCG dP	0.088 ± 0.0046 (5)		2.69 ± 0.87 (5)	92.05 ± 1.53 (5)

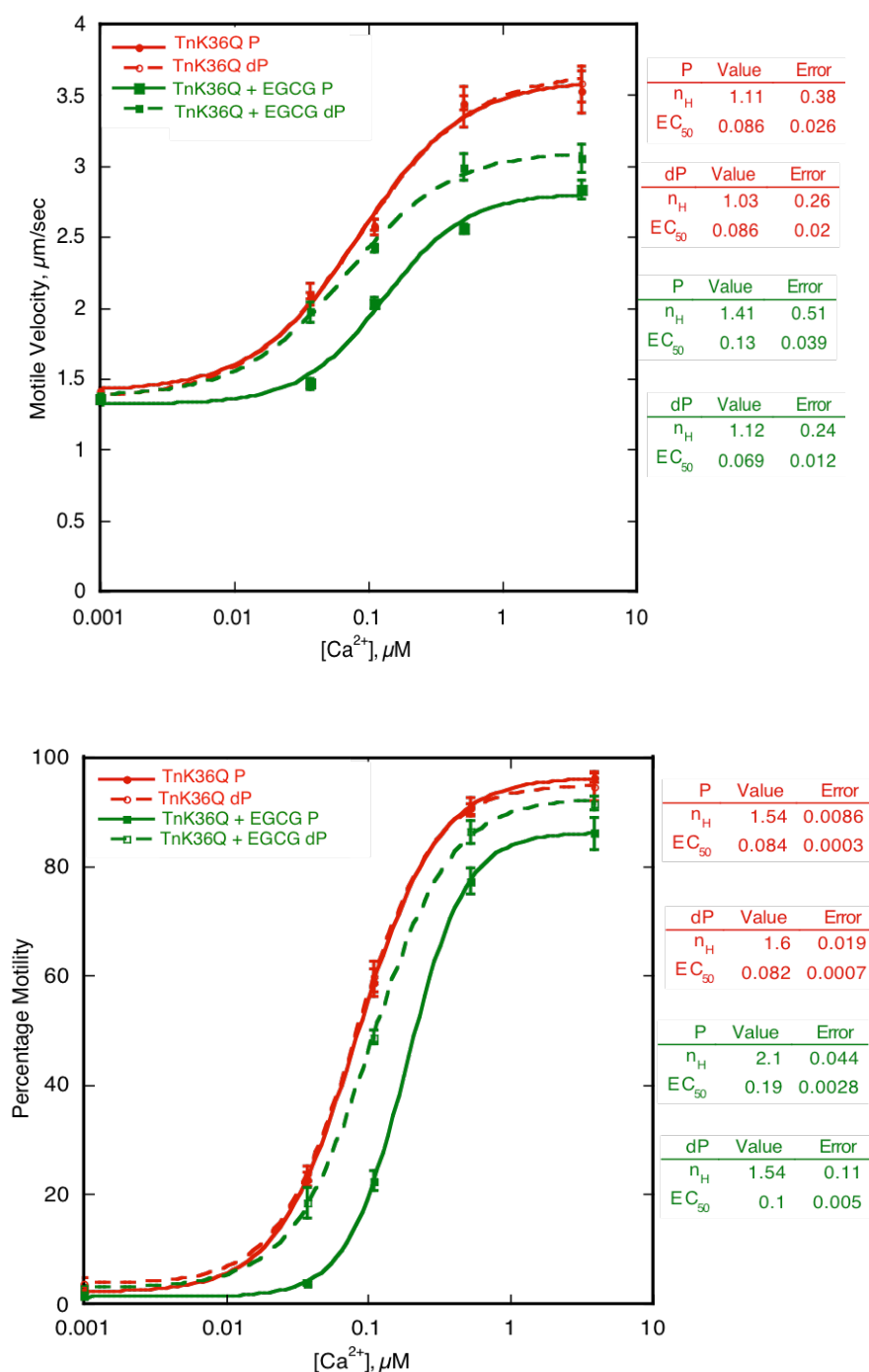
* $p < 0.05$; for presence and absent of reagent. † $p < 0.05$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.4.2 The effect of EGCG on *TNNI3* K36Q DCM mutant thin filaments

The mutation in TnI Lys36Gln (K36Q) was identified by Carballo et al, 2009. K36Q in *TNNI3* was one autosomal dominant missense mutations linked to the disease. A cardiac muscle sample of the transplanted heart from the original patient has been provided to the Marston's lab. K36Q is a DCM mutation that has been proven to uncouple the Ca^{2+} sensitivity from TnI phosphorylation in the study by Memo et al, therefore it was chosen to study the effects of EGCG on a TnI mutation.

Thin filaments were reconstituted using 60 nM human donor cardiac tropomyosin, 60 nM phosphorylated or dephosphorylated troponin containing the K36Q mutation and 10 nM actin. Due to the limitation of the sample, K36Q troponin was produced by exchange of the native TnI subunit of a donor troponin with mutant recombinant K36Q TnI subunit (See Section 2.2.5.3). Recombinant proteins are naturally non-phosphorylated, thus to perform the experiments the exchanged troponin was divided into two samples, one was phosphorylated and the other dephosphorylated (See Section 2.2.6.1 and 2.2.6.2, APPENDIX). Three Ca^{2+} curves were performed comparing phosphorylated and dephosphorylated K36Q mutant thin filaments treated or not with EGCG, an example of which is shown in Figure 5-10. Additionally two Ca^{2+} curves were produced comparing phosphorylated and dephosphorylated thin filaments reconstituted with exchanged troponin treated with 100 μM EGCG. The data are summarised in Table 5-11 and Table 5-12) Student t test was used to perform the statistical analysis between the groups because not all the data could be used for paired one-way ANOVA. In all the experiments performed, the Ca^{2+} sensitivity was uncoupled from TnI phosphorylation. 100 μM EGCG restored the phosphorylation dependency of Ca^{2+} sensitivity in K36Q mutant thin filaments ($\text{EC}_{50}\text{P/dP} + \text{EGCG} = 1.97 \pm 0.12$, $p < 0.01$, $n = 5$ for percentage motility and $\text{EC}_{50}\text{P/dP} + \text{EGCG} = 1.97 \pm 0.22$, $p = 0.01$, $n = 5$ for motile velocity). EGCG also had an effect on the motile velocity of the K36Q reconstituted thin filaments, decreasing the motile velocity of dephosphorylated thin filaments from $3.69 \pm 0.15 \mu\text{m/sec}$ to $2.99 \pm 0.17 \mu\text{m/sec}$, $p < 0.01$, $n = 5$.

Figure 5-10: Ca^{2+} activation curves for phosphorylated and dephosphorylated K36Q mutant thin filaments treated with 100 μM EGCG.



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with phosphorylated (solid lines) or dephosphorylated (dotted lines) exchanged recombinant K36Q troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent K36Q thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-11: Ca^{2+} sensitivity data for motile velocity of thin filaments reconstituted with phosphorylated or dephosphorylated exchanged K36Q troponin treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
K36Q	P	0.094 ± 0.0076 (3)	1.16 ± 0.13 (3)	1.52 ± 0.46 (3)	3.74 ± 0.23 (3)
K36Q	dP	0.082 ± 0.0025 (3)		2.08 ± 1.045 (3)	3.67 ± 0.15 (3)
EGCG	P	0.14 ± 0.015 (5)	1.97 ± 0.22 (5) $\dagger$	2.01 ± 0.69 (5)	2.71 ± 0.14 (5)
EGCG	dP	0.073 ± 0.007 (5)		1.49 ± 0.44 (5)	2.99 ± 0.17 (5)**

Table 5-12: Ca^{2+} sensitivity data for percentage motility of thin filaments reconstituted with phosphorylated and dephosphorylated exchanged K36Q troponin treated with EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
K36Q	P	0.077 ± 0.0057 (3)	1.15 ± 0.13 (3)	3.32 ± 1.46 (3)	92 ± 3.52 (3)
K36Q	dP	0.069 ± 0.011 (3)		3 ± 1.46 (3)	93.75 ± 0.75 (3)
EGCG	P	0.19 ± 0.018 (5)	1.97 ± 0.12 (5) $\dagger\dagger$	2.69 ± 0.84 (5)	87.11 ± 1.65 (5)
EGCG	dP	0.096 ± 0.0086 (5) $^{*++}$		2.21 ± 0.72 (5)	90.77 ± 1.89 (5)

* $p < 0.05$, ** $p < 0.01$; for presence and absent of reagent. $\dagger$ $p < 0.05$, $\dagger\dagger$ $p < 0.01$; for the effect of phosphorylation. p values were calculated using multiple t tests. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number. All experiments were performed by myself using exchanged recombinant K36Q troponin.

5.4.3 The effect of EGCG on *ACTC* E361G DCM mutant thin filaments

The missense mutation in *ACTC* Glutamine 361 to Glycine (E361G) was first described by Olson et al in a study where two families affected by idiopathic DCM were screened. The E361G mutation is located in subdomain 1 of the actin monomer and it is proposed that it is an altered actin function rather than an loss of function that leads to the disease (Olson et al., 1998).

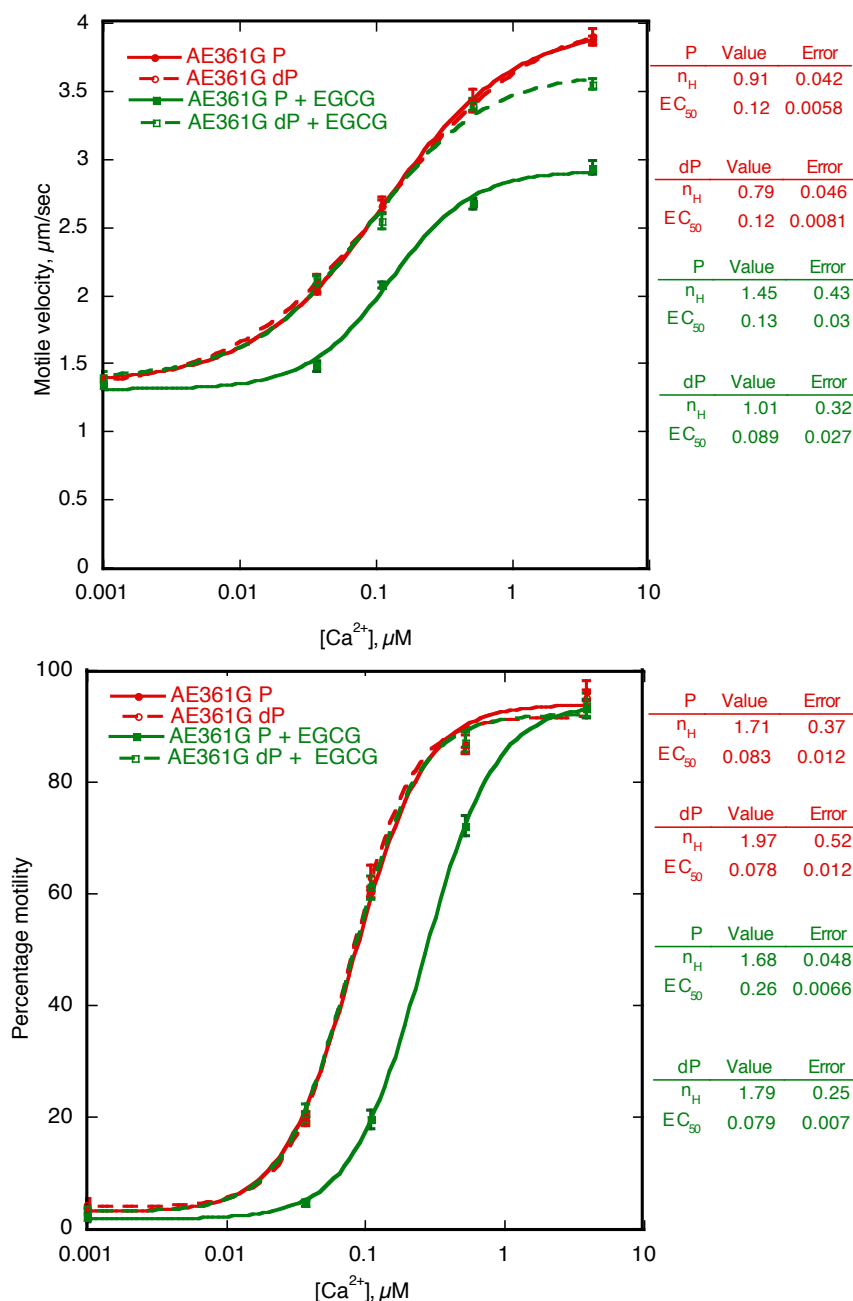
TG mice carrying the *ACTC* E361G mutation were generated in Prof Steve Marston's lab in collaboration with Dr Dominick Wells. TG mice expressed the E361G mutant actin at 50% of the total cardiac actin.

IVMA experiments performed by Dr Massimiliano Memo showed that thin filaments reconstituted with E361G mutant actin had equal Ca^{2+} sensitivity as thin filaments reconstituted with actin extracted from wild type mice (Song et al., 2010). When phosphorylated and dephosphorylated E361G mutant thin filaments were compared, it was found that Ca^{2+} sensitivity was independent from TnI phosphorylation and it was the first demonstration of uncoupling due to mutations. These findings make this mutation of particular importance for the investigation of the effect of EGCG on the uncoupling phenomenon.

Thin filaments were reconstituted with 60 nM human cardiac tropomyosin, 60 nM phosphorylated or dephosphorylated troponin from donor heart and 60 nM actin isolated from E361G transgenic mice. Five Ca^{2+} curves were performed comparing phosphorylated and dephosphorylated E361G DCM mutant thin filaments treated with 100 μM EGCG. A representative curve can be shown in Figure 5-11 and data are summarized in Table 5-13 and Table 5-14. As we can see from the Table, the Ca^{2+} sensitivity is uncoupled from TnI phosphorylation in E361G mutant thin filaments (Ratio $\text{EC}_{50} \text{ P/dP} = 1.08 \pm 0.024$, $n = 5$ for percentage motility, ratio $\text{EC}_{50} \text{ P/dP} = 1.04 \pm 0.04$, $n = 5$ for motile velocity). EGCG was able to restore the Ca^{2+} sensitivity upon TnI phosphorylation, as it decreased the Ca^{2+} sensitivity of phosphorylated thin filaments by 2.26 ± 0.27 for percentage motility and by 1.22 ± 0.042 for motile velocity ($\text{EC}_{50} \text{ P} = 0.087 \pm 0.0023$, $\text{EC}_{50} \text{ P} + \text{EGCG} = 0.2 \pm 0.0022$, $p = 0.03$, $n = 5$, all data for percentage motility). EGCG did not have an effect on dephosphorylated E361G thin filaments ($\text{EC}_{50} \text{ dP} = 0.08 \pm 0.0017$, $\text{EC}_{50} \text{ dP} + \text{EGCG} = 0.081 \pm 0.0016$, $n = 5$). The ratio $\text{EC}_{50} \text{ P/dP} + \text{EGCG}$ was 2.41 ± 0.28 , $n = 5$, $p < 0.001$ for percentage motility and 1.51 ± 0.0062 , $n = 5$, $p < 0.001$ for motile velocity. EGCG also had an effect on the maximum velocity of the filaments, reducing the velocity of

phosphorylated thin filaments from $3.66 \pm 0.11 \mu\text{m/sec}$ to $2.71 \pm 0.089 \mu\text{m/sec}$, $n = 5$, $p < 0.01$ and this of dephosphorylated thin filaments from $3.56 \pm 0.15 \mu\text{m/sec}$ to $2.2 \pm 0.17 \mu\text{m/sec}$, $n = 5$, $p < 0.001$.

Figure 5-11: Ca^{2+} activation curves comparing phosphorylated and dephosphorylated E361G mutant thin filaments treated with $100 \mu\text{M}$ EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with phosphorylated (solid lines) or dephosphorylated (dotted lines) donor troponin and E361G mutant actin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Red lines represent E361G thin filaments and green lines represent thin filaments treated with $100 \mu\text{M}$ EGCG.

Table 5-13: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E361G mutant thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E361G	P	0.094 ± 0.0067 (5)	1.04 ± 0.04 (5)	1.72 ± 0.41 (5)	3.66 ± 0.11 (5)
E361G	dP	0.092 ± 0.0095 (5)		1.78 ± 0.47 (5)	3.56 ± 0.15 (5)
EGCG	P	0.11 ± 0.0073 (5) *	$1.51 \pm$ 0.0062 (5) ††	2.91 ± 0.74 (5)	2.71 ± 0.089 (5)**
EGCG	dP	0.077 ± 0.0062 (5) ††		2.3 ± 0.74 (5)	2.2 ± 0.17 (5) **†

Table 5-14: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E361G thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E361G	P	0.087 ± 0.0023 (5)	$1.08 \pm$ 0.024 (5)	2.91 ± 0.81 (5)	92.8 ± 2.262 (5)
E361G	dP	0.08 ± 0.0017 (5)		2.3 ± 0.74 (5)	91.45 ± 2.07 (5)
EGCG	P	0.2 ± 0.022 (5) *	$2.41 \pm$ 0.28 (5) ††	2.87 ± 1.11 (5)	84.3 ± 4.37 (5)
EGCG	dP	0.081 ± 0.0016 (5) †		2.44 ± 0.28 (5)	88.07 ± 3.07 (5)

* $p < 0.05$, for presence and absent of reagent. † $p < 0.05$, †† $p < 0.01$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.5 The effect of EGCG on two HCM mutations

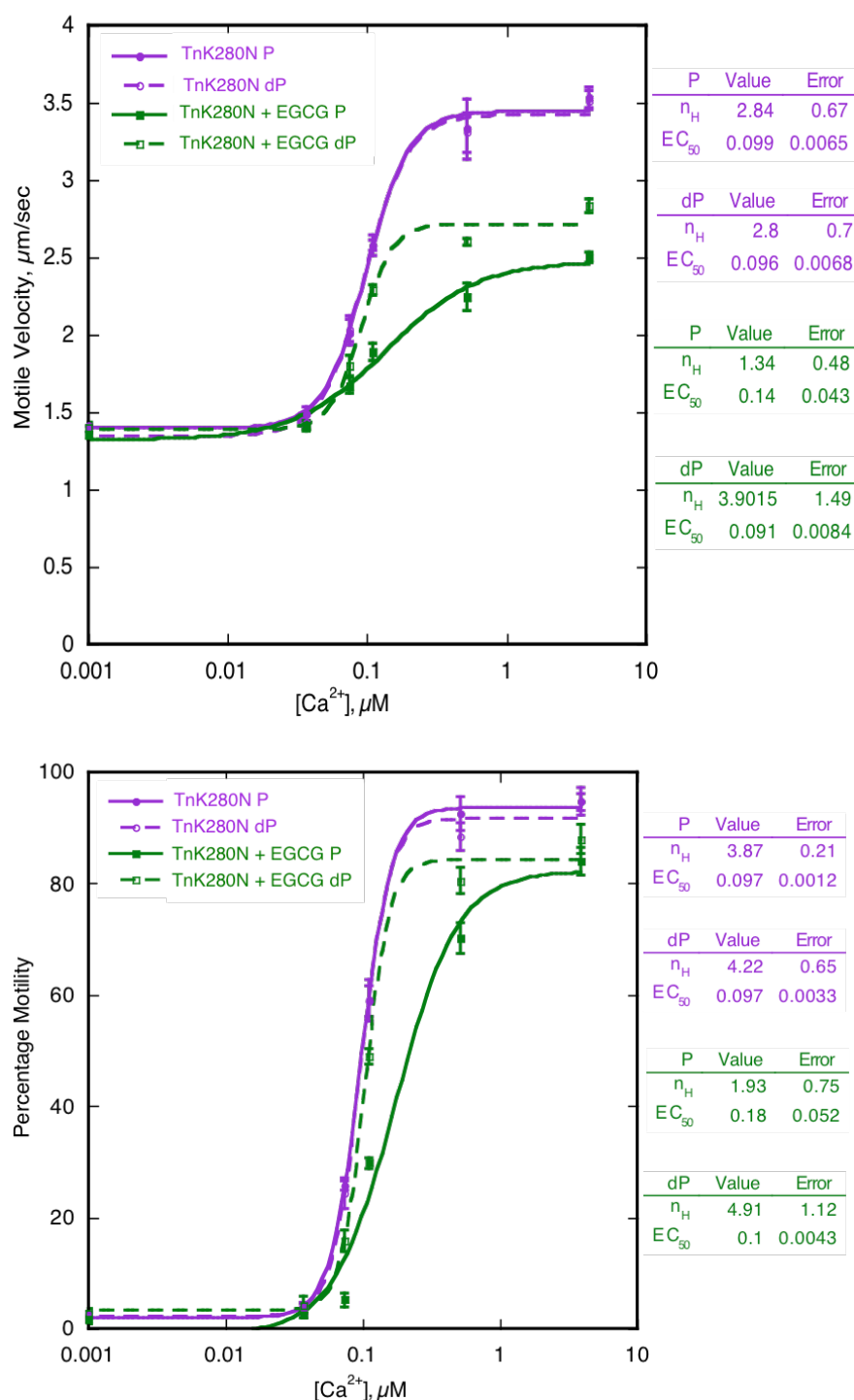
5.5.1 The effect of EGCG on *TNNT2* K280N HCM mutant thin filaments

In the previous section the re-coupling properties of EGCG on DCM was investigated. EGCG has also been able to restore the Ca^{2+} sensitivity difference upon phosphorylation in E180G HCM mutant thin filaments as well. The aim of this section is to investigate the re-coupling properties of EGCG on two further HCM mutations that have been found to be uncoupled. These mutations are K280N in TnT and E99K in actin. The missense mutation of Lysine to Asparagine at position 280 in *TNNT2* (K280N) is novel and has been identified in a biopsy sample from the left ventricle of a 26-year old male who had a heart transplant by Dr Jolanda Van der Velden. The patient was homozygous for the mutation and had previously undergone a myectomy operation. Prof Cris Dos Remedios has made the sample available to our lab, in a collaboration with Dr Jolanda Van der Velden and Prof Corrado Poggesi. The rate constant of active tension after maximal Ca^{2+} activation (K_{ACT}) as well as the rate constant of isometric relaxation (K_{REL}) of myofibrils extracted from the K280N tissue sample was faster than the control, suggesting that the K280N mutation acts by altering cross-bridge kinetics of the sarcomere (Ferrara et al., 2015). Dr Christopher Bayliss and Dr Andrew Messer in the Marston lab have been working on this sample for many years. It has been found that the phosphorylation status of the sample was the same as the donor patients. IVMA experiments comparing phosphorylated and dephosphorylated K280N troponin extracted from the sample have been performed and it was found that the Ca^{2+} sensitivity was independent of TnI phosphorylation. Furthermore, experiments were performed comparing phosphorylated and dephosphorylated troponin from donor sample, where the TnT was fully exchanged with recombinant mutant K280N TnT. In that case, the Ca^{2+} sensitivity was also independent of TnI phosphorylation. Since the patient was homozygous for the mutation, exchanging the TnT subunit entirely with K280N mutant TnT represents the physiological situation. In this section I will investigate the effect of EGCG on the coupling between TnI phosphorylation and Ca^{2+} sensitivity.

Thin filaments were reconstituted using troponin extracted from donor heart where the TnT subunit was exchanged with mutant K280N TnT (See Section 2.2.6.3).

Troponin was then divided into two samples, whereby one was phosphorylated (See Section 2.2.6.2) and the other one remained dephosphorylated (see APPENDIX). In that way, phosphorylated and dephosphorylated thin filaments could be compared. Three Ca^{2+} activation curves were performed comparing phosphorylated and dephosphorylated K280N mutant thin filaments treated or untreated with 100 μM EGCG, a representative experiment shown in Table 5-10. Two further experiments were performed comparing phosphorylated and dephosphorylated K280N thin filaments treated with 100 μM EGCG. Two experiments were also included in the analysis performed by Dr Andrew Messer only comparing phosphorylated and dephosphorylated thin filaments. Data for K280N thin filaments are summarized in Table 5-15 and Table 5-16. We can see from the Ca^{2+} activation curves, that in K280N mutant thin filaments the Ca^{2+} sensitivity is uncoupled from the TnI phosphorylation ($\text{EC}_{50} \text{ P/dP} = 1.08 \pm 0.042$ for percentage motility and 0.06 ± 0.0067 for motile velocity, $n = 5$), supporting previous results. In the presence of 100 μM EGCG, the Ca^{2+} was re-coupled to TnI phosphorylation ($\text{EC}_{50} \text{ P/dP} + \text{EGCG} = 1.67 \pm 0.24$, $p = 0.02$, $n = 5$ for percentage motility and $\text{EC}_{50} \text{ P/dP} + \text{EGCG} = 3.27 \pm 1.41$, $p = 0.1$, $n = 5$). EGCG also had an effect on the motile velocity of the K280N reconstituted thin filaments, decreasing the motile velocity of phosphorylated thin filaments from $3.22 \pm 0.27 \mu\text{m/sec}$ to $2.49 \pm 0.021 \mu\text{m/sec}$, $p = 0.03$, $n = 5$.

Figure 5-12: Ca^{2+} activation curves for phosphorylated and dephosphorylated thin filaments reconstituted with exchanged K280N HCM mutant troponin treated with 100 μM



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with phosphorylated (solid lines) or dephosphorylated (dotted lines) exchanged recombinant K280N troponin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Purple lines represent K280N thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-15: Ca^{2+} sensitivity data summary for motile velocity of phosphorylated and dephosphorylated thin filaments reconstituted with K280N HCM mutant troponin treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
K280N	P	0.096 ± 0.0046 (5)	1.06 ± 0.067 (5)	3.25 ± 1.32 (5)	3.22 ± 0.27 (5)
K280N	dP	0.093 ± 0.0095 (5)		2.14 ± 0.49 (5)	3.11 ± 0.22 (5)
EGCG	P	0.26 ± 0.1 (5)	3.27 ± 1.41 (5) $\dagger$	1.62 ± 0.44 (5)	2.49 ± 0.021 (5)*
EGCG	dP	0.081 ± 0.0037 (5)		3.51 ± 0.5 (5)	2.78 ± 0.15 (5)

Table 5-16: Ca^{2+} sensitivity data summary for percentage motility of phosphorylated and dephosphorylated thin filaments reconstituted with K280N HCM mutant troponin treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
K280N	P	0.11 ± 0.0073 (5)	1.08 ± 0.042 (5)	2.76 ± 0.53 (5)	94.25 ± 0.92 (5)
K280N	dP	0.1 ± 0.004 (5)		3.45 ± 0.8 (5)	94.25 ± 0.45 (5)
EGCG	P	0.2 ± 0.031 (5)	1.67 ± 0.24 (5) $\dagger$	2.59 ± 0.86 (5)	84.85 ± 2.95 (5)
EGCG	dP	0.1 ± 0.004 (5) * $\dagger$		4.56 ± 0.091 (5)	88.85 ± 0.71 (5)

* $p < 0.05$, for presence and absent of reagent. $\dagger$ $p < 0.05$; for the effect of phosphorylation. p values were calculated using multiple t tests. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

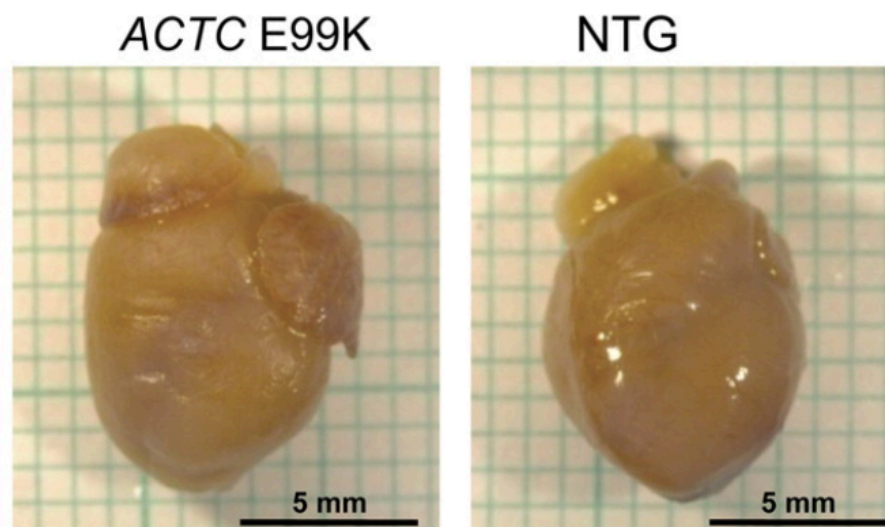
5.5.2 The effect of EGCG on *ACTC* E99K HCM mutant thin filaments

The last HCM mutation explored in this study is *ACTC* Glu99Lys (E99K). This mutation was first identified as a cause of HCM by Olson et al (Olson et al., 2000). Monserrat et al have characterized the mutation in 76 individuals from 10 families in Spain and these patients present symptoms of HCM such as apical hypertrophy, left ventricular non- compaction and septal defects (Monserrat et al., 2007).

A TG mouse line has been developed in Prof Steve Marston's lab carrying the E99K mutation. TG mice express 50% of E99K mutant actin of the total actin (Song et al., 2011)

The E99K mouse model recapitulated many of the same characteristics of HCM in human. At the molecular level, the E99K mutation was associated with increased Ca^{2+} sensitivity, as shown by the IVMA and also by testing skinned papillary muscle directly. Increased Ca^{2+} sensitivity is a characteristic common to all HCM mutations tested so far (Marston, 2011). 21-week-old mice showed enlarged atria, myocyte disarray and increased interstitial fibrosis, also common characteristics of HCM (See Figure 5-13). Hypertrophy was also observed mainly in the apex of the heart, as shown by MRI and end-systolic volume and pressure were found to be increased (Song et al., 2011). Contractile force of isolated papillary muscles and also of myofibrils from E99K transgenic mouse was 4 times greater and the rate of muscle relaxation was slower. In E99K myofibrils the Ca^{2+} sensitivity of force was increased, suggesting that the increased Ca^{2+} sensitivity observed in E99K mutant mice causes the hypercontractility of the cardiac muscle (Song et al., 2013).

Figure 5-13: Heart morphology of 21-week-old E99K mouse



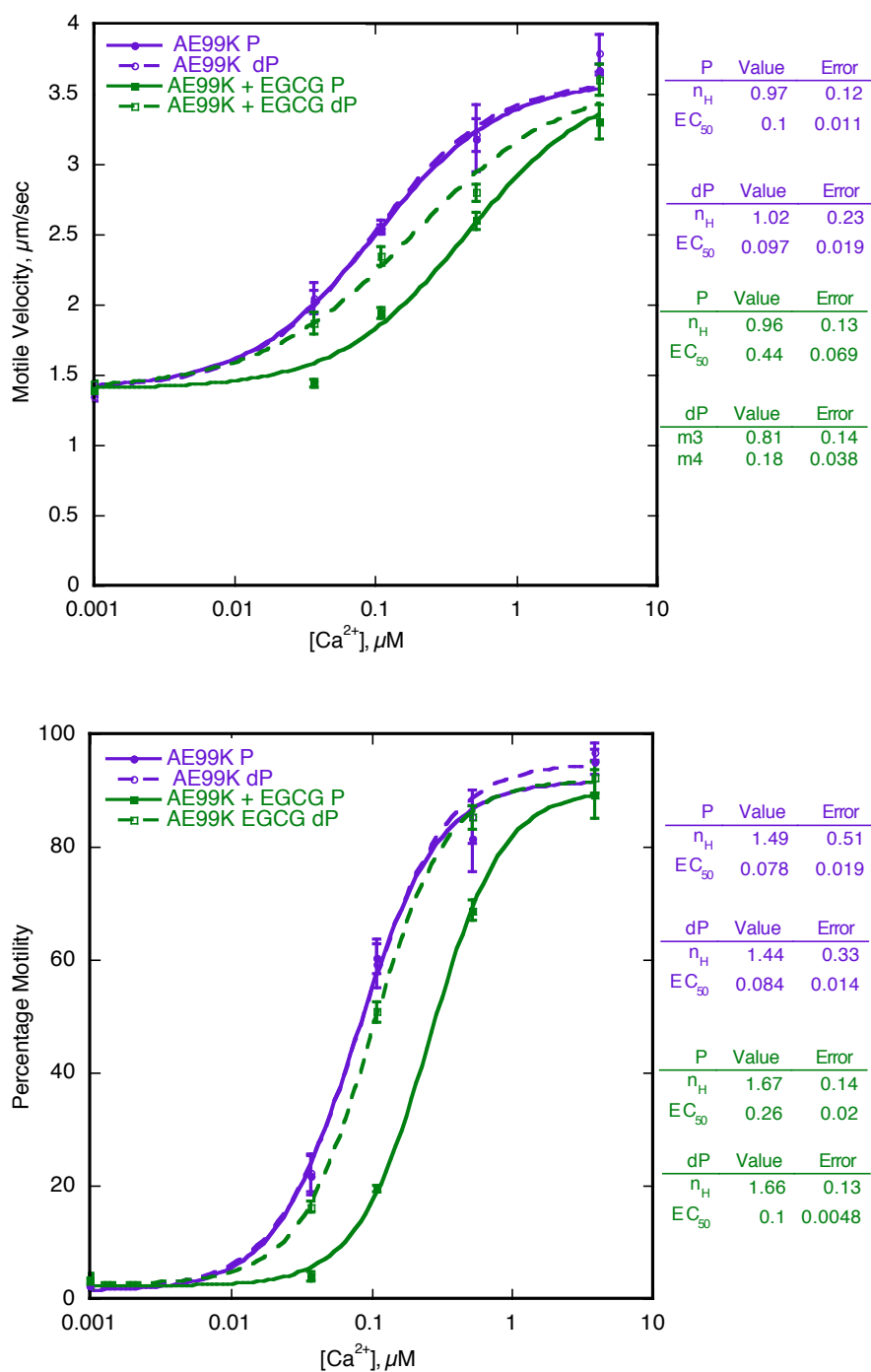
E99K transgenic mouse hearts have enlarged atria but normal ventricles. Adapted from Song et al (Song et al., 2011).

Dr Christopher Bayliss and Dr Andrew Messer have performed IVMA experiments comparing phosphorylated and dephosphorylated thin filaments containing E99K mutant actin. It was found that Ca^{2+} sensitivity was uncoupled from TnI phosphorylation. This finding was supported by echocardiography experiments on the E99K transgenic mice, whereby 21 and 38 week old mice showed a blunted response to β adrenergic stimulation. Since uncoupling has been clearly demonstrated in both actin mutations, in this chapter I will demonstrate the effect of EGCG on the uncoupling phenomenon of thin filaments reconstituted E99K mutant actin.

Thin filaments were reconstituted using 60 nM human cardiac tropomyosin, 60 nM phosphorylated or dephosphorylated troponin extracted from donor heart and 10 nM mutant actin extracted from E99K mouse. Five Ca^{2+} activation curves were created with phosphorylated or dephosphorylated thin filaments treated with 100 μM EGCG and a representative curve is shown in Figure 5-14. Data are summarized in Table 5-17 and Table 5-18 and full data for all the experiments can be found in APPENDIX. Consistent with previous measurements, E99K mutant thin filaments were uncoupled (Ratio $\text{EC}_{50} \text{ P/dP} = 0.99 \pm 0.021$, $n = 5$, $p = 0.8$ for percentage motile and Ratio $\text{EC}_{50} \text{ P/dP} = 0.99 \pm 0.028$, $n = 5$, $p = 0.8$ for motile velocity). 100 μM EGCG reduced the EC_{50} of phosphorylated E99K thin filaments by 2.9 ± 0.28 fold, but not this of

dephosphorylated thin filaments. As a result, the Ca^{2+} sensitivity upon TnI phosphorylation was restored ($\text{EC}_{50} \text{ P/dP} + \text{EGCG} = 2.02 \pm 0.21$, $n = 5$, $p < 0.01$ for percentage motility and $\text{EC}_{50} \text{ P/dP} + \text{EGCG} = 2.25 \pm 0.55$, $n = 5$, $p = 0.08$). Likewise E361G, EGCG had an effect on motile velocity on E99K thin filaments as well: the maximum motile velocity was reduced in phosphorylated thin filaments from $3.52 \pm 0.44 \mu\text{m/sec}$ to $2.9 \pm 0.13 \mu\text{m/sec}$, $n = 5$, $p = 0.02$.

Figure 5-14: Ca^{2+} activation curve comparing phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG



Motile velocity (top) and percentage motility (bottom) of thin filaments reconstituted with phosphorylated (solid lines) or dephosphorylated (dotted lines) donor troponin and E99K mutant actin was measured over a range of Ca^{2+} concentrations in the same experiment of IVMA. Purple lines represent E99K thin filaments and green lines represent thin filaments treated with 100 μM EGCG.

Table 5-17: Ca^{2+} sensitivity data for percentage motility of phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max motile velocity, $\mu\text{m}/\text{sec}$
E99K	P	0.073 ± 0.0088 (5)	0.99 ± 0.028 (5)	1.21 ± 0.18 (5)	3.52 ± 0.44 (5)
E99K	dP	0.073 ± 0.0074 (5)		1.36 ± 0.22 (5)	3.62 ± 0.11 (5)
EGCG	P	0.25 ± 0.067 (5)	2.25 ± 0.55 (5)	1.46 ± 0.26 (5)	2.9 ± 0.13 (5)*
EGCG	dP	0.11 ± 0.019 (5)		1.55 ± 0.15 (5)	3.34 ± 0.14 (5) †

Table 5-18: Ca^{2+} sensitivity data for motile velocity of phosphorylated and dephosphorylated E99K mutant thin filaments treated with 100 μM EGCG

Treatment		EC_{50} , μM	Ratio EC_{50} P/unP	n_H	Max % motile
E99K	P	0.074 ± 0.0052 (5)	0.99 ± 0.021 (5)	1.9 ± 0.26 (5)	94.9 ± 0.26 (5)
E99K	dP	0.074 ± 0.0054 (5)		1.93 ± 0.24 (5)	94.45 ± 0.77 (5)
EGCG	P	0.21 ± 0.026 (5) *	2.02 ± 0.21 (5) ††	1.74 ± 0.16 (5)	85.35 ± 3.14 (5)
EGCG	dP	0.1 ± 0.0069 (5) †		2.3 ± 0.76 (5)	90.55 ± 1.91 (5)

* $p < 0.05$, for presence and absent of reagent. † $p < 0.05$, †† $p < 0.05$; for the effect of phosphorylation. p values were calculated using one-way ANOVA. p values for the ratios were calculated using single value t test compared to 1. EC_{50} was rounded to two significant figures. In brackets the n number.

5.6 DISCUSSION

5.6.1 The uncoupling phenomenon and its relationship to EC₅₀

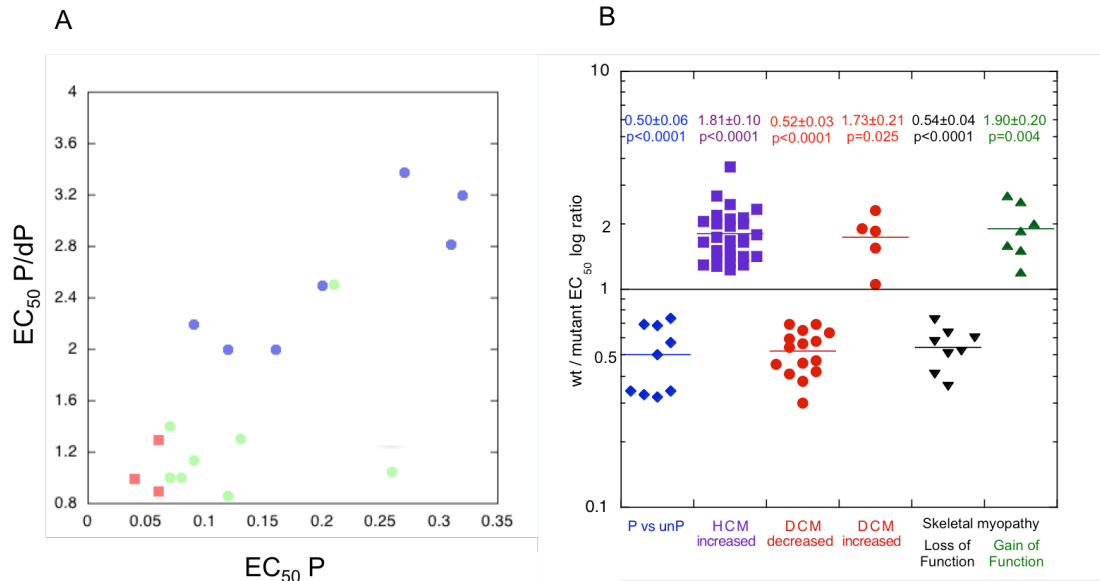
In this chapter, I have tested the effect of the Ca²⁺ desensitiser EGCG on the coupling between TnI phosphorylation and Ca²⁺ sensitivity on DCM and HCM mutant thin filaments. The coupling between Ca²⁺ sensitivity and TnI phosphorylation has been previously studied in most of the mutations that I investigated, and this was the reason behind the selection of the particular mutations. In this study, only mutations in the thin filament proteins have been considered. Uncoupling of Ca²⁺ sensitivity to TnI phosphorylation by PKA has been previously found by other members of Steve Marston's group in all of the mutations described in this chapter: G159D in TnC, K280N in TnT, K36Q in TnI, E50K and E54K in Tm and E361G and E99K in actin. My experiments further confirmed these results, as it was found that Ca²⁺ sensitivity was the same in phosphorylated and dephosphorylated DCM or HCM mutant thin filaments. Uncoupling in the Tpm1.1 E180G mutation was a novel finding. The table from Messer and Marston shows that this phenomenon has been noticed in other labs (see Section 1.4 for more details and review by Messer and Marston, 2014).

The absolute Ca²⁺ sensitivity was not necessarily similar across all experiments. For example, with K36Q mutation in TnI, different Ca²⁺ sensitivities were observed when using troponin extracted from the K36Q tissue sample (experiments performed by Dr Massimiliano Memo and Dr Andrew Messer, (Memo, 2012)) than when K36Q mutant troponin was produced by exchanging the TnI subunit of donor troponin with a mutant recombinant TnI subunit containing the K36Q mutation. Thus, the diverse source of the sample can account for the differences in the absolute Ca²⁺ sensitivity that were observed. However, uncoupling was observed independently of the absolute value of the Ca²⁺ sensitivity.

Myofibrillar experiments have been performed for the two actin mutations whereby the Ca²⁺ sensitivity of myofibrillar force was measured from myofibrils extracted from mouse hearts. The Ca²⁺ sensitivity of single myofibres in E361G transgenic mice is calculated to be $0.38 \pm 0.05 \mu\text{M}$ for phosphorylated and $0.43 \pm 0.06 \mu\text{M}$ for dephosphorylated myofibres. These values do not correspond to the values I found using IVMA ($0.087 \pm 0.0023 \mu\text{M}$ for phosphorylated and $0.08 \pm 0.0017 \mu\text{M}$ for

dephosphorylated thin filaments) but in both cases phosphorylated and dephosphorylated thin filaments have equal Ca^{2+} sensitivities compared to control (Vikhorev et al., 2014), so the Ca^{2+} sensitivity was uncoupled to TnI phosphorylation in both IVMA and in the myofibrillar assay. The EC_{50} found in the IVMA system is much smaller than the EC_{50} produced in the myofibrillar assay. This is due to the fact that the myofibrillar assay is force dependent whereas the IVMA measures unloaded filaments. In order to investigate this matter more, the relationship between the coupling constant (defined as the EC_{50} ratio of phosphorylated to dephosphorylated filaments) and the EC_{50} , which represents the Ca^{2+} sensitivity, was plotted (Figure 5-15 A). We can observe that the coupling constant is related to the Ca^{2+} sensitivity in the case of treatment of donor thin filaments with EMD 57033, meaning that it is a possibility that EMD 57033 pushes the equilibrium on the Tn complex so far towards the N terminal bound conformation that the phosphorylation of troponin cannot have an effect. (See Section 4.4.3). On the other hand, the coupling constant is not related to Ca^{2+} sensitivity in the case of mutations, where absolute Ca^{2+} sensitivity seems to be independent from uncoupling.

When ratios were plotted for the EC_{50} WT/mutant to investigate the effect of a mutation on the absolute Ca^{2+} sensitivity. We can see from Figure 5-15 B that mutations can either increase or decrease the absolute Ca^{2+} sensitivity but there is always a limit at around 2.0 and 0.5 fold. This is consistent with the fact that the Ca^{2+} sensitivity decreases by 2-3 fold upon phosphorylation. It is very interesting to point out that EGCG restored the ratio of $\text{EC}_{50}\text{P/dP}$ to these values.

Figure 5-15: The relationship between EC_{50} and uncoupling

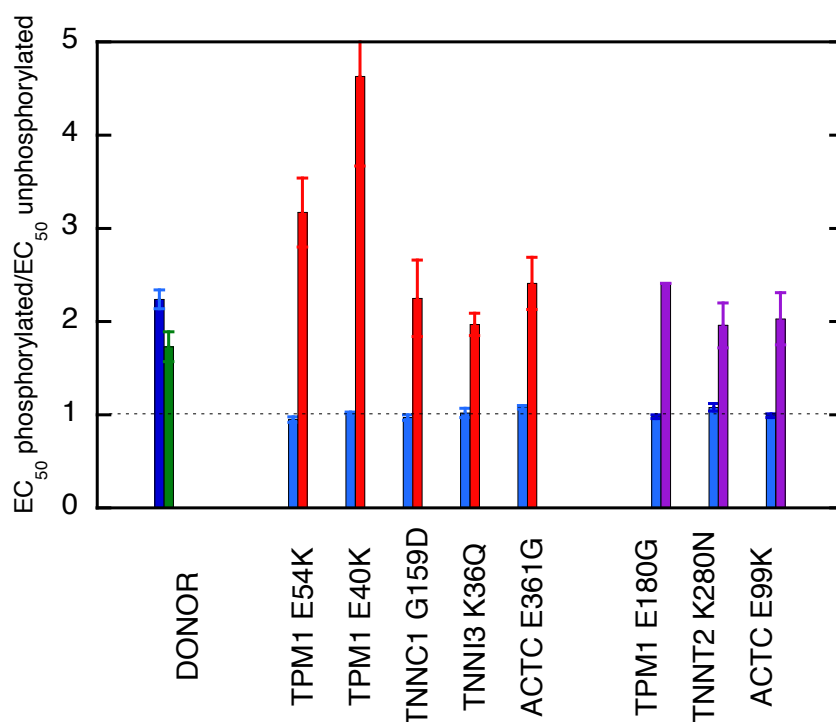
A) Coupling constant (EC_{50} P/dP) is plotted against the EC_{50} P value. Blue symbols represent untreated donor thin filaments or single myofibrils from wild type mice treated with EMD 57033 and green symbols represent HCM or DCM mutations treated with EGCG. Red represents donor thin filaments treated with Ca^{2+} sensitizers B) The EC_{50} WT/mutant ratio is plotted for P/dP (blue), HCM (purple), DCM mutations that decrease the Ca^{2+} sensitivity (red), DCM mutations that increase the Ca^{2+} sensitivity (red), loss of function mutations in skeletal muscle (black) and gain of function mutations in skeletal muscle (green). Values are shown $\pm$ SEM and p values were calculated using a single value t test against 1.

5.6.2 The Ca^{2+} desensitizer EGCG restores coupling to thin filaments with HCM and DCM mutations.

The observation that EGCG reduces Ca^{2+} sensitivity in human donor thin filaments with small effects on sliding speed or Hill coefficient is also in accord with previous measurements in skinned cardiac muscle fibres (Liou et al., 2008, Tadano et al., 2010). The phosphorylation-dependent change in Ca^{2+} sensitivity was unaffected by EGCG in reconstituted thin filaments, in contrast to Ca^{2+} sensitizers. For the first time, I show that EGCG was able to restore the Ca^{2+} sensitivity change upon TnI phosphorylation, which is otherwise uncoupled in thin filament cardiomyopathies. This was because EGCG was able to decrease the Ca^{2+} sensitivity of phosphorylated thin filaments containing DCM or HCM causing mutations, but it had different effects

on phosphorylated and dephosphorylated thin filaments. EGCG either had no effect or increased (in the case of the three tropomyosin mutations) the Ca^{2+} sensitivity of dephosphorylated mutant thin filaments. This resulted in the reversal of uncoupling of the TnI phosphorylation to Ca^{2+} sensitivity in all the DCM and HCM thin filament mutations studied (Table 5-19 and Figure 5-16). The effect of EGCG was also tested in myofibrils of E361G mice. EGCG decreased the Ca^{2+} sensitivity of myofibrils from wild type mice and also from heterozygous E361G mice (experiments performed by Dr Petr Vikhorev). EGCG was also able to restore the Ca^{2+} sensitivity difference upon TnI phosphorylation in myofibrils extracted from the E361G transgenic mice, confirming the results from the IVMA (Papadaki et al., 2015)(see Figure 5-17)

Figure 5-16: The effect of EGCG on the coupling between TnI phosphorylation and Ca^{2+} sensitivity on DCM and HCM mutations



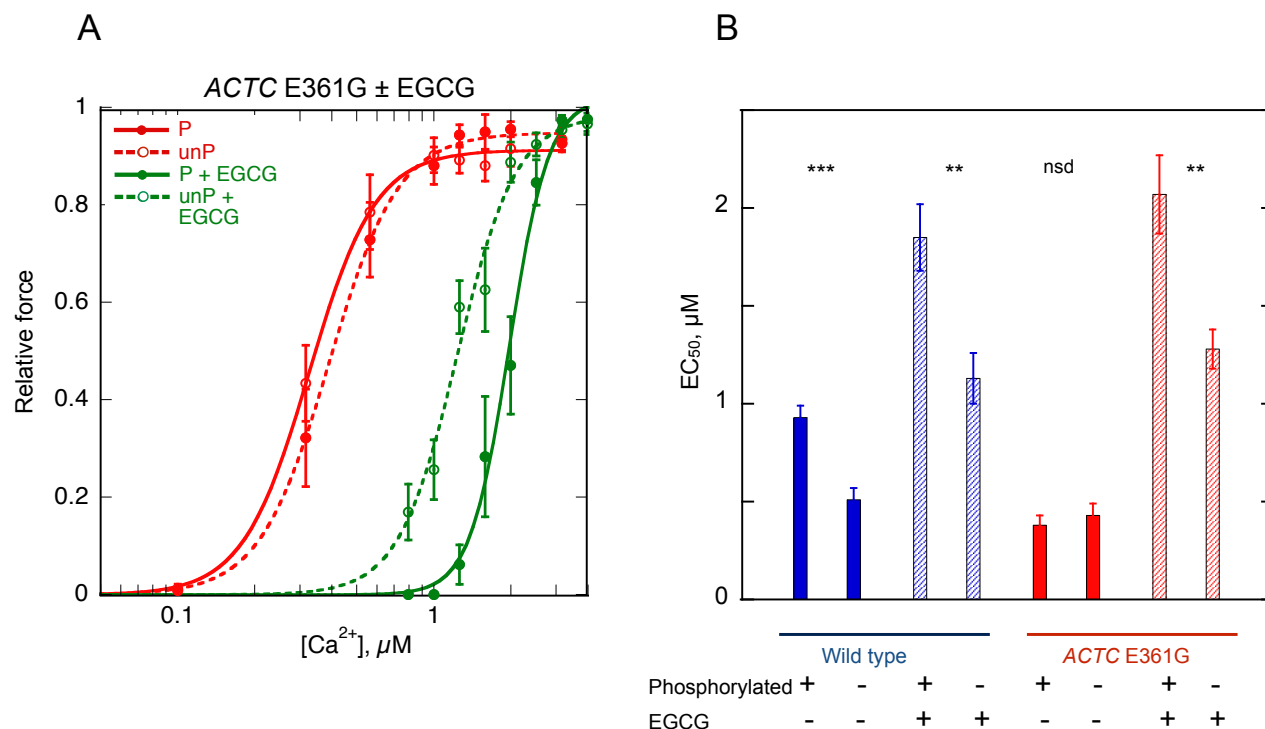
The Ratio of EC_{50} P/dP thin filaments is plotted with (red, green or purple) and without EGCG (blue). In red the DCM mutations are shown, in purple the HCM mutations and the donor are indicated with the color green. We can see that in all the mutant thin filaments treated with EGCG the EC_{50} P/dP ratio is restored to normal value.

Table 5-19: Ca²⁺ sensitivity data for DCM and HCM thin filaments treated with EGCG

Mutation	EC ₅₀ of thin filaments containing phosphorylated troponin μM ± SEM	EC ₅₀ of thin filaments containing unphosphorylated troponin μM ± SEM	Ratio EC ₅₀ P / unP ± SEM	EC ₅₀ of thin filaments treated with EGCG containing phosphorylated troponin μM ± SEM	EC ₅₀ of thin filaments treated with EGCG containing unphosphorylated troponin μM ± SEM	Ratio EC ₅₀ P + EGCG / unP + EGCG ± SEM	Ratio EC ₅₀ P + EGCG / P ± SEM	Ratio EC ₅₀ unP + EGCG / unP ± SEM
WT	0.14 ± 0.03(7)	0.059 ± 0.011(6)††	2.24 ± 0.10(6)††	0.26 ± 0.02(7)**	0.15 ± 0.02(6)**††	1.73 ± 0.16(6)††	2.15 ± 0.45(7)	2.80 ± 0.48(6)
DCM								
<i>TPM1</i> E54K	0.11 ± 0.013(5)	0.11 ± 0.02(5)	0.95 ± 0.03(5)	0.23 ± 0.03(5)*	0.071 ± 0.005(5)††	3.17 ± 0.37(5)††	2.33 ± 0.53(5)	0.68 ± 0.10(5)
<i>TPM1</i> E40K	0.18 ± 0.03(8)	0.17 ± 0.03(8)	1.02 ± 0.007(8)	0.22 ± 0.04(5)	0.052 ± 0.01(5)*†	4.63 ± 0.96(5)†	1.70 ± 0.43(3)	0.34 ± 0.05(3)
<i>TNNC1</i> G159D	0.092 ± 0.004(5)	0.095 ± 0.005(5)	0.97 ± 0.03(5)	0.19 ± 0.03(5)*	0.088 ± 0.005(5)*†	2.25 ± 0.41(5)†	2.13 ± 0.36(5)	0.93 ± 0.02(5)
<i>TNNI3</i> K36Q	0.18 ± 0.03(10)	0.18 ± 0.04(10)	1.02 ± 0.05(10)	0.19 ± 0.02(5)	0.10 ± 0.009(5)*†	1.97 ± 0.12(5)†	2.32 ± 0.29(3)	1.28 ± 0.04(3)
<i>ACTC</i> E361G	0.087 ± 0.002(5)	0.080 ± 0.002(5)	1.08 ± 0.02(5)	0.20 ± 0.02(5)**	0.081 ± 0.002(5)††	2.41 ± 0.28(5)††	2.27 ± 0.28(5)	1.01 ± 0.02(5)
HCM								
<i>TPM1</i> E180G	0.086 ± 0.013(3)	0.087 ± 0.011(3)	0.98 ± 0.02(3)	0.11 ± 0.01(5)*	0.048 ± 0.003(5)*†	2.41 ± 0.32(5)†	1.36 ± 0.10(3)	0.51 ± 0.06(3)
<i>TNNT2</i> K280N	0.11 ± 0.007(5)	0.10 ± 0.004(5)	1.08 ± 0.04(5)	0.20 ± 0.03(5)*	0.10 ± 0.004(5)†	1.96 ± 0.24(5)†	2.15 ± 0.18(3)	1.09 ± 0.006(3)
<i>ACTC</i> E99K	0.074 ± 0.005(5)	0.074 ± 0.005(5)	0.99 ± 0.02(5)	0.21 ± 0.03(5)**	0.10 ± 0.007(5)*††	2.03 ± 0.21(5)††	2.85 ± 0.28(5)	1.43 ± 0.13(5)

* p<0.05, ** p<0.01; for presence and absence of EGCG, † p<0.05, †† p<0.01; unphosphorylated compared with phosphorylated using paired t test. Ratios: single value t test compared with 1. ANOVA analysis of this dataset is shown in the APPENDIX. EC₅₀ values rounded to 2 significant figures. In brackets is the number of experiments.

Figure 5-17: The effect of EGCG on myofibrils from E361G transgenic mice



A) Ca²⁺ activation curve for myofibrils extracted from the E361G transgenic mouse in the absence (red) and presence (green) of 10 μM EGCG. Solid lines represent phosphorylated myofibrils and dotted lines represent dephosphorylated myofibrils. B) Effects of EGCG mutation, phosphorylation and EGCG treatment on the EC₅₀ of myofibrillar contraction. *** indicates $p < 0.001$ and ** indicates $p < 0.01$ between phosphorylated and dephosphorylated myofibrils calculated by unpaired student t test. Data produced by Dr Petr Vikhorev (Papadaki et al., 2015).

5.6.3 Mechanistic insights on the action of EGCG

The uncoupling phenomenon was first discovered as a common molecular mechanism for DCM caused by thin filament mutations, in a study by Memo et al. This study considered the mutations *TPM1* E54K, E40K and D230N, *TNNI3* K36Q, *TNNT2* R141W and ΔK210, *TNNC2* G159D and *ACTC* E361G; some of them are also included in this study. Thin filaments carrying any of these mutations showed independence of Ca²⁺ sensitivity to the TnI phosphorylation. The fact that EGCG was able to restore the phosphorylation dependent relationship of Ca²⁺ sensitivity in the case of a mutation in any protein of the thin filament equally, is compatible with a common mechanism for uncoupling and re-coupling by EGCG.

Several studies have shown that EGCG binds to C terminal domain of cTnC that is responsible for the desensitizing action of EGCG to the myofilament (Liou et al., 2008). Solution NMR structures of TnC-EGCG have shown that EGCG binds to the hydrophobic cleft of cTnC C terminal domain, in a position similar to this of EMD 57033. However, unlike EMD 57033 that binds deep into the hydrophobic core of cTnC, EGCG can bind closer to the surface of the pocket in the same way as cTnI₃₄₋₇₁. EGCG binds in complex with TnI₃₄₋₇₁ with a KD of $1.64 \pm 0.24 \mu\text{M}$. (Robertson et al., 2009). Molecular Dynamics simulations also show that EGCG is able to bind in complex with TnC and TnI₃₄₋₇₁ in different interchangeable orientations (Botten et al., 2013).

I suggest that it is possible that EGCG can re-stabilise the TnC- TnI interaction that is hypothesized to be unstable in uncoupled DCM and HCM mutants, restoring the Ca^{2+} response to phosphorylation in thin filament cardiomyopathies. Molecular Dynamics simulations have shown that the DCM mutation G159D that has also been found to uncouple strengthens the binding of TnI₃₄₋₇₁ peptide to the hydrophobic cleft of TnC, whilst EGCG weakens this interaction (Botten et al., 2013), compatible with their opposite effects on coupling.

Dr Petr Vikhorev showed that in myofibrils from wild type mice, EGCG slightly decreased ($p = 0.059$) the k_{ACT} (rate of force development measured at high Ca^{2+}) in unphosphorylated TnI. EGCG also had an effect on k_{ACT} in phosphorylated and dephosphorylated myofibrils from E361G mice (Papadaki et al, 2015). In IVMA EGCG also had an effect on the maximum velocity for some of the mutations, suggesting that unlike EMD 57033, EGCG might have an effect on the cross-bridge activation kinetics of the sarcomere. It is possible that EGCG reduces Ca^{2+} sensitivity by slowing Ca^{2+} activation kinetics. EGCG did not affect the EC_{50} P/dP or T_{LIN} in wild type myofibrils but changed this of E361G myofibrils suggest that EGCG can restore coupling independently of its Ca^{2+} desensitizing action (see Table 5-20)

Table 5-20: Effect of E180G mutation, phosphorylation and EGCG on myofibrillar contractility

		$F_{\max}$, kN/m ²	EC_{50} , μ M	n_H	k_{ACT} , s ⁻¹	t_{LIN} , ms	k_{REL} , s ⁻¹
WT¶	P	100.9±6.3(14)	0.93±0.06(11)	10.43±1.84(10)	4.16±0.43(11)	50.8±3.5(12)	35.0±4.0(10)
	unP	87.1±6.0(16)	0.51±0.06(14)†††	4.74±0.66(13)††	4.50±0.24(15)	67.0±4.2(16)†	23.2±2.8(11)†††
WT+EGCG	P	92.5±5.0(15)	1.85±0.17(9)***	4.30±0.85(8)**	4.44±0.24(15)	46.7±3.9(12)	41.0±4.0(12)
	unP	92.6±8.9(8)	1.13±0.13(5)†††***	5.08±1.24(5)	3.47±0.55(6)	88.9±9.9(7)†††*	22.3±1.2(7)††
E361G¶	P	93.5±8.9(11)	0.38±0.05(12)§§§	5.39±1.26(9)§	4.51±0.32(13)	75.4±6.5(10)§§	21.6±2.8(10)§§§
	unP	89.3±8.6(10)	0.43±0.06 (10)	4.48±0.70(8)	4.12±0.32(11)	70.8±9.7(11)	21.6±2.1(10)
E361G+EGCG	P	89.1±4.5(12)	2.07±0.20(5)*** †††	7.18±1.54(5)	3.43±0.20(13)*§ ††	49.7±3.3(12)**	31.0±2.6(12)*§
	unP	96.3±11.1(11)	1.28±0.10(8)†††*** †††	4.14±0.42(7)†	3.25±0.26(11)*** ††	63.0±4.7(12)†§	31.0±2.0(12)**§§ ††

* p<0.05, ** p<0.01, *** p<0.001; EGCG treated compared with no EGCG,

† p<0.05, †† p<0.01, ††† p<0.001; unphosphorylated compared with phosphorylated,

§ p<0.05, §§ p<0.01, §§§ p<0.001; *ACTC* E361G compared with wild-type,

‡ p<0.05, ‡‡ p<0.01, ‡‡‡ p<0.001; *ACTC* E361G EGCG treated compared with no EGCG wild-type, ¶ Vikhorev et al. 2014.

In brackets is the number of experiments. Statistical analysis carried out by un-paired t test (equal variance).

5.6.4 Clinical relevance of re-coupling by EGCG

EGCG is an extract from green tea, the consumption of which has been found to be beneficial for cardiovascular diseases in epidemiological studies (Hertog et al., 1993). Common treatment for heart failure can include agents that work through the β receptor such as dobutamine but in long term they can lead to arrhythmias. Drugs that act on the myofilament directly and alter the Ca^{2+} sensitivity thus target the effect of the mutations directly can provide a good alternative.

Such an example is EGCG, which acts directly on thin filament. EGCG can be beneficial especially for the treatment of HCM, as it is always associated with increased Ca^{2+} sensitivity and data from this study also show that additionally the Ca^{2+} sensitivity is uncoupled to TnI phosphorylation. Ca^{2+} antagonists have been suggested as a potential treatment for HCM (Semsarian et al., 2002). EGCG is able to both decrease the Ca^{2+} sensitivity and re-couple the Ca^{2+} sensitivity to phosphorylation. The ability to re-couple DCM mutant could also be favourable, since uncoupling seems to be the primary cause of DCM and also the Ca^{2+} sensitivity of phosphorylated and dephosphorylated thin filaments was restored to normal values. EGCG is a polyphenol that has been described as a promiscuous molecule and has many pharmacological properties thus it is not specific for troponin (Ingólfsson et al., 2014). In fact, EGCG in nM concentrations has a positive inotropic action because it can bind to ryanodine receptor type 2, Na^+/K^+ ATPase and Na/Ca^{2+} exchanger (Feng et al., 2012), which may override the desensitising action on the contractile apparatus. Although EGCG is too non-specific to be a viable drug for treatment of HCM this study has demonstrated an important proof of principle: that it is possible to directly reverse the primary effects found in thin filament cardiomyopathies pharmacologically.

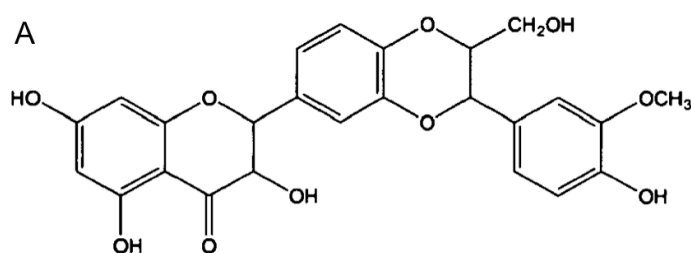
6 The effect of EGCG analogues on human heart thin filaments

6.1 Introduction

In the previous chapter I showed the action of the desensitiser Epigallocatechin-3-gallate (EGCG) and its potential role as a therapeutic candidate for thin filament cardiomyopathies. However, EGCG is a promiscuous molecule, limiting its potential as a therapeutic agent. As part of collaboration with Dr Brian Blagg from the University of Kansas we received 30 compounds that have a similar structure to EGCG. These compounds have been originally developed as Hsp90 inhibitors, as EGCG has been found to inhibit Hsp90 by directly binding to it (Khandelwal et al., 2013b).

Silibinin or Silybin is constituent of silimarine, which is an extract of the plant *silybum* (milk thistle), shown in Figure 6-1 and is another Hsp90 inhibitor (Zhao et al, 2011). Two of the given compounds were structural analogues of Silybin.

Figure 6-1: Chemical structure of Silybin



B



A. Chemical structure of Silybin. B. *Silybum maritimum* (milk thistle) from where silybin is extracted

The aim of this chapter is to test EGCG analogues identified as Hsp90 inhibitors as potential re-couplers of Ca^{2+} sensitivity to troponin I (TnI) phosphorylation. In order to address this specific aim, a quick screening method needed to be developed to identify the drugs that recouple TnI phosphorylation to changes in Ca^{2+} sensitivity. Then, the most potent re-couplers were selected for further analysis. Miss Alice Sheehan contributed to the majority of the experiments described in this chapter, as part of her MRes project in Biomedical Research under my direction.

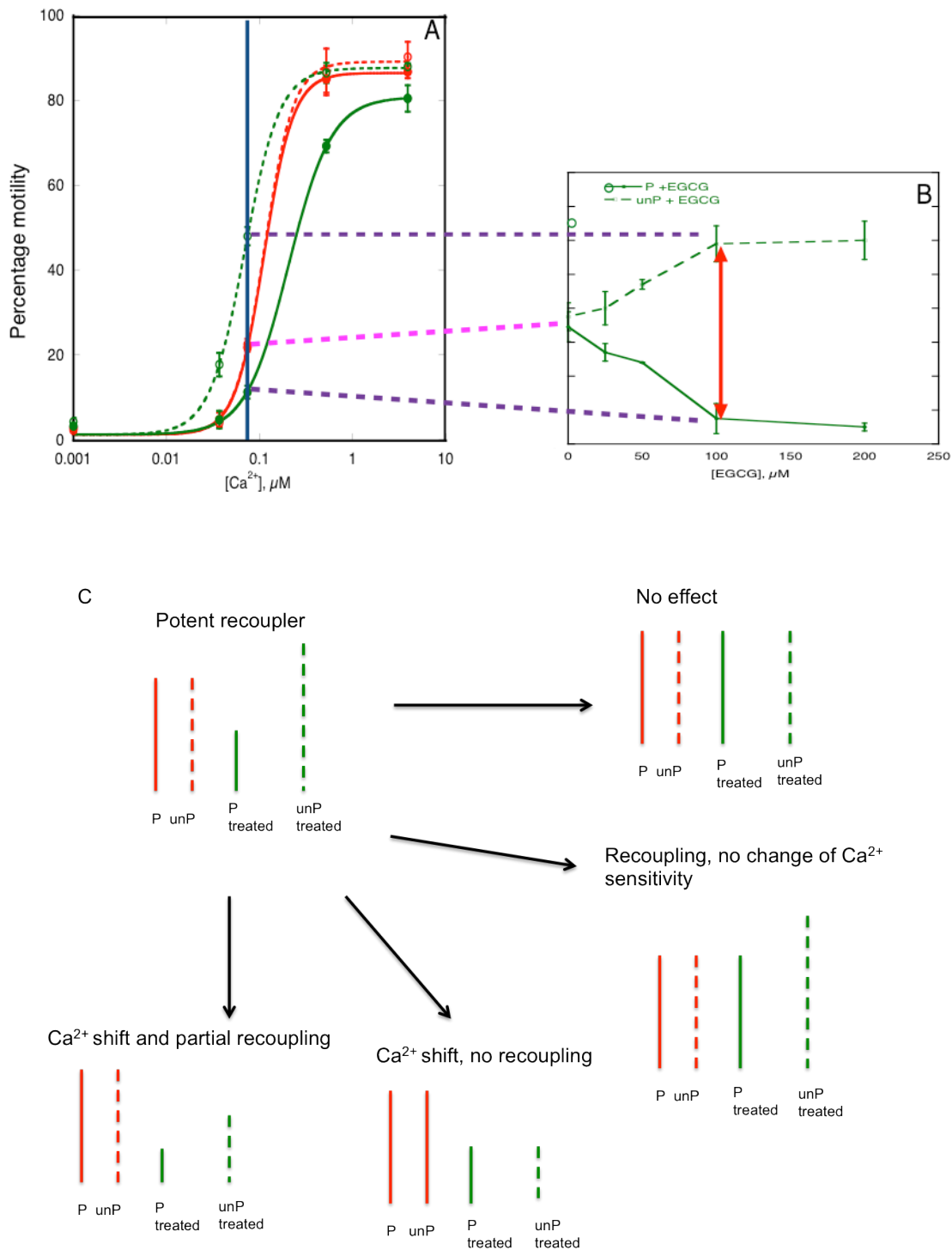
6.2 Rapid screening assay to identify re-couplers

6.2.2 Developing a screening methodology

To assess the recoupling properties of the analogues, the *in vitro* motility assay (IVMA) was used. However, a different methodology was used for the screening, which resulted in a quick way to detect small molecules with promising re-coupling abilities. The analogues were tested directly on mutant thin filaments to screen for their desensitising action and their recoupling properties. As the potential desensitising and re-coupling properties would be beneficial for Hypertrophic Cardiomyopathy (HCM), an HCM-causing mutation was chosen for the screening assay. The HCM-causing mutation of choice is the *TPMI* E180G mutation, because recombinant E180G tropomyosin from baculovirus/*sf9* was available in large stocks and could be combined with troponin from human donor heart, saving time in the preparation for the IVMA.

The assay is based on the observation that at an intermediate Ca^{2+} concentration of the Ca^{2+} activation curve, percentage motility and motile velocity of phosphorylated and dephosphorylated HCM thin filaments is equal because of uncoupling, but in case of recoupling due to the addition of drug they would be separate. Figure 6-2 shows the concept behind the screening assay and the possible outcomes of the analogues regarding a change in Ca^{2+} sensitivity and re-coupling of the mutant thin filaments. This screen would highlight small molecules that have recoupling properties as candidates for further investigation. The Ca^{2+} concentration chosen for the screening assay is $0.037\ \mu\text{M}$. Initially, a single compound concentration of $100\ \mu\text{M}$ was tested as this concentration was optimal for the activity of EGCG in the IVMA (see Section 5.3.4).

Figure 6-2: Drug screening assay concept and possible outcomes



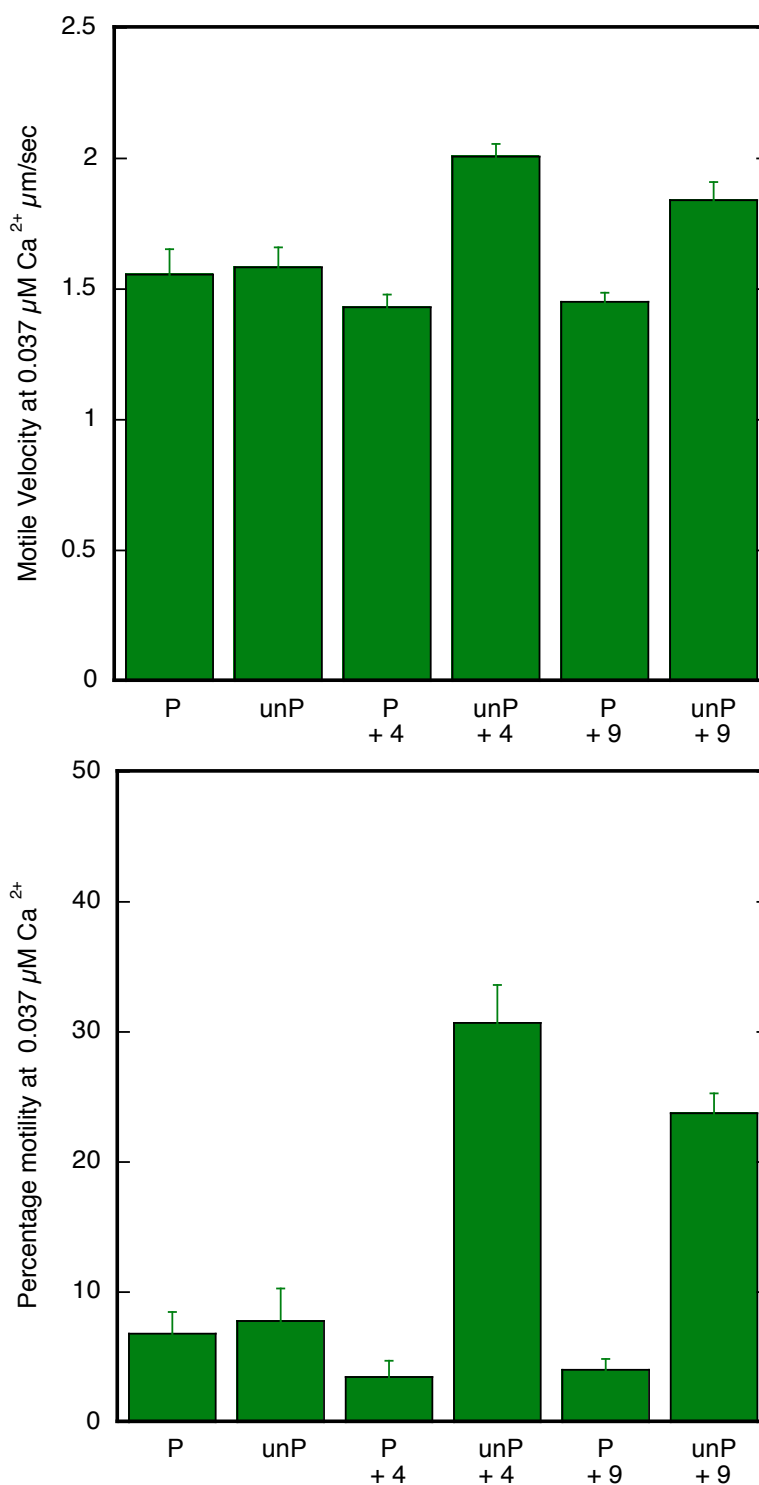
A) The effect of EGCG on Ca^{2+} activation curves of thin filaments reconstituted with E54K mutant tropomyosin. B) EGCG dose response curve of percentage motility for E54K thin filaments at $0.037 \mu M$ Ca^{2+} concentration corresponding to the Ca^{2+} EC_{50} . Purple and pink broken lines illustrate the relationship between the percentage motility of phosphorylated and dephosphorylated thin filaments at a constant Ca^{2+} concentration. C) shows possible outcomes of the assay for percentage motility and motile velocity at a specific Ca^{2+} concentration ($0.037 \mu M$). Red lines represent mutant thin filaments and green lines represent thin filaments treated with EGCG or compounds. Solid lines represent phosphorylated thin filaments and broken lines represent dephosphorylated thin filaments.

6.2.3 Results of the rapid screening assay

Thin filaments were reconstituted using 60 nM phosphorylated or dephosphorylated troponin, 60 nM E180G mutant tropomyosin and 10 nM rabbit skeletal actin. After ensuring that the motility assay was working using only mutant tropomyosin and actin as control, troponin was also tested with low and high Ca^{2+} buffers to make sure that filaments are regulated correctly with Ca^{2+} . Thin filaments reconstituted with phosphorylated or dephosphorylated troponin, tropomyosin and actin were incubated with 100 μM of the different compounds. For the screening experiments a single Ca^{2+} concentration was used, 0.037 μM Ca^{2+} , and in every flow motility cell a different compound was tested. One flow motility chamber contained phosphorylated thin filaments and the other flow motility chamber contained dephosphorylated thin filaments. In that way, a direct comparison between phosphorylated and dephosphorylated thin filaments can be made. A control cell containing only phosphorylated and dephosphorylated E180G thin filaments was performed every 4-5 drugs screened. An example of drug screening is shown in Figure 6-3. Dr Brian Blagg had suggested several compounds most likely to re-couple based on their structural similarity to EGCG and these compounds were tested first. Drugs number 4 and 9 (shown in the Figure) were re-couplers at 100 μM .

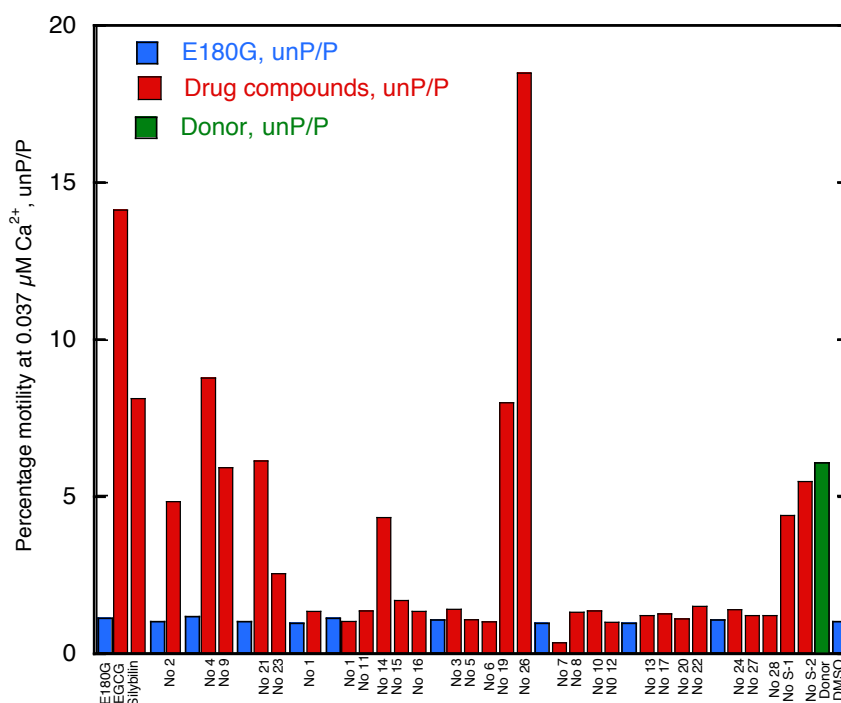
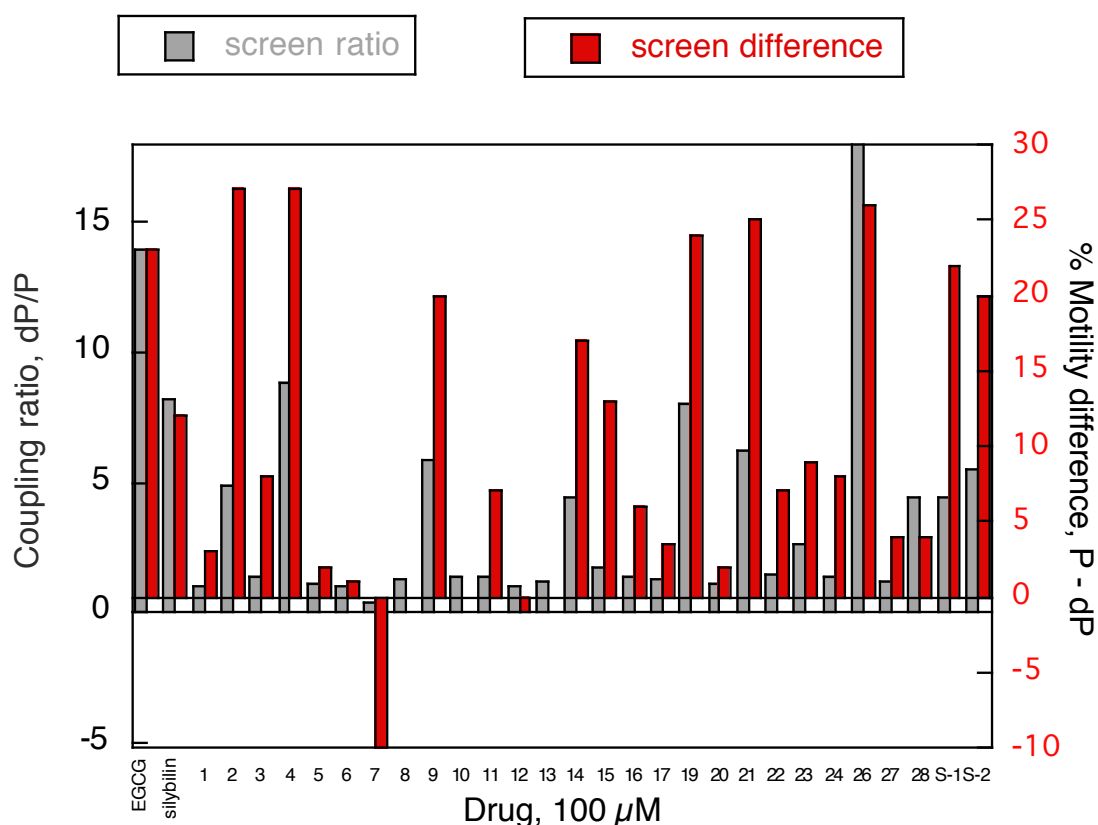
Figure 6-4 shows the data for the preliminary drug screen for all the compounds. Because the compounds were dissolved in DMSO according to the instructions given by their manufacturers, the effect of DMSO alone was also tested in thin filaments. DMSO had no effect on the motility of the filaments. The coupling constant as indicated by the ratio P/dP (screen ratio) and the motility difference between phosphorylated and dephosphorylated thin filaments (screen difference) were plotted. The screen ratio and the screen difference had a similar trend across the compounds, although difference in motility gave a more pronounced difference compared to ratios. For this reason, data are represented as the motility difference between phosphorylated and dephosphorylated thin filaments (P-dP) for the following study.

Figure 6-3: A screening example of two different compounds using the E180G HCM model



Motile velocity (top) and percentage motility (bottom) of phosphorylated and dephosphorylated E180G mutant thin filaments treated with 100 μM of drugs number 4 and number 9 were measured at 0.037 μM Ca^{2+} . Motility parameters of phosphorylated and dephosphorylated E180G mutant filaments without treatment were also tested. Each motility flow cell contained a different combination of the parameters (phosphorylation and compound).

Figure 6-4: Screening summary of all the compounds tested with IVMA



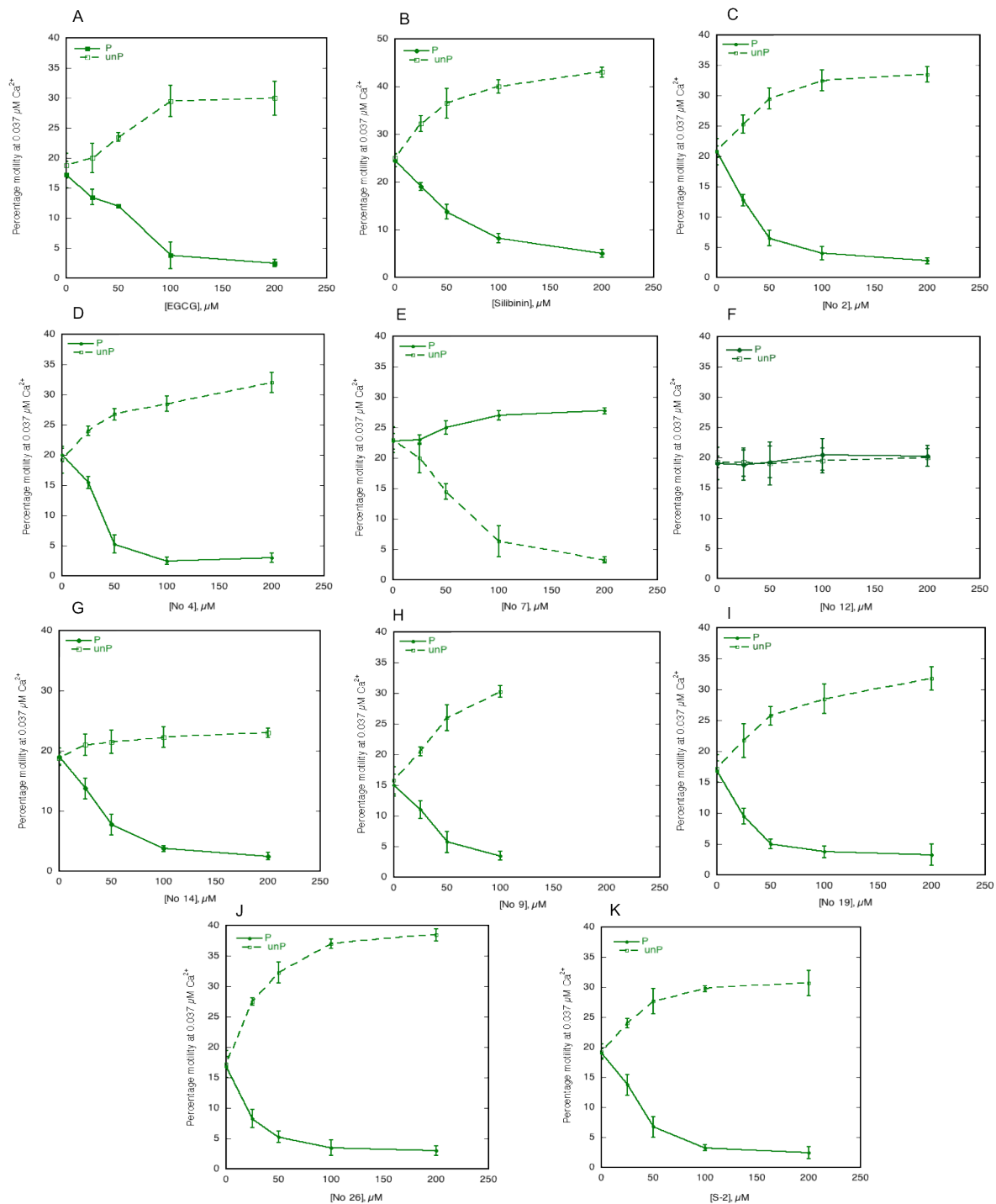
Top: Coupling ratio (screen ratio: dP/P) in grey and motility difference (screen difference: percentage motility P-dP) for all the drug compounds in the tested order. Bottom: Ratio dP/P for all the compounds, as well as the DMSO control. Red bars represent thin filaments treated with different drugs or DMSO, blue bars represent E180G HCM mutant thin filaments and green bars represent donor thin filaments. Percentage motility was measured at constant 0.037 μ M Ca^{2+} .

6.3 Evaluation of best candidates on *TPM1* E180G HCM mutant thin filaments

From the screening assay described, the 10 analogues that were the most potent re-couplers (i.e. gave the biggest coupling ratio and motility difference) were selected for further analysis. The selected drugs were: Silybin and drug numbers 2, 4, 7, 9, 12, 14, 19, 26 and S2. Drugs number 2, 4, 9, 12, 19 and 26 (EGCG analogues) were chosen because they gave the largest screen difference. Number 7 was chosen because it gave an interesting “negative” screen ration and S2 was the Silybin analogue that gave the best difference in the screening assay. Number 12 had a screening ratio of 1 so it was randomly chosen as a control analogue. Dose-response curves were created for these drugs using reconstituted E180G thin filaments. Thin filaments were reconstituted using 60 nM phosphorylated or dephosphorylated troponin, 60 nM E180G recombinant mutant tropomyosin from baculovirus/*sf9* and 10 nM rabbit skeletal actin. Thin filaments were incubated with the analogues mentioned in concentrations between 25-200 μM . The dose response curves for percentage motility and motile velocity can be found in Figure 6-5 and Figure 6-6. The difference in percentage motility between phosphorylated and dephosphorylated thin filaments treated with the analogues was also plotted and is shown in Figure 6-7. The Hill equation was fitted on the data for the percentage motility difference between phosphorylated and dephosphorylated thin filaments and the values of the EC_{50} , the nH and the magnitude difference (motility difference P-dP, $\Delta \%$) were calculated for each of the drugs (See Figure 6-8 and Table 6-1). In some cases where data for a certain dose was not available (for example number 9) the m_2 (max-min value) of the Hill equation was fixed according to the raw data of maximum and minimum values from IVMA to obtain the EC_{50} . We can observe that the most potent drug was number 26, with an EC_{50} value of $24.6 \pm 1.19 \mu\text{M}$ and magnitude of $37.8 \pm 1.4 \%$. EGCG, Silybin and drug numbers 2, 4, 9, 14, 19 and S2 showed similar levels of potency, the EC_{50} ranging between 30.5 ± 0.4 and $47.2 \pm 2.66 \mu\text{M}$. Drug number 12 was the control drug, as it had no effect on either phosphorylated or dephosphorylated thin filaments at the tested concentrations. Drug number 12 had no effect also in the preliminary screen, giving more confidence on the screening results. Drug number 7 had a unique property, that increased the motility parameters of phosphorylated thin

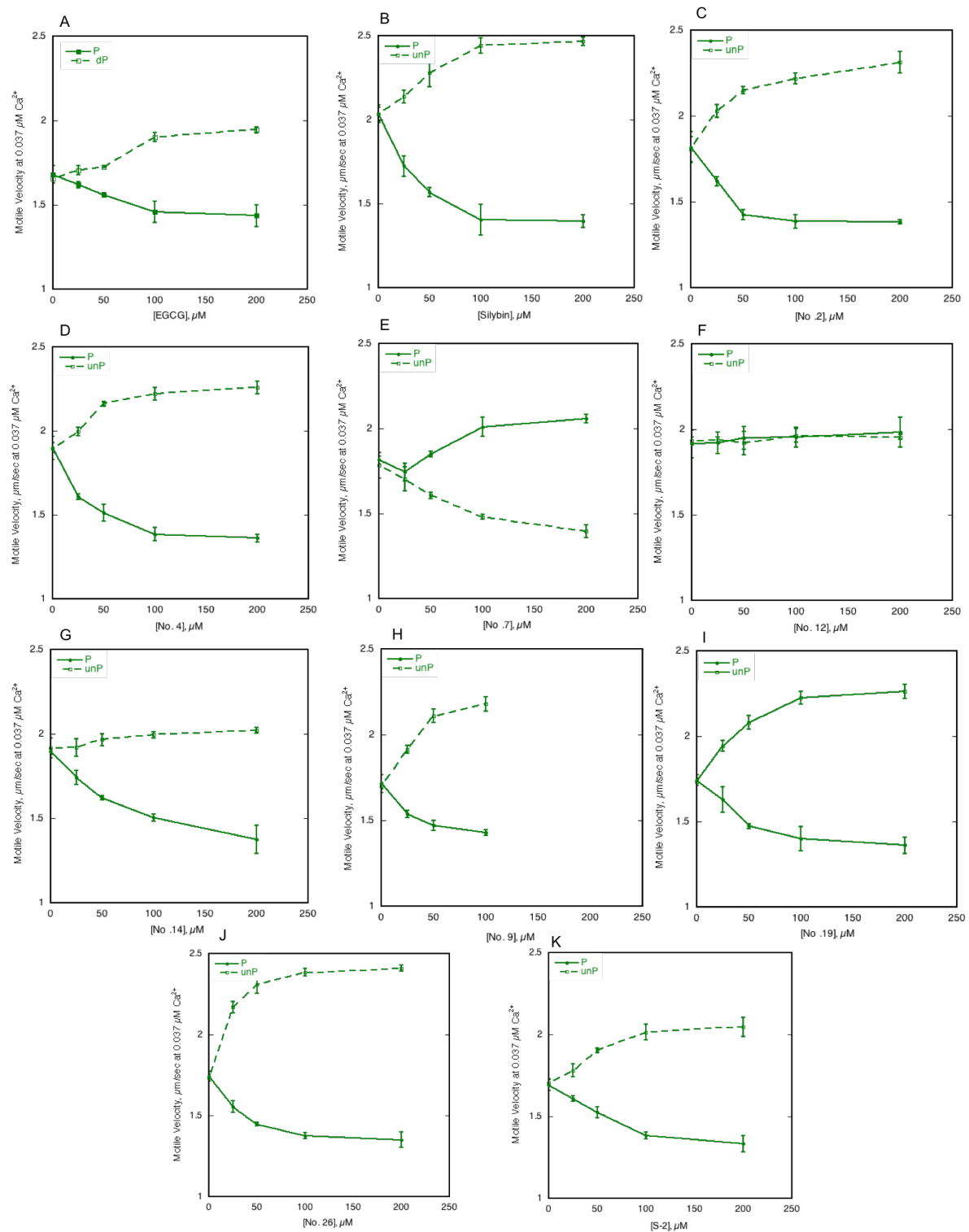
filaments and decreased the motility parameters of the dephosphorylated thin filaments, resulting in “reverse recoupling”.

Figure 6-5: Small molecule dose response data for percentage motility in *TPM1* E180G mutant thin filaments



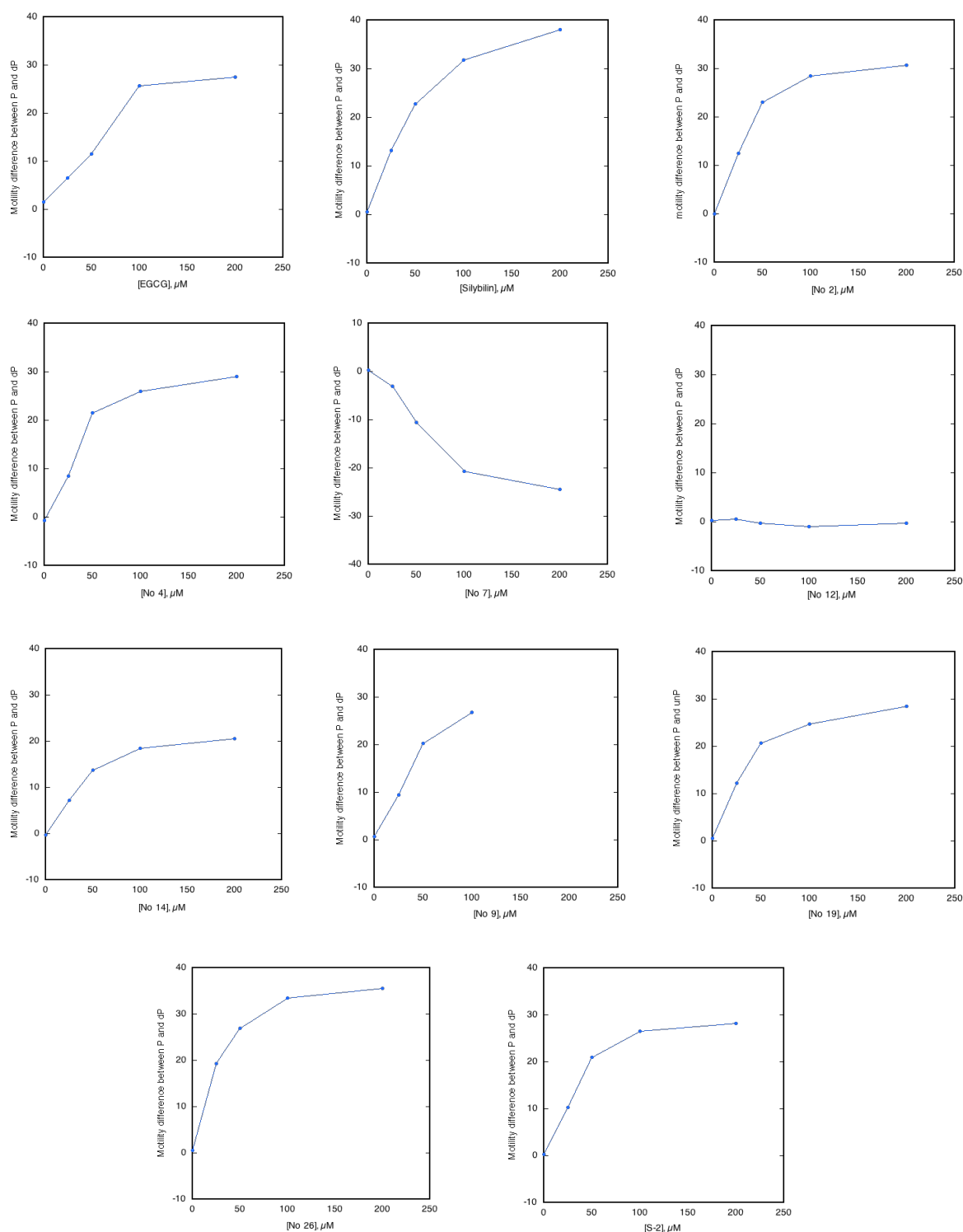
Percentage motility of phosphorylated or dephosphorylated E180G thin filaments was measured at a constant Ca^{2+} concentration of $0.037 \mu\text{M}$ with increasing drug doses. A) EGCG B) Silybin C) No 2 D) No 4 E) No 7 F) No 12 G) No 14 H) No 9 I) No 19 J) No 26 K) S-2. The drug compounds are in order of the screening experiments performed.

Figure 6-6: Small molecule dose response data for motile velocity of *TPM1* E180G mutant thin filaments



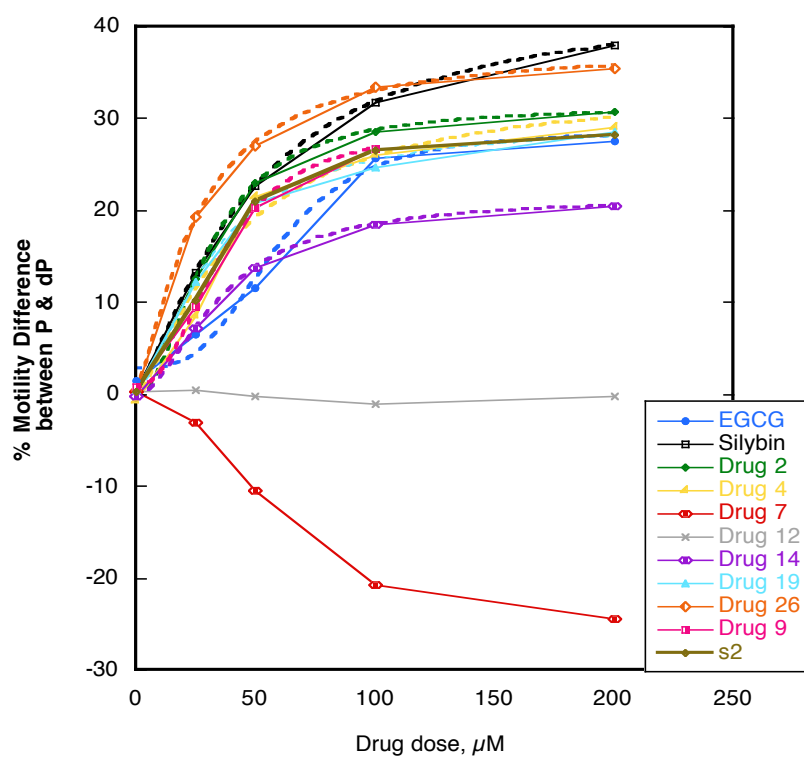
Motile velocity of phosphorylated or dephosphorylated E180G mutant thin filaments was measured at a constant Ca^{2+} concentration of $0.037 \mu\text{M}$ with increasing drug doses. A) EGCG B) Silybin C) No 2 D) No 4 E) No 7 F) No 12 G) No 14 H) No 9 I) No 19 J) No 26 K) S-2

Figure 6-7: Dose response data for percentage motility difference between phosphorylated and dephosphorylated *TPM1* E180G mutant thin filaments treated with different compounds



Percentage motility difference between phosphorylated or dephosphorylated E180G mutant thin filaments. Motility was measured at a constant Ca^{2+} concentration of $0.037 \mu\text{M}$ with increasing drug doses. A) EGCG B) Silybin C) No 2 D) No 4 E) No 7 F) No 12 G) No 14 H) No 9 I) No 19 J) No 26 K) S-2

Figure 6-8: Dose response data summary for percentage motility difference between phosphorylated and dephosphorylated *TPM1* E180G mutant thin filaments treated with different compounds



Percentage motility difference of phosphorylated and dephosphorylated thin filaments treated with different compounds. Percentage motility was measured at $0.037 \mu\text{M Ca}^{2+}$ and the Hill equation was fitted to the data to create the curves (dotted lines). Different colored lines represent different compounds.

Table 6-1: Data summary for the difference between phosphorylated and dephosphorylated *TPMI* E180G HCM mutant thin filaments treated with different compounds

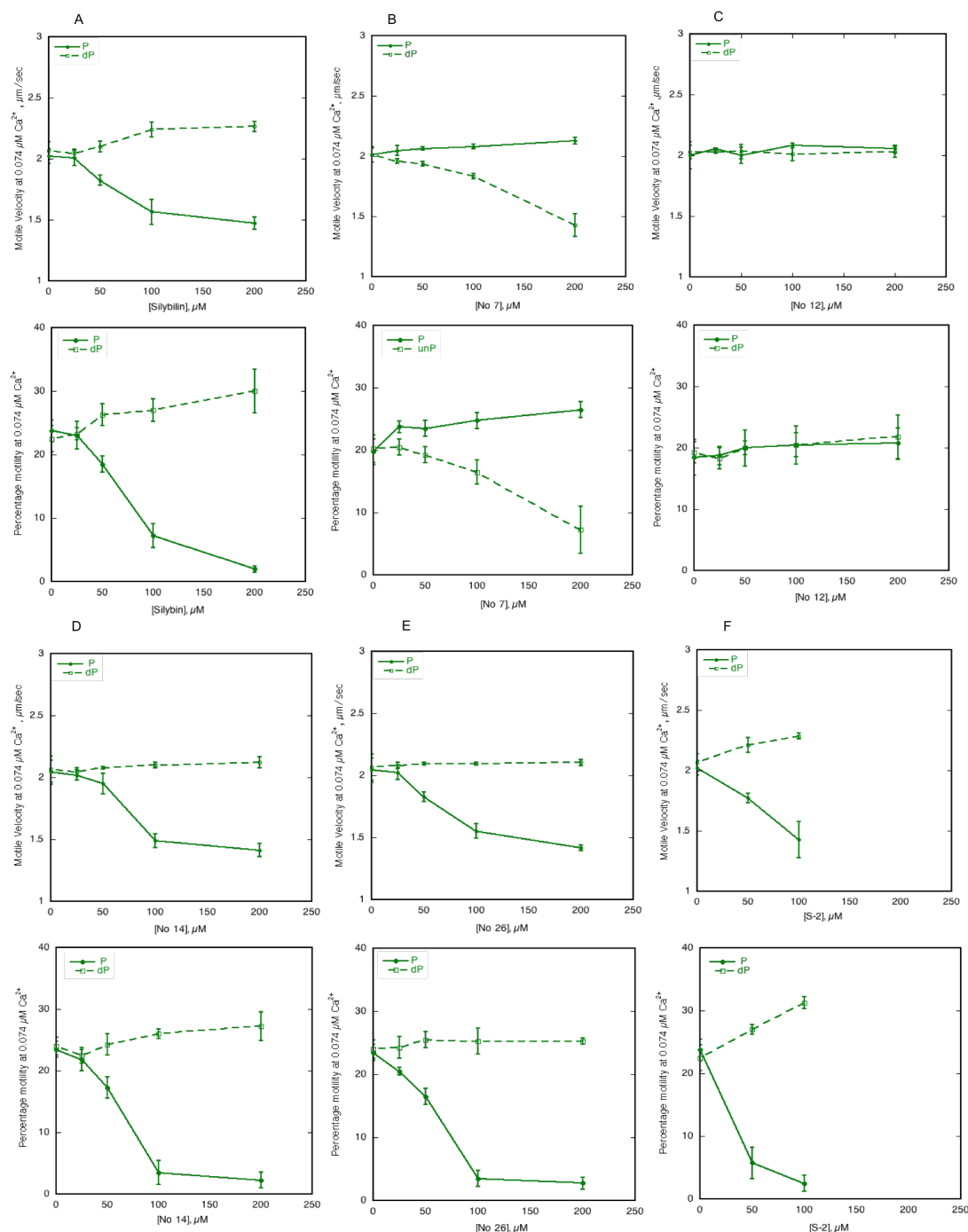
E180G + Drug	EC₅₀, μM	n_H	Magnitude (% difference)
EGCG	58.83 $\pm$ 13.31	2.15 $\pm$ 0.89	30.23 $\pm$ 4.81
Silibinin	47.21 $\pm$ 2.66	1.3 $\pm$ 0.078	43.74 $\pm$ 1.29
2	30.5 $\pm$ 0.41	2.02 $\pm$ 0.067	31.31 $\pm$ 2.24
4	33.98 $\pm$ 2.13	2.69 $\pm$ 0.43	28.52 $\pm$ 1.05
7	67.05 $\pm$ 20.31	1.71 $\pm$ 0.57	28.16 $\pm$ 5.3
9	34.83 $\pm$ 2.91	2.22 $\pm$ 0.37	29.32 $\pm$ 1.9
12	No Effect	No Effect	No Effect
14	36.23 $\pm$ 0.82	1.81 $\pm$ 0.08	21.44 $\pm$ 0.29
19	30.79 $\pm$ 2.9	1.54 $\pm$ 0.3	29.68 $\pm$ 1.63
26	24.59 $\pm$ 1.49	1.37 $\pm$ 0.2	37.8 $\pm$ 1.4
S-2	32.24 $\pm$ 0.4	2.28 $\pm$ 0.071	28.57 $\pm$ 0.21

Percentage motility difference between phosphorylated and dephosphorylated E64K mutant thin filaments treated with Silybin or drug numbers 2, 4, 7, 9, 12, 14, 19, 26 and S2. The effect of reverse recoupling by drug number 7 is also shown. EC₅₀ was calculated from the Hill equation fitted to the percentage motility difference between phosphorylated and dephosphorylated filaments and was rounded in two significant figures.

6.4 Evaluation of the best drug candidates in *TPM1* E54K DCM mutant thin filaments

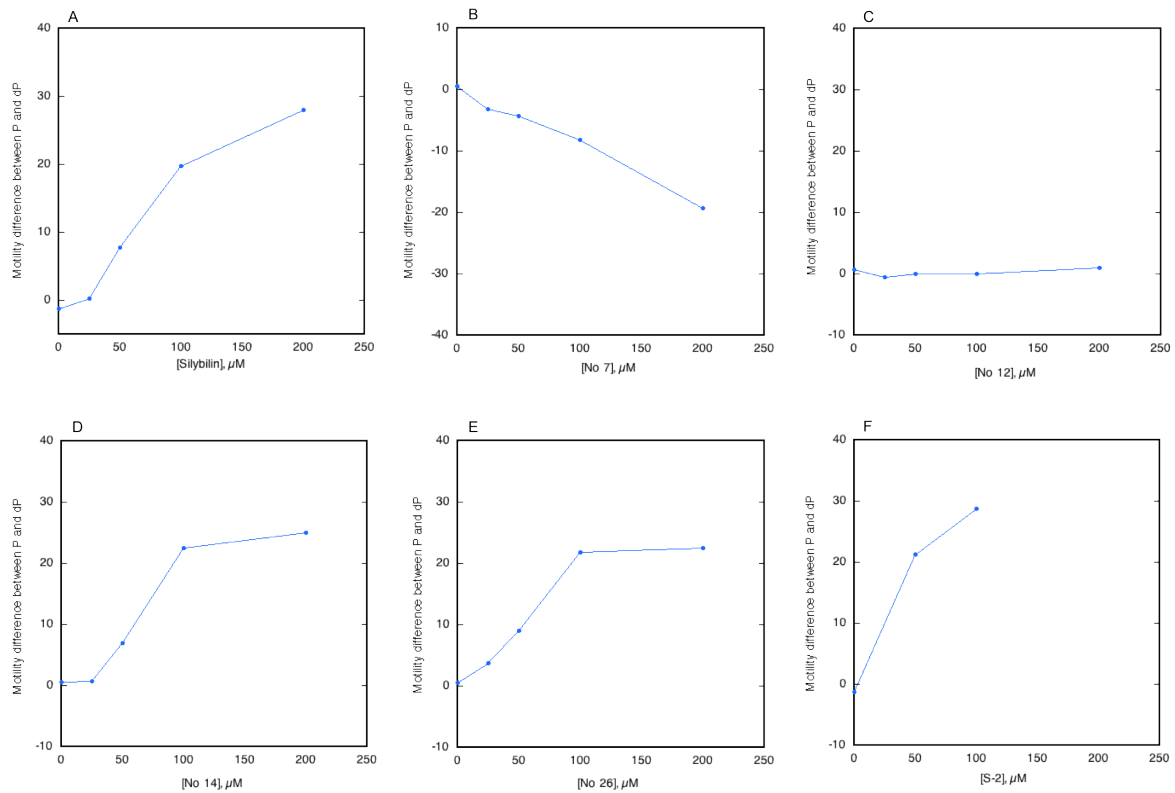
Next, the effect of the best candidate drugs was examined in Dilated Cardiomyopathy (DCM) thin filaments. The selected DCM mutation was *TPM1* E54K because of the availability of recombinant E54K tropomyosin. From the careful examination of the dose response data with the E180G mutation, the most potent drugs were selected: Silybin, and drug numbers 26, 14 and S2. The interesting drug number 7 (“reverse recoupling”) was also selected and drug number 12 was the control (no effect). Thin filaments were reconstituted using 60 nM E54K recombinant tropomyosin, 60 nM phosphorylated or dephosphorylated troponin extracted from donor heart and 10 nM rabbit skeletal actin. Different concentrations of the drugs from 25 to 200 μM were tested in phosphorylated and dephosphorylated thin filaments at a fixed Ca^{2+} concentration of 0.074 μM . The dose response results for the percentage motility and the motile velocity can be found in Figure 6-9 and the percentage motility difference between phosphorylated and dephosphorylated thin filaments in Figure 6-10. A summary of the differences between phosphorylated and dephosphorylated E54K DCM mutant thin filaments for each drug concentration is illustrated in Figure 6-11. For each of the drug, the Hill equation was fitted to the data and the n_H , EC_{50} and the magnitude difference ($\Delta \%$) was calculated (Table 6-2). Comparable to the E180G mutation, the uncoupling of Ca^{2+} sensitivity to TnI phosphorylation was restored with similar magnitude for Silybin and drug numbers 14, 26 and S-2. Drug number 26 had the largest effect of coupling, with a magnitude of 37.8 ± 1.4 while S-2 was the most potent with an EC_{50} of $36.6 \pm 5.46 \mu\text{M}$, n_H of 1.9 ± 0.54 and magnitude 28.75. Silybin and drug numbers 14 and 26 showed similar levels of potency when comparing the values of EC_{50} , where the EC_{50} range was from $55.18 \pm 7.47 \mu\text{M}$ to $77.1 \pm 7.93 \mu\text{M}$. As expected, drug number 12 did not have any effect on the percentage motility and motile velocity of the thin filaments, consistent with the E180G dose response curve and also the initial screening. Drug number 7 had also the same effect with E180G, where it reverse coupled the phosphorylated and dephosphorylated mutant thin filaments. Drug number 7 increased the motility parameters of the dephosphorylated E54K thin filaments, while decreased the parameters of phosphorylated E54K thin filaments.

Figure 6-9: Small molecule dose response data for motile velocity and percentage motility of phosphorylated and dephosphorylated *TPMI* E54K DCM mutant thin filaments



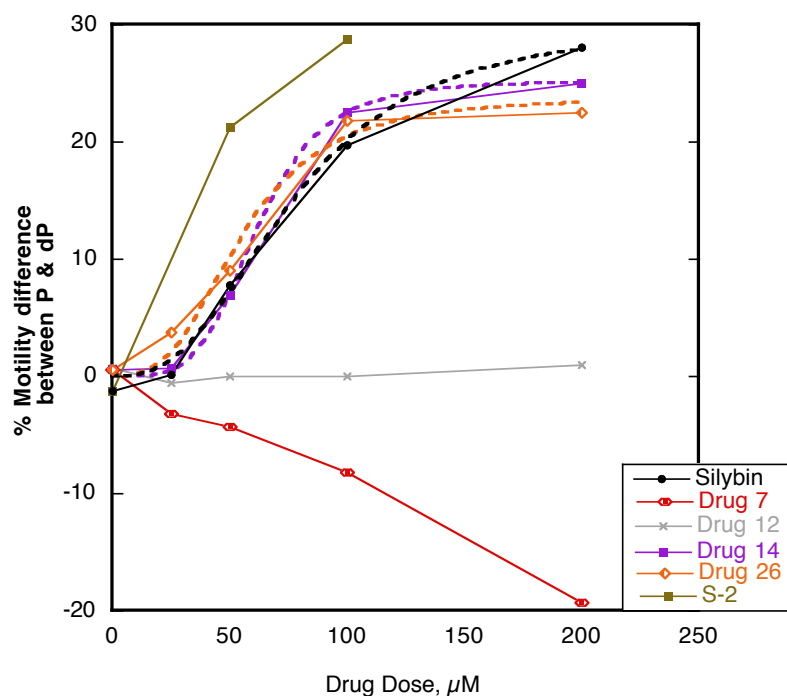
Motile velocity and percentage motility difference between phosphorylated or dephosphorylated E54K mutant thin filaments. Motility was measured at a constant Ca²⁺ concentration of 0.074 µM with increasing drug doses. A) Silybin B) No 7 C) No 12 D) No 14 E) 26 F) S-2

Figure 6-10: Percentage motility difference between phosphorylated and dephosphorylated *TPM1* E54K DCM mutant thin filaments dose response curves



Percentage motility difference between phosphorylated or dephosphorylated E54K mutant thin filaments. Motility was measured at a constant Ca^{2+} concentration of $0.074 \mu\text{M}$ with increasing drug doses. A) Silybin B) No 7 C) No 12 D) No 14 E) 26 F) S-2

Figure 6-11: Summary of percentage motility difference between phosphorylated and dephosphorylated *TPMI* E54K DCM mutant thin filaments treated with different compounds



Percentage motility difference of phosphorylated and dephosphorylated thin filaments treated with different compounds. Percentage motility was measured at $0.074 \mu\text{M Ca}^{2+}$ and the Hill equation was fitted to the data to create the curves. Different colored lines represent different compounds.

Table 6-2: Data summary for the percentage motility difference between phosphorylated and dephosphorylated *TPMI* E54K DCM mutant thin filaments treated with different compounds

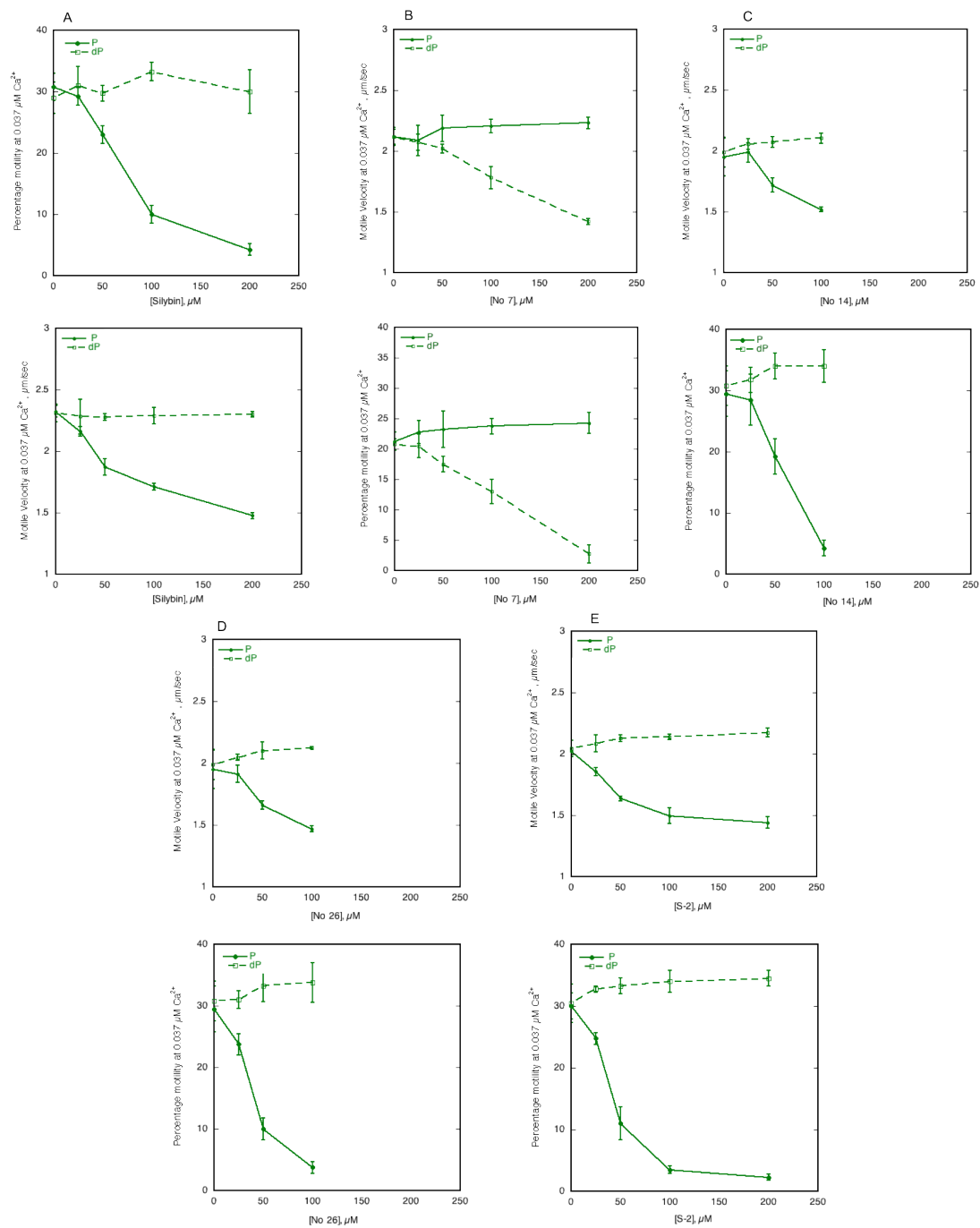
E54K + Drug	EC ₅₀ , μM	n _H	Magnitude (% difference)
Silybin	77.13 ± 7.93	2.69 ± 0.52	30.03 ± 2.47
7	~ 100	N/A	N/D
12	N/D	N/D	No effect
14	61.99 ± 1.51	4.39 ± 0.35	25.19 ± 0.46
26	55.18 ± 7.47	1.37 ± 0.2	37.8 ± 1.4
S-2	36.62 ± 5.46	1.9 ± 0.54	28.75

Percentage motility difference between phosphorylated and dephosphorylated E64K mutant thin filaments treated with Silybin or drug numbers 14, 26 and S2. The effect of reverse recoupling by drug number 7 is also shown. EC₅₀ was calculated from the Hill equation fitted to the percentage motility difference between phosphorylated and dephosphorylated filaments and was rounded in two significant figures. N/A= Not Applicable, N/D= Not Determined.

6.5 Evaluation of the best drug candidates on *ACTC* E99K HCM mutant thin filaments

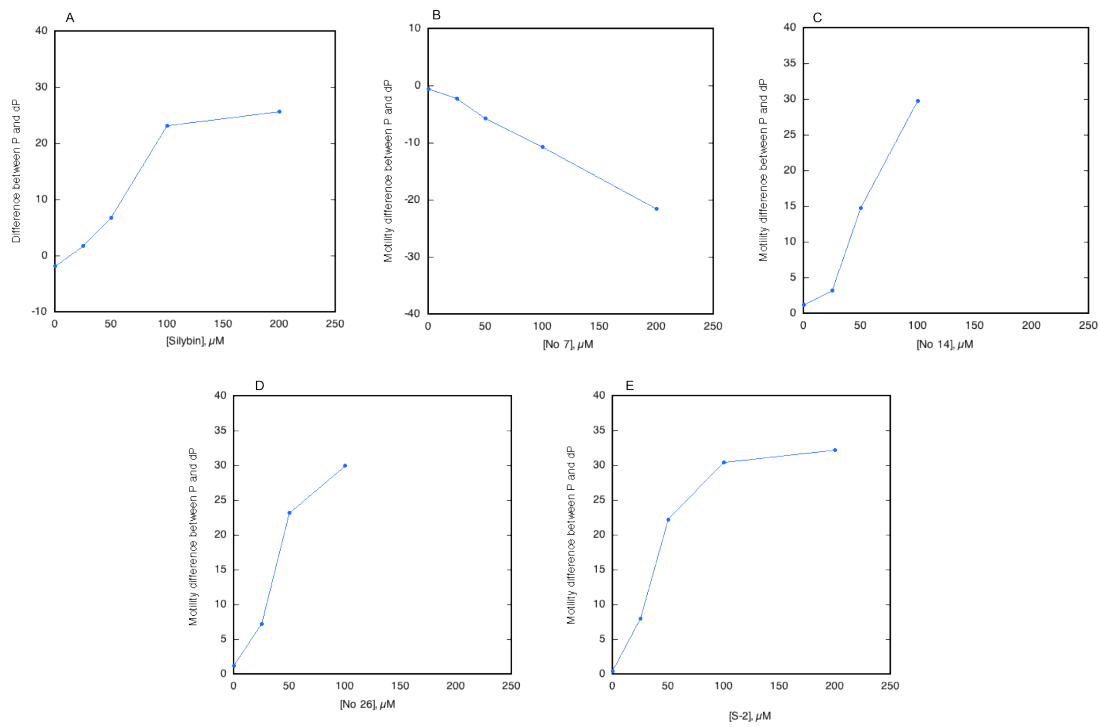
The analogues were then tested in HCM *ACTC* E99K mutant thin filaments. This mutation was selected because we have the E99K transgenic mouse line in the laboratory. The E99K mice have blunted response to adrenaline so the experiments described in this chapter would provide preliminary data for potentially treating the animals with the compounds (Song et al, 2011). Thin filaments were reconstituted with 60 nM human cardiac tropomyosin, 60 nM phosphorylated or dephosphorylated troponin extracted from donor human heart and 10 nM actin extracted from transgenic E99K mice. Percentage motility and motile velocity of the thin filaments was calculated at $0.037 \mu\text{M Ca}^{2+}$. Silybin and drugs number 7, 14, 26 and S-2 were tested in E99K thin filaments at different concentrations. Percentage motility and motile velocity of phosphorylated and dephosphorylated E99K thin filaments treated with the different drugs can be shown in Figure 6-12. The percentage motility difference between phosphorylated and dephosphorylated thin filaments treated with increasing concentrations of the different compounds is shown in Figure 6-13. A summary of the percentage motility difference results is shown in Figure 6-14 where all the different compounds were plotted on the same graph. Like previously, the Hill equation was fitted to the data so that the EC_{50} , the n_H and the $\Delta \%$ (magnitude of percentage motility difference) was determined. Table 6-3 summarises the results. Similar effects to E180G and E54K mutations of the different drugs were observed in the E99K mutant thin filaments as well. Silybin, drug numbers 14, 26 and S-2 were able to re-couple the uncoupled E99K mutant thin filaments in concentrations more than $50 \mu\text{M}$. The most potent drug was found to be Silybin with an EC_{50} of $62.58 \pm 3.14 \mu\text{M}$. Unfortunately, the EC_{50} and n_H could not be determined accurately for drug numbers 14 and 26 due to lack of experimental data on high drug concentrations. 26 showed a large difference in the motility parameters between phosphorylated and dephosphorylated thin filaments with a magnitude of 30. Drug S-2 showed the highest magnitude, 38.26 ± 0.0082 . Drug number 7 reversed coupled the thin filaments, decreasing the motility of dephosphorylated E99K thin filaments and increasing slightly the motility of E99K phosphorylated thin filaments.

Figure 6-12: Small molecule dose response summary for percentage motility and motile velocity of *ACTC* E99K HCM mutant thin filaments



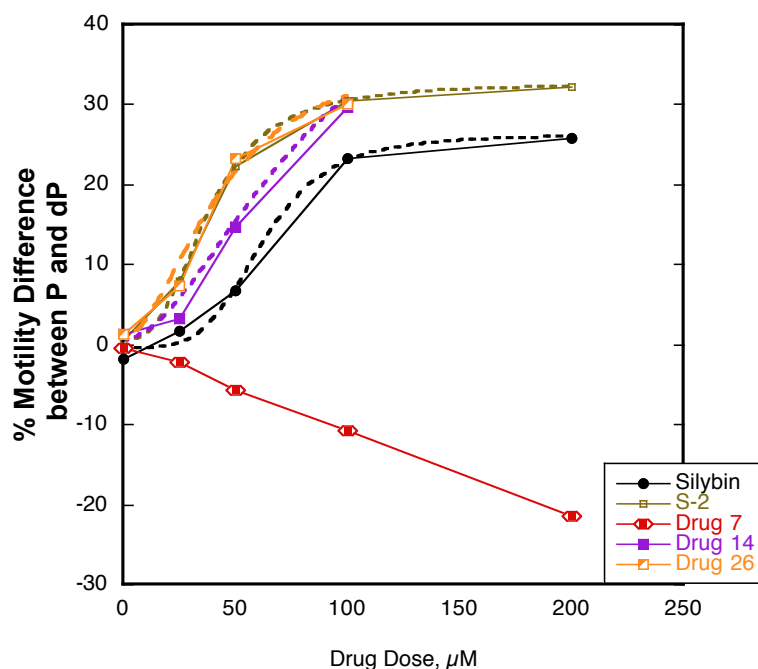
Motile velocity and percentage motility difference between phosphorylated or dephosphorylated E99K mutant thin filaments. Motility was measured at a constant Ca^{2+} concentration of $0.037 \mu\text{M}$ with increasing drug doses. A) Silybin B) No 7 C) No 14 D) No 26 E) S-2.

Figure 6-13: Small molecule data for percentage motility difference between phosphorylated and dephosphorylated *ACTC* E99K HCM mutant thin filaments



Percentage motility difference between phosphorylated or dephosphorylated E99K mutant thin filaments. Motility was measured at a constant Ca^{2+} concentration of $0.037 \mu\text{M}$ with increasing drug doses. A) Silybin B) No 7 C) No14 D) No 26 E) S-2

Figure 6-14: Summary of the percentage motility difference between phosphorylated and dephosphorylated *ACTC* E99K HCM mutant thin filaments treated with different compounds



Percentage motility difference of phosphorylated and dephosphorylated thin filaments treated with different compounds. Percentage motility was measured at $0.037 \mu\text{M Ca}^{2+}$ and the Hill equation was fitted to the data to create the curves. Different coloured lines represent different compounds.

Table 6-3: Data summary of the percentage motility difference between phosphorylated and dephosphorylated *ACTC* E99K HCM mutant thin filaments treated with different compounds

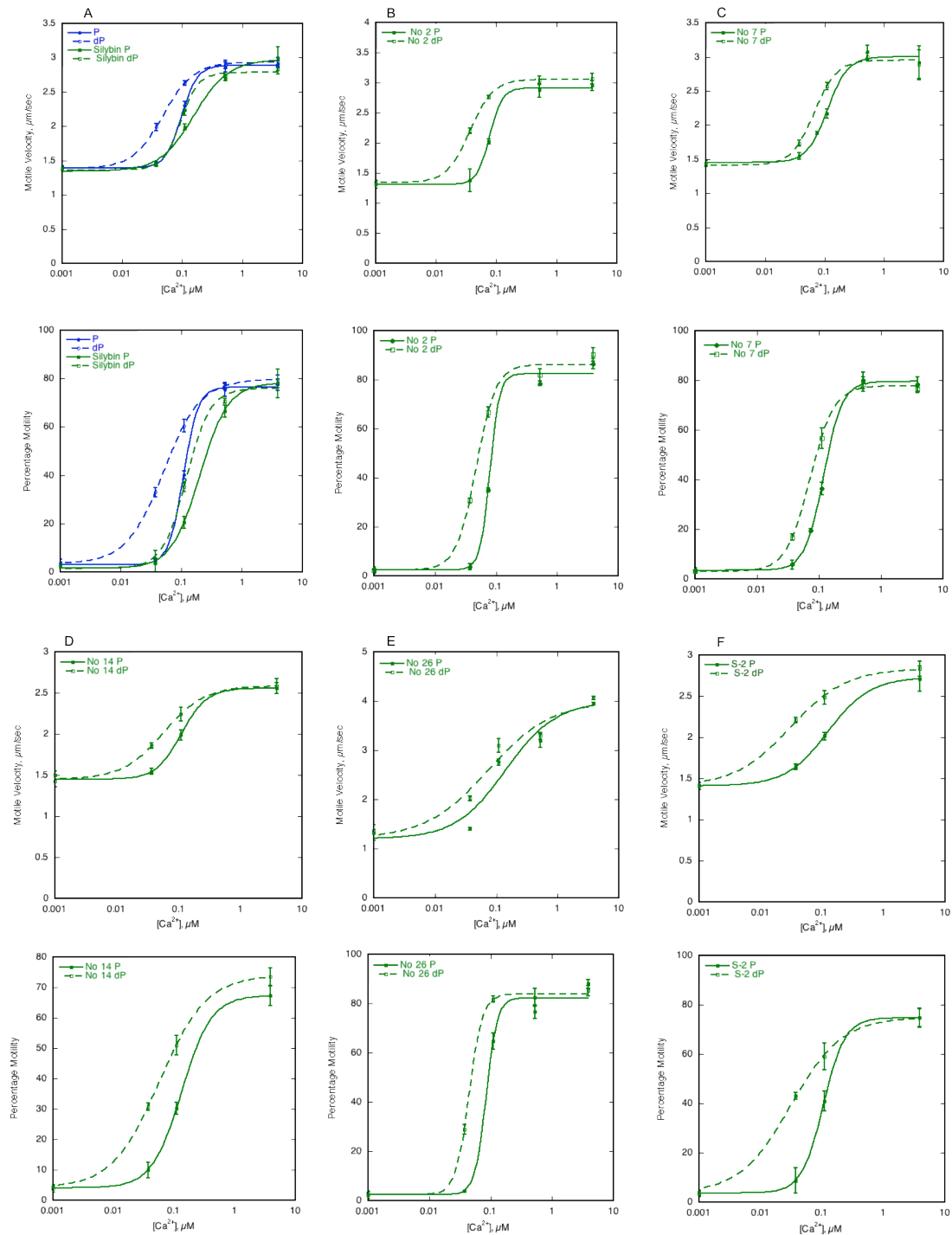
E99K + Drug	EC ₅₀ , μM	n _H	Magnitude (% difference)
Silybin	62.58 ± 3.14	4.18 ± 1.64	26.56 ± 7.82
7	~ 100	N/A	N/D
14	N/D	N/D	29.75
26	N/D	N/D	30
S-2	32.06 ± 0.005	2.79 ± 0.0013	38.26 ± 0.0082

Percentage motility difference between phosphorylated and dephosphorylated E64K mutant thin filaments treated with Silybin or drug numbers 14, 26 and S2. The effect of reverse recoupling by drug number 7 is also shown. EC₅₀ was calculated from the Hill equation fitted to the percentage motility difference between phosphorylated and dephosphorylated filaments and was rounded in two significant figures. N/A= Not Applicable, N/D= Not Determined.

6.6 The effect of the compounds on donor thin filaments.

The initial property of EGCG is its desensitising action on donor thin filaments (decrease in Ca^{2+} sensitivity). In this section, the best candidate drugs as well as the anomalous drug number 7 were tested for their effect as desensitisers. Thin filaments were reconstituted using 60 nM human cardiac tropomyosin, 60 nM phosphorylated or dephosphorylated troponin extracted from donor heart and 10 nM rabbit skeletal actin. From the previous experiments it was shown that 100 μM was sufficient to produce the effect of the drug. For this reason, 100 μM of Silybin and drug numbers: 2, 14, 7, 26 and S-2 were tested. Full Ca^{2+} activation curves were performed for the percentage motility and the motile velocity of phosphorylated and dephosphorylated donor thin filaments treated with the different drugs. The Ca^{2+} activation curves are shown in Figure 6-15. The EC_{50} of phosphorylated and dephosphorylated donor thin filaments, which is an indicator of the Ca^{2+} sensitivity, was measured for the percentage motility of different drugs as well as the EC_{50} P/dP ratios (Table 6-4). Interestingly, from the EC_{50} P data we can observe that drug numbers: 2, 7, 14, 26 and S-2 did not decrease the Ca^{2+} sensitivity of donor thin filaments. The EC_{50} of the donor phosphorylated thin filaments was $0.11 \pm 0.0018 \mu\text{M}$ and the EC_{50} for the phosphorylated donor thin filaments treated with the different drugs ranged from 0.079 ± 0.0059 to $0.11 \pm 0.00067 \mu\text{M}$. The Ca^{2+} sensitivity remained coupled to the TnI phosphorylation in all cases as the EC_{50} P/dP ratio was greater than one. This is an unexpected result because EGCG was originally identified as a re-coupler due to its known action as a Ca^{2+} desensitiser. Silybin has the same properties as EGCG, where it decreased the Ca^{2+} sensitivity of phosphorylated thin filaments (EC_{50} P = 0.21 ± 0.013) and the Ca^{2+} sensitivity remained coupled to the TnI phosphorylation (EC_{50} P/dP ratio = 0.75).

Figure 6-15: Ca^{2+} activation curves for phosphorylated and dephosphorylated donor thin filaments treated with different compounds



Motile velocity and percentage motility was measured over a range of Ca^{2+} concentrations to create Ca^{2+} activation curves for both phosphorylated (solid lines) and dephosphorylated (dotted lines). A) Silybin (green) and donor untreated (blue) B) No 2 C) No 7 D) No 14 E) No 26 F) S-2

Table 6-4: Summary of Ca^{2+} sensitivity for percentage motility of phosphorylated and dephosphorylated donor thin filaments treated with different compounds

Drug	EC_{50} P	EC_{50} dP	EC_{50} P/dP Ratio
Donor untreated	0.11 ± 0.0018	0.051 ± 0.0006	2.16
+ EGCG*	0.26 ± 0.03	0.15 ± 0.02	1.7
+ Silybin	0.21 ± 0.013	0.12 ± 0.011	1.75
+ No 2	0.079 ± 0.0059	0.048 ± 0.034	1.65
+ No 7	0.12 ± 0.01	0.07 ± 0.01	1.7
+ No 14	0.13 ± 0.0027	0.058 ± 0.0033	2.24
+ No 26	0.08 ± 0.005	0.05 ± 0.01	1.6
+ No S-2	0.11 ± 0.00067	0.032 ± 0.0038	3.44

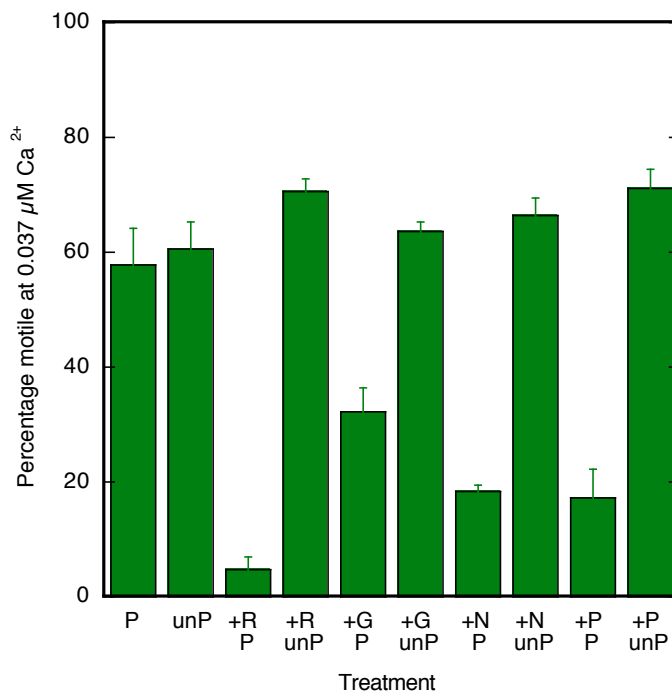
Summary showing the Ca^{2+} sensitivity for percentage motility and motile velocity of donor thin filaments treated with EGCG, Silybin or drug numbers 2, 7, 14, 26 and S2. EC_{50} values were calculated from the Ca^{2+} activation curves for the percentage motility of thin filaments treated with different drugs. * EGCG data was taken from measurements described in the previous chapter

6.7 The re-coupling effect of known Hsp90 inhibitors

The compounds given to us by Dr Brian Blagg have the common characteristic that they are all inhibitors of Hsp90 by directly binding to it. Preliminary experiments were performed in order to investigate whether other known inhibitors of Hsp90 can also re-couple uncoupled HCM mutant thin filaments. Known inhibitors of Hsp90 include geldanamycin (Stebbins et al., 1997), novobiocin (Matts et al., 2011), resveratrol (Sahin et al., 2012) and pterostilbene (Kostin et al., 2012). E180G HCM mutant thin filaments were reconstituted using 60 nM phosphorylated or dephosphorylated troponin extracted from donor heart, 60 nM E180G recombinant Tpm1 from baculovirus/*sf9* cells and 10 nM rabbit skeletal actin. Thin filaments were treated with 100 μM of the four Hsp90 inhibitors and their percentage motility and motile velocity was measured at 0.037 μM Ca^{2+} . The percentage motility of E180G

thin filaments treated with Hsp90 inhibitors is shown in Figure 6-16. We can observe from the preliminary experiments that all four of these compounds restore the coupling between TnI phosphorylation and Ca^{2+} sensitivity, like EGCG, Silybin and other structural analogues.

Figure 6-16: Percentage motility of Tpm1 E180G phosphorylated and dephosphorylated thin filaments treated with different known Hsp90 inhibitors



Percentage motility of either phosphorylated (P) or dephosphorylated (unP) thin filaments at $0.037 \mu\text{M Ca}^{2+}$ reconstituted with donor troponin, E180G recombinant mutant tropomyosin and rabbit skeletal actin treated with Hsp90 inhibitors ($100 \mu\text{M}$): Resveratrol (R), Geldanamycin (G), Novobiocin (N) and Pterostilbene (P).

6.8 Discussion

6.8.1 Rapid screening methodology

As part of our collaboration with Dr Brian Blagg, we received 30 compounds structurally similar to EGCG or Silybin to be tested for their recoupling properties. For the purpose of the screening experiments, a screening assay needed to be developed using the IVMA. The methodology was based on the fact that re-coupling can be observed in a single Ca^{2+} concentration and a full Ca^{2+} activation curve was not necessary, permitting for a rapid experimental procedure. HCM-causing mutation *TPMI* E180G was the choice of the first screening, because the mutant tropomyosin is already made from baculovirus/*sf9* and thin filaments can be reconstituted easily. From the first screening, several potent re-couplers could be identified for thorough examination. For the initial screening, only 100 μM of the compounds were used in the assay. It is possible that some of the drugs that found to have no effect from the rapid screening might still be re-couplers at a higher dose. Drug number 12 gave a negative result in the rapid screening and was selected as a negative control for the dose response curves for E180G and E54K mutant thin filaments. 25-200 μM of drug number 12 had no effect on neither or these mutations, confirming the results of the rapid screen. Four known Hsp90 inhibitors were also tested for their recoupling properties in E180G thin filaments and it was found that all of them re-coupled the uncoupled thin filaments, suggesting a potential structural similarity between Hsp90 and troponin.

6.8.2 Further analysis of potent re-couplers allows for insights about the uncoupling phenomenon

Testing the most potent compounds selected from the rapid screening assay in different backgrounds and doses, gave some very interesting insights about the mechanism of the uncoupling phenomenon. A summary of the results for all the compounds can be found in Table 6-5.

The more detailed dose response assay gave important information about the potency of the compounds to re-couple Ca^{2+} sensitivity from TnI phosphorylation. Silybin, which is also a natural occurring compound, re-coupled E180G mutant thin filaments

with a similar potency to EGCG (EC_{50} of Silybin $47.2 \pm 2.66 \mu\text{M}$ compared to that of EGCG $58.8 \pm 13.3 \mu\text{M}$). Drug numbers 2, 4, 9, 14, 19, 26 and S-2 also re-coupled E180G mutant thin filaments with similar or slightly higher potency. The fact that some of the compounds had an effect and others did not, implies that there are key functional groups that account for the re-coupling and/or the desensitising properties. These have been removed or replaced from the drugs that had no effect on the Ca^{2+} sensitivity on mutant thin filaments, such as drug number 12. The structures of the compounds are unknown (Brian Blagg lab proprietary information) and the important functional groups could not be identified.

We can observe that drugs have very similar effects across the three mutations studied. For example, all the compounds that re-coupled E180G HCM thin filaments, also re-coupled E54K DCM and E99K HCM thin filaments. Drug number 12 had no effect on the Ca^{2+} sensitivity of E180G and E54K mutant thin filaments. In the E54K thin filaments Silybin and drug numbers 14 and 26 showed re-coupling properties with an EC_{50} roughly 50% weaker than this of the same drugs in the E180G mutation. Drug S-2 had very similar EC_{50} across all the three mutations, with an EC_{50} of $36.6 \pm 5.46 \mu\text{M}$ for the E54K mutation, $32.2 \pm 0.398 \mu\text{M}$ for the E180G mutation and $32.06 \pm 0.005 \mu\text{M}$ for the E99K mutation. Silybin was a potent re-coupler also for the E99K mutant thin filaments, but with a greater EC_{50} value of $62.58 \pm 3.14 \mu\text{M}$ comparable to an EC_{50} of $77.13 \pm 7.93 \mu\text{M}$ for the E54K mutation. Silybin had an EC_{50} of $47.21 \pm 2.66 \mu\text{M}$ for the E180G mutation. From these results we can conclude that the overall effect of the drugs was preserved across the three mutations studied, however the potency of the drugs slightly changed within the three mutations. This prevented us from finding a single compound that was the most potent for all three cardiomyopathy mutations.

From this study it was discovered a novel reverse recoupling molecule number 7 that decreased the Ca^{2+} sensitivity of dephosphorylated thin filaments and slightly increased the Ca^{2+} sensitivity of phosphorylated thin filaments in all three mutations. This is an unexpected result because the reverse recoupling has never been observed physiologically. Reverse recoupling elucidates the chemical differences between drug number 7 and other EGCG related compounds. All the small molecules provided by Dr Brian Blagg had similar structure to EGCG but some of the chemical groups were slightly changed. The chemical differences between number 7 and EGCG can provide new insights about the structure-function relationship of the uncoupling mechanism as

well as leading to the design of tailored drugs that could modulate the coupling of TnI phosphorylation and Ca^{2+} sensitivity in thin filament cardiomyopathy.

The last but equally important finding from the detailed examination of the potent re-couplers is the effect of the re-couplers on the regulation of Ca^{2+} sensitivity of the donor thin filaments. Silybin and drugs number 2, 4, 7, 26 and S-2 were tested for their effect on the Ca^{2+} sensitivity of phosphorylated and dephosphorylated donor thin filaments. Ca^{2+} sensitivity was coupled to TnI phosphorylation, which is also a property of the donor thin filaments. Although Silybin and EGCG decreased the Ca^{2+} sensitivity of donor thin filaments, the rest of the small molecules did not alter the Ca^{2+} sensitivity of the donor thin filaments. This was an unpredictable result because the lead compound EGCG was chosen on the basis that it was a Ca^{2+} desensitiser. These results suggest that the coupling between TnI and Ca^{2+} sensitivity might be independent of the thin filament Ca^{2+} sensitivity and that it is possible to act on the coupling without altering the absolute Ca^{2+} sensitivity of the thin filaments. In that aspect, it was lucky that EGCG, which was a known Ca^{2+} desensitiser also re-coupled the Ca^{2+} sensitivity to the TnI phosphorylation in thin filament cardiomyopathies.

Table 6-5: Small molecule screening summary

Drug	E180G P/dP difference (initial screen)	E180G EC ₅₀ , μ M	E54K EC ₅₀ , μ M	E99K EC ₅₀ , μ M
No drug	-1	58.7 $\pm$ 11.9 *		
EGCG	23	58.83 $\pm$ 13.31		
Silybin	12	47.21 $\pm$ 2.66	77.13 $\pm$ 7.93	62.99 $\pm$ 5.59
1	1			
2	27	30.5 $\pm$ 0.41		
3	8			
4	27	33.98 $\pm$ 2.21		
5	2			
6	1			
7	-11	67.05 $\pm$ 20.31	~ 100	~100
8	No Effect			
9	20	34.83 $\pm$ 2.9		
10	No Effect			
11	7			
12	-1	No Effect	No Effect	
13	No Effect			
14	17	36.23 $\pm$ 0.82	61.99 $\pm$ 1.5	
15	13			
16	6			
17	3.5			
19	24	30.79 $\pm$ 0.82	62 $\pm$ 1.5	
20	2			
22	25			
22	7			
23	9			
24	8			
26	26	24.59 $\pm$ 1.49	55.18 $\pm$ 7.47	
27	4			
28	4			
S-1	22			
S-2	20	32.23 $\pm$ 0.4	36.62 $\pm$ 5.46	32.06 $\pm$ 0.005

Summary of the compound screening experiments. Motility difference between phosphorylated and dephosphorylated E180G mutant thin filaments as calculated from the initial screen is shown and EC₅₀ for mutations E180G (HCM), E54K (DCM) and E99K (HCM), which was calculated from the Hill equation fitted to the percentage motility difference between phosphorylated and dephosphorylated filaments dose response curves. * calculated from the previous chapter

6.8.3 Limitations

Although this study gave positive outcomes in terms of potent re-couplers and provided some very interesting insights about the uncoupling phenomenon in a short period of time, there are several limitations that need to be taken into account. Firstly, although it was possible to shorten the experimental time with the developments of the screening assay, IVMA is not a high- throughput assay for testing many different compounds simultaneously. In order to develop such an assay, a different experimental method needs to be developed, such as using DCM or HCM mutant cell lines where such assay is possible.

Due to the nature of the IVMA screening assay and the limitation of time, only $n=1$ was performed, because I wanted to quickly identify re-couplers. Therefore, statistical analysis could not be performed. However, it is noted that in general the IVMA is quite reproducible and there is not much experimental error in the previous findings using the IVMA. More experimental repeats are needed in order to draw conclusions. Finally, the structures of the small molecules are not known to us, so no conclusions can be made about the structure-function mechanism of the small molecules. All the compounds were identified by Dr Brian Blagg originally as Hsp90 inhibitors. The reason why these compounds work as both Hsp90 inhibitors and as re-couplers of TnI phosphorylation to Ca^{2+} sensitivity in the cardiac thin filaments remains unknown. It is important to find out the structure of these compounds to identify the functional groups that account for the re-coupling properties. Also it would be important to identify the place where these compounds bind to troponin in relationship with EGCG and Silybin.

6.8.4 Clinical perspectives

In this chapter I investigated the effect of different compounds structurally similar to EGCG for their potential to re-couple uncoupled DCM and HCM thin filaments. Using a rapid screening methodology in the IVMA some of the compounds showed potent re-coupling properties. Furthermore, it was made clear that uncoupling and Ca^{2+} sensitivity act by two separate mechanisms as some of the re-couplers did not alter the Ca^{2+} sensitivity of donor thin filaments. This has implications for new therapeutic agents capable of targeting the coupling of the sensitivity to

phosphorylation with or without altering the absolute native Ca^{2+} sensitivity if required. The finding of potent re-couplers indicates that it is possible to have a targeted therapy for thin filament cardiomyopathies because the promiscuous groups have been removed from all the compounds (Brian Blagg personal communication).

7 FINAL DISCUSSION

7.1 Aims of the thesis

The aim of this thesis was to investigate the molecular mechanisms of the uncoupling phenomenon. The initial hypothesis was based on previous work by Memo et al, showing that uncoupling Ca^{2+} sensitivity from troponin I (TnI) phosphorylation is the molecular mechanism behind Dilated Cardiomyopathy (DCM) (Memo et al., 2013). Uncoupling has also been demonstrated in some cases of Hypertrophic Cardiomyopathy (HCM) (reviewed by Messer and Marston). Uncoupling has also been reported in human myectomy samples where there is no thin filament protein mutation (Bayliss et al., 2012).

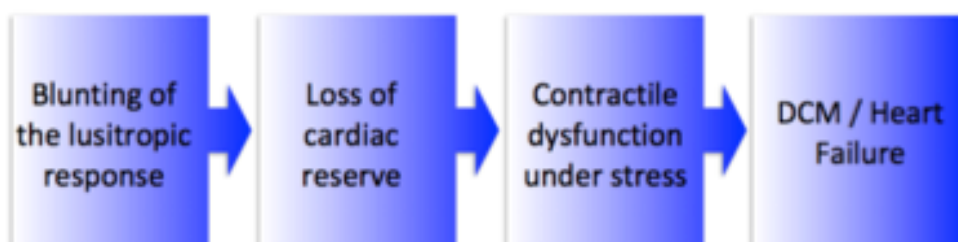
Cardiac TnI has a unique N terminal extension that is phosphorylated by PKA. The consequence of this is that the myofibrillar Ca^{2+} sensitivity and rate of Ca^{2+} release is modulated 2-3 fold in response to β adrenergic stimulation via PKA dependent phosphorylation of TnI Serine 22 and 23. In that way, Ca^{2+} sensitivity of the myofilament can decrease and the muscle can relax upon the action of adrenaline. Based on the structural models it has been suggested that there are specific interactions between the N terminal lobe of troponin C (TnC) and TnI₁₋₃₀ that are phosphorylation dependent and this modulates the Ca^{2+} sensitivity change upon TnI phosphorylation (Ward et al., 2004, Howarth et al., 2007, Cheng et al., 2014, Solaro et al., 2008). Memo et al hypothesised that these interactions are unstable and can be disrupted or changed by many perturbations of which HCM/DCM-causing mutations are the best-known examples. As a result, troponin is in the same state as when phosphorylated, independently of the phosphorylation level (uncoupled). The molecular mechanism of uncoupling has been examined in this thesis, by testing several pharmacological agents that bind to TnC and alter the Ca^{2+} sensitivity of the thin filament, like mutations do, and therefore might affect the coupling between Ca^{2+} sensitivity and TnI phosphorylation. At the same time, the nature of TnI and TnC interactions upon phosphorylation has been unravelled using molecular dynamics by the molecular dynamics group (Dr Ian Gould, Mr Juan Eiros Zamora, Miss Alice Sheehan and Mr David Wilson).

7.2 Uncoupling by mutations as a disease-causing process.

Uncoupling occurs as a consequence of a mutation in any protein of the thin filament, which would be the genetic cause of DCM or HCM. It is now known that uncoupling is the widespread mechanism for DCM, having important implications for the disease progression (Memo et al., 2013, Messer and Marston, 2014). The decrease of the Ca^{2+} sensitivity upon TnI phosphorylation is a downstream effect of the β adrenergic response. Uncoupling of Ca^{2+} sensitivity to TnI phosphorylation would lead to blunting the lusitropic response and as a consequence this would affect also the inotropic response. The physiological consequences of the uncoupling phenomenon have been demonstrated with the *ACTC* E361G transgenic (TG) mice as a model for DCM. Extracted myofibrils from the TG animals have shown increased Ca^{2+} sensitivity of force compared to wild type. Dephosphorylated transgenic mice did not show any alteration in the Ca^{2+} sensitivity of isometric force or the relaxation parameter, T_{LIN} and k_{REL} upon phosphorylation, showing that the DCM mutation blunts the β adrenergic response through phosphorylation without affecting other parameters such as length-dependent activation (Vikhorev et al., 2014). *In vivo* and *ex vivo* experiments with these animals further support these findings; papillary muscle relaxation of E361G animals was unaffected by β_1 adrenergic stimulation treatment with dobutamine showing blunting of the lusitropic response. The inotropic effect was also blunted, as shown by the reduced effect of dobutamine in end-systolic pressure and end-systolic volume (Wilkinson et al., 2015).

The blunting of lusitropic response which is a downstream physiological effect of uncoupling would lead to a loss of cardiac reserve that could eventually lead to heart failure (Figure 7-1). Wilkinson et al showed this, by chronic angiotensin II treatment whereby E361G TG mice develop hypertrophy initially and then dilation with reduced cardiac output compared to the WT mice (Wilkinson et al., 2015). Other DCM transgenic animals have shown blunted response to β agonists (reviewed by Messer and Marston, 2014). Uncoupling has also been observed in studies using cardiomyocytes from induced Pluripotent Stem Cells (iPSCs) from patients with DCM. The experiment of Sun et al with iPSC- cardiomyocytes containing the *TNNT2* R173W DCM mutation has shown a reduced initial response to nor epinephrine followed by a loss of contractility over 6 hours (Sun et al., 2012).

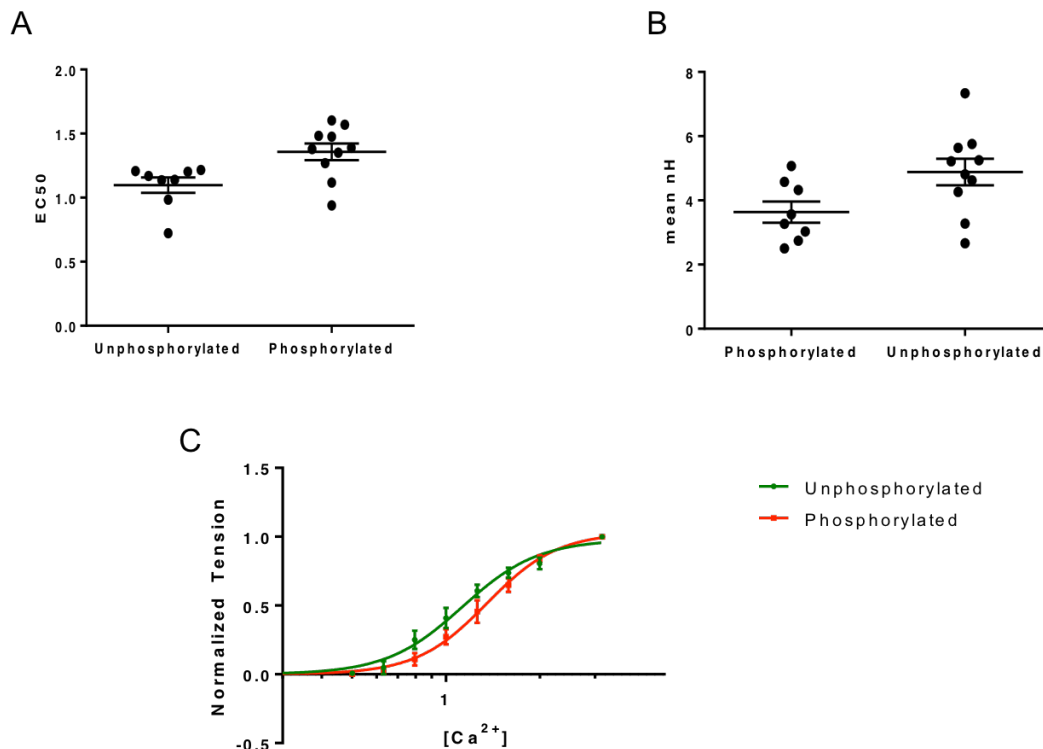
Figure 7-1: The physiological consequences of uncoupling in DCM



Flow chart showing the physiological effects of uncoupling and how it leads to DCM/heart failure. Diagram kindly provided by Mr Ross Wilkinson.

The situation of uncoupling in HCM is more complicated. Cases of uncoupling in HCM have been found *in vitro* (Deng et al., 2001, Dong et al., 2008, Gomes et al., 2005, Schmidtman et al., 2005) including the *TPM1* mutation E180G shown for the first time in this thesis. However, in whole muscle system using explanted heart tissue several cases have been observed where normal coupling occurs (Sequeira et al., 2013). Furthermore, a study with the *TPM1* E180G transgenic mouse have shown normal coupling comparing phosphorylated and pseudophosphorylated mouse skinned fibres (Alves et al., 2014) contradicting my own results in *in vitro* motility assay (IVMA). These discrepancies can occur because there might be a compensatory mechanism in the whole organism that is masking the uncoupling phenomenon. Preliminary experiments with *ACTC* E99K myofibrils done by Dr Petr Vikhorev and Mr Yousef Sebzali have also shown discrepancies in uncoupling in HCM, E99K myofibrils being “partially” coupled, even though they showed a clear uncoupling in IVMA (Figure 7-2). The EC_{50} ratio of *ACTC* E99K myofibrils (coupling constant) is 1.22 compared to 0.99 in thin filaments in IVMA and 2.24 in WT mouse myofibrils. This means that for HCM mutations uncoupling of force production may not be an all-or-nothing response, as it is in unloaded assays. This may be very interesting especially for HCM and it needs to be explored further. Also the extent of uncoupling is important to be examined beyond thin filament mutations, for example in case of mutations in titin, myosin or Myosin Binding Protein C (MyBP-C) at both single filament and myofibrillar level.

Figure 7-2: Uncoupling in E99K myofibrils



A) EC_{50} of phosphorylated and dephosphorylated E99K myofibrils B) n_H of phosphorylated and dephosphorylated E99K myofibrils C) A typical Ca^{2+} curve of phosphorylated (red) and dephosphorylated (green) E99K myofibrils. Force is normalized from 0 to 1 with 0 indicating minimal contraction and 1 indicating maximal contraction. Data were kindly provided by Mr Yousef Sebzali and Dr Petr Vikhorev.

7.3 Small molecules can modulate coupling

My main investigation was to test agents that bind to TnC for their ability to induce uncoupling mimicking the mutations. The Ca^{2+} sensitizer EMD 57033 was the first small molecule to be examined and it indeed induced uncoupling in thin filaments reconstituted with donor contractile proteins. This showed that EMD 57033 mimics HCM-causing mutations at the molecular level, by increasing the Ca^{2+} sensitivity and also uncoupling the Ca^{2+} sensitivity from the TnI phosphorylation. The other Ca^{2+} sensitizer tested, Bepridil, showed the same result. This indicates that the coupling of Ca^{2+} sensitivity and TnI phosphorylation is a labile property of troponin and can be

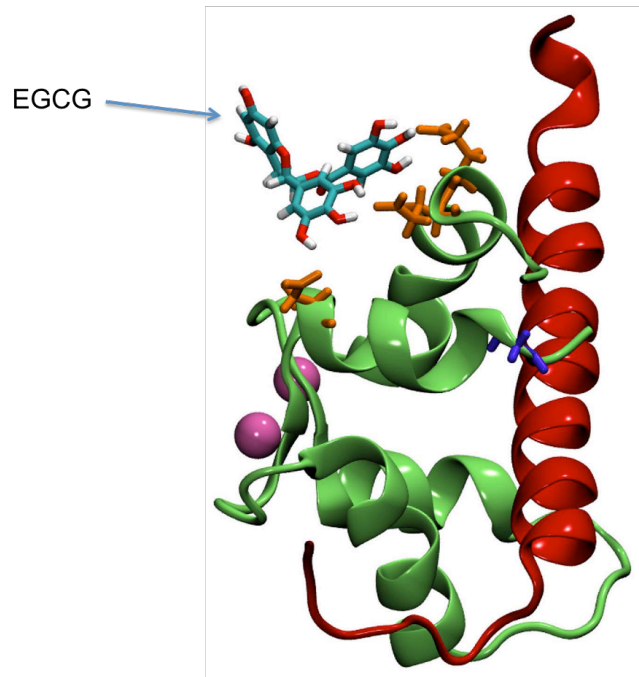
manipulated either by mutations or by small molecules. It is possible that the mechanism of uncoupling is through disruption or change of the TnC-TnI specific interactions, as also confirmed by structural studies showing that EMD 57033 compete with TnI₃₄₋₇₁ (Wang et al., 2011, Botten et al., 2013, Li et al., 2004) Bepridil affects the affinity of TnI₁₄₇₋₁₆₃ with TnC and binds to a different position than EMD 57033, but the effect of Bepridil on the N terminal peptide of TnI has not been looked at so no conclusions can be drawn. In any case, it is interesting that although the two molecules bind to two different positions, the effect on the coupling is the same. These results confirm the original hypothesis; that it is possible to manipulate uncoupling pharmacologically.

7.4 Ca²⁺ desensitisers like EGCG can reverse the uncoupling phenomenon.

The major discovery of my PhD thesis was the fact that green tea extract Epigallocatechin-3-Gallate (EGCG) which is also a Ca²⁺ desensitiser was able to reverse the uncoupling of Ca²⁺ sensitivity to TnI phosphorylation in all the DCM and HCM mutations studied that had previously been found to be uncoupled. This function of EGCG was not expected, as EGCG was initially tested due to its desensitising properties as demonstrated by Tadano et al, (Tadano et al., 2010). EGCG binds to TnC and its effects on coupling were examined because it was predicted that EGCG would also alter the TnC-TnI interactions. It was very surprising when it was discovered that EGCG does not induce uncoupling in donor thin filaments, but rather reverses the uncoupling observed in DCM and HCM mutant thin filaments. IVMA was the most appropriate method for this investigation, because the effect of the small molecules on the proteins can be measured and the phosphorylation level of TnI can be easily controlled. These results were very robust, as the uncoupling was reversed in many mutations, and furthermore it was confirmed in myofibrils of E361G TG mice (experiments performed by Dr Petr Vikhorev). Several structural models have been proposed for the binding of EGCG to TnC, including NMR and molecular dynamics (MD) models. Unlike EMD 57033 it has been suggested that EGCG binds in complex with TnI₃₄₋₇₁ peptide so that would suggest that it is possible that EGCG enhances the signal from TnI to TnC through interactions which is lost in cardiomyopathies. Figure 7-3 shows the binding of

EGCG to TnC as determined by Botten et al. Botten et al in his study showed by MD simulations that EGCG had the opposite effects of the DCM mutation G159D on the TnC-TnI interaction, confirming the results described in this thesis. All these findings demonstrate that the uncoupling phenomenon is a general rather than a specific mechanism, as all the different mutations cause uncoupling, and EGCG restores the uncoupling in all the mutations respectively.

Figure 7-3: EGCG binding to TnC



EGCG binding to TnC/ Ca^{2+} / TnI₃₄₋₇₁. TnC as calculated with MD simulations is shown in green, the TnI₃₄₋₇₁ peptide is shown in red, purple spheres are the Ca^{2+} , residue G159 in TnC which is mutated in DCM is shown in blue and TnC residues that make contact with EGCG in orange. Adapted from Botten et al, 2013

Taking *TPMI* E180G as a paradigm of uncoupling in HCM mutations, it has been found that other small molecules that are structurally similar to EGCG can also recouple uncoupled thin filaments; their effect also confirmed in the DCM mutation *TPMI* E54K and HCM mutation *ACTC* E99K. Another natural derivative that is a desensitiser and re-coupler is Silybin from the plant *Silibum*. For this part of the project, I developed a quick screening method to test the analogues for their recoupling potential using the IVMA.

The only known feature that these compounds have in common is that they are all inhibitors of Hsp90 via its C terminus (Khandelwal et al., 2013, Zhao et al., 2011). This suggests that there might be some structural common features between TnC and Hsp90; perhaps there is sequence homology. It is noted that even though EGCG is a promiscuous molecule acting on many different targets (Ingolfsson et al, 2014), a structural group that gives promiscuity has been removed from some of the analogues without loss of function (Dr Brian Blagg, personal communication). Preliminary experiments have been performed investigating the re-coupling effect of four other Hsp90 inhibitors: geldanamycin, novobiocin, resveratrol and pterostilbene. All four of these compounds restore the coupling between TnI phosphorylation and Ca^{2+} sensitivity, like EGCG, Silybin and other structural analogues. The C terminal small molecule binding site where novobiocin binds has been elucidated (Matts et al., 2011) and it was found that it binds at the interface between the two subunits of Hsp90. This is analogous to EGCG that also acts on the interface between TnC and TnI subunits. Resveratrol and pterostilbin also bind to the C terminal binding site while Geldanamycin binds at the N terminal small molecule binding site of the Hsp90. (Stebbins et al., 1997). Geldanamycin was the least effective of all the molecules studied therefore maybe there is a structural similarity between the C terminal binding site of Hsp90 and troponin. However, all these are just speculations which brings difficulties on drawing conclusions about the structural similarities between troponin and Hsp90.

Another very important finding from the analogues study is the separation of the effect on Ca^{2+} sensitivity from the uncoupling phenomenon. Some agents were able to re-couple uncoupled E180G mutant thin filaments without changing the Ca^{2+} sensitivity of wild type thin filaments. This indicates that the increased or decreased Ca^{2+} sensitivity observed in DCM and HCM is a separate property from uncoupling. In that respect, it has been very fortuitous that EGCG was found to be a desensitiser and also a re-coupler. The fact that uncoupling seems to be independent from Ca^{2+} sensitivity may be important regarding the potential therapy for inherited cardiomyopathies, that it is possible to target uncoupling irrespective of the effect of mutations on the Ca^{2+} sensitivity.

7.5 Insights for troponin regulatory mechanism

As previously mentioned, the results have given rise to some interesting insights about the molecular mechanism of the uncoupling phenomenon and as a result, about the mechanism of regulation of Ca^{2+} sensitivity by the adrenergic response and how it is disrupted in disease. The results from the Ca^{2+} sensitisers and desensitisers indicate that there are structural and functional changes in the interaction between TnI₁₋₃₀ peptide and the N terminal of the TnC, having important effects on the binding of Ca^{2+} to TnC so that the Ca^{2+} sensitivity can change. As discussed in Section 5.6.1, phosphorylation, HCM and DCM mutations in cardiac muscle and gain of function and loss of function mutations in the skeletal muscle only change the Ca^{2+} sensitivity by a certain extent. This implies that the TnI₁₋₃₀ peptide seems to be involved in a fixed transition with a 2-3 fold Ca^{2+} sensitivity change readout. DCM and HCM-causing mutations and Ca^{2+} sensitisers that cause uncoupling block this transition, but the way by which this happens is uncertain.

Opposed to the Ca^{2+} switch, the exact mechanism by which TnI phosphorylation modulates Ca^{2+} sensitivity has been an unsolved problem for many years. The problem arises from the fact that the TnI₁₋₃₀ peptide is unstable and it is not present in the crystal structure by Takeda et al. The first to give some light on this matter was a study by Ward et al, in which the structure of phosphorylated and dephosphorylated troponin including the TnI₁₋₃₀ peptide was elucidated using NMR. According to this model, there is a weak ionic interaction between the TnI N terminal peptide and TnC, which is absent upon phosphorylation (Ward et al., 2004). Following this study, Howarth et al determined the structure of TnI₁₋₃₀ peptide in phosphorylated and unphosphorylated states and used these to build a model of the whole troponin (Howarth et al., 2007). However, this model has been updated with more recent concepts. The N terminal extension of dephosphorylated troponin has been recently described as an intrinsically disorder region interacting with TnC and orienting it relative to the rest of the troponin complex, resulting in an increased Ca^{2+} affinity. This delicate interaction is abolished upon phosphorylation (Hwang et al., 2014). These results are also in line with my data, since the re-couplers seem to affect the phosphorylated state more, which is predicted to be more unstable.

The intrinsic disorder nature of the N terminal extension of TnI is a concept that has not been addressed by structural biologists, which needs to be taken into account in

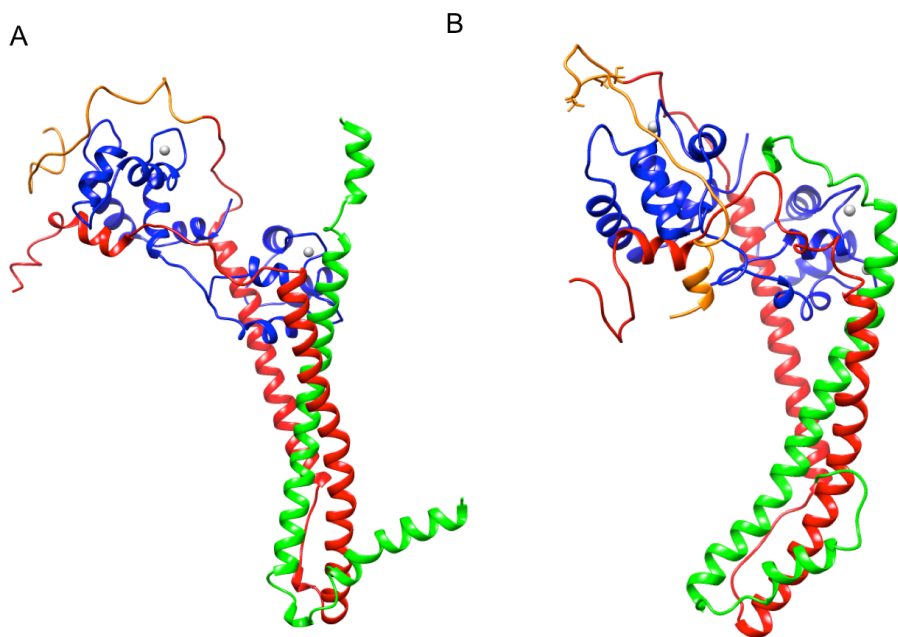
order to determine the mechanism of Ca^{2+} sensitivity changes upon phosphorylation. In fact, being disordered is important for the phosphorylation itself and it has been found that the majority of the proteins that get phosphorylated in nature have domains close to the phosphorylation site similar to proteins that have been characterized as intrinsically disordered (Iakoucheva et al., 2004). Unfortunately, this makes the molecular mechanism of the cardiac muscle regulation upon phosphorylation difficult to study, since structural studies by NMR and crystallography will not give any further information.

The way forward would be to use molecular dynamics (MD) simulations to elucidate the structural changes upon troponin phosphorylation. This technique views the proteins as moving objects rather than as stable peptides like in the case of NMR and crystallography. As part of my PhD thesis I have started a collaboration with Dr Ian Gould from the Department of Chemistry which has provided some insightful data about the matter.

Phosphorylated and unphosphorylated troponin structural model have been developed, containing the complete human sequence of troponin, including the N terminal extension of TnI₁₋₃₀ and ₁₃₇₋₁₄₈ and the entire TnT including the C terminal not present in the crystal structure. Figure 7-4 shows the phosphorylated and unphosphorylated structure of troponin as defined using MD. The multiple simulations run for a long time (7.25 μs for the unphosphorylated and 2.65 μs for the phosphorylated structure) in order for the obtained results to be as reliable as possible. Analysis of the changes upon phosphorylation has been performed measuring the interactions between the different peptides in order to understand the structural changes that occur upon TnI phosphorylation. Interactions between TnI, TnC and TnT have been analysed by measuring the Root Mean Square Fluctuation (RMSF). Figure 7-5 shows the changes between the amino acid interactions in TnC, TnT and TnI. In general it can be observed that no massive changes occur upon phosphorylation in either of the subunits. This is in line with other MD studies that showed that phosphorylation of Serine 22 and 23 did not have significant effects on TnC and Ca^{2+} binding but rather increased the overall fluctuations of the troponin complex and induced interactions between the N terminal extension and the inhibitory peptide of TnI, regulating the switch (Cheng et al, 2014). Furthermore these results are rational considering that the Ca^{2+} sensitivity change upon phosphorylation is just 2-fold indicating that only subtle changes would occur.

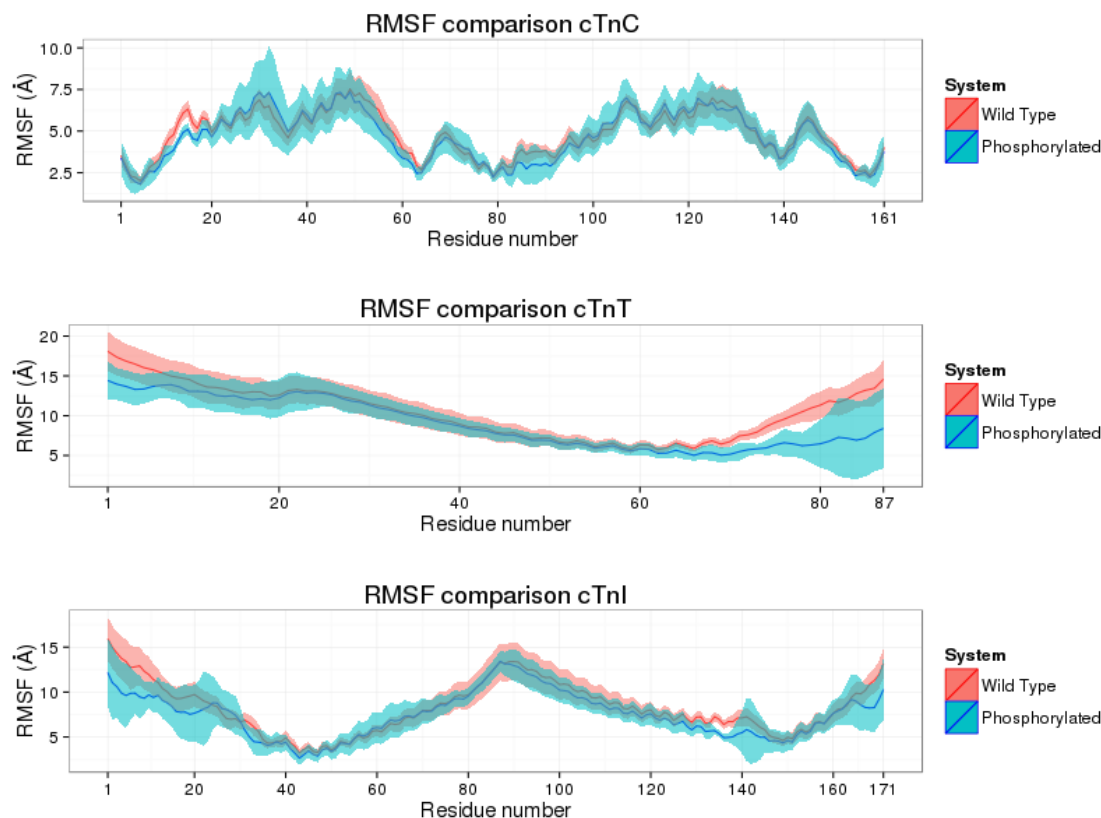
From the MD results it can be observed that the most differences are actually found on the TnI N terminus and the C terminus of TnT. This is very interesting because TnT does not influence Ca^{2+} binding, phosphorylation or thin filament switching directly but rather co-ordinates the whole process, which complicates the model further. This finding is also thought provoking because it can be combined with experimental results in IVMA. When exchange experiments in troponin were performed to observe which of the troponin subunits is responsible for uncoupling it was found that the TnT was necessary for uncoupling in thin filaments reconstituted with troponin from Hypertrophic Obstructive Cardiomyopathy (HOCM). However, the exact abnormality within the TnT could not be defined using standard structural methods (Bayliss et al, 2012). It is also noted that the phosphorylated structure is slightly less mobile than the unphosphorylated structure, which is against the simple trend but is compatible with a shift between disordered states.

Figure 7-4: Structure of phosphorylated and unphosphorylated troponin determined by MD simulations



Ribbon diagram of troponin representing the whole human sequences of TnC (blue), TnI (red), TnT (green). Ca^{2+} is represented by a gray sphere. The N terminal extension of TnI is shown in orange and the phosphorylation is indicated. A) unphosphorylated B) phosphorylated. The figure was created using chimera and the PDBs of the simulations were kindly provided by Mr Juan Eiros Zamora.

Figure 7-5: Amino acid changes in troponin subunits upon phosphorylation



Root Mean Square Fluctuations (RMSF) of phosphorylated and unphosphorylated troponin calculated using AMBER. RMSF of the amino acids in all the troponin subunits is shown (TnC at the top, TnT in the middle and TnI at the bottom). Red represents unphosphorylated (Wild Type) and blue represents phosphorylated. The figure was produced by Mr Juan Eiros Zamora.

From all these studies it can be concluded that troponin has a rather dynamic structure, owing to the intrinsically disorder domains such as the N terminal extension of TnI. This makes it difficult to elucidate its exact structure and thus the mechanisms of its regulation, at least by using the standard structural techniques. Determining the structure of troponin mobile regions will not only lead to understanding the changes that occur upon its regulation, but also to predicting the effect of a mutation or a pharmacological agent.

For example, just by knowing the structure of tropomyosin and its exact interactions with actin it has been possible to predict the effect of mutations. The experiments I did with the tropomyosin gain of function mutations shows what is possible if there is a well-defined structure known. In Chapter 3 I showed that all the tropomyosin mutations that are found in the site of interaction of tropomyosin- actin lead to a gain of function, as demonstrated by an increase in the Ca^{2+} sensitivity. This correlated with a hypercontractile phenotype in patients, showing that it possible to make structure-function predictions when the structure has been unravelled based on the stabilisation of tropomyosin interactions with actin Asp25, 326, 328 and 147. With troponin it is not possible to make such predictions at the moment, as the exact structure is still unresolved. However, MD simulations can be a good way to go forward with troponin, as the molecule is perceived as a moving object and not as a rigid structure.

7.6 Clinical implications of the uncoupling phenomenon

Uncoupling the Ca^{2+} sensitivity from TnI phosphorylation has important implications both for the diagnosis and the treatment of cardiomyopathies.

Idiopathic DCM patients can be divided into two categories; the ones that are responsive to dobutamine treatment, which is a drug used to treat acute heart failure by stimulating the β adrenergic response, and the ones that are not responsive to the therapy (Borow et al., 1988, Dubois-Randé et al., 1992, Naqvi et al., 1999). It might be possible that the ones that are non responsive are the ones with inherited DCM showing uncoupling, thus even if TnI gets phosphorylated the Ca^{2+} sensitivity would not change, giving the patient resistance to dobutamine and poor prognosis. If this is the case it would allow distinguishing the cases of DCM to inherited and idiopathic DCM and will be possible for the clinics to assign the DCM patients the right treatment.

The effects of the EGCG are also translationally important. EGCG was able to restore the uncoupling in all of the DCM and HCM cases with mutations in the thin filament. This has significant implications for the correct therapy of DCM and HCM, as EGCG has the ideal profile *in vitro*. This is especially true for HCM as EGCG can both decrease the Ca^{2+} sensitivity and re-couple, although the DCM cases did not have significantly different Ca^{2+} sensitivity levels to normal after treatment with EGCG.

However, EGCG is quite a promiscuous molecule with increased inotropic effects (Ingolfsson et al., 2014, Feng et al., 2012), which makes EGCG itself not a good candidate for therapy.

With EGCG analogues like the ones provided by Dr Brian Blagg the problem of promiscuity can be overcome, as it might be possible to make the drugs specific for troponin by manipulating the chemical groups of EGCG. It is also possible to separate the Ca^{2+} sensitising property from the re-coupling property, thus it would be likely to “cure” any cardiomyopathy cases that have TnI phosphorylation uncoupled to Ca^{2+} sensitivity irrespective of their effect on absolute Ca^{2+} sensitivity. Knowing the structure of the analogues would be important to understand the functional group which is responsible for re-coupling and thus making drugs that can be used in the clinic. The functional homology with the C terminal of Hsp90 inhibitors is also a worthwhile direction of study since these have been very extensively investigated already (Hall et al., 2014)

7.7 Future work

Although many interesting discoveries have been made in this thesis, many questions have also arisen. First of all, the structural changes of troponin upon phosphorylation need to be found, in order to understand the exact mechanism of regulation of troponin by phosphorylation, which accounts for the β adrenergic response. As explained in the previous sections, a good approach to solve this problem is through MD simulations. DCM and HCM-causing mutations can be incorporated into the simulations in the phosphorylated and unphosphorylated state, and thus the structural mechanisms that account for the uncoupling phenomenon can be revealed. Similarly, it would be possible to model the actions of the different drugs studied in this thesis, such as EGCG, Silybin, EMD 57033 and Bepridil. In that way the molecular mechanism between the uncoupling and how it is reversed by molecules such as EGCG can be discovered. My work has made finding a common mechanism possible, because there are now many different perturbations with derived functional effects that can be tested against structural models.

Another important aspect that needs to be examined is the physiological implication of uncoupling, especially in HCM. As mentioned previously, the uncoupling phenomenon in HCM is not clear, as there are discrepancies between *in vitro* and *in*

vivo studies. Uncoupling in HCM mutations should be investigated using IVMA to see whether all mutations cause uncoupling *in vitro*.

My *in vitro* experiments suggest re-couplers would be good agents to treat HCM and DCM. It is now important to investigate the effect of EGCG and EGCG-like compounds *in vivo*. This can be done using the transgenic mouse lines E361G (DCM) and E99K (HCM). The physiological consequences of the uncoupling phenomenon has been studied in these models *in vivo* by Pressure Volume loops and Echocardiography and *ex vivo* by force measurements of the papillary muscle (Song et al, 2011, Song et al, 2013, Wilkinson et al, 2015). It would be important to inject EGCG and/or its structural analogues into the E99K or the E361G mouse model in the presence of dobutamine and observe whether the disease symptoms are reduced. To view the effect on the muscle alone, the effect of the drugs can be observed on isolated papillary muscle using the AURORA apparatus. Phosphorylation levels in the mouse can also be manipulated by propranolol injections before sacrifice (Vikhorev et al., 2014). Thus, the effect of EGCG and its analogues can be observed in a physiological system. This would give interesting insights for the use of these compounds as treatment for DCM and HCM in mouse, and by extension potentially in humans.

In this thesis a medium-throughput method for screening of candidate compounds for the treatment of DCM and HCM has been developed. In the future, it is important for a high-throughput screening to be developed to find the most potent compound. A way to do this would be to use adult cardiomyocytes with a known DCM or HCM mutation in 96-well plates. Apart from the screening of the drugs, this would also give insights about the uncoupling phenomenon in a cellular system, something that has never been looked at before. The adrenergic response of the cardiomyocyte contractility in response to dobutamine can be assessed as well as the effect of the drugs. If uncoupling occurs then DCM and HCM cells would not respond to dobutamine, as preliminary experiments of Sun et al show (Sun et al., 2012). Cellular contractility can be measured in presence or absence of dobutamine and then in the presence of re-coupling drugs to test if the effect will be reversed. Toxicity of the drugs can be tested by measuring apoptosis of the cells and hypertrophy of the cells can also be measured by cell size and sarcomere alignment therefore reversibility of disease characteristics by re-couplers can be tested.

To assess the compound effect on human heart the more potent drug candidates can also be tested in Engineered Heart Tissue (EHT). Mr Thomas Owen in our lab has already optimized the EHT development and is now in the process of producing EHT from iPSC-derived cardiomyocytes with the *ACTC* E99K mutation. It will be interesting to see the effect of the compounds at the force of the mutant muscle. All these together will shed light into all aspects of the uncoupling phenomenon, from the molecular mechanism, the physiological consequences and most importantly the potential to reverse it using EGCG-like compounds.

BIBLIOGRAPHY

- ABUSAMHADNEH, E., ABBOTT, M. B., DVORETSKY, A., FINLEY, N., SASI, S. & ROSEVEAR, P. R. 2001. Interaction of bepridil with the cardiac troponin C/troponin I complex. *FEBS Lett*, 506, 51-54.
- AHMAD, R. S., BUTT, M. S., SULTAN, M. T., MUSHTAQ, Z., AHMAD, S., DEWANJEE, S., DE FEO, V. & ZIA-UL-HAQ, M. 2015. Preventive role of green tea catechins from obesity and related disorders especially hypercholesterolemia and hyperglycemia. *J Transl Med*, 13, 79.
- AKKARI, P. A., SONG, Y., HITCHCOCK-DEGREGORI, S., BLECHYNDEN, L. & LAING, N. 2002. Expression and biological activity of Baculovirus generated wild-type human slow alpha tropomyosin and the Met9Arg mutant responsible for a dominant form of nemaline myopathy. *Biochem Biophys Res Commun*, 296, 300-4.
- ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. 2002. *Molecular Biology of the Cell*, Garland Science
- ALVES, M. L., DIAS, F. A. L., GAFFIN, R. D., SIMON, J. N., MONTMINY, E. M., BIESIADECKI, B. J., HINKEN, A. C., WARREN, C. M., UTTER, M. S., DAVIS, R. T., SAKTHIVEL, S., ROBBINS, J., WIECZOREK, D. F., SOLARO, R. J. & WOLSKA, B. M. 2014. Desensitization of Myofilaments to Ca²⁺ as a Therapeutic Target for Hypertrophic Cardiomyopathy with Mutations in Thin Filament Proteins. *Circ: Cardiovasc Genet*, 7, 132-143.
- ALVES, M. L., GAFFIN, R. D. & WOLSKA, B. M. 2010. Rescue of familial cardiomyopathies by modifications at the level of sarcomere and Ca²⁺ fluxes. *J Mol Cell Cardiol*, 48, 834-42.
- AYAZ-GUNER, S., ZHANG, J., LI, L., WALKER, J. W. & GE, Y. 2009. In Vivo Phosphorylation Site Mapping in Mouse Cardiac Troponin I by High Resolution Top-Down Electron Capture Dissociation Mass Spectrometry: Ser22/23 Are the Only Sites Basally Phosphorylated. *Biochemistry*, 48, 8161-8170.
- BAILIN, G. 1979. Phosphorylation of a bovine cardiac actin complex. *Am J Physiol*, 236, C41-6.
- BARTON, P. J., TOWNSEND, P. J., BRAND, N. J. & YACCOUB, M. H. 1997. Localization of the fast skeletal muscle troponin I gene (TNNI2) to 11p15.5: genes for troponin I and T are organized in pairs. *Ann Hum Genet*, 61, 519-23.
- BAUDENBACHER, F., SCHOBBER, T., PINTO, J. R., SIDOROV, V. Y., HILLIARD, F., SOLARO, R. J., POTTER, J. D. & KNOLLMANN, B. C. 2008. Myofilament Ca²⁺ sensitization causes susceptibility to cardiac arrhythmia in mice. *J Clin Invest*, 118, 3893-903.
- BAYLISS, C. 2012. Dysfunction of contractile proteins in Hypertrophic Cardiomyopathy. *PhD Thesis, Imperial College London*, 1-341.
- BAYLISS, C., MESSER, A., LEUNG, M.-C., WARD, D., VAN DER VELDEN, J., POGGESI, C., REDWOOD, C. & MARSTON, S. 2012a. *Functional investigation of troponin with the homozygous HCM mutation, TNNT2 K280M, obtained from an explanted heart. *Cardiovasc Res*, 93, S107.
- BAYLISS, C. R., JACQUES, A. M., LEUNG, M.-C., WARD, D. G., REDWOOD, C. S., GALLON, C. E., COPELAND, O., MCKENNA, W. J., DOS REMEDIOS, C., MARSTON, S. B. & MESSER, A. E. 2012b. Myofibrillar Ca²⁺-

- Sensitivity Is Uncoupled From Troponin I Phosphorylation In Hypertrophic Obstructive Cardiomyopathy Due To Abnormal Troponin T. *Cardiovasc Res*, 97, 500-508.
- BEHRMANN, E., MÜLLER, M., PENCZEK, P. A., MANNHERZ, H. G., MANSTEIN, D. J. & RAUNSER, S. 2012. Structure of the rigor actin-tropomyosin-Myosin complex. *Cell*, 150, 327-338.
- BERS, D. M. 2002. Cardiac excitation-contraction coupling. *Nature*, 415, 198-205.
- BIESIADECKI, B. J., KOBAYASHI, T., WALKER, J. S., JOHN SOLARO, R. & DE TOMBE, P. P. 2007. The troponin C G159D mutation blunts myofilament desensitization induced by troponin I Ser23/24 phosphorylation. *Circ Res*, 100, 1486-93.
- BING, W., KNOTT, A., REDWOOD, C., ESPOSITO, G., PURCELL, I., WATKINS, H. & MARSTON, S. 2000. Effect of hypertrophic cardiomyopathy mutations in human cardiac muscle alpha -tropomyosin (Asp175Asn and Glu180Gly) on the regulatory properties of human cardiac troponin determined by in vitro motility assay. *J Mol Cell Cardiol*, 32, 1489-1498.
- BING, W., REDWOOD, C. S., PURCELL, I. F., ESPOSITO, G., WATKINS, H. & MARSTON, S. B. 1997. Effects of two hypertrophic cardiomyopathy mutations in a-tropomyosin, Asp175Asn and Glu180Gly, on Ca^{2+} regulation of thin filament motility. *Biochem Biophys Res Commun*, 236, 760-764.
- BODOR, G. S., OAKELEY, A. E., ALLEN, P. D., CRIMMINS, D. L., LADENSON, J. H. & ANDERSON, P. A. W. 1997. Troponin I phosphorylation in the normal and failing adult human heart. *Circulation*, 96, 1495-1500.
- BOROW, K. M., LANG, R. M., NEUMANN, A., CARROLL, J. D. & RAJFER, S. I. 1988. Physiologic mechanisms governing hemodynamic responses to positive inotropic therapy in patients with dilated cardiomyopathy. *Circulation*, 77, 625-637.
- BOTTEN, D., FUGALLO, G., FRATERNALI, F. & MOLTENI, C. 2013. A computational exploration of the interactions of the green tea polyphenol (-)-epigallocatechin 3-gallate with cardiac muscle troponin C. *PLoS ONE*, 8, e70556.
- CARBALLO, S., ROBINSON, P., OTWAY, R., FATKIN, D., JONGBLOED, J. D., DE JONGE, N., BLAIR, E., VAN TINTELEN, J. P., REDWOOD, C. & WATKINS, H. 2009. Identification and functional characterization of cardiac troponin I as a novel disease gene in autosomal dominant dilated cardiomyopathy. *Circ Res*, 105, 375-82.
- CHEN, D., WAN, S. B., YANG, H., YUAN, J., CHAN, T. H. & DOU, Q. P. 2011. EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. *Adv Clin Chem*, 53, 155-77.
- CHENG, Y., LINDERT, S., KEKENES-HUSKEY, P., RAO, V. S., SOLARO, R. J., ROSEVEAR, P. R., AMARO, R., MCCULLOCH, A. D., MCCAMMON, J. A. & REGNIER, M. 2014. Computational Studies of the Effect of the S23D/S24D Troponin I Mutation on Cardiac Troponin Structural Dynamics. *Biophys J*, 107, 1675-1685.
- CLARKE, N. F., DOMAZETOVSKA, A., WADDELL, L., KORNBERG, A., MCLEAN, C. & NORTH, K. N. 2009. Cap disease due to mutation of the beta-tropomyosin gene (TPM2). *Neuromuscul Disord*, 19, 348-351.
- CLARKE, N. F., KOLSKI, H., DYE, D. E., LIM, E., SMITH, R. L., PATEL, R., FAHEY, M. C., BELLANCE, R., ROMERO, N. B., JOHNSON, E. S., LABARRE-VILA, A., MONNIER, N., LAING, N. G. & NORTH, K. N.

2008. Mutations in TPM3 are a common cause of congenital fiber type disproportion. *Ann Neurol*, 63, 329-337.
- CLARKE, N. F. & NORTH, K. N. 2003. Congenital fiber type disproportion--30 years on. *J Neuropathol Exp Neurol*, 62, 977-89.
- COPELAND, O., NOWAK, K., LAING, N., RAVENSCROFT, G., MESSER, A. E., BAYLISS, C. R. & MARSTON, S. B. 2010. Investigation of changes in skeletal muscle alpha-actin expression in normal and pathological human and mouse hearts. *J Musc Res Cell Motil*, 31, 207-14.
- CUISSET, J. M., MAURAGE, C. A., PELLISSIER, J. F., BAROIS, A., URTIZBEREA, J. A., LAING, N., TAJSHARGHI, H. & VALLEE, L. 2006. 'Cap myopathy': case report of a family. *Neuromuscul Disord*, 16, 277-81.
- CUNNINGHAM, J. G. 2002. *Textbook of Veterinary Physiology*, Philadelphia, P.A., W.B. Saunders Co.
- DAVIDSON, A. E., SIDDIQUI, F. M., LOPEZ, M. A., LUNT, P., CARLSON, H. A., MOORE, B. E., LOVE, S., BORN, D. E., ROPER, H., MAJUMDAR, A., JAYADEV, S., UNDERHILL, H. R., SMITH, C. O., VON DER HAGEN, M., HUBNER, A., JARDINE, P., MERRISON, A., CURTIS, E., CULLUP, T., JUNGBLUTH, H., COX, M. O., WINDER, T. L., ABDEL SALAM, H., LI, J. Z., MOORE, S. A. & DOWLING, J. J. 2013. Novel deletion of lysine 7 expands the clinical, histopathological and genetic spectrum of TPM2-related myopathies. *Brain*, 136, 508-521.
- DAVIES, M. J. 2000. The cardiomyopathies: an overview. *Heart*, 83, 469-74.
- DENG, Y., SCHMIDTMANN, A., REDLICH, A., WESTERDORF, B., JAQUET, K. & THIELECZEK, R. 2001. Effects of Phosphorylation and Mutation R145G on Human Cardiac Troponin I Function. *Biochemistry*, 40, 14593-14602.
- DOMINGUEZ, R. & HOLMES, K. C. 2011. Actin structure and function. *Annu Rev Biophys*, 40, 169-86.
- DONG, W., XING, J., OUYANG, Y., AN, J. & CHEUNG, H. C. 2008. Structural kinetics of Cardiac troponin C mutants linked to familial hypertrophic and dilated cardiomyopathy in troponin complexes. *J Biol Chem*, 283, 3424-3432.
- DONG, W. J., JAYASUNDAR, J. J., AN, J., XING, J. & CHEUNG, H. C. 2007. Effects of PKA phosphorylation of cardiac troponin I and strong crossbridge on conformational transitions of the N-domain of cardiac troponin C in regulated thin filaments. *Biochemistry*, 46, 9752-61.
- DONKERVOORT, S., PAPADAKI, M., DE WINTER, J. M., NEU, M. B., KIRSCHNER, J., BOLDUC, V., YANG, M. L., GIBBONS, M. A., HU, Y., DASTGIR, J., LEACH, M. E., RUTKOWSKI, A., FOLEY, A. R., KRUGER, M., WARTCHOW, E. P., MCNAMARA, E., ONG, R., NOWAK, K. J., LAING, N. G., CLARKE, N. F., OTTENHEIJM, C. A., MARSTON, S. B. & BONNEMANN, C. G. 2015. TPM3 deletions cause a hypercontractile congenital muscle stiffness phenotype. *Ann Neurol*. doi:10.1002/ana24535.
- DOS-REMEDIOS, C. G. & MOENS, P. D. 1995. Actin and the actomyosin interface: a review. *Biochimica et Biophysica Acta*, 1228, 99-124.
- DUBOIS-RANDÉ, J. L., MERLET, P., ROUDOT, F., BENVENUTI, C., ADNOT, S., HITTINGER, L., DUVAL, A. M., SYROTA, A., CASTAIGNE, A. & LOISANCE, D. 1992. Beta-adrenergic contractile reserve as a predictor of clinical outcome in patients with idiopathic dilated cardiomyopathy. *American Heart Journal*, 124, 679-685.
- DYER, E., JACQUES, A., HOSKINS, A., WARD, D., GALLON, C., MESSER, A., KASKI, J., BURCH, M., KENTISH, J. & MARSTON, S. 2009. Functional

- Analysis of a Unique Troponin C Mutation, Gly159Asp that Causes Familial Dilated Cardiomyopathy, Studied in Explanted Heart Muscle. *Circ Heart Fail*, 2, 456-464.
- EFTHIMIADIS, I., SKENDROS, P., SARANTOPOULOS, A. & BOURA, P. 2011. CD4+/CD25+ T-Lymphocytes and Th1/Th2 regulation in dilated cardiomyopathy. *Hippokratia*, 15, 335-42.
- EL-ARMOUCHE, A. & ESCHENHAGEN, T. 2009. Beta-adrenergic stimulation and myocardial function in the failing heart. *Heart Fail Rev*, 14, 225-41.
- ELLIOTT, P. & MCKENNA, W. J. 2004. Hypertrophic cardiomyopathy. *Lancet*, 363, 1881-91.
- ESCHENHAGEN, T., FINK, C., REMMERS, U., SCHOLZ, H., WATTCHOW, J., WEIL, J., ZIMMERMANN, W., DOHMEN, H. H., SCHAFER, H., BISHOPRIC, N., WAKATSUKI, T. & ELSON, E. L. 1997. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. *FASEB J*, 11, 683-94.
- FENG, J. J. & MARSTON, S. 2009. Genotype-Phenotype Correlations in ACTA1 Mutations That Cause Congenital Myopathies. *Neuromusc Disord*, 19, 6-16.
- FENG, W., HWANG, H. S., KRYSHAL, D. O., YANG, T., PADILLA, I. T., TIWARY, A. K., PUSCHNER, B., PESSAH, I. N. & KNOLLMANN, B. C. 2012. Coordinated Regulation of Murine Cardiomyocyte Contractility by Nanomolar (-)-Epigallocatechin-3-Gallate, the Major Green Tea Catechin. *Mol Pharmacol*, 82, 993-1000.
- FERRARA, C., WITJAS-PAALBERENDS, E. R., PIRODDI, N., SCELLINI, B., FERRANTINI, C., WIHNER, P. J., SEQUIERA, V., DOS REMEDIOS, C., SCHLOSSAREK, S., LEUNG, J., TESI, C., CARRIER, L., REDWOOD, C. S., MARSTON, S., VAN DER VELDEN, J. & POGGESI, C. 2015. A novel HCM mutation in cardiac troponin T primarily alters cross-bridge kinetics and increases the energy cost of tension generation in human cardiac myofibrils. *In prep*.
- FLASHMAN, E., REDWOOD, C., MOOLMAN-SMOOK, J. & WATKINS, H. 2004. Cardiac myosin binding protein C: its role in physiology and disease. *Circ Res*, 94, 1279-89.
- FRASER, I. D. & MARSTON, S. B. 1995a. In vitro motility analysis of actin-tropomyosin regulation by troponin and calcium. The thin filament is switched as a single cooperative unit. *J Biol Chem*, 270, 7836-7841.
- FRASER, I. D. & MARSTON, S. B. 1995b. In vitro motility analysis of smooth muscle caldesmon control of actin-tropomyosin filament movement. 270, 19688-19693.
- FUCHS, F. & GRABAREK, Z. 2013. The green tea polyphenol (-)-epigallocatechin-3-gallate inhibits magnesium binding to the C-domain of cardiac troponin C. *J Muscle Res Cell Motil*, 34, 107-113.
- GEEVES, M. A., HITCHCOCK-DEGREGORI, S. E. & GUNNING, P. W. 2015. A systematic nomenclature for mammalian tropomyosin isoforms. *J Muscle Res Cell Motil*, 36, 147-53.
- GEEVES, M. A. & HOLMES, K. C. 2005. The molecular mechanism of muscle contraction. *Adv Protein Chem*, 71, 161-93.
- GEISTERFER-LORANCE, A. A., KASS, S., TANIGAWA, G., VOSBERG, H. P., MCKENNA, W., SEIDMAN, C. E. & SEIDMAN, J. G. 1990. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. *Cell*, 62, 999-1006.

- GOMES, A. V., HARADA, K. & POTTER, J. D. 2005. A mutation in the N-terminus of troponin I that is associated with hypertrophic cardiomyopathy affects the Ca(2+)-sensitivity, phosphorylation kinetics and proteolytic susceptibility of troponin. *J Mol Cell Cardiol*, 39, 754-765.
- GORDON, A. M., HOMSHER, E. & REGNIER, M. 2000. Regulation of contraction in striated muscle. *Physiol Rev*, 80, 853-924.
- GUINTO, P. J., HAIM, T. E., DOWELL-MARTINO, C. C., SIBINGA, N. & TARDIFF, J. C. 2009. Temporal and mutation-specific alterations in Ca²⁺ homeostasis differentially determine the progression of cTnT-related cardiomyopathies in murine models. *AJP: Heart and Circulatory Physiology*, 297, H614-26.
- HAIM, T. E., DOWELL, C., DIAMANTI, T., SCHEUER, J. & TARDIFF, J. C. 2007. Independent FHC-related cardiac troponin T mutations exhibit specific alterations in myocellular contractility and calcium kinetics. *J Mol Cell Cardiol*, 42, 1098-110.
- HALL, J. A., FORSBERG, L. K. & BLAGG, B. S. 2014. Alternative approaches to Hsp90 modulation for the treatment of cancer. *Future Med Chem*, 6, 1587-605.
- HARVEY, P. A. & LEINWAND, L. A. 2011. The cell biology of disease: cellular mechanisms of cardiomyopathy. *J Cell Biol*, 194, 355-65.
- HERMAN, D. S., LAM, L., TAYLOR, M. R. G., WANG, L., TEEKAKIRIKUL, P., CHRISTODOULOU, D., CONNER, L., DEPALMA, S. R., MCDONOUGH, B., SPARKS, E., TEODORESCU, D. L., CIRINO, A. L., BANNER, N. R., PENNELL, D. J., GRAW, S., MERLO, M., DI LENARDA, A., SINAGRA, G., BOS, J. M., ACKERMAN, M. J., MITCHELL, R. N., MURRY, C. E., LAKDAWALA, N. K., HO, C. Y., BARTON, P. J. R., COOK, S. A., MESTRONI, L., SEIDMAN, J. G. & SEIDMAN, C. E. 2012. Truncations of titin causing dilated cardiomyopathy. *New Eng J Med*, 366, 619-628.
- HERSHBERGER, R. E., HEDGES, D. J. & MORALES, A. 2013. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. *Nat Rev Cardiol*, 10, 531-547.
- HERSHBERGER, R. E. & SIEGFRIED, J. D. 2011. Update 2011: clinical and genetic issues in familial dilated cardiomyopathy. *J Am Coll Cardiol*, 57, 1641-9.
- HERTOG, M. G., FESKENS, E. J., HOLLMAN, P. C., KATAN, M. B. & KROMHOUT, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet*, 342, 1007-11.
- HERZBERG, O. & JAMES, M. N. G. 1985. Structure of the calcium regulatory muscle protein troponin-C at 2.8Å resolution. *Nature*, 313, 653-659.
- HINSON, J. T., CHOPRA, A., NAFISSI, N., POLACHECK, W. J., BENSON, C. C., SWIST, S., GORHAM, J., YANG, L., SCHAFER, S., SHENG, C. C., HAGHIGHI, A., HOMSY, J., HUBNER, N., CHURCH, G., COOK, S. A., LINKE, W. A., CHEN, C. S., SEIDMAN, J. G. & SEIDMAN, C. E. 2015. HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science*, 349, 982-6.
- HITCHCOCK DEGREGORI, S. E. & VARNELL, T. A. 1990. Tropomyosin has discrete actin-binding sites with sevenfold and fourteenfold periodicities. *J.Mol.Biol.*, 214, 885-896.

- HODGKINSON, J. L. 2000. Actin and the smooth muscle regulatory proteins: a structural perspective. *Journal of Muscle Research and Cell motility*, 21, 115-130.
- HOLMES, K. C., POPP, D., GEBHARD, W. & KABSCH, W. 1990. Atomic model of the actin filament. *Nature*, 347, 44-49.
- HOLTHAUZEN, L. M., CORREA, F. & FARAH, C. S. 2004. Ca²⁺-induced rolling of tropomyosin in muscle thin filaments: the alpha- and beta-band hypothesis revisited. *J Biol Chem*, 279, 15204-13.
- HOWARTH, J. W., MELLER, J., SOLARO, R. J., TREWHELLA, J. & ROSEVEAR, P. R. 2007. Phosphorylation-dependent conformational transition of the cardiac specific N-extension of troponin I in cardiac troponin. *J Mol Biol*, 373, 706-22.
- HUXLEY, A. F. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem.*, 7, 255-318.
- HWANG, P. M., CAI, F., PINEDA-SANABRIA, S. E., CORSON, D. C. & SYKES, B. D. 2014. The cardiac-specific N-terminal region of troponin I positions the regulatory domain of troponin C. *Proc Natl Acad Sci U S A*, 111, 14412-7.
- IAKOUCHEVA, L. M., RADIVOJAC, P., BROWN, C. J., O'CONNOR, T. R., SIKES, J. G., OBRADOVIC, Z. & DUNKER, A. K. 2004. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res*, 32, 1037-49.
- INGLES, J., DOOLAN, A., CHIU, C., SEIDMAN, J., SEIDMAN, C. & SEMSARIAN, C. 2005. Compound and double mutations in patients with hypertrophic cardiomyopathy: implications for genetic testing and counselling. *J Med Genet*, 42, e59.
- INGÓLFSSON, H. I., THAKUR, P., HEROLD, K. F., HOBART, E. A., RAMSEY, N. B., PERIOLE, X., DE JONG, D. H., ZWAMA, M., YILMAZ, D., HALL, K., MARETZKY, T., HEMMINGS, H. C., BLOBEL, C., MARRINK, S. J., KOÇER, A., SACK, J. T. & ANDERSEN, O. S. 2014. Phytochemicals perturb membranes and promiscuously alter protein function. *ACS chemical biology*, 9, 1788-1798.
- JAENICKE, T., DIEDERICH, K. W., HAAS, W., SCHLEICH, J., LICHTER, P., PFORDT, M., BACH, A. & VOSBERG, H. P. 1990. The complete sequence of the human beta-myosin heavy chain gene and a comparative analysis of its product. *Genomics*, 8, 194-206.
- JAIN, R. K., JAYAWANT, S., SQUIER, W., MUNTONI, F., SEWRY, C. A., MANZUR, A., QUINLIVAN, R., LILLIS, S., JUNGBLUTH, H., SPARROW, J. C., RAVENSCROFT, G., NOWAK, K. J., MEMO, M., MARSTON, S. B. & LAING, N. G. 2012. Nemaline myopathy with stiffness and hypertonía associated with an ACTA1 mutation. *Neurology*, 78, 1100-1103.
- KAMISAGO, M., SHARMA, S. D., DEPALMA, S. R., SOLOMON, S., SHARMA, P., SMOOT, L., MULLEN, M. P., WOOLF, P. K., WIGLE, E. D., SEIDMAN, J. G., SEIDMAN, C. E., JARCHO, J. & SHAPIRO, L. R. 2000. Mutations in Sarcomere Protein Genes as a Cause of Dilated Cardiomyopathy. *N Engl J Med*, 343, 1688-1696.
- KASKI, J. P., BURCH, M. & ELLIOTT, P. M. 2007. Mutations in the cardiac Troponin C gene are a cause of idiopathic dilated cardiomyopathy in childhood. *Cardiol Young*, 17, 675-7.
- KASS, D. A. & SOLARO, R. J. 2006. Mechanisms and use of calcium-sensitizing agents in the failing heart. *Circulation*, 113, 305-315.

- KATRUKHA, A. G., BEREZNIKOVA, A. V., ESAKOVA, T. V., FILATOV, V. L., BULGARINA, T. V. & GUSEV, N. B. 1995. A new method of human cardiac troponin I and troponin T purification. *Biochem Mol Biol Int*, 36, 195-202.
- KATRUKHA, I. A. 2013. Human cardiac troponin complex. Structure and functions. *Biochemistry (Mosc)*, 78, 1447-65.
- KATZ, A. M. 2006. *Physiology of the heart*, Philadelphia, PA, Williams and Wilkins.
- KAWASAKI, H., NAKAYAMA, S. & KRETSINGER, R. H. 1998. Classification and evolution of EF-hand proteins. *Biometals*, 11, 277-95.
- KHANDELWAL, A., HALL, J. A. & BLAGG, B. S. 2013a. Synthesis and structure-activity relationships of EGCG analogues, a recently identified Hsp90 inhibitor. *J Org Chem*, 78, 7859-84.
- KHANDELWAL, A., HALL, J. A. & BLAGG, B. S. J. 2013b. Synthesis and Structure-Activity Relationships of EGCG Analogues, a Recently Identified Hsp90 Inhibitor. *J. Org. Chem.*, 78, 7859-7884.
- KINOSHITA, E., KINOSHITA-KIKUTA, E., TAKIYAMA, K. & KOIKE, T. 2006. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics*, 5, 749-57.
- KLEEREKOPER, Q., LIU, W., SYKES, B. D. & PUTKEY, J. A. 1998. Cardiac troponin C: Correlation of solution structure with drug binding sites. *Biophysical Journal*, 74, A143-A143.
- KOBAYASHI, T. & SOLARO, R. J. 2005. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol*, 67, 39-67.
- KOOIJ, V., SAES, M., JAQUET, K., ZAREMBA, R., FOSTER, D. B., MURPHY, A., REMEDIOS, C., VELDEN, J. & STIENEN, G. J. 2010. Effect of troponin I Ser23/24 phosphorylation on Ca²⁺-sensitivity in human myocardium depends on the phosphorylation background. *J Mol Cell Cardiol*, 48, 954-963.
- KOSTIN, S. F., MCDONALD, D. E. & MCFADDEN, D. W. 2012. Inhibitory effects of (-)-epigallocatechin-3-gallate and pterostilbene on pancreatic cancer growth in vitro. *J Surg Res*, 177, 255-62.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.
- LEGRICE, I. J., SMAILL, B. H., CHAI, L. Z., EDGAR, S. G., GAVIN, J. B. & HUNTER, P. J. 1995. Laminar structure of the heart: ventricular myocyte arrangement and connective tissue architecture in the dog. *Am J Physiol*, 269, H571-82.
- LEHMAN, W. & CRAIG, R. 2008. Tropomyosin and the steric mechanism of muscle regulation. *Adv Exp Med Biol*, 644, 95-109.
- LEHMAN, W., GALIŃSKA-RAKOCZY, A., HATCH, V., TOBACMAN, L. S. & CRAIG, R. 2009. Structural basis for the activation of muscle contraction by troponin and tropomyosin. *Journal of Molecular Biology*, 388, 673-681.
- LEHMAN, W., ORZECOWSKI, LI, FISCHER, S. & RAUNSER, S. 2013. Gestalt-binding of tropomyosin on actin during thin filament activation. *J Muscle Res Cell Motil*, 1-24.
- LEHRER, S. S. & GEEVES, M. A. 1998. The muscle thin filament as a classical cooperative/allosteric regulatory system. *J Mol Biol*, 277, 1081-1089.
- LEHTOKARI, V.-L., CEUTERICK-DE GROOTE, C., DE JONGHE, P., MARTTILA, M., LAING, N. G., PELIN, K. & WALLGREN-PETTERSSON, C. 2007. Cap disease caused by heterozygous deletion of the beta-tropomyosin gene TPM2. *Neuromuscul Disord*, 17, 433-442.
- LEVICK, J. R. 2000. *An Introduction to Cardiovascular Physiology*, Arnold.

- LI, L., DESANTIAGO, J., CHU, G., KRANIAS, E. G. & BERS, D. M. 2000a. Phosphorylation of phospholamban and troponin I in beta-adrenergic-induced acceleration of cardiac relaxation. *Am J Physiol Heart Circ Physiol*, 278, H769-79.
- LI, M. X. & HWANG, P. M. 2015. Structure and function of cardiac troponin C (TNNC1): Implications for heart failure, cardiomyopathies, and troponin modulating drugs. *Gene*, 571, 153-66.
- LI, M. X., ROBERTSON, I. M. & SYKES, B. D. 2008. Interaction of cardiac troponin with cardiotonic drugs: a structural perspective. *Biochem Biophys Res Commun*, 369, 88-99.
- LI, M. X., WANG, X. & SYKES, B. D. 2004. Structural based insights into the role of troponin in cardiac muscle pathophysiology. *J Muscle Res Cell Motil*, 25, 559-79.
- LI, X. E., LEHMAN, W. & FISCHER, S. 2010. The relationship between curvature, flexibility and persistence length in the tropomyosin coiled-coil. *J Struct Biol*, 170, 313-318.
- LI, X. E., TOBACMAN, L. S., MUN, J. Y., CRAIG, R., FISCHER, S. & LEHMAN, W. 2011. Tropomyosin position on F-actin revealed by EM reconstruction and computational chemistry. *Biophys J*, 100, 1005-1013.
- LI, Y., LOVE, M. L., PUTKEY, J. A. & COHEN, C. 2000b. Bepridil opens the regulatory N-terminal lobe of cardiac troponin C. *Proc Natl Acad Sci U S A*, 97, 5140-5.
- LIU, Y.-M., KUO, S.-C. & HSIEH, S.-R. 2008. Differential effects of a green tea-derived polyphenol (-)-epigallocatechin-3-gallate on the acidosis-induced decrease in the Ca(2+) sensitivity of cardiac and skeletal muscle. *Pflügers Arch*, 456, 787-800.
- LOMPRE, A. M., NADAL-GINARD, B. & MAHDAVI, V. 1984. Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated. *J Biol Chem*, 259, 6437-46.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurements with Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275.
- LYMN, R. W. & TAYLOR, E. W. 1971. Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry*, 10, 4617-4623.
- MACLACHLAN, L. K., REID, D. G., MITCHELL, R. C., SALTER, C. J. & SMITH, S. J. 1990. Binding of a Calcium Sensitizer, Bepridil, to Cardiac Troponin-C - a Fluorescence Stopped-Flow Kinetic, Circular-Dichroism, and Proton Nuclear-Magnetic-Resonance Study. *Journal of Biological Chemistry*, 265, 9764-9770.
- MACLENNAN, D. H. & KRANIAS, E. 2003. Phospholamban: a crucial regulator of cardiac contractility. *Nature reviews Molecular cell biology*, 4, 566-77.
- MALNIC, B., FARAH, C. S. & REINACH, F. C. 1998. Regulatory properties of the NH2- and COOH-terminal domains of troponin T. ATPase activation and binding to troponin I and troponin C. *J Biol Chem*, 273, 10594-601.
- MANNING, E. P., GUINTO, P. J. & TARDIFF, J. C. 2012a. Correlation of molecular and functional effects of mutations in cardiac troponin T linked to familial hypertrophic cardiomyopathy: an integrative in silico/in vitro approach. *J Biol Chem*, 287, 14515-23.
- MANNING, E. P., TARDIFF, J. C. & SCHWARTZ, S. D. 2012b. Molecular effects of familial hypertrophic cardiomyopathy-related mutations in the TNT1 domain of cTnT. *J Mol Biol*, 421, 54-66.

- MARGOSSIAN, S. S. & LOWEY, S. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol*, 85, 55-71.
- MARON, B. J. 1997. Hypertrophic cardiomyopathy. *Lancet*, 350, 127-33.
- MARON, B. J., GARDIN, J. M., FLACK, J. M., GIDDING, S. S., KUROSAKI, T. T. & BILD, D. E. 1995. Prevalence of hypertrophic cardiomyopathy in a general population of young adults: echocardiographic analysis of 4122 subjects in the CARDIA study. *Circulation*, 92, 785-789.
- MARON, B. J., NICHOLS, P. F., 3RD, PICKLE, L. W., WESLEY, Y. E. & MULVIHILL, J. J. 1984. Patterns of inheritance in hypertrophic cardiomyopathy: assessment by M-mode and two-dimensional echocardiography. *Am J Cardiol*, 53, 1087-94.
- MARON, B. J., OLIVOTTO, I., SPIRITO, P., CASEY, S. A., BELLONE, P., GOHMAN, T. E., GRAHAM, K. J., BURTON, D. A. & CECCHI, F. 2000. Epidemiology of hypertrophic cardiomyopathy-related death: revisited in a large non-referral-based patient population. *Circulation*, 102, 858-64.
- MARON, B. J. & PELLICCIA, A. 2006. The heart of trained athletes: cardiac remodeling and the risks of sports, including sudden death. *Circulation*, 114, 1633-44.
- MARON, M. S., OLIVOTTO, I., BETOCCHI, S., CASEY, S. A., LESSER, J. R., LOSI, M. A., CECCHI, F. & MARON, B. J. 2003. Effect of left ventricular outflow tract obstruction on clinical outcome in hypertrophic cardiomyopathy. *N Engl J Med*, 348, 295-303.
- MARSTON, S. 2003. Random walks with thin filaments: application of in vitro motility assay to the study of actomyosin regulation. *J Musc Res Cell motil*, 24, 149-156.
- MARSTON, S. 2015. Important announcement: a rational nomenclature for tropomyosin variants. *J Muscle Res Cell Motil*, 36, 145.
- MARSTON, S., MEMO, M., MESSER, A., PAPADAKI, M., NOWAK, K., MCNAMARA, E., ONG, R., EL-MEZGUELDI, M., LI, X. & LEHMAN, W. 2013. Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients. *Hum Mol Genet*, 22, 4978-4987.
- MARSTON, S. B. 2011. How Do Mutations in Contractile Proteins Cause the Primary Familial Cardiomyopathies? *J Cardiovasc Transl Res*, 4, 245-255.
- MARSTON, S. B., FRASER, I. D. C., WU, B. & ROPER, G. 1996. A simple method for automatic tracking of actin filaments in the motility assay. *J Musc Res Cell Motil*, 17, 497-506.
- MARSTON, S. B. & REDWOOD, C. S. 2003. Modulation of thin filament activation by breakdown or isoform switching of thin filament proteins: physiological and pathological implications. *Circ Res*, 93, 1170-8.
- MARTTILA, M., LEHTOKARI, V. L., MARSTON, S., NYMAN, T. A., BARNERIAS, C., BEGGS, A. H., BERTINI, E., CEYHAN-BIRSOY, O., CINTAS, P., GERARD, M., GILBERT-DUSSARDIER, B., HOGUE, J. S., LONGMAN, C., EYMARD, B., FRYDMAN, M., KANG, P. B., KLINGE, L., KOLSKI, H., LOCHMULLER, H., MAGY, L., MANEL, V., MAYER, M., MERCURI, E., NORTH, K. N., PEUDENIER-ROBERT, S., PIHKO, H., PROBST, F. J., REISIN, R., STEWART, W., TARATUTO, A. L., DE VISSER, M., WILICHOWSKI, E., WINER, J., NOWAK, K., LAING, N. G., WINDER, T. L., MONNIER, N., CLARKE, N. F., PELIN, K., GRONHOLM, M. & WALLGREN-PETTERSSON, C. 2014. Mutation update and genotype-

- phenotype correlations of novel and previously described mutations in TPM2 and TPM3 causing congenital myopathies. *Hum Mutat*, 35, 779-90.
- MARTTILA, M., LEMOLA, E., WALLEFELD, W., MEMO, M., DONNER, K., LAING, N. G., MARSTON, S., GRÖNHOLM, M. & WALLGREN-PETTERSSON, C. 2012. Abnormal actin binding of aberrant β -tropomyosins is a molecular cause of muscle weakness in TPM2-related nemaline and cap myopathy. *Biochem J*, 442, 231-239.
- MATTS, R. L., DIXIT, A., PETERSON, L. B., SUN, L., VORUGANTI, S., KALYANARAMAN, P., HARTSON, S. D., VERKHIVKER, G. M. & BLAGG, B. S. 2011. Elucidation of the Hsp90 C-terminal inhibitor binding site. *ACS Chem Biol*, 6, 800-7.
- MAYTUM, R., LEHRER, S. & GEEVES, M. 1999. Cooperativity and switching within the Three-state model of muscle regulation. *Biochemistry*, 38, 1102-1110.
- MCGUIGAN, J. A., LUTHI, D. & BURI, A. 1991. Calcium buffer solutions and how to make them: a do it yourself guide. *Can J Physiol Pharmacol*, 69, 1733-49.
- MCKILLOP, D. F. A. & GEEVES, M. A. 1993. Regulation of the interaction between actin and myosin subfragment-1: evidence for three states of the thin filament. *Biophys.J.*, 65, 693-701.
- MEMO, M. 2012. *Molecular Mechanisms of Myopathies*. PhD, Imperial College London.
- MEMO, M., LEUNG, M.-C., WARD, D. G., DOS REMEDIOS, C., MORIMOTO, S., ZHANG, L., RAVENSCROFT, G., MCNAMARA, E., NOWAK, K. J., MARSTON, S. B. & MESSER, A. E. 2013. Mutations in thin Filament Proteins that Cause Familial Dilated Cardiomyopathy Uncouple Troponin I Phosphorylation from Changes in Myofibrillar Ca^{2+} -Sensitivity. *Cardiovasc Res*, 99, 65-73.
- MEMO, M. & MARSTON, S. 2013. Skeletal muscle myopathy mutations at the actin tropomyosin interface that cause gain- or loss-of-function. *J Muscle Res Cell Motil*, 34, 165-169.
- MERELES, D. & HUNSTEIN, W. 2011. Epigallocatechin-3-gallate (EGCG) for clinical trials: more pitfalls than promises? *Int J Mol Sci*, 12, 5592-603.
- MESSER, A. 2007. *Structural and functional polymorphisms of troponin in failing heart*. PhD Thesis . PhD, London.
- MESSER, A., GALLON, C., MCKENNA, W., ELLIOTT, P., DOS REMEDIOS, C. & MARSTON, S. 2009. The use of phosphate-affinity SDS-PAGE to measure the troponin I phosphorylation site distribution in human heart muscle. *Proteomics Clin Appl*, 3, 1371-1382.
- MESSER, A. & MARSTON, S. 2014. Investigating the role of uncoupling of Troponin I phosphorylation from changes in myofibrillar Ca^{2+} -sensitivity in the pathogenesis of Cardiomyopathy. *Front Physiol*, 5, 315.
- MESSER, A. E., JACQUES, A. M. & MARSTON, S. B. 2007. Troponin phosphorylation and regulatory function in human heart muscle: Dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. *J Mol Cell Cardiol*, 42, 247-259.
- MESSER, A. E., MEMO, M., BAYLISS, C. R., LEUNG, M.-C. & MARSTON, S. B. 2012. *Does Uncoupling of Troponin I Phosphorylation from Changes in Myofibrillar Ca^{2+} -Sensitivity Play a Role in the Pathogenesis of Cardiomyopathy? *Biophys J*, 102, 556a.

- MIRZA, M. 2005. *Pathological mutations in thin filament proteins investigated by in vitro motility assay*. Imperial College London.
- MIRZA, M., MARSTON, S., WILLOTT, R., ASHLEY, C., MOGENSEN, J., MCKENNA, W., ROBINSON, P., REDWOOD, C. & WATKINS, H. 2005. Dilated cardiomyopathy mutations in three thin filament regulatory proteins result in a common functional phenotype. *J Biol Chem*, 280, 28498-28506.
- MIRZA, M., ROBINSON, P., KREMNEVA, E., COPELAND, O., NIKOLAEVA, O., WATKINS, H., LEVITSKY, D., REDWOOD, C., EL-MEZGUELDI, M. & MARSTON, S. 2007. The effect of mutations in alpha tropomyosin (E40K and E54K), that cause familial dilated cardiomyopathy, on the regulatory mechanism of cardiac muscle thin filaments. *J Biol Chem*, 282, 13487-13497.
- MITTMANN, K., JAQUET, K. & HEILMEYER, L. M., JR. 1990. A common motif of two adjacent phosphoserines in bovine, rabbit and human cardiac troponin I. *FEBS Lett*, 273, 41-5.
- MOGENSEN, J., MURPHY, R. T., KUBO, T., BAHL, A., MOON, J. C., KLAUSEN, I. C., ELLIOTT, P. M. & MCKENNA, W. J. 2004a. Frequency and clinical expression of cardiac troponin I mutations in 748 consecutive families with hypertrophic cardiomyopathy. *J Am Coll Cardiol*, 44, 2315-25.
- MOGENSEN, J., MURPHY, R. T., SHAW, T., BAHL, A., REDWOOD, C., WATKINS, H., BURKE, M., ELLIOTT, P. M. & MCKENNA, W. J. 2004b. Severe disease expression of cardiac troponin C and T mutations in patients with idiopathic dilated cardiomyopathy. *J Am Coll Cardiol*, 44, 2033-40.
- MOKBEL, N., ILKOVSKI, B., KREISSL, M., MEMO, M., JEFFRIES, C. M., MARTTILA, M., LEHTOKARI, V. L., LEMOLA, E., GRONHOLM, M., YANG, N., MENARD, D., MARCORELLES, P., ECHANIZ-LAGUNA, A., REIMANN, J., VAINZOF, M., MONNIER, N., RAVENSCROFT, G., MCNAMARA, E., NOWAK, K. J., LAING, N. G., WALLGREN-PETTERSSON, C., TREWHELLA, J., MARSTON, S., OTTENHEIJM, C., NORTH, K. N. & CLARKE, N. F. 2013. K7del is a common TPM2 gene mutation associated with nemaline myopathy and raised myofibre calcium sensitivity. *Brain*, 136, 494-507.
- MONSERRAT, L., HERMIDA-PRIETO, M., FERNANDEZ, X., RODRIGUEZ, I., DUMONT, C., CAZON, L., CUESTA, M. G., GONZALEZ-JUANATEY, C., PETEIRO, J., ALVAREZ, N., PENAS-LADO, M. & CASTRO-BEIRAS, A. 2007. Mutation in the alpha-cardiac actin gene associated with apical hypertrophic cardiomyopathy, left ventricular non-compaction, and septal defects. *Eur Heart J*, 28, 1953-1961.
- MOPE, L., MCCLELLAN, G. B. & WINEGRAD, S. 1980. Calcium sensitivity of the contractile system and phosphorylation of troponin in hyperpermeable cardiac cells. *J Gen Physiol*, 75, 271-282.
- MORIMOTO, S. 2008. Sarcomeric proteins and inherited cardiomyopathies. *Cardiovasc Res*, 77, 659-66.
- MOSS, R. L., FITZSIMONS, D. P. & RALPHE, J. C. 2015. Cardiac MyBP-C regulates the rate and force of contraction in mammalian myocardium. *Circ Res*, 116, 183-92.
- MUNTONI, F., CAU, M., GANAU, A., CONGIU, R., ARVEDI, G., MATEDDU, A., MARROSU, M. G., CIANCHETTI, C., REALDI, G., CAO, A. & ET AL. 1993. Brief report: deletion of the dystrophin muscle-promoter region associated with X-linked dilated cardiomyopathy. *N Engl J Med*, 329, 921-5.

- MURAKAMI, K., STEWART, M., NOZAWA, K., TOMII, K., KUDOU, N., IGARASHI, N., SHIRAKIHARA, Y., WAKATSUKI, S., YASUNAGA, T. & WAKABAYASHI, T. 2008. Structural basis for tropomyosin overlap in thin (actin) filaments and the generation of a molecular swivel by troponin-T. *Proc Natl Acad Sci U S A*, 105, 7200-5.
- NAQVI, T. Z., GOEL, R. K., FORRESTER, J. S. & SIEGEL, R. J. 1999. Myocardial contractile reserve on dobutamine echocardiography predicts late spontaneous improvement in cardiac function in patients with recent onset idiopathic dilated cardiomyopathy. *JAC*, 34, 1537-1544.
- NEIVA-SOUSA, M., ALMEIDA-COELHO, J., FALCAO-PIRES, I. & LEITE-MOREIRA, A. F. 2015. Titin mutations: the fall of Goliath. *Heart Fail Rev*, 20, 579-88.
- OCHALA, J., GOKHIN, D. S., PÉNISSON-BESNIER, I., QUIJANO-ROY, S., MONNIER, N., LUNARDI, J., ROMERO, N. B. & FOWLER, V. M. 2012. Congenital myopathy-causing tropomyosin mutations induce thin filament dysfunction via distinct physiological mechanisms. *Human Molecular Genetics*, 21, 4473-4485.
- OCHALA, J., LI, M., OHLSSON, M., OLDFORS, A. & LARSON, L. 2008. Defective regulation of contractile function in muscle fibres carrying an E41K β -tropomyosin mutation. *J Physiol*, in press.
- OHLSSON, M., FIDZIANSKA, A., TAJSHARGHI, H. & OLDFORS, A. 2009. TPM3 mutation in one of the original cases of cap disease. *Neurology*, 72, 1961-3.
- OHLSSON, M., QUIJANO-ROY, S., DARIN, N., BROCHIER, G., LACROIX, E., AVILA-SMIRNOW, D., FARDEAU, M., OLDFORS, A. & TAJSHARGHI, H. 2008. New morphologic and genetic findings in cap disease associated with beta-tropomyosin (TPM2) mutations. *Neurology*, 71, 1896-901.
- OLSON, T. M., DOAN, T. P., KISHIMOTO, N. Y., WHITBY, F. G., ACKERMAN, M. J. & FANANAPAZIR, L. 2000. Inherited and de novo mutations in the cardiac actin gene cause hypertrophic cardiomyopathy. *J Mol Cell Cardiol*, 32, 1687-94.
- OLSON, T. M., KISHIMOTO, N. Y., WHITBY, F. G. & MICHELS, V. V. 2001. Mutations that Alter the Surface Charge of Alpha-tropomyosin are Associated with Dilated Cardiomyopathy. *J Mol Cell Cardiol*, 33, 723-32.
- OLSON, T. M., MICHELS, V. V., THIBODEAU, S. N., TAI, Y. S. & KEATING, M. T. 1998. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. *Science*, 280, 750-2.
- ORZECOWSKI, M., FISCHER, S. & LEHMAN, W. 2013. Influence of Actin Mutation on the Energy Landscape of Actin-Tropomyosin Filaments. *Biophys J*, 104, 480a.
- PALM, T., GREENFIELD, N. J. & HITCHCOCK-DEGREGORI, S. E. 2003. Tropomyosin ends determine the stability and functionality of overlap and troponin T complexes. *Biophys J*, 84, 3181-9.
- PAN, B. S. & JOHNSON, R. G., JR. 1996. Interaction of cardiotonic thiadiazinone derivatives with cardiac troponin C. *J Biol Chem*, 271, 817-23.
- PAPADAKI, M., VIKHOREV, P. G., MARSTON, S. B. & MESSER, A. E. 2015. Uncoupling of myofilament Ca²⁺ sensitivity from troponin I phosphorylation by mutations can be reversed by epigallocatechin-3-gallate. *Cardiovasc Res*, 108, 99-110.

- PERRY, S. V. 1998. Troponin T: genetics, properties and function. *J Muscle Res Cell Motil*, 19, 575-602.
- PERRY, S. V. 1999. Troponin I: inhibitor or facilitator. *Mol Cell Biochem*, 190, 9-32.
- PERRY, S. V. 2001. Vertebrate tropomyosin: distribution, properties and function. *J Musc Res Cell motil*, 22, 5-49.
- PI, Y.-Q., KEMNITZ, K. R., ZHANG, D., KRANIAS, E. G. & WALKER, J. W. 2002. Phosphorylation of troponin I controls cardiac twitch dynamics. Evidence from phosphorylation site mutants expressed on a troponin I-null background in mice. *Circ Res*, 90, 649-656.
- PINTO, J. R., SIEGFRIED, J. D., PARVATIYAR, M. S., LI, D., NORTON, N., JONES, M. A., LIANG, J., POTTER, J. D. & HERSHBERGER, R. E. 2011. Functional Characterization of TNNC1 Rare Variants Identified in Dilated Cardiomyopathy. *J Biol Chem*, 286, 34404-12.
- PRABHAKAR, R., BOIVIN, G. P., GRUPP, I. L., HOIT, B., ARTEAGA, G., SOLARO, R. J. & WIECZOREK, D. F. 2001. A familial hypertrophic cardiomyopathy alpha-tropomyosin mutation causes severe cardiac hypertrophy and death in mice. *J Mol Cell Cardiol*, 33, 1815-28.
- RADKE, M. B., TAFT, M. H., STAPEL, B., HILFIKER-KLEINER, D., PRELLER, M. & MANSTEIN, D. J. 2014. Small molecule-mediated refolding and activation of myosin motor function. *Elife*, 3, e01603.
- RAJAN, S., AHMED, R. P., JAGATHEESAN, G., PETRASHEVSKAYA, N., BOIVIN, G. P., URBONIENE, D., ARTEAGA, G. M., WOLSKA, B. M., SOLARO, R. J., LIGGETT, S. B. & WIECZOREK, D. F. 2007. Dilated cardiomyopathy mutant tropomyosin mice develop cardiac dysfunction with significantly decreased fractional shortening and myofilament calcium sensitivity. *Circ Res*, 101, 205-14.
- RAY, K. P. & ENGLAND, P. J. 1976. Phosphorylation of the inhibitory subunit of troponin and its effect on the calcium dependence of cardiac myofibril adenosine triphosphatase. *FEBS Lett*, 70, 11-16.
- REDWOOD, C. S., MOOLMAN-SMOOK, J. C. & WATKINS, H. 1999. Properties of mutant contractile proteins that cause hypertrophic cardiomyopathy. *Cardiovasc Res*, 44, 20-36.
- ROBERTSON, I. M., LI, M. X. & SYKES, B. D. 2009. Solution structure of human cardiac troponin C in complex with the green tea polyphenol, (-)-epigallocatechin 3-gallate. *J Biol Chem*, 284, 23012-23.
- ROBERTSON, S. P., JOHNSON, J. D., HOLROYDE, M. J., KRANIAS, E. G., POTTER, J. D. & SOLARO, R. J. 1982. The effect of troponin I phosphorylation on the Ca²⁺-binding properties of the Ca²⁺-regulatory site of bovine cardiac troponin. *J Biol Chem*, 257, 260-3.
- ROBINSON, P., LIPSCOMB, S., PRESTON, L. C., ALTIN, E., WATKINS, H., ASHLEY, C. C. & REDWOOD, C. S. 2007. Mutations in fast skeletal troponin I, troponin T, and beta-tropomyosin that cause distal arthrogryposis all increase contractile function. *Faseb J*, 21, 896-905.
- ROBINSON, P., MIRZA, M., KNOTT, A., ABDULRAZZAK, H., WILLOTT, R., MARSTON, S., WATKINS, H. & REDWOOD, C. 2002. Alterations in thin filament regulation induced by a human cardiac troponin T mutant that causes dilated cardiomyopathy are distinct from those induced by troponin T mutants that cause hypertrophic cardiomyopathy. *J Biol Chem*, 277, 40710-40716.
- SAHIN, K., ORHAN, C., AKDEMIR, F., TUZCU, M., IBEN, C. & SAHIN, N. 2012. Resveratrol protects quail hepatocytes against heat stress: modulation of the

- Nrf2 transcription factor and heat shock proteins. *J Anim Physiol Anim Nutr (Berl)*, 96, 66-74.
- SANOUDOU, D. & BEGGS, A. H. 2001. Clinical and genetic heterogeneity in nemaline myopathy--a disease of skeletal muscle thin filaments. *Trends Mol Med*, 7, 362-8.
- SCHMIDTMANN, A., LINDOW, C., VILLARD, S., HEUSER, A., MÜGGE, A., GESSNER, R., GRANIER, C. & JAQUET, K. 2005. Cardiac troponin C-L29Q, related to hypertrophic cardiomyopathy, hinders the transduction of the protein kinase A dependent phosphorylation signal from cardiac troponin I to C. *FEBS J*, 272, 6087-6097.
- SEIDMAN, J. G. & SEIDMAN, C. 2001. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell*, 104, 557-67.
- SEMSARIAN, C., AHMAD, I., GIEWAT, M., GEORGAKOPOULOS, D., SCHMITT, J. P., MCCONNELL, B. K., REIKEN, S., MENDE, U., MARKS, A. R., KASS, D. A., SEIDMAN, C. E. & SEIDMAN, J. G. 2002. The L-type calcium channel inhibitor diltiazem prevents cardiomyopathy in a mouse model. *J Clin Invest*, 109, 1013-20.
- SEQUEIRA, V., WIJNKER, P. J. M., NIJENKAMP, L. L. A. M., KUSTER, D. W. D., NAJAFI, A., WITJAS-PAALBERENDS, E. R., REGAN, J. A., BOONTJE, N., TEN CATE, F. J., GERMANS, T., CARRIER, L., SADAYAPPAN, S., VAN SLEGTENHORST, M. A., ZAREMBA, R., FOSTER, D. B., MURPHY, A. M., POGGESI, C., DOS REMEDIOS, C., STIENEN, G. J. M., HO, C. Y., MICHELS, M. & VAN DER VELDEN, J. 2013. Perturbed Length-Dependent Activation in Human Hypertrophic Cardiomyopathy With Missense Sarcomeric Gene Mutations. *Circ Res*, 112, 1491-1505.
- SOLARO, R. J., GAMBASSI, G., WARSHAW, D. M., KELLER, M. R., SPURGEON, H. A., BEIER, N. & LAKATTA, E. G. 1993. Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments. *Circ Res*, 73, 981-990.
- SOLARO, R. J., MOIR, A. G. J. & PERRY, S. V. 1976. Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart. *Nature*, 262, 615-616.
- SOLARO, R. J., ROSEVEAR, P. & KOBAYASHI, T. 2008. The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. *Biochem Biophys Res Commun*, 369, 82-7.
- SONG, W., DYER, E., STUCKEY, D., COPELAND, O., LEUNG, M., BAYLISS, C., MESSER, A. E., WILKINSON, R., TREMOLEDA, J., SCHNEIDER, M., HARDING, S., REDWOOD, C., CLARKE, K., NOWAK, K., MONSERRAT, L., WELLS, D. & MARSTON, S. 2011. Molecular mechanism of the Glu99Lys mutation in cardiac actin (ACTC gene) that causes apical hypertrophy in man and mouse. *J Biol Chem*, 286, 27582-27593.
- SONG, W., DYER, E., STUCKEY, D., LEUNG, M.-C., MEMO, M., MANSFIELD, C., FERENCZI, M., LIU, K., REDWOOD, C., NOWAK, K., HARDING, S., CLARKE, K., WELLS, D. & MARSTON, S. 2010. Investigation of a transgenic mouse model of familial dilated cardiomyopathy. *J Mol Cell Cardiol*, 49, 380-389.
- SONG, W., VIKHOREV, P. G., KASHYAP, M. N., ROWLANDS, C., FERENCZI, M. A., WOLEDGE, R. C., MACLEOD, K., MARSTON, S. & CURTIN, N. A. 2013. Mechanical and energetic properties of papillary muscle from ACTC

- E99K transgenic mouse models of hypertrophic cardiomyopathy. *Am J Physiol Heart Circ Physiol*, 304, H1513-H1524.
- SPIRITO, P., SEIDMAN, C. E., MCKENNA, W. J. & MARON, B. J. 1997. The management of hypertrophic cardiomyopathy [see comments]. *N Engl J Med*, 336, 775-85.
- SPUDICH, J. A. 2001. The myosin swinging cross-bridge model. *Nat Rev Mol Cell Biol*, 2, 387-92.
- SPUDICH, J. A. 2014. Hypertrophic and dilated cardiomyopathy: four decades of basic research on muscle lead to potential therapeutic approaches to these devastating genetic diseases. *Biophys J*, 106, 1236-49.
- SPUDICH, J. A. & WATT, S. 1971. The regulation of rabbit skeletal muscle contraction. *Journal of Biological Chemistry*, 246, 4866-4871.
- STEBBINS, C. E., RUSSO, A. A., SCHNEIDER, C., ROSEN, N., HARTL, F. U. & PAVLETICH, N. P. 1997. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell*, 89, 239-50.
- STRAUB, F. B. 1942. Actin. *Studies from the Institute of Medical Chemistry, University of Szeged*, 2, 3-16.
- SUN, N., YAZAWA, M., LIU, J., HAN, L., SANCHEZ-FREIRE, V., ABILEZ, O. J., NAVARRETE, E. G., HU, S., WANG, L., LEE, A., PAVLOVIC, A., LIN, S., CHEN, R., HAJJAR, R. J., SNYDER, M. P., DOLMETSCH, R. E., BUTTE, M. J., ASHLEY, E. A., LONGAKER, M. T., ROBBINS, R. C. & WU, J. C. 2012. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci Transl Med*, 4, 130ra47.
- SUNG, S. S., BRASSINGTON, A. M., GRANNATT, K., RUTHERFORD, A., WHITBY, F. G., KRAKOWIAK, P. A., JORDE, L. B., CAREY, J. C. & BAMSHAD, M. 2003. Mutations in genes encoding fast-twitch contractile proteins cause distal arthrogryposis syndromes. *Am J Hum Genet*, 72, 681-90.
- TADANO, N., DU, C., YUMOTO, F., MORIMOTO, S., OHTA, M., XIE, M., NAGATA, K., ZHAN, D., LU, Q., MIWA, Y., TAKAHASHI-YANAGA, F., TANOKURA, M., OHTSUKI, I. & SASAGURI, T. 2010. Biological actions of green tea catechins on cardiac troponin C. *Brit J Pharmacol*, 161, 1034-1043.
- TAKEDA, N., YAMASHITA, A., MAEDA, K. & MAEDA, Y. 2003. Structure of the core domain of human cardiac troponin in the Ca²⁺-saturated form. *Nature*, 424, 35-41.
- THIERFELDER, L., WATKINS, H., MACRAE, C., LAMAS, R., MCKENNA, W., VOSBERG, H. P., SEIDMAN, J. G. & SEIDMAN, C. E. 1994. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell*, 77, 701-12.
- TOBACMAN, L. S. 1996. Thin filament-mediated regulation of cardiac contraction. *Ann.Rev.Physiol.*, 58, 447-481.
- TOWNSEND, P. J., YACOUB, M. H. & BARTON, P. J. 1997a. Assignment of the human cardiac/slow skeletal muscle troponin C gene (TNNC1) between D3S3118 and GCT4B10 on the short arm of chromosome 3 by somatic cell hybrid analysis. *Ann Hum Genet*, 61, 375-7.
- TOWNSEND, P. J., YACOUB, M. H. & BARTON, P. J. 1997b. Assignment of the human fast skeletal muscle troponin C gene (TNNC2) between D20S721 and GCT10F11 on chromosome 20 by somatic cell hybrid analysis. *Ann Hum Genet*, 61, 457-9.

- VAN DER VELDEN, J., PAPP, Z., BOONTJE, N. M., ZAREMBA, R., DE JONG, J. W., JANSSEN, P. M., HASENFUSS, G. & STIENEN, G. J. 2003. The effect of myosin light chain 2 dephosphorylation on Ca^{2+} -sensitivity of force is enhanced in failing human hearts. *Cardiovas Res* 57, 505-14.
- VAN DRIEST, S. L., OMMEN, S. R., TAJIK, A. J., GERSH, B. J. & ACKERMAN, M. J. 2005. Yield of genetic testing in hypertrophic cardiomyopathy. *Mayo Clin Proc*, 80, 739-44.
- VARNAVA, A. M., ELLIOTT, P. M., MAHON, N., DAVIES, M. J. & MCKENNA, W. J. 2001. Relation between myocyte disarray and outcome in hypertrophic cardiomyopathy. *Am J Cardiol*, 88, 275-9.
- VIKHOREV, P. G., SONG, W., WILKINSON, R., COPELAND, O., MESSER, A. E., FERENCZI, M. A. & MARSTON, S. B. 2014. The Dilated Cardiomyopathy-Causing Mutation ACTC E361G in Cardiac Muscle Myofibrils Specifically Abolishes Modulation of Ca^{2+} Regulation by Phosphorylation of Troponin I. *Biophys J*, 107, 2369-2380.
- VINDIN, H. & GUNNING, P. 2013. Cytoskeletal tropomyosins: choreographers of actin filament functional diversity. *J Muscle Res Cell Motil*, 34, 261-74.
- VON DER ECKEN, J., MULLER, M., LEHMAN, W., MANSTEIN, D. J., PENCZEK, P. A. & RAUNSER, S. 2015. Structure of the F-actin-tropomyosin complex. *Nature*, 519, 114-7.
- WALKER, J. M., KLAKOTSKAIA, D., AJIT, D., WEISMAN, G. A., WOOD, W. G., SUN, G. Y., SERFOZO, P., SIMONYI, A. & SCHACHTMAN, T. R. 2015. Beneficial effects of dietary EGCG and voluntary exercise on behavior in an Alzheimer's disease mouse model. *J Alzheimers Dis*, 44, 561-72.
- WANG, X., LI, M. X., SPYRACOPOULOS, L., BEIER, N., CHANDRA, M., SOLARO, R. J. & SYKES, B. D. 2001. Structure of the C-domain of human cardiac troponin C in complex with the Ca^{2+} sensitizing drug EMD 57033. *J Biol Chem*, 276, 25456-66.
- WANG, X., LI, M. X. & SYKES, B. D. 2002. Structure of the regulatory N-domain of human cardiac troponin C in complex with human cardiac troponin I147-163 and bepridil. *J Biol Chem*, 277, 31124-33.
- WANG, Y., PINTO, J., SANCHEZ SOLIS, R., DWECK, D., LIANG, J., DIAZ-PEREZ, Z., GE, Y., WALKER, J. & POTTER, J. 2011. The generation and functional characterization of knock in mice harboring the cardiac Troponin I-R21C mutation associated with hypertrophic cardiomyopathy. *J Biol Chem*, 287, 2156-2167.
- WARD, D. G., BREWER, S. M., GALLON, C. E., GAO, Y., LEVINE, B. A. & TRAYER, I. P. 2004. NMR and mutagenesis studies on the phosphorylation region of human cardiac troponin I. *Biochemistry*, 43, 5772-5781.
- WATTANASIRICHAIGOON, D., SWOBODA, K. J., TAKADA, F., TONG, H. Q., LIP, V., IANNACCONE, S. T., WALLGREN-PETTERSSON, C., LAING, N. G. & BEGGS, A. H. 2002. Mutations of the slow muscle alpha-tropomyosin gene, TPM3, are a rare cause of nemaline myopathy. *Neurology*, 59, 613-7.
- WEEDS, A. G. & TAYLOR, R. S. 1975. Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. *Nature*, 257, 54-56.
- WIGLE, E. D., SASSON, Z., HENDERSON, M. A., RUDDY, T. D., FULOP, J., RAKOWSKI, H. & WILLIAMS, W. G. 1985. Hypertrophic cardiomyopathy. The importance of the site and the extent of hypertrophy. A review. *Prog Cardiovasc Dis*, 28, 1-83.

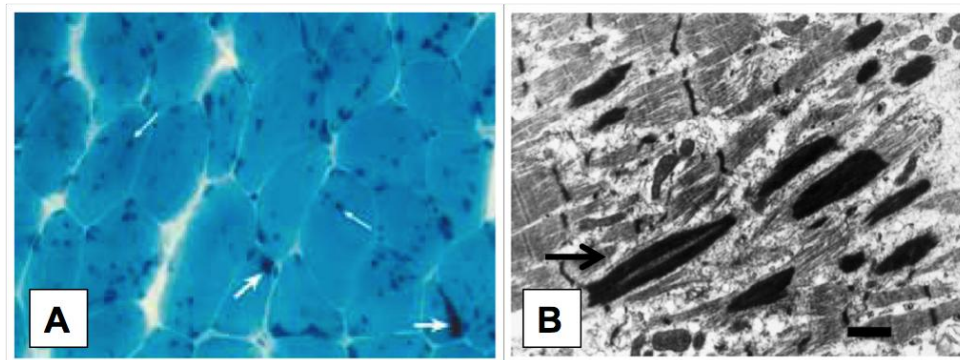
- WILKINSON, R., SONG, W., SMOKTUNOWICZ, N. & MARSTON, S. 2015. A dilated cardiomyopathy mutation blunts adrenergic response and induces contractile dysfunction under chronic angiotensin II stress. *Am J Physiol Heart Circ Physiol*, 309, 1936-46.
- WOLFF, M. R., MCDONALD, K. S. & MOSS, R. L. 1995. Rate of tension development in cardiac muscle varies with level of activator calcium. *Circ.Res.*, 76, 154-160.
- XU, Q., DEWEY, S., NGUYEN, S. & GOMES, A. V. 2010. Malignant and benign mutations in familial cardiomyopathies: insights into mutations linked to complex cardiovascular phenotypes. *J Mol Cell Cardiol*, 48, 899-909.
- YASUDA, S., COUTU, P., SADAYAPPAN, S., ROBBINS, J. & METZGER, J. M. 2007. Cardiac transgenic and gene transfer strategies converge to support an important role for troponin I in regulating relaxation in cardiac myocytes. *Circ Res*, 101, 377-86.
- ZARAGOZA, C., GOMEZ-GUERRERO, C., MARTIN-VENTURA, J. L., BLANCO-COLIO, L., LAVIN, B., MALLAVIA, B., TARIN, C., MAS, S., ORTIZ, A. & EGIDO, J. 2011. Animal models of cardiovascular diseases. *J Biomed Biotechnol*, 2011, 497841.
- ZHANG, J., GUY, M. J., NORMAN, H. S., CHEN, Y. C., XU, Q., DONG, X., GUNER, H., WANG, S., KOHMOTO, T., YOUNG, K. H., MOSS, R. L. & GE, Y. 2011. Top-down quantitative proteomics identified phosphorylation of cardiac troponin I as a candidate biomarker for chronic heart failure. *J Proteome Res*, 10, 4054-65.
- ZHANG, R., ZHAO, J., MANDVENO, A. & POTTER, J. D. 1995. Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. *Biophys J*, 76, 1028-1035.
- ZHAO, H., BRANDT, G. E., GALAM, L., MATTS, R. L. & BLAGG, B. S. 2011. Identification and initial SAR of silybin: an Hsp90 inhibitor. *Bioorg Med Chem Lett*, 21, 2659-64.
- ZHONG, W., HUAN, X. D., CAO, Q. & YANG, J. 2015. Cardioprotective effect of epigallocatechin-3-gallate against myocardial infarction in hypercholesterolemic rats. *Exp Ther Med*, 9, 405-410.

Drugbank website: <http://www.drugbank.ca/drugs/DB01244>

APPENDIX

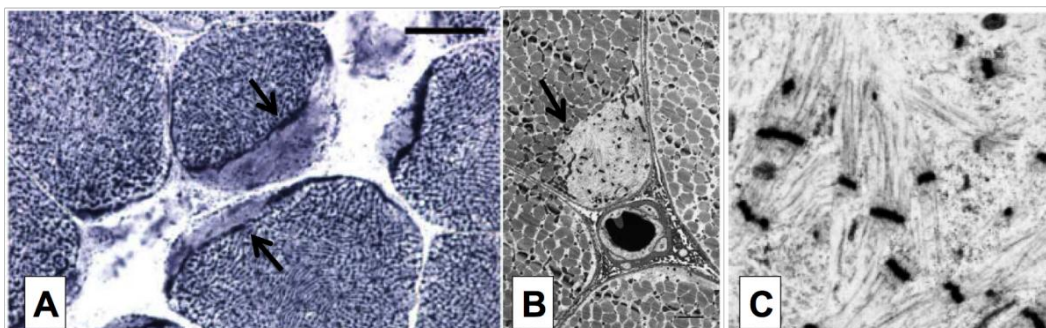
From Section 3.1:

Nemaline Myopathy histological characteristics:



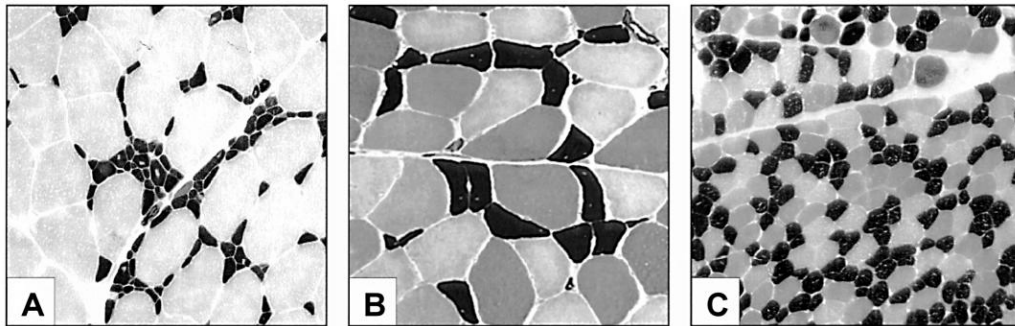
A) Gomori trichrome triple stain of a histological section of skeletal muscle biopsy of a patient with skeletal muscle myopathy. Rods are found across the cytoplasm (dark circles indicated with small arrows) or underneath the sarcolemma (dark circles indicated with large arrows) (Sanoudou and Beggs, 2001) B) Electron microgram showing typical rods found in Nemaline myopathy patients developing in the Z line and disrupting the skeletal muscle structure (Wattanasirichaigoon et al., 2002).

Cap disease histological characteristics:



A) Light microscopy of a skeletal muscle biopsy of a patient with Cap disease. B) Electron microscopy of the same sample where the cap structure is indicated with an arrow. C) Magnification of the cap structure in B. It consists of myofibrils lacking thick filaments and enlarged Z lines. (Cuisset et al., 2006)

CFTD histology



A, B and C show varying degrees of fibre size disproportion. Muscle biopsies from CFTD patients and stained with ATPase. Type 1 fibres are dark, type 2A are light and type 2B are intermediate (Clarke and North, 2003)

From Section 4.2.2.1

Ca²⁺ sensitivity data for donor troponin treated with different concentrations of EMD 57033

Motile Velocity	EC50, μ M							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	0.066	0.013	0.06	0.0045	0.053	0.0042	0.11	0.018
Exp2	0.11	0.013	0.054	0.011	0.047	0.013	0.11	0.011
Mean	0.088		0.057		0.050		0.110	
SE	0.022		0.003		0.003		0.00	

Percentage Motility	EC50, μ M							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	0.18	0.05	0.2	0.069	0.1	0.0075	0.16	0.0061
Exp2	0.26	0.027	0.19	0.069	0.14	0.036	0.22	0.05
Mean	0.22		0.20		0.12		0.19	
SE	0.04		0.01		0.02		0.03	

Motile Velocity	Hill Coefficient							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	2.12	0.61	1.11	0.094	2.21	0.29	2.22	0.76
Exp2	2.12	0.5	1.36	0.37	1.3	0.48	3.08	1.19
Mean	2.12		1.235		1.76		2.65	
SE	0.00		0.125		0.46		0.43	

Percentage Motility	Hill Coefficient							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	2.5	1.46	1.78	0.95	1.87	0.22	3.03	0.27
Exp2	4.12	1.87	1.21	0.48	1.07	0.28	2.34	0.98
Mean	3.31		1.50		1.47		2.69	
SE	0.81		0.29		0.40		0.35	

Motile Velocity	Max Motile Velocity, μ m/sec							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	2.52	0.025	2.78	0.06	2.95	0.034	2.85	0.12
Exp2	2.78	0.059	2.83	0.022	2.99	0.13	2.81	0.047
Mean	2.65		2.81		2.97		2.83	
SE	0.13		0.025		0.02		0.02	

Percentage Motility	Max % Motile							
	Donor	SD	Donor + 15 μ M EMD	SD	Donor + 30 μ M EMD	SD	Donor + 60 μ M EMD	SD
Exp1	69.25	4.65	84.25	4.35	89	2	83.5	5.2
Exp2	76.75	2.99	83.5	3	89.8	2.08	78.25	2.22
Mean	73.00		83.88		89.40		80.88	
SE	3.75		0.38		0.40		2.63	

From Section 4.2.3

Analysis of measurements of the Ca^{2+} -dependence of fraction motile and motile velocity measured by *in vitro* motility assay. The effects of TnI phosphorylation on donor thin filaments in the presence and absence of EMD 57033. EC_{50} , n_{H} , maximum fraction motile and maximum sliding speed are shown.

Effect of EMD on wild type troponin

Percentage motility																												
	P	EC50, μM		SD	Ratio P/unP	P	Hill coefficient		SD	P	Max % Motile		SD	P + EMD	EC50, μM		SD	Ratio P+E/unP+E	P + EMD	Hill coefficient		SD	P + EMD	Max % Motile		SD	Ratio P/P+EMD	Ratio unP/unP+EMD
		SD	unP				SD	unP			SD	unP			SD	unP + EMD					SD			SD				
Exp1	0.12	0.01	0.06	0.01	2.00	2.34	0.75	1.97	0.33	81.75	3.59	83.00	3.74	0.03	0.003	0.03	0.002	1.07	1.16	0.14	1.19	0.13	87.00	1.41	85.75	1.71	3.44	1.84
Exp2	0.15	0.04	0.11	0.02	1.44	1.72	0.52	1.19	0.21	81.25	1.71	80.75	2.22	0.08	0.017	0.07	0.011	1.01	1.16	0.29	1.09	0.18	81.75	3.86	80.75	1.71	2.05	1.44
Exp3	0.13	0.01	0.08	0.01	1.71	2.28	0.29	1.88	0.21	77.50	3.70	80.25	1.89	0.07	0.002	0.06	0.004	1.12	1.23	0.05	1.37	0.11	84.25	3.10	84.50	1.29	1.87	1.23
Exp4	0.23	0.01	0.12	0.02	1.93	1.98	0.08	0.98	0.15	78.25	3.20	73.50	2.12	0.05	0.002	0.04	0.003	1.11	1.47	0.14	1.72	0.69	77.25	5.56	76.25	4.79	4.91	2.83
Mean	0.16		0.09		1.77	2.08		1.51		79.69		79.38		0.06		0.05		1.08	1.26		1.34		82.56		81.81		3.07	1.83
Std Error	0.03		0.01		0.13	0.14		0.25		1.06		2.05		0.01		0.01		0.03	0.07		0.14		2.07		2.14		0.71	0.36
Motile velocity																												
	P	EC50, μM		SD	Ratio P/unP	P	Hill coefficient		SD	P	ΔV_{max} , $\mu\text{m/sec}$		SD	P + EMD	EC50, μM		SD	Ratio P+E/unP+E	P + EMD	Hill coefficient		SD	P + EMD	ΔV_{max} , $\mu\text{m/sec}$		SD	Ratio P/P+EMD	Ratio unP/unP+EMD
		SD	unP				SD	unP			SD	unP			SD	unP + EMD					SD			SD				
Exp1	0.08	0.01	0.04	0.005	2.08	5.11	2.21	1.94	0.57	1.23	0.02	1.30	0.05	0.03	0.01	0.03	0.01	1.06	0.93	0.32	1.00	0.39	1.70	0.12	1.69	0.12	1.38	1.30
Exp2	0.09	0.01	0.04	0.003	2.06	3.02	1.28	1.60	0.22	1.38	0.07	1.33	0.03	0.03	0.01	0.03	0.01	1.14	0.82	0.20	0.96	0.28	1.58	0.09	1.51	0.08	1.14	1.14
Exp3	0.09	0.02	0.05	0.005	1.74	4.50	2.13	1.65	0.30	1.26	0.08	1.38	0.04	0.03	0.01	0.03	0.01	0.96	0.89	0.24	0.90	0.21	1.59	0.09	1.63	0.09	1.26	1.18
Exp4	0.29	0.07	0.18	0.074	1.58	1.03	0.22	0.50	0.10	1.73	0.35	1.19	0.13	0.05	0.03	0.05	0.03	1.11	0.82	0.35	0.88	0.43	1.32	0.15	1.33	0.16	0.76	1.12
Mean	0.14		0.08		1.87	3.42		1.42		1.40		1.30		0.04		0.04		1.07	0.87		0.94		1.55		1.54		1.14	1.18
Std Error	0.01		0.01		0.13	0.46		0.15		0.14		0.20		0.01		0.004		0.11	0.17		0.16		0.15		0.16		0.13	0.04

From Section 4.3.3

Analysis of measurements of the Ca^{2+} -dependence of fraction motile and motile velocity measured by *in vitro* motility assay. The effects of TnI phosphorylation on donor thin filaments in the presence and absence of Bepridil. EC_{50} , n_H , maximum fraction motile and maximum sliding speed are shown.

Effect on Bepridil on wild type troponin

Fraction motile	EC50, μM				Ratio		Hill coefficient				Max % Motile					EC50, μM				Ratio		Hill coefficient				Max % Motile					Ratio	
	P	SD	unP	SD	P/unP	P	SD	unP	SD	P	SD	unP	SD	P+B/unP+B		P+Bepridil	SD	unP+Bepridil	SD	P+B/unP+B	P+Bepridil	SD	unP+Bepridil	SD	P+B/unP+B	P+Bepridil	SD	unP+Bepridil	SD		P+B/P	Ratio unP+B/unP
Exp1	0.11	0.002	0.06	0.00720	1.74	3.44	0.59	1.74	0.330	94.50	5.97	97.75	2.63		0.04	0.0011	0.04	0.001	1.06	1.87	0.11	1.69	0.31	98.75	2.50	99.50	1.00		2.56	1.57		
Exp2	0.14	0.029	0.08	0.00340	1.83	1.81	0.58	2.08	0.160	97.25	2.50	97.50	2.08		0.05	0.0015	0.04	0.001	1.04	1.77	0.12	2.37	0.13	97.33	3.06	95.25	2.22		3.06	1.73		
Exp3	0.14	0.016	0.08	0.00056	1.74	1.94	0.43	1.86	0.021	93.75	2.75	92.50	1.91		0.04	0.0041	0.04	0.001	1.00	1.92	0.37	1.86	0.12	97.25	0.96	95.75	2.22		3.19	1.84		
Exp4	0.13	0.006	0.07	0.01300	1.77	2.36	0.31	1.81	0.490	91.50	0.71	92.25	2.22		0.04	0.0003	0.04	0.001	1.05	1.71	0.02	1.73	0.09	93.25	2.06	94.00	0.82		2.92	1.72		
Exp5	0.12	0.002	0.07	0.00003	1.68	3.28	0.38	2.25	0.002	94.00	0.82	94.25	0.96		0.05	0.0005	0.05	0.003	1.01	2.23	0.04	2.32	0.24	96.00	1.83	96.00	1.63		2.35	1.41		
Exp6	0.11	0.017	0.07	0.00094	1.62	2.23	1.04	2.03	0.043	97.75	1.50	96.50	1.29		0.05	0.0007	0.05	0.003	1.01	2.31	0.08	2.22	0.28	96.25	1.50	98.25	0.96		2.44	1.52		
Mean	0.13		0.07		1.73	2.51		1.96		94.79		95.13			0.05		0.04		1.03	1.97		2.03		96.47		96.46			2.75	1.63		
Std Error	0.01		0.002		0.03	0.48		0.08		1.93		1.01			0.001		0.001		0.01	0.08		0.13		1.27		0.83			0.35	0.16		
Motile velocity	EC50, μM				Ratio		Hill coefficient				ΔV_{max} , $\mu\text{m/sec}$					EC50, μM				Ratio		Hill coefficient				ΔV_{max} , $\mu\text{m/sec}$					Ratio	
	P	SD	unP	SD	P/unP	P	SD	unP	SD	P	SD	unP	SD	P+B/unP+B		P+Bepridil	SD	unP+Bepridil	SD	P+B/unP+B	P+Bepridil	SD	unP+Bepridil	SD	P+B/unP+B	P+Bepridil	SD	unP+Bepridil	SD		P+B/P	Ratio unP+B/unP
Exp1	0.12	0.001	0.09	0.022	1.34	2.91	0.18	1.17	0.34	2.29	0.01	2.41	0.18		0.06	0.013	0.05	0.0095	1.28	1.13	0.36	1.85	1.56	2.70	0.18	2.57	0.08		1.99	1.74		
Exp2	0.18	0.012	0.09	0.013	1.91	1.12	0.69	0.89	0.12	2.44	0.64	2.58	0.14		0.07	0.049	0.06	0.0150	1.13	1.32	0.52	1.17	0.35	2.95	0.87	2.59	0.25		2.48	1.46		
Exp3	0.10	0.007	0.06	0.003	1.71	1.95	0.27	1.40	0.08	1.73	0.06	1.78	0.03		0.03	0.002	0.04	0.0026	0.93	1.46	0.13	1.41	0.18	2.06	0.04	1.86	0.06		3.24	1.76		
Exp4	0.14	0.019	0.07	0.005	2.10	2.06	0.53	1.43	0.17	1.45	0.07	1.38	0.04		0.02	0.011	0.05	0.0110	0.49	0.91	0.39	0.93	0.26	1.76	0.15	1.79	0.11		5.91	1.38		
Exp5	0.12	0.005	0.09	0.031	1.33	2.40	0.34	1.14	0.44	1.84	0.05	1.98	0.27		0.05	0.001	0.05	0.0015	1.02	1.39	0.05	1.32	0.07	1.93	0.02	2.04	0.03		2.53	1.94		
Exp6	0.11	0.002	0.07	0.006	1.64	2.21	0.11	1.98	0.25	2.55	0.03	2.64	0.11		0.05	0.002	0.05	0.0001	1.01	1.96	0.13	1.90	0.01	2.67	0.05	2.61	0.00		2.40	1.48		
Mean	0.13		0.08		1.67	2.11		1.34		2.05		2.13			0.05		0.05		0.98	1.36		1.43		2.35		2.24			3.09	1.63		
Std Error	0.01		0.01		0.13	0.46		0.15		0.08		0.04			0.01		0.004		0.11	0.17		0.16		0.05		0.08			1.44	0.22		

From Section 5.2.1

Analysis of measurements of the Ca^{2+} -dependence of fraction motile and motile velocity measured by *in vitro* motility assay. The effects of TnI phosphorylation on donor thin filaments in the presence and absence of Bepridil. EC_{50} , n_H , maximum fraction motile and maximum sliding speed are shown.

Effect of EGCG on wild type troponin

Percentage motility																																					
	P	EC50, μM		SD	Ratio P/unP	P	Hill coefficient		SD	P	Max % Motile		SD	P + EGCG	EC50, μM		SD	Ratio P+E/unP+E	P + EGCG	Hill coefficient		SD	P + EGCG	Max % Motile		SD		Ratio P+EGCG/P	Ratio unP+EGCG/unP								
		SD	unP				SD	unP			SD	unP			SD	unP + EGCG					SD			SD	unP + EGCG												
Exp1	0.18	0.055			1.30	0.44				77.00	3.16			0.27	0.0660			1.51	0.530				60.25	2.22			1.51										
Exp2	0.15	0.020	0.06	0.009	2.42	2.59	0.88	1.72	0.42	77.75	0.96	85.25	3.77	0.18	0.0120	0.14	0.02	1.33	3.24	0.380	3.27	1.36	86.00	4.24	82.75	3.30	1.24	2.25									
Exp3	0.27	0.026	0.11	0.063	2.53	1.39	0.16	0.75	0.36	71.25	3.77	65.75	1.71	0.34	0.0540	0.21	0.04	1.61	1.65	0.470	1.53	0.31	65.75	2.36	64.25	1.26	1.24	1.95									
Exp4	0.08	0.013	0.04	0.045	1.91	0.98	0.24	2.06	1.08	92.25	2.63	93.00	2.16	0.35	0.0350	0.20	0.03	1.70	2.39	0.540	1.39	0.17	65.25	1.26	72.67	2.08	4.58	5.15									
Exp5	0.13	0.017	0.06	0.003	2.15	1.49	0.26	1.87	0.13	95.00	1.41	95.50	2.38	0.22	0.0120	0.14	0.02	1.54	1.60	0.099	1.31	0.17	85.50	4.04	92.00	2.71	1.69	2.36									
Exp6	0.07	0.010	0.03	0.007	2.04	1.82	0.40	1.01	0.28	95.75	1.50	95.50	1.91	0.20	0.0350	0.08	0.01	2.50	1.49	0.290	1.53	0.19	84.75	3.10	92.00	1.83	2.77	2.26									
Exp7	0.12	0.001	0.05	0.001	2.39	1.68	0.03	1.61	0.03	97.00	1.41	95.50	1.29	0.24	0.0003	0.14	0.02	1.72	1.87	0.003	1.68	0.35	91.75	3.50	94.25	1.89	2.05	2.84									
Mean	0.14		0.06		2.24	1.61		1.50		86.57		88.42		0.26		0.15		1.73	1.96		1.79		77.04		82.99		2.15	2.80									
Std Error	0.03		0.01		0.10	0.19		0.21		4.08		4.81		0.02		0.02		0.16	0.24		0.30		4.82		4.99		0.45	0.48									
Motile velocity																																					
	P	EC50, μM		SD	Ratio P/unP	P	Hill coefficient		SD	P	ΔV_{max} , $\mu\text{m/sec}$		SD	P + EGCG	EC50, μM		SD	Ratio P+E/unP+E	P + EGCG	Hill coefficient		SD	P + EGCG	ΔV_{max} , $\mu\text{m/sec}$		SD		Ratio P+EGCG/P	Ratio unP+EGCG/ unP								
		SD	unP				SD	unP			SD	unP			SD	unP + EGCG					SD			SD	unP + EGCG												
Exp1	0.14	0.0370			1.31	0.38				1.51	0.19			0.27	0.0520			1.42	0.380				0.84	0.090			1.96										
Exp2	0.12	0.0130	0.05	0.020	2.21	2.72	0.59	1.76	0.56	1.43	0.12	1.45	0.27	0.15	0.0064	0.11	0.003	1.35	1.94	0.140	1.87	0.09	1.35	0.030	1.34	0.02	1.28	2.09									
Exp3	0.13	0.0280	0.04	0.006	3.00	1.52	0.40	1.91	0.51	1.45	0.15	1.25	0.08	0.24	0.0570	0.12	0.009	2.09	2.13	0.690	1.92	0.26	1.27	0.150	1.23	0.05	1.90	2.74									
Exp4	0.11	0.0430	0.04	0.014	2.53	0.86	0.31	1.26	0.79	2.09	0.22	1.85	0.10	0.57	0.1600	0.14	0.056	3.98	0.84	0.190	0.75	0.23	1.22	0.160	1.19	0.14	5.06	3.21									
Exp5	0.11	0.0028	0.06	0.014	1.70	1.17	0.03	1.07	0.01	2.01	0.02	2.04	0.18	0.32	0.0170	0.08	0.006	4.09	0.89	0.210	1.38	0.14	1.50	0.160	1.64	0.05	2.96	1.23									
Exp6	0.07	0.0004	0.05	0.004	1.29	2.80	0.03	2.38	0.29	2.67	0.01	2.74	0.11	0.24	0.0003	0.09	0.019	2.84	1.63	0.002	1.69	0.57	1.84	0.001	1.95	0.21	3.52	1.59									
Exp7	0.11	0.0110	0.06	0.001	1.94	1.30	0.16	1.41	0.05	2.18	0.10	2.18	0.02	0.27	0.0500	0.09	0.009	3.12	1.11	0.190	1.63	0.26	1.63	0.120	1.74	0.09	2.52	1.57									
Mean	0.11		0.05		2.11	1.67		1.63		1.91		1.92		0.30		0.10		2.91	1.42		1.54		1.38		1.52		2.74	2.07									
Std Error	0.01		0.003		0.25	0.29		0.20		0.18		0.22		0.05		0.01		0.44	0.19		0.18		0.12		0.13		0.48	0.31									

Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients

Steven Marston^{1,*}, Massimiliano Memo¹, Andrew Messer¹, Maria Papadaki¹, Kristen Nowak², Elyshia McNamara², Royston Ong², Mohammed El-Mezguedi³, Xiaochuan Li⁴ and William Lehman⁴

¹NHLI, Imperial College London, London, W12 0NN, UK, ²Centre for Medical Research, University of Western Australia, WA Institute for Medical Research, Nedlands, Australia, ³Department of Biochemistry, University of Leicester, Leicester, UK and ⁴Department of Physiology and Biophysics, Boston University School of Medicine, Boston, MA, USA

Received June 14, 2013; Revised and Accepted July 17, 2013

The congenital myopathies include a wide spectrum of clinically, histologically and genetically variable neuromuscular disorders many of which are caused by mutations in genes for sarcomeric proteins. Some congenital myopathy patients have a hypercontractile phenotype. Recent functional studies demonstrated that *ACTA1* K326N and *TPM2* ΔK7 mutations were associated with hypercontractility that could be explained by increased myofibrillar Ca²⁺ sensitivity. A recent structure of the complex of actin and tropomyosin in the relaxed state showed that both these mutations are located in the actin–tropomyosin interface. Tropomyosin is an elongated molecule with a 7-fold repeated motif of around 40 amino acids corresponding to the 7 actin monomers it interacts with. Actin binds to tropomyosin electrostatically at two points, through Asp25 and through a cluster of amino acids that includes Lys326, mutated in the gain-of-function mutation. Asp25 interacts with tropomyosin K6, next to K7 that was mutated in the other gain-of-function mutation. We identified four tropomyosin motifs interacting with Asp25 (K6–K7, K48–K49, R90–R91 and R167–K168) and three E–E/D–K/R motifs interacting with Lys326 (E139, E181 and E218), and we predicted that the known skeletal myopathy mutations ΔK7, ΔK49, R91G, ΔE139, K168E and E181K would cause a gain of function. Tests by an *in vitro* motility assay confirmed that these mutations increased Ca²⁺ sensitivity, while mutations not in these motifs (R167H, R244G) decreased Ca²⁺ sensitivity. The work reported here explains the molecular mechanism for 6 out of 49 known disease-causing mutations in the *TPM2* and *TPM3* genes, derived from structural data of the actin–tropomyosin interface.

INTRODUCTION

The congenital myopathies include a wide spectrum of clinically, histologically and genetically variable neuromuscular disorders, many of them caused by mutations in genes for sarcomeric proteins (1). These myopathies are generally defined on the basis of muscle weakness and histological abnormalities in the muscle fibres. Nemaline myopathy, characterized by nemaline (rod) bodies on muscle biopsy, is the most widely studied but congenital myopathy can also be associated with cap-like structures located under the sarcolemma (Cap disease) or with congenital fibre-type disproportion (CFTD). Mutations in the skeletal muscle α-actin gene (*ACTA1*) account for ~20% of congenital

myopathies and over 200 different mutations have been identified. Recently, disease-causing mutations have also been found in β- and γ- tropomyosins encoded by *TPM2* and *TPM3* genes, respectively; 27 mutations have been reported in the *TPM2* gene and 22 mutations have been reported in the *TPM3* gene (1–14) (see Supplementary Material, figures.)

A small proportion of congenital myopathies are associated with a hypercontractile phenotype; this is a very heterogeneous disease category that includes distal arthrogryposis, trismus-pseudocamptodactyly syndrome and Escobar syndrome, and some of these cases are reported to be due to mutations in *ACTA1*, *TPM2* or *TPM3* (12–16). These apparent ‘gain-of-function’ mutations are particularly interesting because of their

*To whom correspondence should be addressed at: Myocardial function, Imperial Centre for Translational and Experimental Medicine, Hammersmith Campus, Du Cane Road, London, W12 0NN. Tel: 44 2075942732, Fax: 44 7941135583; Email: s.marston@imperial.ac.uk

parallels with hypertrophic cardiomyopathy that also presents as a hypercontractile phenotype and is associated with mutations in sarcomeric proteins, including actin and tropomyosin (*ACTC* and *TPM1* genes (17)). Enhanced contractility indicates that the abnormality caused by the mutation is likely to be within the force-producing contractile machinery, whereas a loss of function could be due to defects in force production, force transmission, force sensing or sarcomere assembly.

Tropomyosin, together with actin and troponin, constitutes the basic thin filament structural and Ca^{2+} -regulatory machinery that interacts with myosin when muscle contracts. Tropomyosin forms a 40 nm long parallel coiled-coil dimer and is able to polymerize head-to-tail with other tropomyosin molecules into long strands spanning the whole thin filament length. Each tropomyosin molecule binds to seven different actin monomers along the helical actin chain involving seven quasi repeats of about 40 amino acids each. The key function of tropomyosin is in cooperatively switching the location of the actin–tropomyosin interface between active and relaxed states under the control of troponin, Ca^{2+} and myosin heads.

Previously, we studied the effect of actin and tropomyosin mutations on Ca^{2+} regulation of muscle contractility at the single filament level in order to establish a genotype–phenotype relationship. In most biopsies from patients with *ACTA1* mutations, it was not possible to establish any mechanism (18–20) but two mutations are of particular note, since they showed distinctive abnormalities in their regulatory interaction with tropomyosin. The *ACTA1* D292V mutation resulted in an actin with normal activity that was irreversibly switched off by tropomyosin (19), whilst the *ACTA1* K326N mutation from a patient with stiff muscles was indistinguishable from wild-type actin on its own but, when incorporated into thin filaments, showed an increase in Ca^{2+} sensitivity and a crossbridge turnover rate consistent with the hypercontractile phenotype (21).

Since tropomyosin has a relatively simple structure and interacts only with itself, actin, tropomodulin and troponin, we expected that it would be easier to make a molecular explanation of the disease phenotype than for actin mutations. Most of the tropomyosin mutations reported to date are linked to different skeletal muscle myopathies characterized by generalized muscle weakness and, at the molecular level, a reduced crossbridge cycling rate and Ca^{2+} sensitivity. Examples of these mutations on β -tropomyosin are E41K and E117K linked to nemaline myopathy or cap disease (11,22).

Some tropomyosin mutations have been associated with arthrogryposis and a gain of function (15). *TPM2* R91G was the first investigated at the molecular level and this showed a hypercontractile phenotype (23). Recently, we have investigated in detail a common β -tropomyosin mutation, Δ K7, that has been described in a total of nine families (13,24). A histological inspection of biopsies taken from patients of all families showed nemaline bodies as a common feature. Most of the patients were therefore diagnosed with nemaline myopathy, although some were independently diagnosed with core-rod myopathy or distal arthrogryposis VII. Lysine 7 is placed in a region of crucial importance for tropomyosin, as it participates in the head-to-tail polymerisation of tropomyosin (25), is close to a residue binding to actin (26) and may also be involved in the binding of troponin T (27). Mokbel *et al.* showed that the mutation strongly impairs the ability of the protein to incorporate in the sarcomere and causes its

accumulation in the nemaline bodies in transfected C2C cells. Nevertheless, the expressed mutant is incorporated into muscle thin filaments and it acts as a poison peptide *in vivo*. Studies with chemically skinned muscle from patient biopsies and isolated thin filaments using an *in vitro* motility assay showed that this mutation increases the Ca^{2+} sensitivity, the cross-bridge turnover rate and maximum force, producing a gain of function. In retrospect, it was noted that all the patients with this mutation presented a hypercontractile phenotype in childhood. Distal arthrogryposis, exhibited by some of the patients, is characterized by hypercontraction and most of the Δ K7 patients did not present evident myopathic symptoms until their teens, proving that their weak muscles were not congenital.

Recently, a high resolution structure of α -tropomyosin bound to actin has been proposed, from a study involving electron microscopy and computational chemistry (26), which highlights the electrostatic interactions holding tropomyosin on F-actin. In this structure (corresponding to the closed-state, roughly equivalent to the OFF functional state (28)), tropomyosin blocks myosin's strong binding in accordance with the well-known observation that skeletal muscle tropomyosin on its own inhibits actin activation of myosin ATPase. This structure provides the framework for understanding how the mutation could cause the gain of function.

In this manuscript, we have examined the location of tropomyosin and actin mutations in the new structure of the actin–tropomyosin interface. The K7 residue is positioned just on the side of the K6 residue, which binds to Asp25 on the actin molecule, therefore we can propose that the mutation Δ K7 destabilizes this inhibitory interaction.

Moreover, since tropomyosin is a modular protein, these binding motifs are repeated in some of the other actin-binding repeats, thus the structure gives us a unique opportunity to predict where other mutations might cause a similar phenotype. We found that four out of the seven quasi-repeats, present in all tropomyosin isoforms, share the feature of a second basic residue downstream of the basic residue binding to Asp25: K6-K7, K48-K49, R90-R91, R167-K168. It is interesting to note that mutations linked to skeletal muscle myopathies have been reported in all the four downstream basic amino acids: Δ K7, Δ K49 and R91G on β -tropomyosin and K168E on γ -tropomyosin and thus, we predict they will cause a gain of function like Δ K7. A second actin–tropomyosin interface involves Lys326 of actin, the amino acids mutated in the 'stiff' patient and, interestingly, mutations have been reported in the acidic amino acids of tropomyosin (Δ E139, E181K, Δ E218) that interact with actin K326 so we predict these would lead to the same effect as the K326N gain-of-function mutation. In this study, we have tested the effect of tropomyosin mutations on the actomyosin interaction by measuring Ca^{2+} -regulation of skeletal muscle thin filaments containing skeletal myopathy-causing tropomyosin mutations and have confirmed the molecular phenotype predicted.

RESULTS

Structural analysis of the actin–tropomyosin interface predicts gain-of-function mutations in tropomyosin

Li *et al.* (26) determined the structure of α -tropomyosin bound to actin, nevertheless tropomyosin isoforms are highly conserved, and in fact the sequences around the actin contacts studied here

are identical in the α -, β -, and γ -isoforms (see Supplementary Material). The Li *et al.* structure shows that tropomyosin is not closely bound to actin and makes electrostatic contact at just two points on every actin; at D25 and with a cluster of basic residues, K326, K328 and R147. This pattern is illustrated in Figure 1. The amino acids in tropomyosin that interact with actin are mapped in Figure 1B, in which the sequence is divided into the seven actin-binding repeats proposed by McLachlan and Stewart (29), based on the analysis of the amino acid sequence. They predicted that the alpha band sequences bound to actin when actin–tropomyosin was in the relaxed state. It will be seen that the basic amino acids interacting with D25 are found at or near the beginning of the alpha bands and the acidic amino acids interacting with the K326, K328, R147 cluster occur at two places near the end of the alpha band. In the latter case, the first site, 12–17 amino acids into the alpha band showed interactions in every period of tropomyosin, while the second site, 16–20 amino acids into the alpha band showed interaction in only six of the seven periods.

Tropomyosin interaction with residue Aspartic acid 25 of actin
We noted that the gain-of-function mutation $\Delta K7$ was one amino acid downstream from K6 that interacts with actin D25 as described above. Figure 2A shows the interface at high resolution. Our initial hypothesis was that disruption of one of a cluster of basic amino acids (K5, K6, K7) would perturb the

actin–tropomyosin interface, destabilizing this structure but not have such a large effect that the mutation would be lethal. This structure corresponds to a functionally relaxed state of muscle, since it is well established that skeletal muscle tropomyosin inhibits actin-activated ATPase and this position of tropomyosin relative to actin is present with troponin in the absence of myosin heads (30). Consequently destabilizing the structure would shift the equilibrium towards the active state of actin–tropomyosin, thus accounting for the gain of function observed with the $\Delta K7$ mutation.

The motif observed with K6 and K7 is repeated in four of the periods of tropomyosin: K6–K7, K48–K49, R90–R91 and R167–K168, and the structures of the interface for the four motifs is very similar (Fig. 2A–D). The other three periods have a single basic amino acid that interacts with D25 (see Fig. 1). If our hypothesis on the mechanism of the gain of function due to the $\Delta K7$ mutation is correct, we would expect charge-change mutations at K49, R91 and K168 to also cause a gain of function. In fact, the mutation R91G in Period 3 of TPM2 is known to cause a hypercontractile phenotype, distal arthrogryposis and an *in vitro* investigation of the effect of this mutation did indeed find that it causes enhanced actomyosin ATPase and incomplete relaxation, although Ca^{2+} sensitivity was not very different from wild type (23) (Fig. 2C). A survey of the published mutations in TPM2 and TPM3 reveals that mutations $\Delta K49$ (Period 2) and K168E (Period 5) have been identified in

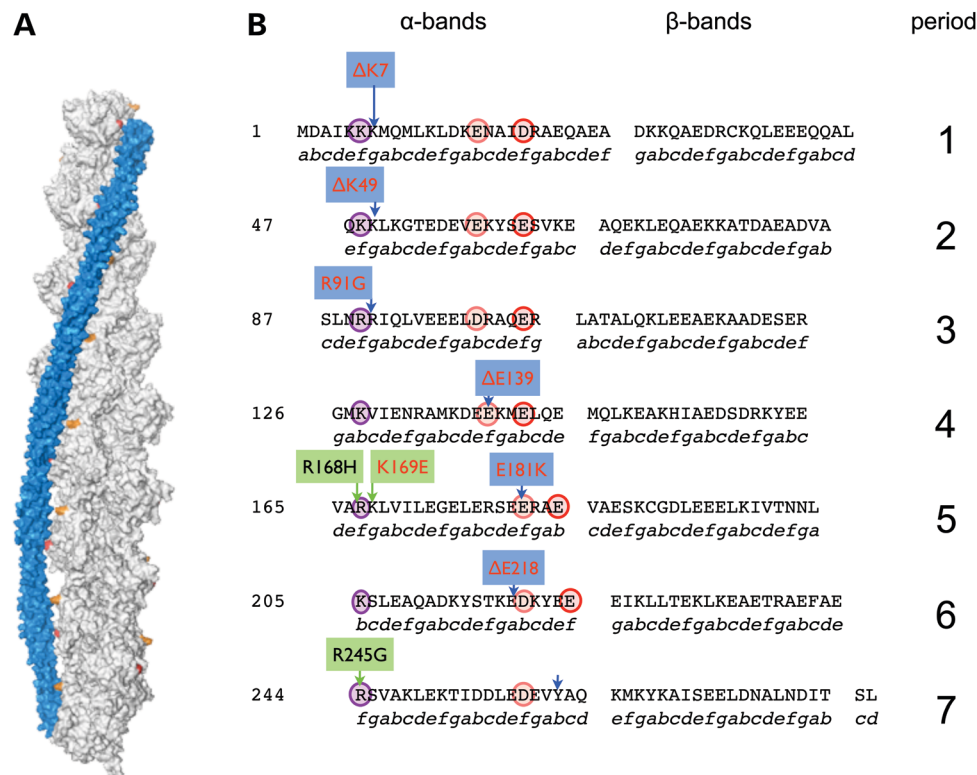


Figure 1. Structure of the actin–tropomyosin interface. (A) Structure of one of the two tropomyosin molecules (cyan) bound to the actin double helix (grey). Surface rendering using PyMol with coordinates from Li *et al.* (26). Actin Asp25 is coloured red and Lys 326 is coloured blue. (B) The β -tropomyosin sequence divided in the seven quasi-repeating periods and α - and β -bands as defined by McLachlan and Stewart (29). The purple circles highlight residues interacting with actin Asp25; the orange and red circles highlight the residues interacting with actin R147, K326 and K328 as defined by Li *et al.* (26). The β -tropomyosin (TPM2) mutations are indicated in blue boxes, and the γ -tropomyosin (TPM3) mutations are indicated in green boxes. The mutations increasing the Ca^{2+} sensitivity are written in red, while those decreasing it are written in black.

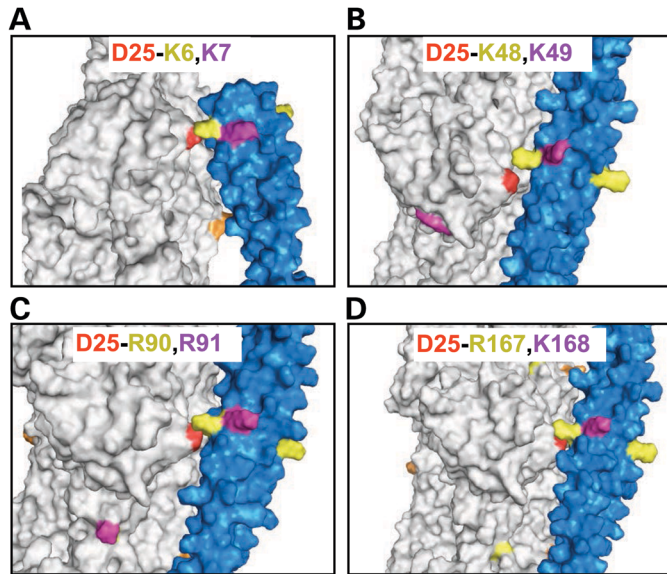


Figure 2. Details of the interactions of tropomyosin with actin Asp25. Magnified views of the Li *et al.* structure from Figure 1A show the locations of proposed gain-of-function mutations in tropomyosin relative to actin Asp 25 for the first (K6, K7), second (K46, K47), third (R90, R91) and fifth (R167, K168) periods of tropomyosin.

single patients (Fig. 2B and D) (7,31), but not associated with a hypercontractile phenotype, whereas no mutations have been reported in the equivalent positions of Periods 4, 6 and 7. Mutations at the amino acid directly interacting with D25 were reported in skeletal myopathy patients: K128E in TPM2 and R90C, R167H and R244G in TPM3, and we would predict that these would have a different, perhaps opposite, effect on muscle function. In order to test our hypothesis, we have determined the effect of these mutations on Ca^{2+} regulation of muscle thin filaments.

Tropomyosin interaction with actin residues 326, 328 and 147
This cluster of amino acids on the surface of actin forms a second interface presenting basic amino acids to tropomyosin (Fig. 3) with at least one of the three residues involved in interaction in every period of tropomyosin (Fig. 1). The actin (*ACTA1*) K326N mutation has been found in a baby suffering from a hypercontractile phenotype characterized by generalized stiffness (21). In that study, *in vitro* motility assay studies showed that the mutation was sufficient to generate an increased Ca^{2+} sensitivity that explains the hypercontractile phenotype. We proposed that the loss of positive charge in the K326N mutant actin destabilized the inhibitory interaction with tropomyosin in the same way as the ΔK7 TPM2 mutation. Consequently, we would expect that charge-change mutations in the cognate amino acids of tropomyosin would have a similar effect. K326 binds to tropomyosin in five tropomyosin periods and one of these involves the residue E181 (Fig. 1B). The mutation E181K in TPM2 was reported in two cases of distal arthrogryposis, and this mutation caused an increase in Ca^{2+} sensitivity of force production in skinned fibres from the patient (12,32). Figure 4 shows the interface between actin K326 and tropomyosin E181 as defined by the Li *et al.* structure. Thus, destabilization of this interaction by loss of charge at either the actin or the

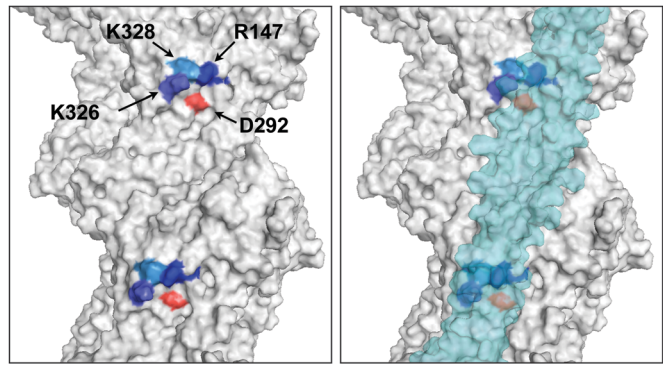


Figure 3. Location of actin residues Lys326, Lys328, Arg147 and Asp272. Magnified views of the Li *et al.* structure from Figure 1A. Left, actin alone is shown; right, tropomyosin is overlaid at 50% transparency. Amino acids are identified in the figure. The lower cluster is viewed face on while the upper cluster is rotated to the right.

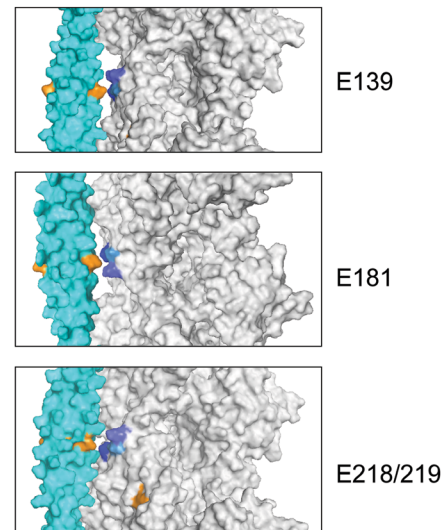


Figure 4. Details of the interactions of tropomyosin with actin Lys326, Lys328 and Arg147. Magnified views of the Li *et al.* structure from Figure 1A show the locations of proposed gain-of-function mutations in tropomyosin relative to actin Lys326, Lys328 and Arg147 in the fourth (E139), fifth (E181) and sixth (E218, D219) periods.

tropomyosin residue produces the same increase in Ca^{2+} sensitivity. There is an EEK (or EDK or EER) motif in tropomyosin homologous to the actin K326-Tm 181 interface in just three of the seven periods (See Fig. 1). We examined published myopathy mutations in these motifs in the other periods of the tropomyosin sequence and found ΔE139 and ΔE218 in TPM2 (Fig. 4) (8,10). It is remarkable that there are three such motifs and three reported mutations, while none have been reported at the equivalent position in other periods that do not have this motif. To test the predictions, we investigated whether these mutations also caused a gain of function *in vitro*.

Actin D292 mutation

The mutation *ACTA1* D292V was found in a patient with weak muscles and congenital fibre-type disproportion (19,33). *In vitro* studies of actin purified from this patient's skeletal muscle showed a very strong molecular phenotype. The

patient sample contained ~45% mutant actin and with actin alone the interaction with myosin in the *in vitro* motility assay was the same as normal actin; however, when tropomyosin was added, the filament motility appeared to be completely switched off. Li *et al.*'s structure provides a possible reason for this effect. The acidic amino acid D292 is located on the surface of actin close to the K326, K328, R147 cluster (Fig. 4); it does not participate in binding but is close to tropomyosin. It is possible that the acidic residue in this position may act to moderate the binding affinity of the K326, K328, R147 cluster so that the binding is not so tight that the transition of tropomyosin from OFF to ON state has a physiologically impossibly high energy barrier. If so, one could hypothesize that the loss of negative charge in the D292V mutation would strengthen binding and stabilize the OFF state. We have investigated this mutation further to test the hypothesis.

Effect of mutations studied by an *in vitro* motility assay

Tropomyosin mutations

We expressed wild-type β -tropomyosin and the mutations Δ K49, R91G and Δ E139 and wild-type γ -tropomyosin and the

mutations R167H, K168E and R244G in the baculovirus/*sf9* system that preserves native N-terminal acetylation. Thin filaments were reconstituted using the expressed tropomyosin with rabbit skeletal muscle actin and troponin and the regulation of their movement over immobilized heavy meromyosin (HMM) was studied in the *in vitro* motility assay. Thin filaments containing mutant tropomyosin species were fully functional in this assay. When Ca^{2+} regulation of mutant thin filaments was compared with thin filaments, containing the appropriate wild-type tropomyosin isoform, it was observed that all the predicted gain-of-function mutations produced a higher Ca^{2+} sensitivity (curve shifted to the left for Δ K49, R91G, Δ E139 and K168E; Fig. 5) and a slightly higher maximum sliding speed similar to the previously investigated gain-of-function mutations, Δ K7 and E181 K (24,32). In contrast, the four skeletal muscle myopathy mutations that were expected to give a hypocontractile phenotype showed lower Ca^{2+} sensitivity (Fig. 5) and lower sliding speeds (β -tropomyosin E41K, E117K and γ -tropomyosin R167H and R245G; Table 1). This also corresponds to previously published data on E41K and R167H contraction in biopsy samples from Ochala's laboratory (22,32). It is interesting to note that thin filaments containing 100 or 50% mutant

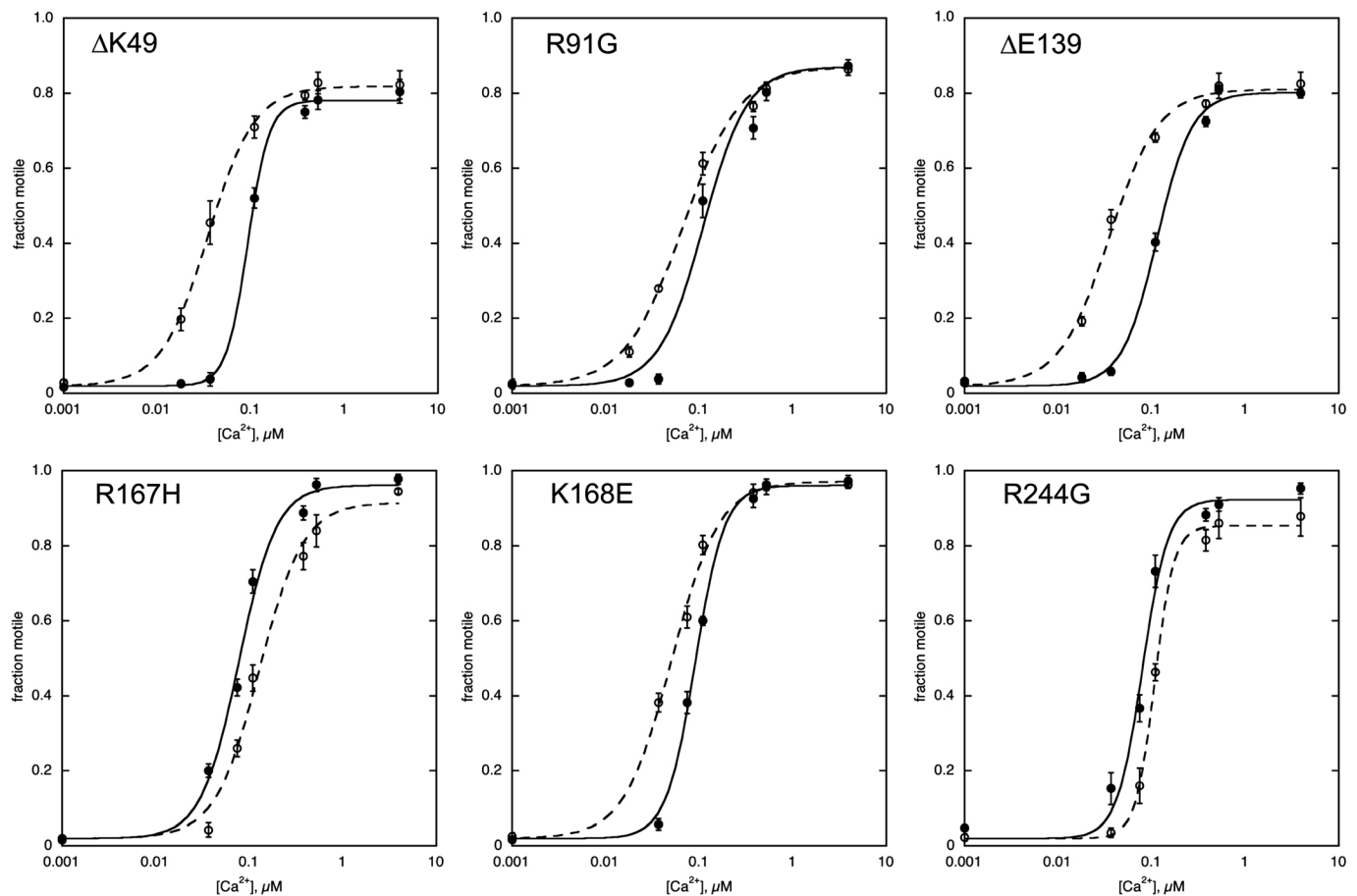


Figure 5. Comparison of Ca^{2+} regulation of thin filaments containing wild-type and mutant tropomyosin. Thin filament motility was measured by an *in vitro* motility assay over a range of (Ca^{2+}) in paired cells. The fraction of filaments motile is plotted as a function of (Ca^{2+}) for a typical experiment. The points are the mean $\pm$ SEM of four determinations of fraction motile measured in one motility cell. The curves are fits of the data to the Hill equation. Solid lines and points, wild-type thin filaments, dashed lines and open points, thin filaments with mutant tropomyosin. The mean values of EC_{50} from replicate experiments are plotted in Figure 6 and summarized in Table 1.

Table 1. The effect of tropomyosin mutations on Ca²⁺ sensitivity and the maximum sliding speed in reconstituted skeletal muscle thin filaments measured by an *in vitro* motility assay

Mutation	EC ₅₀ Mut, $\mu\text{M} \pm \text{SEM}, n, P$	EC ₅₀ WT, $\mu\text{M} \pm \text{SEM}, n, P$	Ratio EC ₅₀ WT/EC ₅₀ Mut $\pm \text{SEM}, n, P$	Max sliding speed relative to WT, $\mu\text{m/s} \pm \text{SEM}, n, P$
ΔK49 100%	0.04 ± 0.002	0.08 ± 0.01	$2.42 \pm 0.25, n = 6, P = -0.002$	$98.1 \pm 2.7\%, n = 3, P = 0.555$
50%	0.03 ± 0.002	0.09 ± 0.003	$2.71 \pm 0.06, n = 6, P < 0.0001$	$101.0 \pm 0.6\%$
R91G 100%	0.05 ± 0.01	0.09 ± 0.01	$1.83 \pm 0.18, n = 6, P = 0.006$	$102.2 \pm 1.0\%, n = 3, P = 0.160$
50%	0.06 ± 0.01	0.09 ± 0.004	$1.55 \pm 0.05, n = 6, P = 0.0002$	$100.2 \pm 0.6\%$
ΔE139 100%	0.03 ± 0.004	0.12 ± 0.03	$2.95 \pm 0.43, n = 6, P = 0.006$	$101.1 \pm 2.4\%, n = 3, P = 0.687$
50%	0.03 ± 0.004	0.11 ± 0.01	$2.58 \pm 0.49, n = 6, P = 0.003$	$104.2 \pm 1.1\%$
R167H 100%	0.16 ± 0.02	0.07 ± 0.01	$0.59 \pm 0.08, n = 6, P = 0.004$	$92.5 \pm 2.6\%, n = 3, P = 0.101$
50%	0.13 ± 0.02	0.08 ± 0.01	$0.59 \pm 0.01, n = 2$	95.0%
K168E 100%	0.05 ± 0.01	0.08 ± 0.01	$2.17 \pm 0.32, n = 8, P = 0.008$	$107.2 \pm 1.6\%$
50%	0.05 ± 0.003	0.09 ± 0.001	$1.84 \pm 0.07, n = 12, P < 0.0001$	$105.4 \pm 2.7\%$
R245G 100%	0.12 ± 0.02	0.05 ± 0.02	$0.46 \pm 0.08, n = 6, P = 0.001$	$97.1 \pm 4.7\%, n = 3, P = 0.598$
50%	0.11 ± 0.003	0.08 ± 0.01	$0.67 \pm 0.07, n = 2$	93.6%

Combined data from *n* replicate Ca²⁺-activation curve measurements similar to those shown in Figure 5. The significance, *P*, of deviation of the EC₅₀ ratio from 1 was determined by a single value *t*-test.

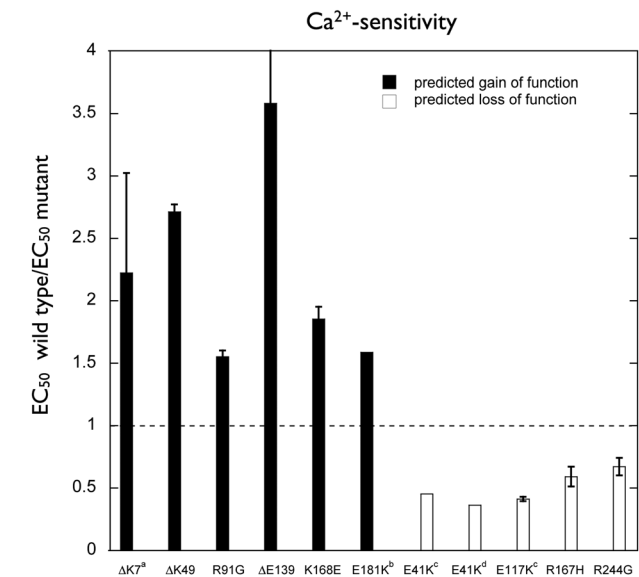


Figure 6. The effect of skeletal myopathy mutations in tropomyosin on thin filament Ca²⁺ sensitivity. Data from this paper and published work are combined. Black, predicted gain-of-function mutations, white, predicted loss-of-function mutations. Ratio of EC₅₀ of mutant thin filaments relative to EC₅₀ of wild-type troponin–tropomyosin is plotted. Error bars show SEM of up to 12 replicate comparative measurements (see Table 1). (A) from Mokbel *et al.* (24). (B) from Ochala *et al.* (32). (C) from Marttila *et al.* (11). (D) from Ochala *et al.* (22)

tropomyosin gave similar alterations in thin filament function (Table 1). (Fig. 6)

ACTA1 D292V mutation

In a previous study, the ACTA1 D292V mutation appeared to stabilize the inactive state of actin–tropomyosin. (19). We investigated the properties of the D292V mutation using actin extracted from a patient biopsy containing the mutation. Addition of tropomyosin caused the D292V actin filaments to stop moving, while it did not affect the fraction motile and slightly increased the sliding speed of wild-type actin (Fig. 7). In the presence of activating concentrations of Ca²⁺, troponin did not re-activate the thin filaments containing D292V actin and tropomyosin. We

then added *N*-ethylmaleimide-treated myosin subfragment 1 (NEM-S-1) to actin–tropomyosin; this modified S-1 forms rigor bonds with actin even in the presence of ATP, therefore a small quantity will cooperatively switch on thin filaments. However, NEM-S-1 was incapable of switching on D292V actin-containing filaments at double the concentration that switched on native thin filaments in the absence of Ca²⁺ (34,35). An actin mutation, E93K, which causes thin filaments to be inhibited by tropomyosin has been observed before, but this mutation was reactivated by either troponin or NEM-S-1 (34). Thus, the D292V mutation seems to have induced a very profound change in the ON–OFF equilibrium towards the OFF state since neither of these treatments restored motility (Fig. 7).

DISCUSSION

Structure of the actin–tropomyosin interface predicts gain-of-function mutations in skeletal muscle

Our investigation of gain-of-function mutations was sparked by the observation that lysine 326 of actin, mutated to asparagine in the muscles of the ‘stiff’ child, was in a critical position in the interface between actin and tropomyosin, according to the structure of Li *et al.* (21,26). The next observation was that, unexpectedly, the TPM2 ΔK7 mutation also caused a gain of function, and that it was located near another critical part of the actin–tropomyosin interface close to Asp25 (24). The detailed information contained in Li *et al.*’s structure combined with the repeating sequence of tropomyosin enabled us to identify a total of four similar motifs interacting with Asp25 and three motifs interacting with Lys326, and we anticipated that charge-change mutations at these sites would also cause a gain of function. Indeed, mutations at two of these sites, R91G and E181K, have already been reported to cause distal arthrogryposis, a hypercontractile phenotype, and enhanced contractility, consistent with a gain-of-function phenotype (23,32). In the current study, we have investigated the effect of the remaining predicted gain-of-function mutations, and confirmed that they did indeed increase Ca²⁺ sensitivity. The structure–function connection that we have observed is further supported by our observation that

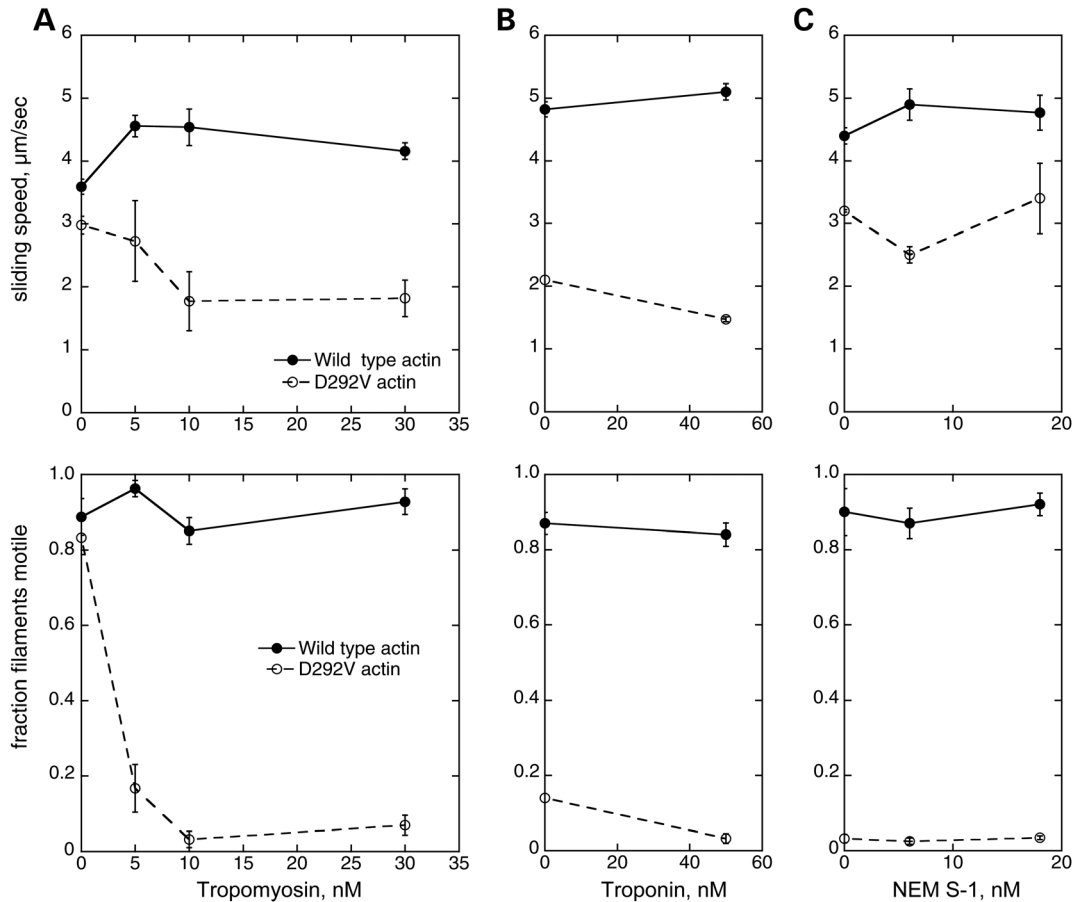


Figure 7. The effect of the actin Asp292Val mutation on actin-tropomyosin interaction measured by the *in vitro* motility assay. Actin movement over immobilized HMM was recorded and analysed. Two parameters were calculated: fraction of filament motile and the sliding speed of motile filaments. Rhodamine-phalloidin labelled actin concentration was 10 nM. Solid lines: wild-type actin, dotted lines D292V actin. (A) The effect of up to 30 nM tropomyosin on wild-type and mutant actin motility. (B) The effect of adding 50 nM troponin to actin-tropomyosin at 3.6×10^{-5} M Ca^{2+} . (C) The effect of adding 18 nM NEM S-1 to actin-tropomyosin.

charge-change mutations at positions adjacent to the predicted gain-of-function sites (R167H and R244G) produced the opposite effect, namely a reduced Ca^{2+} sensitivity.

The finding that the Li *et al.* structure can accurately predict seven gain-of-function mutations in tropomyosin strongly supports the validity of actin-tropomyosin interface structure on which it is based.

Implications for diagnosis of skeletal muscle disease-histopathology is an inappropriate criterion

Skeletal muscle myopathies are historically defined by the histopathology of the muscle; however, the determination of the disease-causing mutations and the advent of functional studies suggest a different approach to diagnosis is needed (36).

No relationship between the conventional disease classification and the gain-of-function phenotype could be ascribed. Among the seven mutations investigated, diagnoses include cap myopathy, nemaline myopathy, CFTD, rod-core disease, distal arthrogryposis and trismus-pseudocamptodactyly. In fact, the single mutation ΔK7 studied in 10 families included diagnoses of rod-core disease, CFTD and trismus pseudocamptodactyly (13,24). A similar range of diagnoses was given for the

R167 mutations found in 14 families where nemaline myopathy, CFTD, cap disease and mixed pathologies were reported (14).

While muscle histology provides no clues about the basis of the myopathy, consideration of muscle contractility is more predictive, especially for the gain-of-function mutations. This aspect was thoroughly investigated for the ΔK7 mutation, where some patients were re-evaluated and re-diagnosed in the light of contractility measurements (24). Besides the overt hypercontractility seen in arthrogryposis (E181K, R91G, ΔK7) there was a tendency for the gain-of-function patients to have contractures in infancy and abnormal gait whilst major symptoms of muscle disorders were delayed; for instance the ΔE139 patient was first examined at 11 years for a muscular-skeletal disorder (10). A clearly hypocontractile phenotype from birth would rule out a gain-of-function type of mutation. These features may not be considered in most neuromuscular clinics: it will be noted that the patients with the three gain-of-function mutations investigated here (ΔK49 , ΔE139 and K168E) were not diagnosed with a hypercontractile phenotype (7,10,31).

This situation somewhat parallels hypertrophic cardiomyopathy (HCM), which is also associated with increased Ca^{2+} -sensitivity, where symptoms frequently do not develop until an individual's second and third decades. In both cases, an initially

hypercontractile phenotype can trigger secondary muscle dysfunction leading to a hypocontractile phenotype mimicking the more common loss-of-function mutations as discussed by Mokbel *et al.* for the $\Delta K7$ mutation (24). In HCM, enhanced Ca^{2+} sensitivity is the primary defect that triggers hypertrophy but biopsies of interventricular septum from such patients invariably have a hypocontractile phenotype, indeed up to 30% of untreated patients subsequently develop symptoms of heart failure (17,37).

Implications for the structural analysis of tropomyosin regulation

The control of striated muscle activity is mediated through Ca^{2+} regulation of the thin filament and this has been shown to be due to a series of allosteric-cooperative transitions between activity states involving every protein component of the thin filament and myosin, largely defined by the position of tropomyosin on the surface of actin (38). Measurement of Ca^{2+} sensitivity is therefore a very sensitive method to detect changes in equilibria between states. The Li *et al.* structure of skeletal muscle tropomyosin on the surface of actin filaments indicates that, when freed of constraints imposed by troponin, myosin or other actin-binding proteins, tropomyosin localizes to a favoured position on F-actin defined primarily by electrostatic interactions that corresponds most closely to the blocked and closed states as defined by Lehman *et al.* in 2009 (26,39). Recent energy landscape calculations confirm that tropomyosin is located in a shallow energy well (28) and that the 'open' conformation is energetically unfavoured, except in the presence of strong myosin binding to actin, as demonstrated in the recent determination of the actin–tropomyosin-S-1 structure (40).

It is logical to propose that the mutations in tropomyosin and actin that cause a gain of function by de-stabilizing the actin–tropomyosin B/C-states and therefore shifting the equilibrium slightly in favour of the open state. The shift in equilibrium is detected as a higher Ca^{2+} sensitivity since the change in activity state and Ca^{2+} switching of the thin filament are linked equilibria (38). There is, in fact, direct evidence for this: first, the affinity of tropomyosin for actin is reported to be reduced in the gain-of-function mutations $\Delta K7$, $\Delta K49$, R91G and $\Delta E139$ (11,23,24), and second, energy landscape calculations of the effect of the actin K326N gain-of-function mutation actually show that the interaction energy (van der Waals + electrostatic) is reduced from 3286 to 2306 kcal/mol, and the energetically most favoured position of tropomyosin is shifted in the direction of the open state (41).

A similar analysis may explain why the actin D292V mutation is so profoundly inhibitory; the location of negative charge relative to the three basic amino acids K326, K328 and R147 may be crucial in defining the shallow energy minimum of tropomyosin in the C state that is necessary to allow the regulatory transition to the other states with a minimal energy expenditure. Preliminary energy landscape calculations indicate that actin–tropomyosin interaction energy is increased when D292 is mutated to valine from -3286 to -3442 kcal/mol based on the charge change alone. The mutation of the negatively charged amino acid to a hydrophobic amino acid could also allow a conformational change that makes the energy well deeper thus trapping tropomyosin in the C-state.

The information provided by these studies gives new insights into the structure–function properties at the actin–tropomyosin interface. One important implication of these results is the oblique requirement of charged residues in key positions to achieve normal regulation.

It is more difficult to explain why tropomyosin mutations causing a gain of function are located not in the amino acid that interacts directly with actin Asp25, but in the adjacent amino acid. There is no other pattern: two are deletions, one is a charge reversal and one is a charge loss. One might hypothesize that a positive charge-reducing mutation at the interaction site itself would have such a large effect as to be lethal and therefore not be seen for that reason; however, this argument fails since mutations of Arg167 to Cys, His or Gly are one of the commonest of skeletal myopathy-causing mutations, having been identified in 14 families.

Likewise, there is no obvious explanation of why the gain-of-function mutations involved with interaction with Lys326 in actin are located at the amino acid in tropomyosin that interacts with actin.

On a more general point, these studies indicate that when the molecular structure and function of a protein–protein interface is well understood, it is possible to make quite precise and testable predictions about the effect of disease-causing mutations, especially in a repeating structure like tropomyosin. The work reported here suggests a molecular mechanism for just 6 out of 49 known *TPM2* and *TPM3* mutations. It is interesting to note that none of the 26 mutations in *TPM1* identified as causes of cardiomyopathy are involved in the actin-binding motifs described here (see Supplementary Material), indicating a different mechanism of modification of function for these phenotypes. The effect of mutations in tropomyosin and actin on the interface in the ON state as described by Behrman *et al.* (40) has not yet been investigated, and the study of mutations in tropomyosin's interfaces with troponin and tropomodulin awaits further structural analysis. In the future, such studies could define which other residues in the protein are key for optimal regulation and potentially highlight therapeutic targets or treatments.

METHODS

Sources of contractile proteins

Skeletal muscle myosin, HMM, troponin and α -actin were prepared as described by Bing *et al.* in 1997 (35). Tropomyosin was expressed in a baculovirus/*Sf9* system and purified with a protocol based on that of Akkari *et al.* (42) as described by Memo *et al.* (43). Age-matched normal and D292V human skeletal muscle actin was obtained from muscle biopsies as described by Clarke *et al.* (19).

Quantitative *in vitro* motility assay

Thin filaments were reconstituted with 10 nM rabbit skeletal muscle α -actin (labelled with TRITC phalloidin) (44), tropomyosin (20–30 nM) and troponin (10–30 nM) to study Ca^{2+} regulation of filament motility by the quantitative *in vitro* motility assay (45,46). Thin filament movement over a bed of immobilized rabbit fast skeletal muscle HMM (100 $\mu\text{g/ml}$) was compared in paired motility cells in which troponin varied by a

single factor (mutation or phosphorylation state). Filament movement was recorded and analysed as previously described (47), yielding two parameters, the fraction of filaments moving and the speed of moving filaments. In our motility system, both these parameters are regulated by Ca^{2+} . The fraction motile changes from <0.1 to >0.8 in the range 1 nmol/l to $3.7 \text{ } \mu\text{mol/l}$ free Ca^{2+} . The Ca^{2+} -dependent change of sliding speed was less and more variable (0 – 50% change), as previously noted Song *et al.* (48).

The fraction motile and the sliding speed were measured over a range of Ca^{2+} concentrations to generate Ca^{2+} -activation curves. Each experiment involved the preparation of test and control troponins at the same time. To keep intra-experiment variability as low as possible, test and control thin filaments were reconstituted together and for each Ca^{2+} -concentration point they were pipetted into two channels of the dual chamber motility cell. Motility was then measured in each chamber within a couple of minutes of each other. The data were fitted to the four-variable Hill equation to yield a value for EC_{50} . EC_{50} values from replicate experiments were analysed by a paired *t*-test since the distribution of EC_{50} has been shown to be normal (45,46). The absolute value of EC_{50} was slightly variable between different troponin and myosin preparations, but the ratio of control to test was very consistent (Table 1).

Molecular structures

Structural analysis used a refined version of the structure of the actin–tropomyosin interface published by Li *et al.* (26). PDB files describing the coordinates of 15 actins and one tropomyosin in this structure were rendered using MacPyMol. The pdb files are included in Supplementary Material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We are grateful to Dr Marek Orzechowski, Boston University Medical Centre, for the energy landscape calculations of Actin K326N and D292V mutations.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the British Heart Foundation to S.M., M.M. and M.P. (RG/08/–10/25918, FS/07/057/23834, FS/12/24/29568), NIH grant # R37-HL036153 to W.L. and X.L. and Australian National Health and Medical Research Council Project Grant (1022707) and Australian Research Council Future Fellowship (FT100100734) to K.N.

REFERENCES

- Laing, N.G. and Nowak, K.J. (2005) When contractile proteins go bad: the sarcomere and skeletal muscle disease. *Bioessays*, **27**, 809–822.
- Clarke, N.F., Waddell, L.B., Sie, L.T.L., van Bon, B.W.M., Mclean, C., Clark, D., Kornberg, A., Lammens, M. and North, K.N. (2012) Mutations in

- TPM2 and congenital fibre type disproportion. *Neuromuscul. Disord.*, **22**, 955–958.
- Laing, N.G., Wilton, S.D., Akkari, P.A., Dorosz, S., Boundy, K., Kneebone, C., Blumbergs, P., White, S., Watkins, H. and Love, D.R. (1995) A mutation in the alpha tropomyosin gene TPM3 associated with autosomal dominant nemaline myopathy NEM1. *Nat. Genet.*, **10**, 249.
- Tan, P., Briner, J., Boltshauser, E., Davis, M.R., Wilton, S.D., North, K., Wallgren-Pettersson, C. and Laing, N.G. (1999) Homozygosity for a nonsense mutation in the alpha-tropomyosin slow gene TPM3 in a patient with severe infantile nemaline myopathy. *Neuromuscul. Disord.*, **9**, 573–579.
- Kiphuth, I.C., Krause, S., Huttner, H.B., Dekomien, G., Struffert, T. and Schröder, R. (2010) Autosomal dominant nemaline myopathy caused by a novel alpha-tropomyosin 3 mutation. *J. Neurol.*, **257**, 658–660.
- Tajsharghi, H., Ohlsson, M., Palm, L. and Oldfors, A. (2012) Myopathies associated with β -tropomyosin mutations. *Neuromuscul. Disord.*, **22**, 923–933.
- Clarke, N.F., Kolski, H., Dye, D.E., Lim, E., Smith, R.L.L., Patel, R., Fahey, M.C., Bellance, R., Romero, N.B., Johnson, E.S. *et al.* (2008) Mutations in TPM3 are a common cause of congenital fiber type disproportion. *Ann. Neurol.*, **63**, 329–337.
- Clarke, N.F., Domazetovska, A., Waddell, L., Kornberg, A., Mclean, C. and North, K.N. (2009) Cap disease due to mutation of the beta-tropomyosin gene (TPM2). *Neuromuscul. Disord.*, **19**, 348–351.
- Donner, K., Ollikainen, M., Ridanpää, M., Christen, H.-J., Goebel, H.H., de Visser, M., Pelin, K. and Wallgren-Pettersson, C. (2002) Mutations in the beta-tropomyosin (TPM2) gene—a rare cause of nemaline myopathy. *Neuromuscul. Disord.*, **12**, 151–158.
- Lehtokari, V.-L., Ceuterick-de Groote, C., de Jonghe, P., Marttila, M., Laing, N.G., Pelin, K. and Wallgren-Pettersson, C. (2007) Cap disease caused by heterozygous deletion of the beta-tropomyosin gene TPM2. *Neuromuscul. Disord.*, **17**, 433–442.
- Marttila, M., Lemola, E., Wallefeld, W., Memo, M., Donner, K., Laing, N.G., Marston, S., Grönholm, M. and Wallgren-Pettersson, C. (2012) Abnormal actin binding of aberrant β -tropomyosins is a molecular cause of muscle weakness in TPM2-related nemaline and cap myopathy. *Biochem. J.*, **442**, 231–239.
- Jarraya, M., Quijano-Roy, S., Monnier, N., Béhin, A., Avila-Smirnov, D., Romero, N.B., Allamand, V., Richard, P., Barois, A., May, A. *et al.* (2012) Whole-body muscle MRI in a series of patients with congenital myopathy related to TPM2 gene mutations. *Neuromuscul. Disord.*, **22**, S137–S147.
- Davidson, A.E., Siddiqui, F.M., Lopez, M.A., Lunt, P., Carlson, H.A., Moore, B.E., Love, S., Born, D.E., Roper, H., Majumdar, A. *et al.* (2013) Novel deletion of lysine 7 expands the clinical, histopathological and genetic spectrum of TPM2-related myopathies. *Brain*, **136**, 508–521.
- Memo, M. and Marston, S. (2013) Skeletal muscle myopathy mutations at the actin–tropomyosin interface that cause gain- or loss-of-function. *J. Muscle. Res. Cell. Motil.*, doi:10.1007/s10974-013-9344-y.
- Sung, S.S., Brassington, A.M., Grannatt, K., Rutherford, A., Whitby, F.G., Krakowiak, P.A., Jorde, L.B., Carey, J.C. and Bamshad, M. (2003) Mutations in genes encoding fast-twitch contractile proteins cause distal arthrogryposis syndromes. *Am. J. Hum. Genet.*, **72**, 681–690.
- Sung, S.S., Brassington, A.M., Krakowiak, P.A., Carey, J.C., Jorde, L.B. and Bamshad, M. (2003) Mutations in TNNT3 cause multiple congenital contractures: a second locus for distal arthrogryposis type 2B. *Am. J. Hum. Genet.*, **73**, 212–214.
- Marston, S.B. (2011) How Do mutations in contractile proteins cause the primary familial cardiomyopathies? *J. Cardiovasc. Transl. Res.*, **4**, 245–255.
- Feng, J.J. and Marston, S. (2009) Genotype–phenotype correlations in ACTA1 mutations that cause congenital myopathies. *Neuromusc. Disord.*, **19**, 6–16.
- Clarke, N.F., Ilkovski, B., Cooper, S., Valova, V.A., Robinson, P.J., Nonaka, I., Feng, J.J., Marston, S. and North, K. (2007) The pathogenesis of ACTA1-related congenital fiber type disproportion. *Ann. Neurol.*, **61**, 552–561.
- D’Amico, A., Graziano, C., Pacileo, G., Petrini, S., Nowak, K.J., Boldrini, R., Jacques, A., Feng, J.J., Porfiro, B., Sewry, C.A. *et al.* (2006) Fatal hypertrophic cardiomyopathy and nemaline myopathy associated with ACTA1 K336E mutation. *Neuromuscul. Disord.*, **16**, 548–552.
- Jain, R.K., Jayawant, S., Squier, W., Muntioni, F., Sewry, C.A., Manzur, A., Quinlivan, R., Lillis, S., Jungbluth, H., Sparrow, J.C. *et al.* (2012) Nemaline

- myopathy with stiffness and hypertonia associated with an ACTA1 mutation. *Neurology*, **78**, 1100–1103.
22. Ochala, J., Li, M., Ohlsson, M., Oldfors, A. and Larson, L. (2008) Defective regulation of contractile function in muscle fibres carrying an E41K β -tropomyosin mutation. *J. Physiol.*, **586**, 2993–3002.
 23. Robinson, P., Lipscomb, S., Preston, L.C., Altin, E., Watkins, H., Ashley, C.C. and Redwood, C.S. (2007) Mutations in fast skeletal troponin I, troponin T, and beta-tropomyosin that cause distal arthrogryposis all increase contractile function. *FASEB J.*, **21**, 896–905.
 24. Mokbel, N., Ilkovski, B., Kreissl, M., Memo, M., Jeffries, C.M., Marttila, M., Lehtokari, V.L., Lemola, E., Gronholm, M., Yang, N. *et al.* (2013) K7del is a common TPM2 gene mutation associated with nemaline myopathy and raised myofibre calcium sensitivity. *Brain*, **136**, 494–507.
 25. Frye, J., Klenchin, V.A. and Rayment, I. (2010) Structure of the tropomyosin overlap complex from chicken smooth muscle: insight into the diversity of N-terminal recognition. *Biochemistry*, **49**, 4908–4920.
 26. Li, X.E., Tobacman, L.S., Mun, J.Y., Craig, R., Fischer, S. and Lehman, W. (2011) Tropomyosin position on F-actin revealed by EM reconstruction and computational chemistry. *Biophys. J.*, **100**, 1005–1013.
 27. Palm, T., Graboski, S., Hitchcock-DeGregori, S.E. and Greenfield, N.J. (2001) Disease-causing mutations in cardiac troponin T: identification of a critical tropomyosin-binding region. *Biophys. J.*, **81**, 2827–2837.
 28. Lehman, W., Orzechowski, L., Fischer, S. and Raunser, S. (2013) Gestalt-binding of tropomyosin on actin during thin filament activation. *J. Muscle. Res. Cell. Motil.*, doi:10.1007/s10974-013-9342-0.
 29. McLachlan, A.D. and Stewart, M. (1976) The 14-fold periodicity in alpha-tropomyosin and the interaction with actin. *J. Mol. Biol.*, **103**, 271–298.
 30. Vibert, P., Craig, R. and Lehman, W. (1997) Steric-model for activation of muscle thin filaments. *J. Mol. Biol.*, **266**, 8–14.
 31. Ohlsson, M., Quijano-Roy, S., Darin, N., Brochier, G., Lacène, E., Avila-Smirnow, D., Fardeau, M., Oldfors, A. and Tajsharghi, H. (2008) New morphologic and genetic findings in cap disease associated with beta-tropomyosin (TPM2) mutations. *Neurology*, **71**, 1896–1901.
 32. Ochala, J., Gokhin, D.S., Pénisson-Besnier, I., Quijano-Roy, S., Monnier, N., Lunardi, J., Romero, N.B. and Fowler, V.M. (2012) Congenital myopathy-causing tropomyosin mutations induce thin filament dysfunction via distinct physiological mechanisms. *Hum. Mol. Genet.*, **21**, 4473–4485.
 33. Laing, N.G., Clarke, N.F., Dye, D.E., Liyanage, K., Walker, K.R., Kobayashi, Y., Shimakawa, S., Hagiwara, T., Ouvrier, R., Sparrow, J.C. *et al.* (2004) Actin mutations are one cause of congenital fibre type disproportion. *Ann. Neurol.*, **56**, 689–694.
 34. Bing, W., Razaq, A., Sparrow, J. and Marston, S. (1998) Tropomyosin and troponin regulation of wild type and E93K mutant actin filaments from *Drosophila* flight muscle. Charge reversal on actin changes actin–tropomyosin from on to off state. *J. Biol. Chem.*, **273**, 15016–15021.
 35. Bing, W., Fraser, I.D.C. and Marston, S.B. (1997) Troponin I and troponin T interact with troponin C to produce different Ca^{2+} -dependant effects on actin–tropomyosin filament motility. *Biochem. J.*, **327**, 335–340.
 36. Sewry, C.A. (2008) Pathological defects in congenital myopathies. *J. Muscle. Res. Cell. Motil.*, **29**, 231–238.
 37. Elliott, P. and McKenna, W.J. (2004) Hypertrophic cardiomyopathy. *Lancet*, **363**, 1881–1891.
 38. Lehrer, S.S. and Geeves, M.A. (1998) The muscle thin filament as a classical cooperative/allosteric regulatory system. *J. Mol. Biol.*, **277**, 1081–1089.
 39. Lehman, W., Galińska-Rakoczy, A., Hatch, V., Tobacman, L.S. and Craig, R. (2009) Structural basis for the activation of muscle contraction by troponin and tropomyosin. *J. Mol. Biol.*, **388**, 673–681.
 40. Behrmann, E., Müller, M., Penczek, P.A., Mannherz, H.G., Manstein, D.J. and Raunser, S. (2012) Structure of the rigor actin–tropomyosin–myosin complex. *Cell*, **150**, 327–338.
 41. Orzechowski, M., Fischer, S. and Lehman, W. (2013) Influence of actin mutation on the energy landscape of actin–tropomyosin filaments. *Biophys. J.*, **104**, 480a.
 42. Akkari, P.A., Song, Y., Hitchcock-DeGregori, S., Blechynden, L. and Laing, N. (2002) Expression and biological activity of Baculovirus generated wild-type human slow alpha tropomyosin and the Met9Arg mutant responsible for a dominant form of nemaline myopathy. *Biochem. Biophys. Res. Commun.*, **296**, 300–304.
 43. Marston, S.B., Lehman, W., Li, X. and Memo, M. (2013) A repeating structural motif in tropomyosin that is responsible for multiple gain of function skeletal myopathy mutations. *Biophys. J.*, **104**, 646a–647a.
 44. Kron, S.J., Toyoshima, Y.Y., Uyeda, T.Q.P. and Spudich, J.A. (1991) Assays for actin sliding movement over myosin coated surfaces. *Methods Enzymol.*, **196**, 399–416.
 45. Fraser, I.D.C. and Marston, S.B. (1995) *In vitro* motility analysis of actin–tropomyosin regulation by troponin and Ca^{2+} : the thin filament is switched as a single cooperative unit. *J. Biol. Chem.*, **270**, 7836–7841.
 46. Messer, A.E., Jacques, A.M. and Marston, S.B. (2007) Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. *J. Mol. Cell Cardiol.*, **42**, 247–259.
 47. Marston, S.B., Fraser, I.D.C., Wu, B. and Roper, G. (1996) A simple method for automatic tracking of actin filaments in the motility assay. *J. Musc. Res. Cell. Motil.*, **17**, 497–506.
 48. Song, W., Dyer, E., Stuckey, D., Leung, M.-C., Memo, M., Mansfield, C., Ferenczi, M., Liu, K., Redwood, C., Nowak, K. *et al.* (2010) Investigation of a transgenic mouse model of familial dilated cardiomyopathy. *J. Mol. Cell Cardiol.*, **49**, 380–389.

Uncoupling of myofilament Ca^{2+} sensitivity from troponin I phosphorylation by mutations can be reversed by epigallocatechin-3-gallate

Maria Papadaki, Petr G. Vikhorev, Steven B. Marston, and Andrew E. Messer*

National Heart and Lung Institute, Imperial College London, London W12 0NN, UK

Received 12 December 2014; revised 22 May 2015; accepted 11 June 2015

Time for primary review: 40 days

Aims

Heart muscle contraction is regulated via the β -adrenergic response that leads to phosphorylation of Troponin I (TnI) at Ser22/23, which changes the Ca^{2+} sensitivity of the cardiac myofilament. Mutations in thin filament proteins that cause dilated cardiomyopathy (DCM) and some mutations that cause hypertrophic cardiomyopathy (HCM) abolish the relationship between TnI phosphorylation and Ca^{2+} sensitivity (uncoupling). Small molecule Ca^{2+} sensitizers and Ca^{2+} desensitizers that act upon troponin alter the Ca^{2+} sensitivity of the thin filament, but their relationship with TnI phosphorylation has never been studied before.

Methods and results

Quantitative *in vitro* motility assay showed that 30 μM EMD57033 and 100 μM Bepridil increase Ca^{2+} sensitivity of phosphorylated cardiac thin filaments by 3.1- and 2.8-fold, respectively. Additionally they uncoupled Ca^{2+} sensitivity from TnI phosphorylation, mimicking the effect of HCM mutations. Epigallocatechin-3-gallate (EGCG) decreased Ca^{2+} sensitivity of phosphorylated and unphosphorylated wild-type thin filaments equally (by 2.15 ± 0.45 - and 2.80 ± 0.48 -fold, respectively), retaining the coupling. Moreover, EGCG also reduced Ca^{2+} sensitivity of phosphorylated but not unphosphorylated thin filaments containing DCM and HCM-causing mutations; thus, the dependence of Ca^{2+} sensitivity upon TnI phosphorylation of uncoupled mutant thin filaments was restored in every case. In single mouse heart myofibrils, EGCG reduced Ca^{2+} sensitivity of force and k_{ACT} and also preserved coupling. Myofibrils from the ACTC E361G (DCM) mouse were uncoupled; EGCG reduced Ca^{2+} sensitivity more for phosphorylated than for unphosphorylated myofibrils, thus restoring coupling.

Conclusion

We conclude that it is possible to both mimic and reverse the pathological defects in troponin caused by cardiomyopathy mutations pharmacologically. Re-coupling by EGCG may be of potential therapeutic significance for treating cardiomyopathies.

Keywords

Ca^{2+} sensitizers • Epigallocatechin-3-gallate • Ca^{2+} regulation of contractility • Troponin phosphorylation • Cardiomyopathies

1. Introduction

Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are common inherited diseases. HCM is detected in 1 in 500 of the adult population, and the causative mutations are overwhelmingly in the proteins of the cardiac muscle contractile apparatus.^{1,2} At least 30% of cases of idiopathic DCM are of genetic origin, and DCM with no additional complications such as conduction disease is usually caused by mutations in the contractile apparatus.^{3,4} In many cases, the

cardiomyopathies result from missense mutations in one of the proteins of the muscle thin filament (actin, tropomyosin, troponin I, C, or T). Investigations of the mechanisms that cause HCM and DCM have generally found that mutations in the muscle thin filament cause abnormalities in the Ca^{2+} regulatory system of the muscle.^{5–7}

HCM is closely associated with enhanced myofilament Ca^{2+} sensitivity, although the process by which chronically high Ca^{2+} sensitivity leads to the symptoms of HCM, hypertrophy, fibrosis, and arrhythmias, is uncertain. In DCM, the situation is more complex. It has been

* Corresponding author. Tel: +44 0207 594 2732, +44 07722 966655, Email: a.messer@imperial.ac.uk

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suggested that DCM-causing mutations are associated with a reduced Ca^{2+} sensitivity,⁸ but comprehensive surveys now show that DCM-causing mutations in thin filament proteins can increase or decrease Ca^{2+} sensitivity.^{5,9} However, the DCM phenotype is always linked to the absence of modulation of Ca^{2+} sensitivity by troponin I (TnI) phosphorylation (uncoupling), and this has been proposed to be causative of the DCM phenotype.^{9,10} In addition, the coupling between TnI phosphorylation and change of Ca^{2+} sensitivity seems to be lost due to HCM mutations in thin filament proteins when studied by *in vitro* motility assay (IVMA) techniques and by myofibrillar assays,^{11–15} but in permeabilized muscle, this phenomenon is not observed.¹⁶ Thus, in HCM, the relationship between TnI phosphorylation and Ca^{2+} sensitivity is not clear.

Uncoupling appears to be closely associated with mutations in thin filament proteins that cause cardiomyopathies. The decrease in Ca^{2+} sensitivity upon phosphorylation of TnI and the corresponding increase in the rate of Ca^{2+} dissociation from troponin C (TnC) is a key component of the lusitropic response to β 1-adrenergic stimulation in the heart. The uncoupling reported in DCM and HCM is likely to impact on cardiac reserve pathologically, and indeed, most studies of mouse models with cardiomyopathy mutations in thin filament proteins report a blunted response to adrenergic stimulation *in vivo* (reviewed in Ref. 17).

A number of small molecules have been found that alter myofilament Ca^{2+} sensitivity by binding to troponin and act as either Ca^{2+} sensitizers or Ca^{2+} desensitizers. The effect of these reagents on Ca^{2+} sensitivity is well documented, but the effect of small molecules on the coupling between Ca^{2+} sensitivity and TnI phosphorylation has not previously been considered.

EMD57033 and Bepridil are well-established Ca^{2+} sensitizers acting directly upon TnC.^{18–20} Epigallocatechin-3-gallate (EGCG), the principal polyphenol isolated from green tea, is reported to be a Ca^{2+} desensitizer that also acts via a binding site on TnC.^{21–23} Structural studies indicate that the regulatory Ca^{2+} binding site (Site II in the N-terminal lobe of TnC) is closely coupled both to the binding of the TnI switch peptide (147–163), critical for neutralizing the inhibitory action of TnI, and the cardiac-specific N-terminal peptide of TnI (1–30) that contains the phosphorylatable serines 22 and 23.^{24–28} We have proposed that DCM-causing mutations in thin filament proteins uncouple phosphorylation from the change in Ca^{2+} sensitivity by disrupting this coupled allosteric system.^{9,17} It is likely that Ca^{2+} sensitizers and desensitizers binding to TnC would also have an effect on the coupling between Ca^{2+} sensitivity and TnI phosphorylation by modulating the coupled system.

We have therefore investigated how EMD57033, Bepridil, and EGCG affect the Ca^{2+} regulation and its modulation by phosphorylation in native human heart thin filaments and how these reagents interact with mutations in thin filament proteins associated with HCM or DCM. Using IVMA and single myofibril contractility, we have confirmed the Ca^{2+} -sensitizing effects of EMD57033 and Bepridil and demonstrated that they also uncouple Ca^{2+} sensitivity from the TnI phosphorylation level, thus mimicking the effects of HCM-causing mutations. In contrast, EGCG decreases Ca^{2+} sensitivity in native thin filaments while retaining the modulation of Ca^{2+} sensitivity by TnI phosphorylation. Moreover, EGCG has the unique ability to restore the coupling to uncoupled HCM and DCM mutant thin filaments and myofibrils, thus antagonizing the disease-causing defect. This property of EGCG may be of therapeutic significance for treating some cardiomyopathies.

2. Methods

2.1 Sources of contractile proteins

Donor heart tissue, used as control, and end-stage failing heart tissue from explanted hearts were obtained from the Sydney Tissue Bank Sydney, Australia.^{1,2} Ethical approval for collection and use of tissue samples was obtained from the St Vincent's Hospital, Sydney and Brompton, Harefield and NHLI Research Ethics Committees.

Troponin was isolated from 100 mg of human heart muscle using an anti-cardiac TnI mAb affinity column as described by Messer.²⁹ Troponin containing the *TNNC1* G159D mutation was purified from explanted heart samples as previously described.^{9,30,31} Recombinant *TNNI2* K280N and *TNNI3* K36Q was introduced into donor heart troponin by exchange as described.^{29,32} Wild-type α -tropomyosin (Tpm1.1) and the mutants E40K, E54K, and E180G were expressed in a baculovirus/*Sf9* system with a protocol based on that of Akkari et al.^{9,33,34} Native, E361G, and E99K mutant mouse cardiac actins were extracted from transgenic mouse hearts as described by Song et al.³⁵

2.2 Manipulation and measurement of TnI phosphorylation level

Troponin isolated from human heart samples has a high level of phosphorylation, which was reduced by treatment with shrimp AP (Sigma P9088). Recombinant TnI was phosphorylated by treatment with protein kinase A (PKA) catalytic subunit (Sigma, P2645–400) as previously described.^{9,32} To dephosphorylate mouse heart troponin, mice were treated with propranolol as described.¹⁰ TnI phosphorylation levels in myofibrils and isolated troponin were measured by Phosphate affinity SDS–PAGE as described;^{9,36} results are shown in Supplementary material online, SC.

2.3 Quantitative IVMA

Thin filaments were reconstituted with 10 nM rabbit skeletal or mouse cardiac muscle α -actin (labelled with TRITC phalloidin), tropomyosin (40–60 nM), and troponin (60 nM) to study Ca^{2+} regulation of filament motility by the quantitative IVMA.^{29,37} Thin filament movement over a bed of immobilized rabbit fast skeletal muscle heavy meromyosin (100 $\mu\text{g}/\text{mL}$) was compared in paired motility chambers in which troponin varied by a single factor (mutation, phosphorylation state, or treatment with drug). Filament movement was recorded and analysed as previously described,³⁸ yielding two parameters, the fraction of filaments moving, and the speed of moving filaments.

2.4 Contraction of isolated myofibrils

Contraction and relaxation of isolated mouse myofibrils were initiated using a fast-solution change system and sensitive force transducer system recently described.¹⁰ Further details are in Supplementary material online, SA.

3. Results

3.1 Relationship between phosphorylation and Ca^{2+} sensitivity

We measured the Ca^{2+} dependence of thin filaments containing human heart troponin by the IVMA. The assay yields two parameters, fraction of filaments motile and sliding speed of the motile thin filaments. Both parameters are Ca^{2+} dependent, and Ca^{2+} activation curves were plotted (Figure 1). 3.9 μM Ca^{2+} increased the fraction of filaments motile from 0.03 to 0.83 and the sliding speed from 1.5 to 3.0 $\mu\text{m}/\text{s}$. The EC_{50} and Hill co-efficient (n_H) determined by fitting the Hill equation to the data were similar for both parameters under

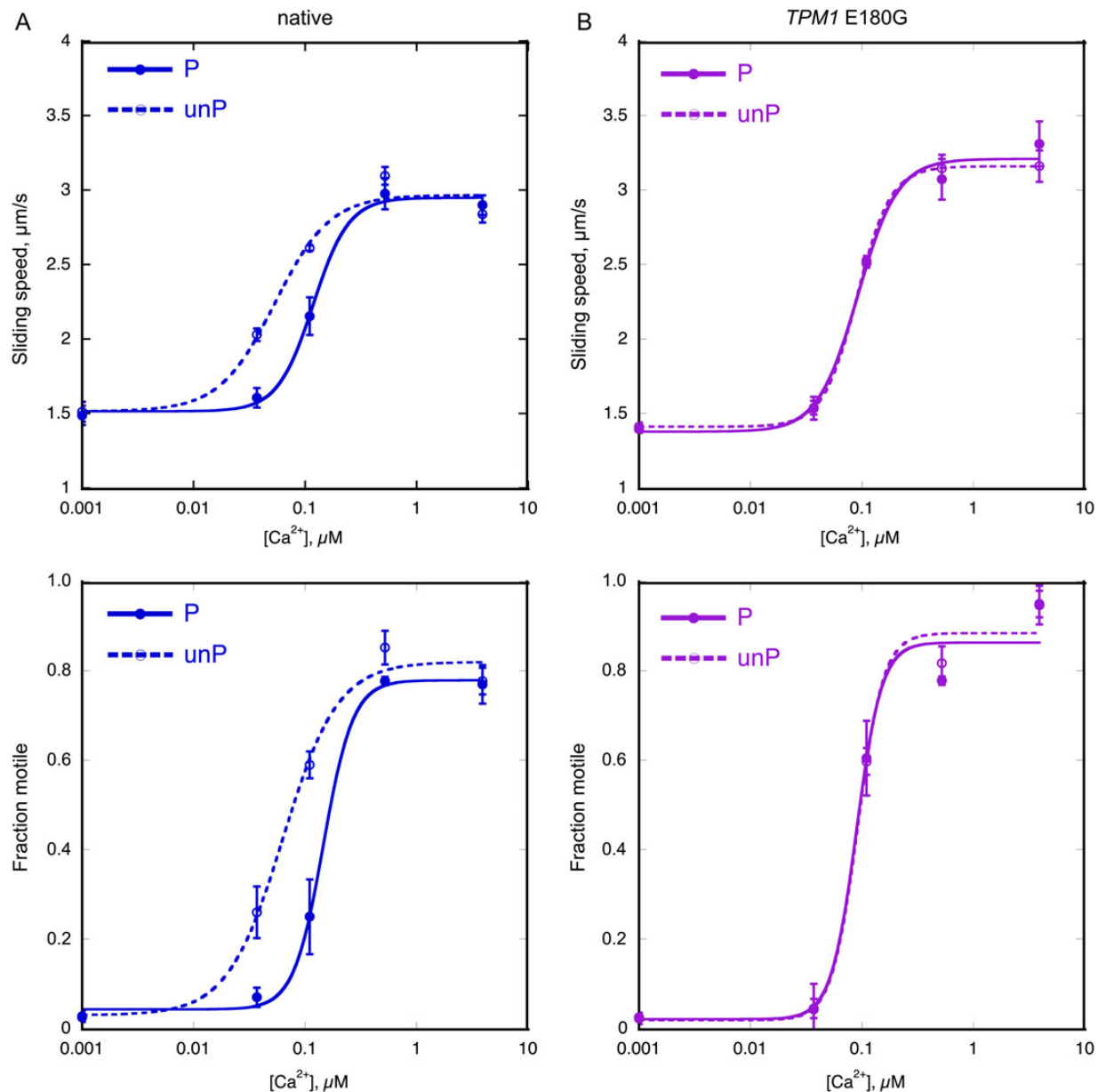


Figure 1 Relationship between EC_{50} and TnI phosphorylation in native human thin filaments and thin filaments containing HCM-causing mutation *TPM1* E180G. Sliding speed and fraction of filaments motile, measured in the same experiment by IVMA, are plotted against $[\text{Ca}^{2+}]$ for representative experiments. Raw data from one experiment are shown here; the mean values of EC_{50} from replicate experiments are shown in Table 2. Solid lines and points, phosphorylated troponin (P); dotted lines and open points, unphosphorylated troponin (unP). Error bars represent SEM of four measurements of motility in the same motility chamber. Blue, native thin filaments; purple, HCM-causing mutation *TPM1* E180G present. (A) Native thin filaments: phosphorylation increased EC_{50} (decreased Ca^{2+} sensitivity) but had no effect on the maximum sliding speed or fraction of filaments motile at saturating Ca^{2+} . (B) Thin filaments containing *TPM1* E180G HCM-causing mutation. The relationship of Ca^{2+} sensitivity to TnI phosphorylation is uncoupled.

all conditions; therefore, in this manuscript we have only shown the fraction motile parameter. Full data for both parameters are shown in Supplementary material online, SB.

Human heart troponin has a high level of TnI phosphorylation in the 1.4–1.8 mol Pi/mol range.³⁶ This is reduced to <0.3 mol Pi/mol by treatment with phosphatase, see Supplementary material online, SC. The Ca^{2+} sensitivity of unphosphorylated thin filaments is 1.88 ± 0.10 ($P < 0.0001$, $n = 16$) times greater than the native phosphorylated

thin filaments as reported previously.²⁹ n_{H} was significantly reduced from 2.11 ± 0.18 for phosphorylated to 1.72 ± 0.11 for unphosphorylated thin filaments, $P = 0.017$, $n = 16$. The maximum sliding speed was not significantly affected by phosphorylation level (Table 1, Figure 1A).

We have investigated thin filaments incorporating six DCM-causing mutations that have been shown to abolish this relationship (uncouple) in previous studies⁹ and three HCM mutations that uncouple *in vitro* (Figure 1B^{15,39}).

Table 1 Effect of EMD57033, Bepridil, and EGCG on Ca^{2+} regulation of motility

Treatment	EC_{50}	n_H	$\Delta V_{\max}$	EC_{50} , TPM1 E54K DCM mutation
Native				
P	0.16 ± 0.03 (4)	2.08 ± 0.14 (4)	1.40 ± 0.11 (4)	0.12 ± 0.02 (3)
unP	0.091 ± 0.014 (4) [†]	1.51 ± 0.25 (4) [†]	1.30 ± 0.04 (4)	0.11 ± 0.008 (3)
+EMD57033				
P	0.057 ± 0.010 (4)**	1.26 ± 0.07 (4)*	1.55 ± 0.08 (4)	0.041 ± 0.002 (3)*
unP	0.053 ± 0.010 (4)	1.34 ± 0.14 (4)	1.54 ± 0.08 (4)*	0.041 ± 0.002 (3)*
Native				
P	0.13 ± 0.007 (8)	2.89 ± 0.48 (8)	2.09 ± 0.14 (8)	0.13 ± 0.014 (3)
unP	0.072 ± 0.002 (6) ^{††}	1.96 ± 0.08 (6)	2.13 ± 0.20 (6)	0.13 ± 0.012 (3)
+Bepridil				
P	0.045 ± 0.001 (8)**	1.94 ± 0.08 (8)	2.40 ± 0.15 (8)**	0.060 ± 0.009 (3)*
unP	0.045 ± 0.001 (6)**	2.03 ± 0.13 (6)	2.24 ± 0.16 (6)	0.058 ± 0.006 (3)**
Native				
P	0.14 ± 0.03 (7)	1.61 ± 0.19 (7)	1.91 ± 0.18 (7)	0.11 ± 0.01 (5)
unP	0.059 ± 0.011 (6) ^{††}	1.50 ± 0.21 (6)	1.92 ± 0.22 (6)	0.11 ± 0.02 (5)
+EGCG				
P	0.26 ± 0.02 (7)**	1.96 ± 0.24 (7)	1.38 ± 0.12 (7)**	0.23 ± 0.03 (5)*
unP	0.15 ± 0.02 (6)** ^{††}	1.79 ± 0.30 (6)	1.52 ± 0.13 (6)*	0.071 ± 0.005 (5) ^{††}

Paired t-test was used for EC_{50} , n_H , and $\Delta V_{\max}$ (the change in velocity due to Ca^{2+}). EC_{50} values rounded to two significant figures. The number of experiments is given in brackets.

* $P < 0.05$, ** $P < 0.01$, for presence and absence of reagent.

[†] $P < 0.05$, ^{††} $P < 0.01$, unphosphorylated compared with phosphorylated.

3.2 EMD57033 and Bepridil increase Ca^{2+} sensitivity and uncouple TnI phosphorylation from changes in Ca^{2+} sensitivity

EMD57033 and Bepridil substantially increased the Ca^{2+} sensitivity (Figure 2A and D) and slightly increased the maximum sliding speed of native human thin filaments as expected.

The effect of EMD57033 on EC_{50} for Ca^{2+} activation of motility was biphasic with a maximum at 30 μM and an EC_{50} in the range of 15–20 μM (Figure 2C). Interestingly, we observed that 30 μM EMD57033 increased the Ca^{2+} sensitivity of thin filaments containing phosphorylated troponin more than unphosphorylated TnI (mean ratio EC_{50} P/ EC_{50} P + EMD57033 = 3.07 ± 0.71 , $P = 0.0001$, $n = 4$ in phosphorylated compared with a mean ratio of EC_{50} unP/ EC_{50} unP + EMD57033 = 1.83 ± 0.36 , $P = 0.07$, $n = 4$ in unphosphorylated, Table 1, Figure 2A, see Supplementary material online, SB). Consequently, in the presence of EMD57033, Ca^{2+} sensitivity of thin filaments was the same, independent of the level of phosphorylation (EC_{50} P + EMD57033/ EC_{50} unP + EMD57033 = 1.08 ± 0.03 , $P = 0.06$, $n = 4$). EMD57033 increased sliding speed slightly at saturating Ca^{2+} concentration in phosphorylated thin filaments and in unphosphorylated thin filaments by $18.4 \pm 4.1\%$ ($P = 0.02$, $n = 4$). n_H was slightly decreased in phosphorylated but not in unphosphorylated thin filaments. EMD57033 also increased the Ca^{2+} sensitivity of thin filaments containing the DCM-causing mutations, TPM1 E40K and E54K. In both these cases, Ca^{2+} sensitivity was independent of troponin phosphorylation (uncoupled) due to the mutation (Table 1, Figure 2B).

A similar pattern of results was observed with Bepridil. The dose–response curve was cooperative with a calculated EC_{50} of 52 μM (Figure 2F). At 100 μM , which is the saturated concentration, the increase

in Ca^{2+} sensitivity of phosphorylated donor thin filaments (measured by the mean ratio EC_{50} P/ EC_{50} P + Bepridil) was 2.8 ± 0.14 -fold for fraction motile ($P < 0.0001$, $n = 6$) and 3.09 ± 0.59 -fold for sliding speed ($P = 0.016$, $n = 6$), see Supplementary material online, SB.

Bepridil increased the Ca^{2+} sensitivity of thin filaments and also uncoupled Ca^{2+} sensitivity from TnI phosphorylation, as shown by the EC_{50} ratios of phosphorylated to dephosphorylated troponin (EC_{50} P/ EC_{50} unP = 1.73 ± 0.03 , $P < 0.0001$; EC_{50} P + Bepridil/ EC_{50} unP + Bepridil = 1.03 ± 0.01 , $P = 0.04$, $n = 6$) (Figure 2D, see Supplementary material online, SB). Bepridil increased sliding speed at saturating Ca^{2+} concentrations significantly in phosphorylated thin filaments ($14.8 \pm 3.2\%$, $P = 0.006$, $n = 6$) but did not have a significant effect in unphosphorylated thin filaments ($7.2 \pm 4.6\%$, $P = 0.18$, $n = 6$); it had no significant effect on n_H . Bepridil also reversibly increased the Ca^{2+} sensitivity of both phosphorylated and unphosphorylated thin filaments containing the DCM-causing TPM1 E54K mutation, and these mutant thin filaments remained uncoupled (Table 1, Figure 2E, see Supplementary material online, SE).

3.3 EGCG decreases Ca^{2+} sensitivity while retaining coupling

EGCG decreased Ca^{2+} sensitivity of human cardiac thin filaments measured by IVMA and at saturating concentrations (100 μM) reduced the maximum sliding speed up to 20% but had no significant effect on n_H (Table 1, Figure 3, see Supplementary material online, SB). Titration of EGCG at 0.074 μM Ca^{2+} yielded a cooperative dose–response curve with a calculated EC_{50} of 70 ± 7 μM for phosphorylated and 60 ± 1 for unphosphorylated (Figure 3C). Unlike EMD57033 and Bepridil, 100 μM EGCG had a similar effect on the EC_{50} of thin filaments with phosphorylated and unphosphorylated troponin, (mean ratio EC_{50} +

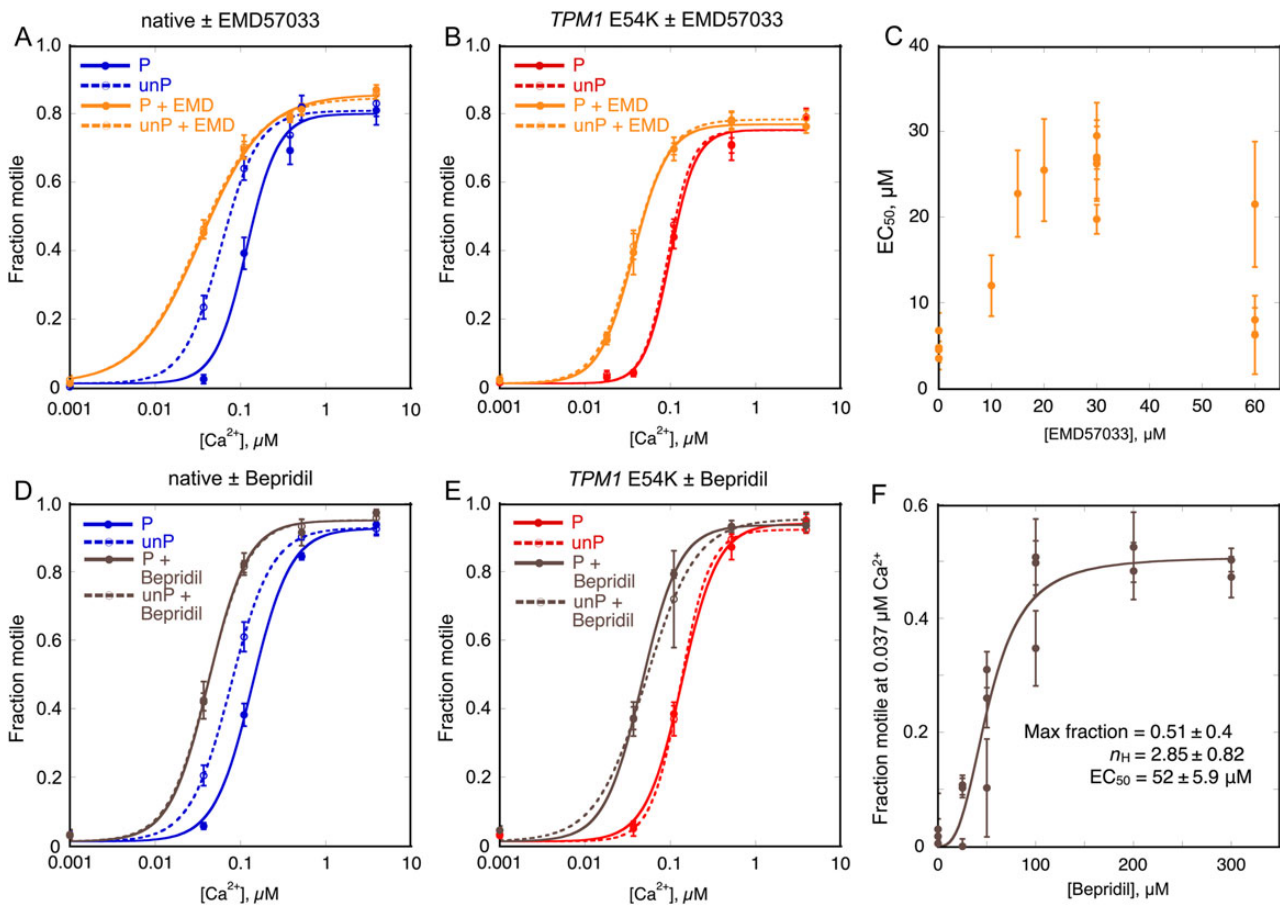


Figure 2 Effect of Ca^{2+} sensitizers on Ca^{2+} control of motility. (A, B, D and E) Fraction of filaments motile, measured by IVMA is plotted against $[\text{Ca}^{2+}]$ details as in Figure 1. (A and D) Effect of Ca^{2+} sensitizers on Ca^{2+} regulation of native thin filaments. Blue, native thin filaments; orange, the presence of $30 \mu\text{M}$ EMD57033; brown, the presence of $100 \mu\text{M}$ Bepridil. (B and E) Effect of Ca^{2+} sensitizers on Ca^{2+} regulation of thin filaments containing the TPM1 E54K DCM-causing mutation. Red, E54K-containing thin filaments; orange, the presence of $30 \mu\text{M}$ EMD57033; brown, the presence of $100 \mu\text{M}$ Bepridil. (C) EMD57033 dose-response curve: EC_{50} was determined at a range of EMD57033 concentrations. The change in EC_{50} ($\pm$ SEM of four measurements of motility in the same motility chamber) is plotted for three separate experiments. (F) Bepridil dose-response curve: motility was measured at a constant of $0.037 \mu\text{M}$ Ca^{2+} with increasing concentrations of Bepridil. The increase in fraction of filaments motile ($\pm$ SEM of four measurements of motility in the same motility chamber) is plotted for three separate experiments and the curve represents the fit of the pooled data to the Hill equation. Values of parameters obtained are shown.

EGCG/ EC_{50} control = 2.15 ± 0.45 -fold for phosphorylated and 2.80 ± 0.48 -fold for unphosphorylated thin filaments, see Supplementary material online, SB). Therefore, the coupling of TnI phosphorylation level to changes in Ca^{2+} sensitivity was retained (EC_{50} P/ EC_{50} unP = 2.24 ± 0.10 , $P < 0.0001$, $n = 6$; EC_{50} P + EGCG/ EC_{50} unP + EGCG = 1.73 ± 0.16 , $P = 0.006$, $n = 6$) (Table 1, Figure 3A, see Supplementary material online, SB).

3.4 EGCG restores coupling to HCM and DCM mutant thin filaments and myofibrils

EGCG also affected Ca^{2+} sensitivity in thin filaments containing mutations associated with HCM or DCM in a phosphorylation-dependent way. This was tested with five DCM-causing mutations and three HCM-causing mutations. EGCG decreased the Ca^{2+} sensitivity of phosphorylated mutant thin filaments in a similar way to wild-type troponin (ratio EC_{50} with/without EGCG was 1.36–2.85). However, with unphosphorylated troponin, EGCG tended to have no effect or, in the case of the three tropomyosin mutations, increased Ca^{2+}

sensitivity (Table 2). As a result, the dependence of Ca^{2+} sensitivity on TnI phosphorylation level was restored. For example, thin filaments containing the DCM-causing TPM1 mutation E54K were uncoupled with EC_{50} of $0.106 \pm 0.013 \mu\text{M}$ for phosphorylated and $0.114 \pm 0.019 \mu\text{M}$ for unphosphorylated giving a ratio of 0.95 ± 0.03 compared with a ratio of 2.24 ± 0.10 for wild-type thin filaments (Table 1). In the presence of $100 \mu\text{M}$ EGCG, the EC_{50} was $0.23 \pm 0.03 \mu\text{M}$ for phosphorylated and $0.071 \pm 0.005 \mu\text{M}$ for unphosphorylated giving a ratio of 3.17 ± 0.37 ($P = 0.004$, $n = 5$) (Table 1, Figure 3B). A dose-response assay for recoupling yielded an EC_{50} of $58.8 \pm 13.3 \mu\text{M}$ (Figure 3D). Similarly, EGCG was able to reversibly restore coupling of all the thin filament HCM and DCM-causing mutations in this study (Table 2, Figures 4 and 5, see Supplementary material online, SF).

3.5 The effect of EGCG on contraction of myofibrils

We studied the effect of EGCG on mouse myofibril contractility in basally phosphorylated and dephosphorylated states, obtained

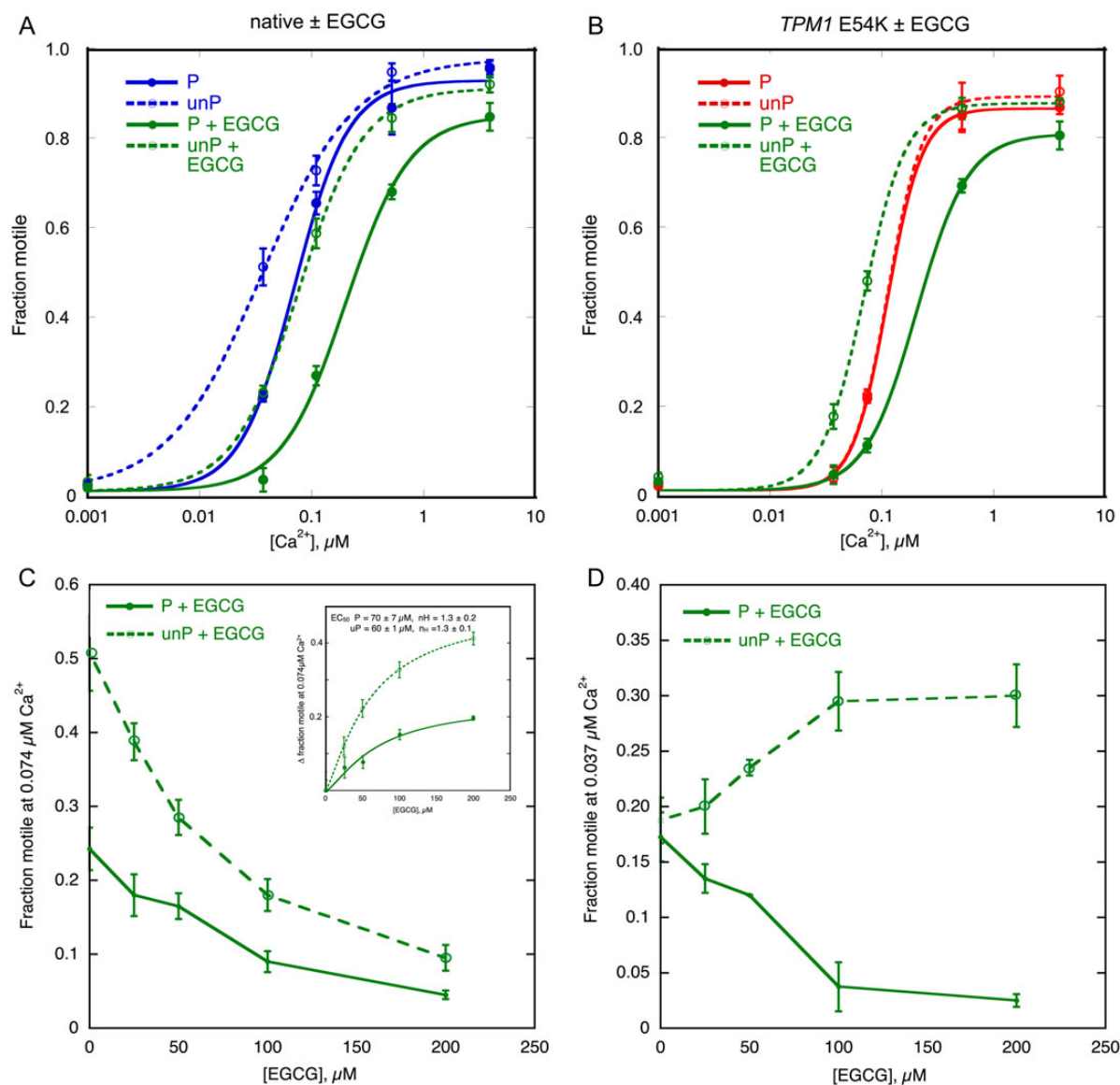


Figure 3 Effect of EGCG on Ca^{2+} control of motility. (A and B) Fraction of filaments motile, measured by IVMA, is plotted against $[\text{Ca}^{2+}]$, details as in Figure 1. Raw data from one experiment are shown here; the mean values of EC_{50} from replicate experiments are shown in Tables 1 and 2. (A) Effect of EGCG on Ca^{2+} regulation of native thin filaments. Blue, native thin filaments; green, the presence of $100 \mu\text{M}$ EGCG. (B) Effect of EGCG on Ca^{2+} regulation of thin filaments containing TPM1 E54K DCM-causing mutation. Red, E54K-containing thin filaments; green, the presence of $100 \mu\text{M}$ EGCG. (C and D) Dose-response curves, the fraction motile was measured at a constant $[\text{Ca}^{2+}]$ with increasing concentrations of EGCG for a representative experiment. (C) Effect of EGCG on donor thin filaments at $0.074 \mu\text{M} \text{Ca}^{2+}$. The inset plots the change in fraction motility with increasing EGCG concentration, and the curve represents the fit of the pooled data to the Hill equation. Values of parameters obtained are shown. (D) Effect of EGCG on thin filaments containing TPM1 E180G tropomyosin at $0.037 \mu\text{M} \text{Ca}^{2+}$. Initially, motility is the same (see Figure 1); the addition of EGCG reduced motility of phosphorylated and increased motility of unphosphorylated thin filaments, indicating recoupling or the restoration of the phosphorylation-dependent Ca^{2+} sensitivity difference (see Figure 3B). Details as in Figure 2F.

by treating mice with propranolol prior to sacrifice (Table 3, Figure 6).

We found that $10 \mu\text{M}$ EGCG decreased Ca^{2+} sensitivity of isometric force for both phosphorylated and unphosphorylated mouse myofibrils equally ($\text{EC}_{50} \text{ P}/\text{EC}_{50} \text{ P} + \text{EGCG} = 0.50 \pm 0.06$, $P < 0.001$, $n = 9$, $\text{EC}_{50} \text{ unP}/\text{EC}_{50} \text{ unP} + \text{EGCG} = 0.45 \pm 0.07$, $P < 0.00$, $n = 5$). Consequently, the effect of phosphorylation on Ca^{2+} sensitivity in wild-type myofibrils was preserved in the presence of EGCG ($\text{EC}_{50} \text{ P} + \text{EGCG}/\text{EC}_{50} \text{ unP} + \text{EGCG} = 1.64 \pm 0.24$, $P < 0.001$, $n = 5$).

As previously shown,¹⁰ in myofibrils from ACTC E361G DCM mice, the Ca^{2+} sensitivity of force is uncoupled (Table 3, Figure 6). EGCG decreased Ca^{2+} sensitivity in myofibrils from ACTC E361G mice; however, this effect was greater in phosphorylated myofibrils than unphosphorylated myofibrils. Thus, EGCG restored the phosphorylation-dependent shift in Ca^{2+} sensitivity for ACTC E361G myofibrils to the same level as in wild-type myofibrils ($\text{EC}_{50} \text{ P} + \text{EGCG}/\text{EC}_{50} \text{ unP} + \text{EGCG} = 1.62 \pm 0.20$, $P < 0.01$, $n = 5$ compared with 1.82 ± 0.24 for wild-type in the absence of EGCG). n_H values were similar to those found in wild-type

Table 2 Effect of EGCG and TnI phosphorylation on the Ca^{2+} sensitivity of thin filaments containing HCM and DCM mutations

Mutation	EC ₅₀ of thin filaments containing phosphorylated troponin $\mu\text{M} \pm \text{SEM}$	EC ₅₀ of thin filaments containing unphosphorylated troponin $\mu\text{M} \pm \text{SEM}$	Ratio EC ₅₀ P/unP $\pm \text{SEM}$	EC ₅₀ of thin filaments treated with EGCG containing phosphorylated troponin $\mu\text{M} \pm \text{SEM}$	EC ₅₀ of thin filaments treated with EGCG containing unphosphorylated troponin $\mu\text{M} \pm \text{SEM}$	Ratio EC ₅₀ P + EGCG/unP + EGCG $\pm \text{SEM}$	Ratio EC ₅₀ P + EGCG/P $\pm \text{SEM}$	Ratio EC ₅₀ unP + EGCG/unP $\pm \text{SEM}$
WT	0.14 $\pm$ 0.03 (7)	0.059 $\pm$ 0.011 (6) ^{††}	2.24 $\pm$ 0.10 (6) ^{††}	0.26 $\pm$ 0.02 (7)**	0.15 $\pm$ 0.02 (6)** ^{††}	1.73 $\pm$ 0.16 (6) ^{††}	2.15 $\pm$ 0.45(7)	2.80 $\pm$ 0.48(6)
DCM								
TPM1 E54K	0.11 $\pm$ 0.013 (5)	0.11 $\pm$ 0.02 (5)	0.95 $\pm$ 0.03 (5)	0.23 $\pm$ 0.03 (5)*	0.071 $\pm$ 0.005 (5) ^{††}	3.17 $\pm$ 0.37 (5) ^{††}	2.33 $\pm$ 0.53(5)	0.68 $\pm$ 0.10(5)
TPM1 E40K	0.18 $\pm$ 0.03 (8)	0.17 $\pm$ 0.03 (8)	1.02 $\pm$ 0.007 (8)	0.22 $\pm$ 0.04 (5)	0.052 $\pm$ 0.01 (5)* [†]	4.63 $\pm$ 0.96 (5) [†]	1.70 $\pm$ 0.43(3)	0.34 $\pm$ 0.05(3)
TNNC1 G159D	0.092 $\pm$ 0.004 (5)	0.095 $\pm$ 0.005 (5)	0.97 $\pm$ 0.03 (5)	0.19 $\pm$ 0.03 (5)*	0.088 $\pm$ 0.005 (5)* [†]	2.25 $\pm$ 0.41 (5) [†]	2.13 $\pm$ 0.36(5)	0.93 $\pm$ 0.02(5)
TNNI3 K36Q	0.18 $\pm$ 0.03 (10)	0.18 $\pm$ 0.04 (10)	1.02 $\pm$ 0.05 (10)	0.19 $\pm$ 0.02 (5)	0.10 $\pm$ 0.009 (5)* [†]	1.97 $\pm$ 0.12 (5) [†]	2.32 $\pm$ 0.29(3)	1.28 $\pm$ 0.04(3)
ACTC E361G	0.087 $\pm$ 0.002 (5)	0.080 $\pm$ 0.002 (5)	1.08 $\pm$ 0.02 (5)	0.20 $\pm$ 0.02 (5)**	0.081 $\pm$ 0.002 (5) ^{††}	2.41 $\pm$ 0.28 (5) ^{††}	2.27 $\pm$ 0.28(5)	1.01 $\pm$ 0.02(5)
HCM								
TPM1 E180G	0.086 $\pm$ 0.013 (3)	0.087 $\pm$ 0.011 (3)	0.98 $\pm$ 0.02 (3)	0.11 $\pm$ 0.01 (5)*	0.048 $\pm$ 0.003 (5)* [†]	2.41 $\pm$ 0.32 (5) [†]	1.36 $\pm$ 0.10(3)	0.51 $\pm$ 0.06(3)
TNNT2 K280N	0.11 $\pm$ 0.007 (5)	0.10 $\pm$ 0.004 (5)	1.08 $\pm$ 0.04 (5)	0.20 $\pm$ 0.03 (5)*	0.10 $\pm$ 0.004 (5) [†]	1.96 $\pm$ 0.24 (5) [†]	2.15 $\pm$ 0.18(3)	1.09 $\pm$ 0.006(3)
ACTC E99K	0.074 $\pm$ 0.005 (5)	0.074 $\pm$ 0.005 (5)	0.99 $\pm$ 0.02 (5)	0.21 $\pm$ 0.03 (5)**	0.10 $\pm$ 0.007 (5)* ^{††}	2.03 $\pm$ 0.21 (5) ^{††}	2.85 $\pm$ 0.28(5)	1.43 $\pm$ 0.13(5)

Ratios: single value t-test compared with 1. ANOVA analysis of this data set is shown in Supplementary material online, SE. EC₅₀ values rounded to two significant figures. The number of experiments is given in brackets.

* $P < 0.05$, ** $P < 0.01$, for the presence and absence of EGCG using paired t-test.

[†] $P < 0.05$, ^{††} $P < 0.01$, unphosphorylated compared with phosphorylated using paired t-test.

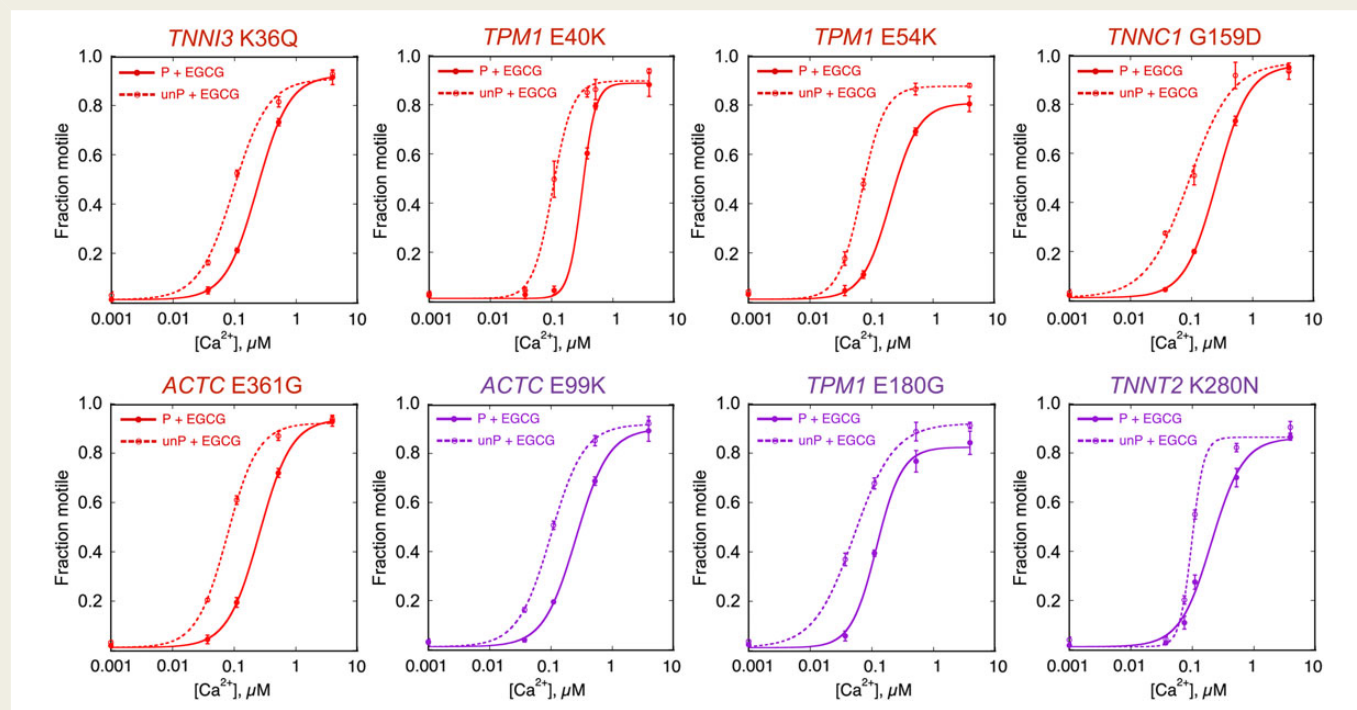


Figure 4 Coupling is restored to HCM and DCM mutations by EGCG. Fraction of filaments motile, measured by IVMA in the presence of 100 μM EGCG, is plotted against $[\text{Ca}^{2+}]$ for representative single experiments. Details as in Figure 1. Red lines, thin filaments containing DCM-causing mutations; Purple lines, thin filaments containing HCM-causing mutations. The mean values of EC_{50} from replicate experiments are plotted in Figure 5 and summarized in Table 2.

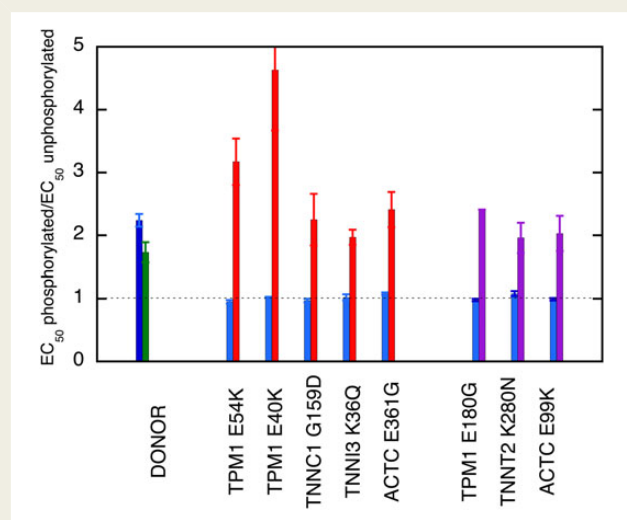


Figure 5 The re-coupling effect of EGCG. The ratio of EC_{50} phosphorylated/ EC_{50} unphosphorylated (coupling constant) is plotted in the absence (blue) and presence (green, red, and purple) of 100 μM EGCG. Green, control troponin; red, DCM-causing mutants; purple, HCM-causing mutants, error bars are $\pm$ SEM. All the mutant samples are uncoupled, but the coupling constant is restored to control levels by EGCG. Data from Table 2.

myofibrils (Table 3). The duration of the initial, nearly isotonic, phase of relaxation, t_{LIN} , in phosphorylated and unphosphorylated ACTC E361G myofibrils was restored to wild-type values (Table 3). EGCG decreased the rate of force development (k_{ACT}) but did not affect maximum force.

k_{ACT} was decreased by EGCG in unphosphorylated wild-type myofibrils (decreased by 22.8%, $P = 0.059$, $n = 15$) as well as in phosphorylated and unphosphorylated ACTC E361G (decreased by 23%, $P < 0.05$, $n = 10$ and by 21%, $P < 0.001$, respectively, $n = 11$).

4. Discussion

In previous studies, it was found that mutations in proteins of the cardiac muscle thin filament that are associated with inherited cardiomyopathies (HCM and DCM) alter myofibrillar Ca^{2+} sensitivity. They also cause the modulation of myofilament Ca^{2+} sensitivity to become independent of the PKA-dependent phosphorylation of TnI. We have named this phenomenon uncoupling. Moreover, this uncoupling effect may be sufficient to generate the disease phenotype of familial DCM.^{9,17} We have investigated whether small molecules might be able to mimic or reverse the molecular effects of mutations.

The Ca^{2+} sensitizers EMD57033 and Bepridil, known to bind to TnC, induce uncoupling in wild-type thin filaments, thus mimicking the effects of HCM mutations. The Ca^{2+} desensitizer EGCG has an opposite effect. It preserves coupling in wild-type troponin and restores coupling to thin filaments with HCM- and DCM-causing mutations in TnI, TnC, TnT, tropomyosin, and actin, thus antagonizing the effect of the HCM or DCM mutation. These findings suggest the potential of EGCG for treating the symptoms of inherited cardiomyopathies.

4.1 Ca^{2+} sensitizers mimic the effects of HCM-causing mutations in thin filaments

Both EMD57033 and Bepridil increase Ca^{2+} sensitivity of phosphorylated thin filaments by 2.8- and 2.9-fold, measured by quantitative

Table 3 Effect of phosphorylation, ACTC E361G mutation, and EGCG on Ca ²⁺ regulation of myofibril contraction						
	<i>F</i> _{max} (kN/m ²)	<i>EC</i> ₅₀ (μM)	<i>n</i> _H	<i>k</i> _{ACT} (s ^{−1})	<i>t</i> _{LIN} (ms)	<i>k</i> _{REL} (s ^{−1})
WT ^a						
P	100.9 ± 6.3 (14)	0.93 ± 0.06 (11)	10.43 ± 1.84 (10)	4.16 ± 0.43 (11)	50.8 ± 3.5 (12)	35.0 ± 4.0 (10)
unP	87.1 ± 6.0 (16)	0.51 ± 0.06 (14) ^{†††}	4.74 ± 0.66 (13) ^{††}	4.50 ± 0.24 (15)	67.0 ± 4.2 (16) [†]	23.2 ± 2.8 (11) ^{†††}
WT + EGCG						
P	92.5 ± 5.0 (15)	1.85 ± 0.17 (9) ^{***}	4.30 ± 0.85 (8) ^{**}	4.44 ± 0.24 (15)	46.7 ± 3.9 (12)	41.0 ± 4.0 (12)
unP	92.6 ± 8.9 (8)	1.13 ± 0.13 (5) ^{††***}	5.08 ± 1.24 (5)	3.47 ± 0.55 (6)	88.9 ± 9.9 (7) ^{†††*}	22.3 ± 1.2 (7) ^{††}
E361G ^a						
P	93.5 ± 8.9 (11)	0.38 ± 0.05 (12) ^{§§§}	5.39 ± 1.26 (9) [§]	4.51 ± 0.32 (13)	75.4 ± 6.5 (10) ^{§§}	21.6 ± 2.8 (10) ^{§§§}
unP	89.3 ± 8.6 (10)	0.43 ± 0.06 (10)	4.48 ± 0.70 (8)	4.12 ± 0.32 (11)	70.8 ± 9.7 (11)	21.6 ± 2.1 (10)
E361G + EGCG						
P	89.1 ± 4.5 (12)	2.07 ± 0.20 (5) ^{***†††}	7.18 ± 1.54 (5)	3.43 ± 0.20 (13) ^{*\$††}	49.7 ± 3.3 (12) ^{**}	31.0 ± 2.6 (12) ^{*\$}
unP	96.3 ± 11.1 (11)	1.28 ± 0.10 (8) ^{††***†††}	4.14 ± 0.42 (7) [†]	3.25 ± 0.26 (11) ^{***††}	63.0 ± 4.7 (12) ^{†\$}	31.0 ± 2.0 (12) ^{**\$§\$††}

The number of experiments is given in brackets. Statistical analysis carried out by un-paired t-test (equal variance).

^aVikhorev et al.¹¹
P* < 0.05, *P* < 0.01, ****P* < 0.001, EGCG treated compared with no EGCG.
[†]*P* < 0.05, ^{††}*P* < 0.01, ^{†††}*P* < 0.001, unphosphorylated compared with phosphorylated.
[§]*P* < 0.05, ^{§§}*P* < 0.01, ^{§§§}*P* < 0.001, ACTC E361G compared with wild type.
[‡]*P* < 0.05, ^{‡†}*P* < 0.01, ^{‡††}*P* < 0.001, ACTC E361G EGCG treated compared with no EGCG wild-type.

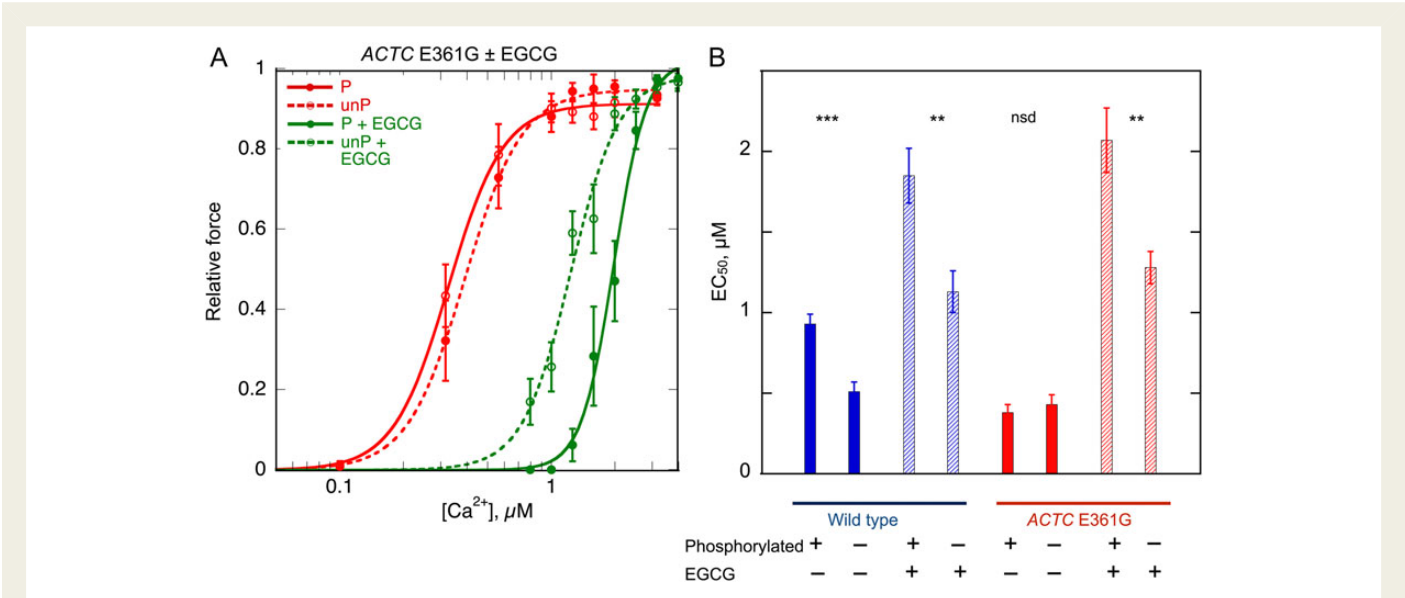


Figure 6 EGCG restores the relationship between Ca²⁺ sensitivity of force production and TnI phosphorylation in wild-type and ACTC E361G mouse myofibrils. Phosphorylation level was reduced by propranolol treatment and measured by phosphate affinity SDS–PAGE. Basally phosphorylated wild-type was 1.02 ± 0.03 and ACTC E361G was 1.08 ± 0.01 mol Pi/mol TnI. For propranolol-treated (dephosphorylated) muscle, the phosphorylation level was 0.30 ± 0.04 in wild type and 0.34 ± 0.07 mol Pi/mol TnI for ACTC E361G. (A) Ca²⁺ activation curves for ACTC E361G myofibrils in the presence (green) and absence (red) of 10 μM EGCG. Solid lines and points, phosphorylated troponin (P); dotted lines and open points, unphosphorylated troponin (unP). The plot shows averaged isometric tension ± SEM from 11 to 14 myofibrils for experiments performed at SL = 2.17 μm. EGCG shifts the activation curve to the right and restores the difference between phosphorylated and unphosphorylated myofibrils. (B) Effects of the ACTC E361G mutation, phosphorylation, and EGCG on the mean *EC*₅₀ (± SEM) for myofibril isometric contraction. Significant differences, calculated by *t*-test, between phosphorylated and unphosphorylated myofibrils are indicated: ***P* < 0.01; ****P* < 0.001. Data from Table 3.

IVMA, in common with previous measurements.^{40,41} However, it has not been shown before that they increase Ca²⁺ sensitivity of unphosphorylated thin filaments considerably less than phosphorylated thin filaments. As a consequence, Ca²⁺ sensitivity becomes independent of the TnI phosphorylation level (uncoupled).

The effect of Ca²⁺ sensitizers is analogous to the effect of HCM-causing mutations *in vitro* (Table 2) where both an increase in the Ca²⁺ sensitivity and uncoupling of the Ca²⁺ sensitivity from the TnI phosphorylation are observed. Uncoupling due to HCM mutations was first reported in 2001¹³ and several more thin filament mutations

have subsequently been demonstrated by *in vitro* assays,^{5,17} as the TPM1 E180G mutation, shown in Figure 1B, demonstrates.

4.2 Mechanistic considerations of Ca²⁺ sensitizers

Since both EMD57033 and Bepridil appear to modulate Ca²⁺ sensitivity with a minimal effect on the sliding speed in IVMA or maximum isometric force in muscle fibres,^{10,42} it is likely that they are acting on troponin rather than the cross-bridge cycling mechanism in our systems.

The Ca²⁺ sensitivity of cardiac troponin is modulated by the unique N-terminal peptide of TnI (1–30) that contains the PKA phosphorylation sites, Ser22 and 23. When unphosphorylated, the peptide interacts with the N-terminal domain of TnC, close to the regulatory Ca²⁺-binding site and this interaction is lost or reduced when the two serines are phosphorylated.^{43,44} The change in Ca²⁺ sensitivity with TnI phosphorylation is a two- to three-fold reduction.

Bepridil is suggested to displace TnI 1–30 and/or the linker helix TnI 31–70.^{20,45} EMD57033 is believed to bind to the C-terminal domain of TnC and also displaces the TnI 31–70 peptide.^{18,19,46} Thus, both these compounds could act allosterically by interfering with the modulation of Ca²⁺ sensitivity due to Ser22/23 phosphorylation, resulting in uncoupling.

Interestingly, in experiments measuring isometric force in cardiac muscle myofibrils, EMD57033 increased Ca²⁺ sensitivity but only caused uncoupling at short sarcomere lengths. Thus, the uncoupling phenomenon may be graded rather than all-or-none. One possibility is that uncoupling may be related to Ca²⁺ sensitivity if the Ca²⁺ sensitizer shifts the conformational equilibrium so far towards the N-terminal bound conformation that it cannot be significantly influenced by phosphorylation of the N-terminal peptide of TnI (see Supplementary material online, SD). However, the Ca²⁺-sensitizing property of these compounds does not necessarily involve the same molecular mechanism as uncoupling.

The actions of small molecules that uncouple highlight the crucial role of the allosteric coupling between the TnI 1–30 peptide and ligand-binding sites that could be remotely located. Likewise, it is notable that DCM-causing mutations that uncouple are distributed in all the proteins of the thin filament, also demonstrating long-range allosteric interactions.^{9,47} We have proposed that these mutations destabilize the unphosphorylated state of the 1–30 peptide.⁹

4.3 The Ca²⁺ desensitizer EGCG restores coupling to thin filaments with HCM and DCM mutations

Our observation that EGCG reduces Ca²⁺ sensitivity two- to three-fold both in human thin filaments and in mouse myofibrils with only small effects on maximum sliding speed, isometric force or Hill coefficient, is in accord with previous measurements in skinned cardiac muscle fibres.^{21,23} Moreover, we have demonstrated that the phosphorylation-dependent shift in myofilament Ca²⁺ sensitivity is unaffected by EGCG in both isolated filaments and intact myofibrils, in contrast to the Ca²⁺ sensitizers (Figures 2, 3, and 6).

EGCG was also able to decrease the Ca²⁺ sensitivity of phosphorylated thin filaments containing HCM- and DCM-causing mutations; however, it had different effects on phosphorylated and unphosphorylated thin filaments. With phosphorylated troponin, EGCG decreased Ca²⁺ sensitivity similarly to wild-type troponin, but EGCG either had

no effect or increased Ca²⁺ sensitivity with unphosphorylated troponin (Table 2). This resulted in the restoration of the coupling of Ca²⁺ sensitivity change to TnI phosphorylation (Figures 3, 4, and 5).

Several studies have located EGCG binding in the C-terminal domain of TnC in the region of the hydrophobic cleft.^{21,22,46} Molecular dynamics (MD) simulation suggests that it can bind in several interchangeable orientations,⁴⁶ and unlike EMD57033, EGCG can bind to the C-terminal domain of cTnC even in the presence of TnI 34–71 helix.⁴⁶ We suggest that it is possible that EGCG can re-stabilize the TnI–TnC interaction, restoring the Ca²⁺ response to phosphorylation found in DCM and HCM. MD calculations indicate that the uncoupling mutation cTnC G159D strengthens TnI 34–71 helix binding to the hydrophobic cleft while EGCG weakens this interaction,⁴⁶ compatible with their opposite effects on coupling.

4.4 Effects of EGCG on myofibrillar contractility

EGCG decreased Ca²⁺ sensitivity in phosphorylated and unphosphorylated wild-type myofibrils by approximately two-fold but did not change the relaxation parameters t_{LIN} and k_{REL} in phosphorylated myofibrils (Table 3). The rate of force development (k_{ACT}), measured at high Ca²⁺, was unchanged in myofibrils with phosphorylated TnI and slightly decreased ($P = 0.059$) in unphosphorylated myofibrils. As k_{ACT} depends strongly on the Ca²⁺ concentration,¹⁰ we conclude that EGCG shifts the $[Ca^{2+}]$ – k_{ACT} relationship towards higher Ca²⁺ concentration in agreement with IVMA data (Table 1). Thus, unlike EMD57033, EGCG decreases cross-bridge activation kinetics.¹⁰

EGCG reduced Ca²⁺ sensitivity and k_{ACT} in both phosphorylated and unphosphorylated ACTC E361G myofibrils. In addition, it restored the lost difference in Ca²⁺ sensitivity between phosphorylated and unphosphorylated myofibrils and also the difference in the relaxation parameter t_{LIN} (Table 3). The observation that EGCG does not affect the EC₅₀ P/EC₅₀ unP or t_{LIN} in wild-type myofibrils but changes them in ACTC E361G suggests that EGCG can restore coupling in ACTC E361G myofibrils independently of its Ca²⁺-desensitizing function.

Although uncoupling can be clearly demonstrated at the level of skinned muscle fibres or myocytes with thin filament mutations causing DCM,^{35,48,49} this does not appear to be true for HCM-causing mutations, despite being indistinguishable at the single filament level. A near-normal response of Ca²⁺ sensitivity to TnI phosphorylation has been reported for *TNNC1* L29Q and *TPM1* E180G in transgenic mice^{50,51} and *TNNI3* R145W and *TNNT2* K280N in human heart myectomy samples.¹⁶ Despite this, both DCM- and HCM-linked mutations in transgenic mice models are associated with an impaired response to β 1-adrenergic stimulation and reduced cardiac reserve as would be expected if uncoupled^{15,35} (reviewed by Messer¹⁷). The physiological manifestations of the uncoupling seen in unloaded filaments require further investigation.

4.5 Clinical relevance of re-coupling by EGCG

Ca²⁺ antagonists have been suggested as being potentially useful for treatment of HCM.⁵² EGCG represents a new class of Ca²⁺ antagonists with a very favourable functional profile. EGCG acts directly on the Ca²⁺ regulatory system of the thin filament that is also the main target of HCM-causing mutations in sarcomeric proteins.⁵ By binding to troponin, it decreases the enhanced Ca²⁺ sensitivity characteristic of HCM while also reversing the uncoupling effect we observed in

HCM mutations. The ability to restore coupling to DCM mutant myofibrils could be beneficial, since this was proposed to be the primary cause of DCM, but the reduction in Ca^{2+} sensitivity in cases where the mutation also reduces Ca^{2+} sensitivity may be deleterious.

EGCG has a wide range of pharmacological properties; indeed, it has been cited as an example of a promiscuous molecule.⁵³ Moreover, low doses of EGCG have a positive inotropic effect in intact heart muscle, due to effects on the ryanodine receptor type 2, Na^+ - K^+ ATPase and Na^+ - Ca^{2+} exchanger, that may override the Ca^{2+} -desensitizing effect in the contractile apparatus.^{54,55}

Although EGCG is too non-specific to be a viable drug for treating HCM, this study has demonstrated a significant proof of principle: it is possible to directly reverse the molecular mechanism of HCM-causing mutations pharmacologically. The coupling of Ca^{2+} sensitivity to cTnI phosphorylation has been demonstrated to be a labile property of troponin. HCM or DCM mutations and Ca^{2+} sensitizers can induce uncoupling, indicating that small perturbations can destabilize troponin.^{9,17,32} It is very unusual to find a reagent that will give a gain of function by apparently restoring the native conformational state in the presence of mutations that destabilized this state.

Our findings provide a starting point for investigating molecules related to EGCG that may be more efficacious and act specifically on troponin.⁵⁶

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We are grateful to Cristobal Dos Remedios (University of Sydney, Sydney, Australia) for the donor heart muscle, *TNNI3* K36Q DCM mutant and *TNNI2* K280N HCM mutant explanted human heart muscle samples. Kristen Nowak, Elyshia McNamara, and Royston Ong (University of Western Australia, Perth, Australia) for the baculovirus-expressed mutant *Tpm1.1* samples. O'Neal Copeland for technical help and Ian Gould (Chemistry Department, Imperial College London, UK) for help with the structure of troponin and Molecular Dynamics simulations. EMD57033 was a gift from E Merck KGaA, Darmstadt.

Conflict of interest: none declared.

Funding

This work was supported by grants from the British Heart Foundation (RG/11/20/29266 and FS/12/24/29568).

References

- Elliott P, McKenna WJ. Hypertrophic cardiomyopathy. *Lancet* 2004;**363**:1881–1891.
- Seidman JG, Seidman C. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell* 2001;**104**:557–567.
- Hershberger E, Hedges DJ, Morales A. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. *Nat Rev Cardiol* 2013;**10**:531–547.
- Kamisago M, Sharma SD, DePalma SR, Solomon S, Sharma P, Smoot L, Mullen MP, Woolf PK, Wigle ED, Seidman JG, Seidman CE, Jarcho J, Shapiro LR. Mutations in sarcomere protein genes as a cause of dilated cardiomyopathy. *N Engl J Med* 2000;**343**:1688–1696.
- Marston SB. How do mutations in contractile proteins cause the primary familial cardiomyopathies? *J Cardiovasc Transl Res* 2011;**4**:245–255.
- Morimoto S. Sarcomeric proteins and inherited cardiomyopathies. *Cardiovasc Res* 2008;**77**:659–666.
- Knollmann BC, Potter JD. Altered regulation of cardiac muscle contraction by troponin T mutations that cause familial hypertrophic cardiomyopathy. *Trends Cardiovasc Med* 2001;**11**:206–212.
- Chang AN, Potter JD. Sarcomeric protein mutations in dilated cardiomyopathy. *Heart Fail Rev* 2005;**10**:225–235.
- Memo M, Leung M-C, Ward DG, dos Remedios C, Morimoto S, Zhang L, Ravenscroft G, McNamara E, Nowak KJ, Marston SB, Messer AE. Mutations in thin filament proteins that cause familial dilated cardiomyopathy uncouple troponin I phosphorylation from changes in myofibrillar Ca^{2+} -sensitivity. *Cardiovasc Res* 2013;**99**:65–73.
- Vikhorev PG, Song W, Wilkinson R, Copeland O, Messer AE, Ferenczi MA, Marston SB. The dilated cardiomyopathy-causing mutation ACTC E361G in cardiac muscle myofibrils specifically abolishes modulation of Ca^{2+} regulation by phosphorylation of troponin I. *Biophys J* 2014;**107**:2369–2380.
- Schmidtman A, Lindow C, Villard S, Heuser A, Mügge A, Gessner R, Granier C, Jaquet K. Cardiac troponin C-L29Q, related to hypertrophic cardiomyopathy, hinders the transduction of the protein kinase A dependent phosphorylation signal from cardiac troponin I to C. *FEBS J* 2005;**272**:6087–6097.
- Dong W, Xing J, Ouyang Y, An J, Cheung HC. Structural kinetics of cardiac troponin C mutants linked to familial hypertrophic and dilated cardiomyopathy in troponin complexes. *J Biol Chem* 2008;**283**:3424–3432.
- Deng Y, Schmidtman A, Redlich A, Westerdorf B, Jaquet K, Thieleczek R. Effects of phosphorylation and mutation R145G on human cardiac troponin I function. *Biochemistry* 2001;**40**:14593–14602.
- Wang Y, Pinto J, Sancho Solis R, Dweck D, Liang J, Diaz-Perez Z, Ge Y, Walker J, Potter J. The generation and functional characterization of knock in mice harboring the cardiac troponin I-R21C mutation associated with hypertrophic cardiomyopathy. *J Biol Chem* 2011;**287**:2156–2167.
- Song W, Dyer E, Stuckey D, Copeland O, Leung M, Bayliss C, Messer AE, Wilkinson R, Tremoleda J, Schneider M, Harding S, Redwood C, Clarke K, Nowak K, Monserrat L, Wells D, Marston S. Molecular mechanism of the Glu99Lys mutation in cardiac actin (ACTC gene) that causes apical hypertrophy in man and mouse. *J Biol Chem* 2011;**286**:27582–27593.
- Sequeira V, Wijner PJM, Nienkamp LLAM, Kuster DWD, Najafi A, Witjas-Paalberends ER, Regan JA, Boontje N, ten Cate FJ, Germans T, Carrier L, Sadayappan S, van Slegtenhorst MA, Zaremba R, Foster DB, Murphy AM, Poggesi C, Dos Remedios C, Stienen GJM, Ho CY, Michels M, van der Velden J. Perturbed length-dependent activation in human hypertrophic cardiomyopathy with missense sarcomeric gene mutations. *Circ Res* 2013;**112**:1491–1505.
- Messer A, Marston S. Investigating the role of uncoupling of troponin I phosphorylation from changes in myofibrillar Ca^{2+} -sensitivity in the pathogenesis of cardiomyopathy. *Front Physiol* 2014;**5**:315.
- Li MX, Robertson IM, Sykes BD. Interaction of cardiac troponin with cardiotonic drugs: a structural perspective. *Biochem Biophys Res Commun* 2008;**369**:88–99.
- Wang X, Li MX, Spyrapoulos L, Beier N, Chandra M, Solaro RJ, Sykes BD. Structure of the C-domain of human cardiac troponin C in complex with the Ca^{2+} sensitizing drug EMD 57033. *J Biol Chem* 2001;**276**:25456–25466.
- Abusamhadneh E, Abbott MB, Dvoretzky A, Finley N, Sasi S, Rosevear PR. Interaction of Bepridil with the cardiac troponin C/troponin I complex. *FEBS Lett* 2001;**506**:51–54.
- Liou Y-M, Kuo S-C, Hsieh S-R. Differential effects of a green tea-derived polyphenol (-)-Epigallocatechin-3-gallate on the acidosis-induced decrease in the Ca^{2+} sensitivity of cardiac and skeletal muscle. *Pflügers Arch* 2008;**456**:787–800.
- Robertson IM, Li MX, Sykes BD. Solution structure of human cardiac troponin C in complex with the green tea polyphenol, (-)-Epigallocatechin 3-gallate. *J Biol Chem* 2009;**284**:23012–23023.
- Tadano N, Du C, Yumoto F, Morimoto S, Ohta M, Xie M, Nagata K, Zhan D, Lu Q, Miwa Y, Takahashi-Yanaga F, Tanokura M, Ohtsuki I, Sasaguri T. Biological actions of green tea catechins on cardiac troponin C. *Brit J Pharmacol* 2010;**161**:1034–1043.
- Takeda N, Yamashita A, Maeda K, Maeda Y. Structure of the core domain of human cardiac troponin in the Ca^{2+} -saturated form. *Nature* 2003;**424**:35–41.
- Solaro RJ, Rosevear P, Kobayashi T. The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. *Biochem Biophys Res Commun* 2008;**369**:82–87.
- Howarth JW, Meller J, Solaro RJ, Trewheella J, Rosevear PR. Phosphorylation-dependent conformational transition of the cardiac specific N-extension of troponin I in cardiac troponin. *J Mol Biol* 2007;**373**:706–722.
- Gould I, Messer AE, Papadaki M, Marston SB. Modulation of the interaction between troponin I N-terminal peptide and troponin C by phosphorylation studied by molecular dynamics. *Biophys J* 2014;**106**:349a.
- Cheng Y, Lindert S, Keken-Huskey P, Rao VS, Solaro RJ, Rosevear PR, Amaro R, McCulloch AD, McCammon JA, Regnier M. Computational studies of the effect of the S23D/S24D troponin I mutation on cardiac troponin structural dynamics. *Biophys J* 2014;**107**:1675–1685.
- Messer AE, Jacques AM, Marston SB. Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. *J Mol Cell Cardiol* 2007;**42**:247–259.
- Dyer E, Jacques A, Hoskins A, Ward D, Gallon C, Messer A, Kaski J, Burch M, Kentish J, Marston S. Functional analysis of a unique troponin C mutation, Gly159Asp that causes familial dilated cardiomyopathy, studied in explanted heart muscle. *Circ Heart Fail* 2009;**2**:456–464.

31. Carballo S, Robinson P, Otway R, Fatkin D, Jongbloed JD, de Jonge N, Blair E, van Tintelen JP, Redwood C, Watkins H. Identification and functional characterization of cardiac troponin I as a novel disease gene in autosomal dominant dilated cardiomyopathy. *Circ Res* 2009;**105**:375–382.
32. Bayliss CR, Jacques AM, Leung M-C, Ward DG, Redwood CS, Gallon CE, Copeland O, McKenna WJ, Dos Remedios C, Marston SB, Messer AE. Myofibrillar Ca^{2+} -sensitivity is uncoupled from troponin I phosphorylation in hypertrophic obstructive cardiomyopathy due to abnormal troponin T. *Cardiovasc Res* 2012;**97**:500–508.
33. Akkari PA, Song Y, Hitchcock-DeGregori S, Blechyniden L, Laing N. Expression and biological activity of Baculovirus generated wild-type human slow alpha tropomyosin and the Met9Arg mutant responsible for a dominant form of nemaline myopathy. *Biochem Biophys Res Commun* 2002;**296**:300–304.
34. Marston S, Memo M, Messer A, Papadaki M, Nowak K, McNamara E, Ong R, El-Mezgueldi M, Li X, Lehman W. Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients. *Hum Mol Genet* 2013;**22**:4978–4987.
35. Song W, Dyer E, Stuckey D, Leung M-C, Memo M, Mansfield C, Ferenczi M, Liu K, Redwood C, Nowak K, Harding S, Clarke K, Wells D, Marston S. Investigation of a transgenic mouse model of familial dilated cardiomyopathy. *J Mol Cell Cardiol* 2010;**49**:380–389.
36. Messer A, Gallon C, McKenna W, Elliott P, Dos Remedios C, Marston S. The use of phosphate-affinity SDS-PAGE to measure the troponin I phosphorylation site distribution in human heart muscle. *Proteomics Clin Appl* 2009;**3**:1371–1382.
37. Fraser IDC, Marston SB. *In vitro* motility analysis of actin-tropomyosin regulation by troponin and Ca^{2+} : the thin filament is switched as a single cooperative unit. *J Biol Chem* 1995;**270**:7836–7841.
38. Marston SB, Fraser IDC, Wu B, Roper G. A simple method for automatic tracking of actin filaments in the motility assay. *J Musc Res Cell Motil* 1996;**17**:497–506.
39. Bayliss C, Messer A, Leung M-C, Ward D, van der Velden J, Poggesi C, Redwood C, Marston S. Functional investigation of troponin with the homozygous HCM mutation, TNNT2 K280M, obtained from an explanted heart. *Cardiovasc Res* 2012;**93**:S107.
40. Baudenbacher F, Schober T, Pinto JR, Sidorov VY, Hilliard F, Solaro RJ, Potter JD, Knollmann BC. Myofilament Ca^{2+} sensitization causes susceptibility to cardiac arrhythmia in mice. *J Clin Invest* 2008;**118**:3893–3903.
41. Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG. Stereoselective actions of thiazidinones on canine cardiac myocytes and myofilaments. *Circ Res* 1993;**73**:981–990.
42. Wolska BM, Kitada Y, Palmiter KA, Westfall MV, Johnson MD, Solaro RJ. CGP-48506 increases contractility of ventricular myocytes and myofilaments by effects on actin-myosin reaction. *Am J Physiol* 1996;**270**:H24–H332.
43. Ward DG, Brewer SM, Calvert MJ, Gallon CE, Gao Y, Trayer IP. Characterization of the interaction between the N-terminal extension of human cardiac troponin I and troponin C. *Biochemistry* 2004;**43**:4020–4027.
44. Baryshnikova OK, Li MX, Sykes BD. Modulation of cardiac troponin C function by the cardiac-specific N-terminus of troponin I: influence of PKA phosphorylation and involvement in cardiomyopathies. *J Mol Biol* 2008;**375**:735–751.
45. Kleerekoper Q. Identification of binding sites for Bepridil and Trifluoperazine on cardiac troponin C. *J Biol Chem* 1998;**273**:8153–8160.
46. Botten D, Fugallo G, Fraternali F, Molteni C. A computational exploration of the interactions of the green tea polyphenol (-)-Epigallocatechin 3-gallate with cardiac muscle troponin C. *PLoS ONE* 2013;**8**:e70556.
47. Manning EP, Tardiff JC, Schwartz SD. Molecular effects of familial hypertrophic cardiomyopathy-related mutations in the TNT1 domain of cTnT. *J Mol Biol* 2012;**421**:54–66.
48. Biesiadecki BJ, Kobayashi T, Walker JS, John Solaro R, de Tombe PP. The troponin C G159D mutation blunts myofilament desensitization induced by troponin I Ser23/24 phosphorylation. *Circ Res* 2007;**100**:1486–1493.
49. Pinto JR, Siegfried JD, Parvatiyar MS, Li D, Norton N, Jones MA, Liang J, Potter JD, Hershberger RE. Functional characterization of TNNT1 rare variants identified in dilated cardiomyopathy. *J Biol Chem* 2011;**286**:34404–34412.
50. Alves ML, Dias FAL, Gaffin RD, Simon JN, Montminy EM, Biesiadecki BJ, Hinken AC, Warren CM, Utter MS, Davis RT, Sakthivel S, Robbins J, Wiecek DF, Solaro RJ, Wolska BM. Desensitization of myofilaments to Ca^{2+} as a therapeutic target for hypertrophic cardiomyopathy with mutations in thin filament proteins. *Circ Cardiovasc Genet* 2014;**7**:132–143.
51. Li AY, Stevens CM, Liang B, Rayani K, Little S, Davis J, Tibbits GF. Familial hypertrophic cardiomyopathy related cardiac troponin C L29Q mutation alters length-dependent activation and functional effects of phosphomimetic troponin I*. *PLoS ONE* 2013;**8**:e79363.
52. Semsarian C, Ahmad I, Giewat M, Georgakopoulos D, Schmitt JP, McConnell BK, Reiken S, Mende U, Marks AR, Kass DA, Seidman CE, Seidman JG. The L-type calcium channel inhibitor Diltiazem prevents cardiomyopathy in a mouse model. *J Clin Invest* 2002;**109**:1013–1020.
53. Ingólfsson HI, Thakur P, Herold KF, Hobart EA, Ramsey NB, Periole X, de Jong DH, Zwama M, Yilmaz D, Hall K, Maretzky T, Hemmings HC, Blobel C, Marrink SJ, Koçer A, Sack JT, Andersen OS. Phytochemicals perturb membranes and promiscuously alter protein function. *ACS Chem Biol* 2014;**9**:1788–1798.
54. Hotta Y, Huang L, Muto T, Yajima M, Miyazeki K, Ishikawa N, Fukuzawa Y, Wakida Y, Tushima H, Ando H, Nonogaki T. Positive inotropic effect of purified green tea catechin derivative in guinea pig hearts: the measurements of cellular Ca^{2+} and nitric oxide release. *Eur J Pharmacol* 2006;**552**:123–130.
55. Feng W, Hwang HS, Kryshtal DO, Yang T, Padilla IT, Tiwary AK, Puschner B, Pessah IN, Knollmann BC. Coordinated regulation of murine cardiomyocyte contractility by nanomolar (-)-Epigallocatechin-3-gallate, the major green tea catechin. *Mol Pharmacol* 2012;**82**:993–1000.
56. Khandelwal A, Hall JA, Blagg BSJ. Synthesis and structure–activity relationships of EGCG analogues, a recently identified Hsp90 inhibitor. *J Org Chem* 2013;**78**:7859–7884.

TPM3 Deletions Cause a Hypercontractile Congenital Muscle Stiffness Phenotype

Sandra Donkervoort, MS, CGC,¹ Maria Papadaki, BSc,^{2*}

Josine M. de Winter, MS,^{3*} Matthew B. Neu, BS,^{1*} Janbernd Kirschner, MD,⁴

Véronique Bolduc, PhD,¹ Michele L. Yang, MD,⁵ Melissa A. Gibbons, MS, CGC,⁶

Ying Hu, MS,¹ Jahannaz Dastgir, DO,¹

Meganne E. Leach, MSN, APRN, PPCNP-BC,^{1,7} Anne Rutkowski, MD,⁸

A. Reghan Foley, MD,¹ Marcus Krüger, MD,⁹ Eric P. Wartchow, BSc,¹⁰

Elyshia McNamara, BS,¹¹ Royston Ong, BSc,¹¹ Kristen J. Nowak, PhD,¹¹

Nigel G. Laing, PhD,¹¹ Nigel F. Clarke, PhD,¹² Coen A. C. Ottenheijm, PhD,³

Steven B. Marston, MA, DPhil, DSc,² and Carsten G. Bönnemann, MD¹

Objective: Mutations in *TPM3*, encoding Tpm3.12, cause a clinically and histopathologically diverse group of myopathies characterized by muscle weakness. We report two patients with novel *de novo* Tpm3.12 single glutamic acid deletions at positions Δ E218 and Δ E224, resulting in a significant hypercontractile phenotype with congenital muscle stiffness, rather than weakness, and respiratory failure in one patient.

Methods: The effect of the Tpm3.12 deletions on the contractile properties in dissected patient myofibers was measured. We used quantitative *in vitro* motility assay to measure Ca^{2+} sensitivity of thin filaments reconstituted with recombinant Tpm3.12 Δ E218 and Δ E224.

Results: Contractility studies on permeabilized myofibers demonstrated reduced maximal active tension from both patients with increased Ca^{2+} sensitivity and altered cross-bridge cycling kinetics in Δ E224 fibers. *In vitro* motility studies showed a two-fold increase in Ca^{2+} sensitivity of the fraction of filaments motile and the filament sliding velocity concentrations for both mutations.

Interpretation: These data indicate that Tpm3.12 deletions Δ E218 and Δ E224 result in increased Ca^{2+} sensitivity of the troponin–tropomyosin complex, resulting in abnormally active interaction of the actin and myosin complex. Both mutations are located in the charged motifs of the actin-binding residues of tropomyosin 3, thus disrupting the electrostatic interactions that facilitate accurate tropomyosin binding with actin necessary to prevent the on-state. The mutations destabilize the off-state and result in excessively sensitized excitation–contraction coupling of the contract-

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.24535

Received Apr 2, 2015, and in revised form Sep 2, 2015. Accepted for publication Sep 5, 2015.

Address correspondence to Dr Bönnemann, National Institutes of Health, Porter Neuroscience Research Center, 35 Convent Drive, Bldg 35, Room 2A-116, Bethesda, MD 20892-3705. E-mail address: carsten.bonnemann@nih.gov

*These authors contributed equally.

From the ¹Neuromuscular and Neurogenetic Disorders of Childhood Section, National Institutes of Health, Bethesda, MD; ²National Heart and Lung Institute, Imperial College London, London, United Kingdom; ³Department of Physiology, VU University Medical Center, Amsterdam, the Netherlands;

⁴Department of Neuropediatrics and Muscle Disorders, University Medical Center Freiburg, Freiburg, Germany; ⁵Section of Child Neurology, Department of Pediatrics and Neurology, University of Colorado School of Medicine, Aurora, CO; ⁶University of Colorado Denver School of Medicine, Aurora, CO; ⁷Children's National Health System, Washington, DC; ⁸Cure CMD, Kaiser Southern California Permanente Medical Group, Olathe, KS;

⁹Department of General Pediatrics, Adolescent Medicine, and Neonatology, University Medical Center Freiburg, Freiburg, Germany; ¹⁰Department of Pathology, Children's Hospital Colorado, Aurora, CO; ¹¹Neuromuscular Diseases Laboratory, Centre for Medical Research, Faculty of Medicine, Dentistry, and Health Sciences, University of Western Australia, Crawley, Western Australia, Australia; and Harry Perkins Institute of Medical Research, Queen Elizabeth II Medical Centre, Perth, Western Australia, Australia; and ¹²Institute for Neuroscience and Muscle Research, Children's Hospital at Westmead, University of Sydney, Sydney, New South Wales, Australia

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ile apparatus. This work expands the phenotypic spectrum of *TPM3*-related disease and provides insights into the pathophysiological mechanisms of the actin–tropomyosin complex.

ANN NEUROL 2015;78:982–994

Tropomyosin 3 (Tpm3.12; α -tropomyosin slow), encoded by the *TPM3* gene (Online Mendelian Inheritance in Man database 191030), belongs to the actin-binding tropomyosin family and is a component of the sarcomeric thin filament troponin–tropomyosin complex. Tpm3.12 is predominantly expressed in type 1 (slow twitch) fibers and forms dimers of linear coiled-coil structures with Tpm2.2 (β -tropomyosin) encoded by *TPM2* and Tpm1.1 (α -tropomyosin) encoded by *TPM1*, which then bind head-to-tail, providing structural stability to actin filaments.^{1,2} This complex acts by regulating Ca^{2+} dependent binding of the myosin head to the actin filament. It serves as an essential component in the regulation of muscle contraction by preventing binding of the myosin head when in the off-state and allowing it in the on-state, in preparation for the force generating power stroke. Mutations in *TPM3* recognized thus far result in a clinically and histopathologically heterogeneous group of neuromuscular disorders, which are characterized by progressive weakness and include cap myopathy,^{3–5} congenital fiber type disproportion (CFTD),⁶ and autosomal dominant as well as recessive nemaline myopathy.^{7–10}

Mutations in *TPM2* also typically result in a phenotype of myopathy, manifesting with weakness. However, the deletion of a single lysine residue at position 7 in Tpm2.2 can manifest clinically with both muscle weakness and moderate progressive muscle contractures. Studies of this specific mutation suggest that the pathogenic mechanism underlying some tropomyosin-related disease may be due to elevated Ca^{2+} sensitivity of the contractile apparatus.^{11,12} Although such a mechanism had been predicted on theoretical molecular grounds to also be applicable for Tpm3.12,¹³ this phenomenon had not yet been shown conclusively to be clinically relevant.^{11,12}

Here we report two unrelated patients with two distinct but closely spaced *de novo* Tpm3.12 single glutamic acid deletions resulting in a marked generalized hypercontractile phenotype with very significant congenital muscle stiffness, in the absence of detectable weakness, and associated with respiratory failure in the more severely affected patient. The effect of the Tpm3.12 glutamic acid deletions was studied using *in vitro* motility assays and mechanics on permeabilized myofibers. These deletions appear to result in impaired maximum force generation with altered cross-bridge cycling kinetics, and increased Ca^{2+} sensitivity of force. This study expands the phenotypic, and pathophysiological spectrum of

TPM3 mutations, which now join a specific *ACTA1* mutation as well as recessive $\alpha\beta$ -crystallin (*CRYAB*) mutations as a myogenic cause for significant neonatal muscle rigidity.^{14,15}

Subjects and Methods

Patient Recruitment and Sample Collection

Two unrelated patients, one from the United States and one from Germany, were identified through their local neurologists. Medical history was obtained and clinical evaluations were performed as part of the standard neurologic evaluation. This study was approved by the institutional review board of the National Institute of Neurological Disorders and Stroke, National Institutes of Health (NIH). Written informed consent was obtained from each family by a qualified investigator. DNA was obtained from blood based on standard procedures. Muscle biopsies were obtained as part of the regular clinical diagnostic testing and were evaluated by standard light and electron microscopy protocols. Banked muscle biopsy samples were transferred to the NIH for further analysis.

Genetic Testing

Whole exome sequencing on blood samples obtained from Patient 1 (P1) and his parents was performed at the NIH Intramural Sequencing Center using the Illumina (San Diego, CA) TruSeq Exome Enrichment Kit and Illumina HiSeq 2000 sequencing instruments. Mutations were analyzed using GEM.app and xBrowse and searched for in dbSNP, NHLBI EVS, ExAC Browser, or GEM.app.¹⁶ Polymerase chain reaction amplification of exon 6 of *TPM3* in P1, Patient 2 (P2), and their parents, was followed by Sanger sequencing on an ABI 3130x1 capillary sequencer, in forward and reverse directions.

Synthesis of Tropomyosin in Sf9 Insect Cells

The wild-type (WT) human *TPM3* cDNA sequence was amplified from reverse transcribed skeletal muscle RNA and then cloned into the BacPak8 plasmid (Clontech Laboratories, Mountain View, CA). The $\Delta E218$ and $\Delta E224$ mutations were introduced into the *TPM3* cDNA by site-directed mutagenesis. The WT and two mutant tropomyosin proteins were expressed in *Sf9* insect cells using the flashBAC baculovirus system (Oxford Expression Technologies, Oxford, UK). Recombinant proteins were purified using protocols based on Akkari et al.¹⁷

Quantitative In Vitro Motility Assay

Thin filaments were reconstituted with 10nM rabbit skeletal muscle α -actin (labeled with tetramethylrhodamine phalloidin),¹⁸ tropomyosin (40–60nM), and skeletal troponin (60nM) to study Ca^{2+} regulation of filament motility by the quantitative *in vitro* motility assay previously described.^{19,20} Thin filament movement over a bed of immobilized rabbit fast skeletal

muscle heavy meromyosin (100 μ g/ml) was then compared in paired motility cells with WT or mutant tropomyosin. Filament movement was recorded and analyzed as previously described,¹⁹ yielding 2 parameters, the fraction of filaments moving and the speed of moving filaments. Fraction motile and sliding speed was measured over a range of Ca^{2+} concentrations to generate Ca^{2+} activation curves as shown previously.^{20–23} The temperature was set to 29°C. Median effective concentration (EC_{50}) values from replicate experiments were analyzed by paired *t*-test, because EC_{50} has been shown to be normally distributed.^{20,24}

Permeabilized Myofiber Contractile Performance

The effect of the Tpm3.12 deletion on the contractile properties in myofibers derived from patients' muscle biopsies was measured. Myofiber bundles from frozen muscle biopsies from P1 and P2 and age-matched controls were isolated as described previously.^{13,25,26} In brief, smaller sections (2 $\times$ 2mm) of the biopsies were isolated in liquid nitrogen. They were then placed for 24 hours at -20°C in 4ml 50% glycerol/relaxing solution containing high concentrations of protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], 0.5mM; leupeptin, 0.04mM; E64, 0.01mM). Subsequently, the sections were placed on a roller band for 24 hours at 4°C , followed by submersion in skinning solution for 24 hours at 4°C . Finally, the skinning solution was substituted by a glycerol/relaxing solution with lower concentrations of protease inhibitors and stored at -20°C until further use. On the day of an experiment, small strips (cross-sectional area [CSA] = 0.005–0.01mm²) were dissected from the glycerinated sections and washed thoroughly with relaxing solution. The strips were mounted using aluminum T-clips between a length motor (ASI 403A; Aurora Scientific, Aurora, ON, Canada) and a force transducer element (ASI 315C-I, Aurora Scientific) in a skinned fiber apparatus (ASI 802D, Aurora Scientific) that was mounted on an inverted microscope (Axio Observer A1; Zeiss, Oberkochen, Germany). Sarcomere length (2.5 μ m) was set using a high-speed VSL camera and ASI 900B software (Aurora Scientific). Six strips were analyzed for P1 (average CSA = 0.01mm²), P2 (average CSA = 0.005mm²), and control (average CSA = 0.006mm²). Fiber width and diameter was measured at 3 points along the fiber and the CSA was determined assuming an elliptical cross section. Fiber dimensions were determined using an inverted microscope ($\times 40$ objective), a high-speed VSL camera with calibrated ASI 900B software (Aurora Scientific), and a custom-made prism that was mounted in the bath. Various types of bathing solutions were used during the experimental protocols: a relaxing solution (40mM balanced electrolyte solution [BES], 10mM ethyleneglycoltetraacetic acid [EGTA], 6.86mM MgCl_2 , 5.96mM Na-adenosine triphosphate [ATP], 3.28mM K-propionate, 33mM creatine phosphate, 1mM dithiothreitol [DTT], 0.5mM PMSF, 0.2mM leupeptin, 0.05mM E64), a preactivating solution with low EGTA concentration (40mM BES, 1mM EGTA, 6.66mM MgCl_2 , 5.98mM Na-ATP, 30.44mM K-propionate, 33mM creatine phosphate, 1mM

DTT, 0.5mM PMSF, 0.2mM leupeptin, 0.05mM E64), and activating solutions with incremental Ca^{2+} concentrations for graded Ca^{2+} response curves (40mM BES, 10mM CaCO_3 -EGTA [for maximal activation]; 6.64mM MgCl_2 , 6.23mM Na-ATP, 2.1mM K-propionate, 15mM creatine phosphate, 1mM DTT, 0.5mM PMSF, 0.2mM leupeptin, 0.05mM E64). The temperature of the bathing solutions was kept constant at 20°C using a temperature controller (ASI 825A, Aurora Scientific).

Absolute forces were normalized to the bundles' CSA to determine the maximal active tension. To determine the Ca^{2+} sensitivity of force generation, permeabilized myofiber bundles were sequentially bathed in a relaxing solution, a preactivation solution, and solutions with pCa values ranging from 9.0 to 4.5. Steady-state forces were measured at all the Ca^{2+} concentrations, and subsequently these steady-state values were normalized to the maximal force obtained at pCa = 4.5. The obtained force-pCa data were fit to the Hill equation ($Y = 1/[1 + 10^{n_H(pCa - pCa_{50})}]$). Cross-bridge cycling kinetics were determined by imposing a rapid release–restretch protocol on an activated fiber based on previously reported methods.²⁷ The rate of tension redevelopment was obtained by fitting a biexponential through the force redevelopment curve. The resulting first-order rate constant k_f reflects cross-bridge cycling kinetics and thus was used in the analysis methods.²⁸ A measure of active stiffness was obtained by applying short-length perturbations on the myofibers, while the myofibers were being maximally activated, and fit to a linear curve through the data points. By dividing the maximal active tension by active stiffness, the force generation per cross-bridge was estimated.²⁹

Myosin Heavy Chain Isoform Composition of Myofibers Used for Contractility Experiments

Because the contractile performance of muscle fibers is affected by the composition of myosin heavy chain isoforms, we used specialized sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis to determine the myosin heavy chain isoforms present in the muscle fibers used for contractile measurements.²⁵ In brief, muscles fibers were denatured by boiling for 2 minutes in SDS sample buffer. The stacking gel contained a 4% acrylamide concentration (pH = 6.7), and the separating gel contained 7% acrylamide (pH = 8.7) with 30% glycerol (vol/vol). The gels were run for 24 hours at 15°C and a constant voltage of 275V. Finally, the gels were silver-stained, scanned, and analyzed with One-D scan EX (Scanalytics, Rockville, MD) software (data not shown).

Results

Clinical Assessment Identified two Patients with a Significant Hypercontractile Phenotype with Marked Congenital Muscle Stiffness

We identified two patients with a history of marked congenital muscle stiffness, which was most significant at the onset. Detailed clinical information is summarized in the Table and Figure 1.

TABLE 1. Clinical Details of Patients 1 and 2

Characteristic	Patient 1	Patient 2
Sex/age, yr	Male/4	Female/3
Tpm3.12 mutation	ΔE224	ΔE218
Presentation	Decreased fetal movements. At birth: stiffness with arthrogryposis multiplex congenita, right clubfoot, bilateral hand fisting, inguinal hernia, bilateral hip dysplasia, and knee contractures.	Decreased fetal movements and polyhydramnios. At birth: pronounced generalized stiffness, limited-to-absent movement, including the thoracic muscles requiring ventilation, hip dysplasia, umbilical hernia, left inguinal hernia. In infancy: recurrent hypoxic crises.
Dysmorphic features	Relative macrocephaly, short stature, and thin habitus. Prominent forehead, small mouth, short neck, and stiff face.	Retrognathia, micrognathia, and short neck.
Cognition	Normal	Normal
Clinical features	Kyphoscoliosis with lower spinal rigidity. Mild head lag, no slip-through, and variable tone distally. Multiple bilateral contractures: shoulders, wrists with ulnar deviation, finger flexors, jaw, hip flexion, knee flexion, and heel cords. Prominent paraspinous muscles. Fine tremor. Reflexes present. Temperature dysregulation.	Kyphosis. Pronounced muscle hypertonia. Limited passive range of motion. Thoracic rigidity. Tracheostomy and ventilator dependency. Feeding tube and bladder catheter dependent. Profuse sweating when excited.
Motor function and strength	Stiff gait marked by exaggerated hip flexion, thoracic kyphosis, and flexion at the knees. Improvement over time. Strong bilateral grip. No weakness.	Limited to absent movement. Unable to sit or roll.
Respiration	Neonatal transient hypoxemia. Increased respiratory rate and occasional grunting. Frequent respiratory infections. Hypoxemia and obstructive sleep apnea requiring supplemental oxygen.	Recurrent hypoxic crises in infancy. Tracheostomy and ventilator dependency.
EMG	Suggestive of a generalized myopathy with denervating features. No myotonia or myokymia observed.	No evidence of spontaneous activity or myotonia.
NCS	Sensory responses of the right median and sural nerves were normal. Motor NCS of the right median nerve showed normal distal latency, moderate reduction in response amplitude, and normal conduction velocity. Motor NCS of the right peroneal nerve showed normal distal latency, severe reduction in response amplitude, and normal conduction velocity.	Not performed.
Other	CK: 205U/l. Normal: EEG, brain MRI, and echocardiogram. Negative: <i>ACTA1</i> , <i>MYH3</i> , <i>TPM2</i> , <i>HSPG2</i> , <i>TNNT3</i> , <i>TNNI2</i> , <i>FKRP</i> , <i>CRYAB</i> , sequencing and microarray.	CK between 200 and 1,000U/l. Osteopenia, shortened phalanges, and clinodactyly on X-ray. Normal: brain MRI, EEG, and metabolic screening. Negative <i>ACTA1</i> and <i>CRYAB</i> sequencing. Increased echogenicity on muscle ultrasound.

CK = creatine kinase; EEG = electroencephalogram; EMG = electromyogram; MRI = magnetic resonance imaging; NCS = nerve conduction studies.



FIGURE 1: (A, B) A 4-year-old male (Patient 1) with relative macrocephaly compared to his short stature and thin habitus. He has a prominent forehead, small mouth, short neck, and stiff face. His shoulders are anteriorly rotated with decreased lumbar lordosis and increased kyphosis, and he has bilateral elbow and knee contractures. He has flat feet that are turned outward, external hip rotation, and a barrel chest with wide-spaced nipples. (C) A 3-year-old female (Patient 2) with pronounced muscle stiffness. She has bilateral contractures of elbows, knees, and ankles. She is ventilated with tracheostomy and has a feeding tube. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

P1 is a 5-year-old male (see Fig 1A, B). Pregnancy was notable for decreased fetal movements but normal amniotic fluid level. He was born with arthrogryposis multiplex congenita with bilateral contractures of the hips, knees, and wrists. He also had bilateral hand fisting, right clubfoot deformity, unilateral hip dislocation, an inguinal hernia, and generalized muscle stiffness. Neonatal course was complicated by transient respiratory distress, particularly during feeding, manifesting with an increased respiratory rate. He also had a history of low oxygen saturations and occasional grunting at rest as a neonate. He rolled from his back to his side at 6 months of age, could manipulate objects in his hands and bring some objects to his mouth by 15 months of age, and walked independently at 25 months of age. He had notable easy fatigability.

On examination at age 4 years, he had a “wooden feel” to his muscles, which were noted to be very stiff, particularly the hamstrings, the paraspinal muscles, and the face muscles. He had well-defined biceps and prominent thigh muscles. There was no muscle weakness noted. Deep tendon reflexes were intact. He had good fine motor control. Active and passive range of motion was restricted at his shoulders, Achilles tendons, elbows, knees, and jaw. He walked on his toes with a stiff, shuf-

fling gait with almost no flexion at the knees or hips appreciated (Supplementary Video). Due to restricted movement at these joints, he could not reach his arms above his head. He had excessive thoracic kyphosis, with a minimal scoliosis, and rigidity of the lower spine. He had short stature with height below the 5th percentile, weight along the 10th percentile, and head circumference along the 50th percentile.

He had a history of inappropriate sweating and was reported to be prone to “overheating” particularly at night during sleep. His muscle stiffness was reported to exacerbate with movement or fatigue and improve with the application of warm water. He had a history of dysphagia and evidence of silent aspiration of liquids on videofluoroscopy. He also had a history of frequent episodes of bronchiolitis. He had been diagnosed with restrictive lung disease and uses a Cough Assist device to help improve expiratory function. A sleep study revealed evidence of hypoventilation. He has been advised to use nighttime noninvasive ventilation in the form of bilevel positive airway pressure (BiPAP), but he could not tolerate the BiPAP mask. After the time of our examination, he received two courses of botulinum toxin injections to the hamstrings and pectoral muscles in an effort to improve his range of motion. He was then able to reach his arms above his head and walk with more fluidity by 5 years of age. Diagnostic testing performed on P1 included electromyography (EMG) at age 6 months of age, which was interpreted as being myopathic with denervating features. Continuous motor unit activity in the form of myotonia and myokymia were not observed.

P2 is a 3-year-old female (see Fig 1C). The pregnancy was notable for polyhydramnios and decreased fetal movement. She was born at term and presented with severe, generalized muscle stiffness immediately after birth. Flexion muscle contractures were present in all large joints, which resulted in an inability for active or passive movements. Apparent involvement of the respiratory muscles contributed to recurrent episodes of apnea that led to the need for invasive ventilation with high inspiratory pressures (up to 40cm H₂O). Treatment with carbamazepine, benzodiazepines, and baclofen reportedly provided no substantial benefit. Due to continuing ventilatory dependency, a tracheostomy was placed. She also had a percutaneous gastrostomy tube placed for feeding. She was discharged from the hospital at the age of 5 months. At the current age of 3 years, she remains fully ventilator-dependent with high inspiratory pressures (up to 25cm H₂O). On examination, she had normal eye movements and appeared mentally alert. Muscle tone was markedly increased, with absent deep tendon reflexes. She demonstrated the ability to use a standing

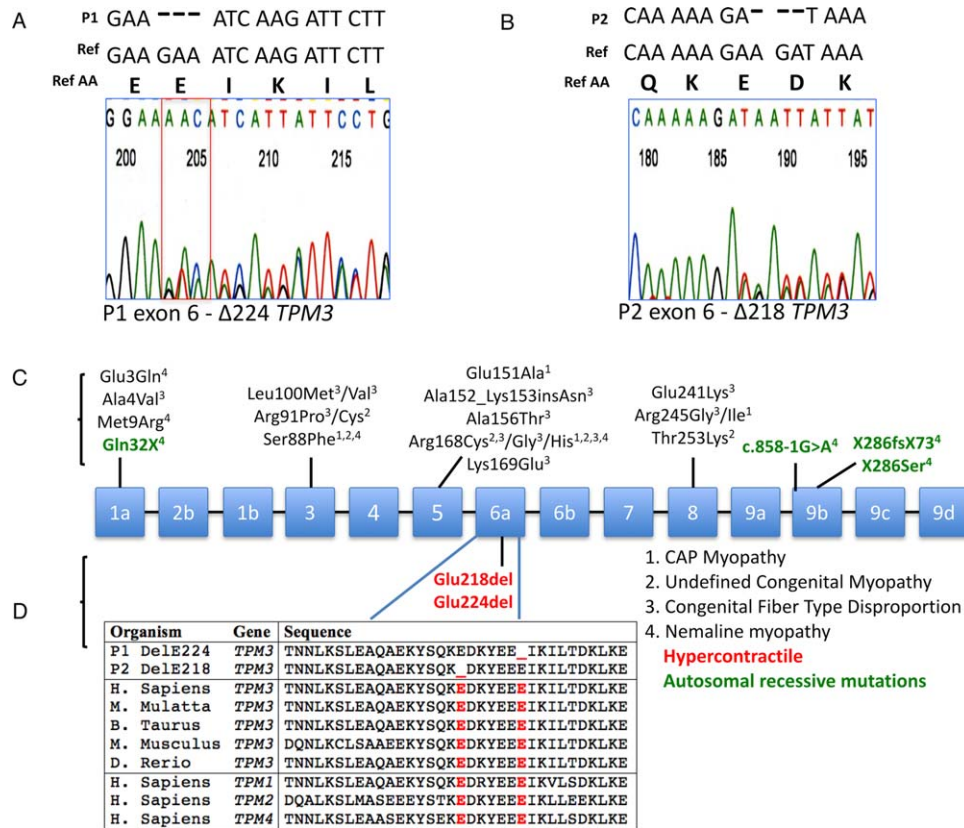


FIGURE 2: (A, B) Sequence chromatogram of Patients 1 and 2 (P1, P2) identifying a glutamic acid deletion in Tpm3.12. (C) Schematic of known mutations in TPM3 along with their disease phenotypes. (D) Illustration of amino acid homology of TPM3 (P1, P2), as well as for TPM1, TPM2, and TPM4.

frame, but had difficulty sitting due to apparent hyperactivity of her hip extensors.

Muscle tone reportedly reduced slightly after intramuscular injections of botulinum toxin, and some limited voluntary movements were appreciated. She has started to use an assistive communication device. Extensive diagnostic evaluations including brain magnetic resonance imaging, electroencephalography, and metabolic screening did not reveal any abnormalities. Serum creatine kinase (CK) levels varied between 200 and 1,000U/l. EMG did not show evidence of myotonia or myokymia. Muscle ultrasound depicted markedly increased echogenicity of all muscles imaged but no evidence of muscle atrophy.

Exome Analysis Identified two Novel De Novo Glutamic Acid Residue Deletions in Tpm3.12

Whole exome sequencing of P1 and both biological parents followed by variant filtering for call quality and rarity yielded a heterozygous c.673-675delGAA (p.Glu224-del, ΔE224) TPM3 variant (reference NM_152263.3). Due to phenotypic similarity noted between P1 and P2, this region was Sanger sequenced in P2, revealing a heterozygous 3 base pair deletion c.657_659delAGA (p.Glu218-

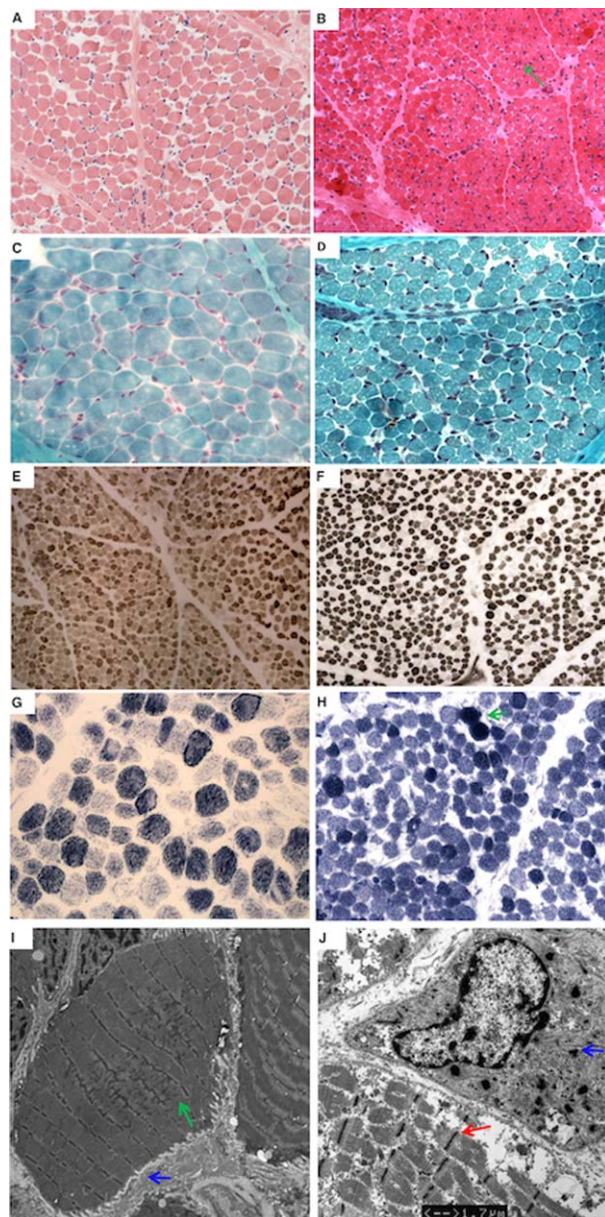
del, ΔE218), resulting in the deletion of glutamic acid at amino acid 218 (Fig 2A, B). Parental segregation studies for P1 and P2 were negative; the mutation was *de novo* in both patients. Neither mutation had been previously reported, and neither was present in dbSNP, NHLBI EVS, ExAC Browser, or GEM.app.¹⁶ Both mutations are located in highly conserved regions of TPM3 (see Fig 2C, D).

Tpm3.12 ΔE218 and ΔE224 Muscle Biopsies Did Not Show Typical Tropomyosin Pathology

Muscle biopsy of P1 was performed at age 17 months and was mildly myopathic in appearance without specific features. There was mild variation in fiber size and a mild type I fiber predominance (Fig 3). Nicotinamide adenine dinucleotide (NADH) staining demonstrated irregular staining in some fibers resulting in some areas of clearing. Modified Gömöri trichrome stain demonstrated irregularities of the myofibrillar apparatus. There was no evidence of clear nemaline rods or a pattern consistent with CFTD. There was ultrastructural evidence of accumulation of mitochondria, increased lipid storage, and Z-line streaming and broadening, which was focal, occurring in rare fibers, and spanning up to 6 to 7 sarcomeres maximally, seen on electron microscopy (EM)

along with some sarcolemmal folding as a result of fiber atrophy.

Muscle biopsy of P2 was performed at age 3 weeks and demonstrated mild myopathic features. Findings included mild variation in fiber size and some rare basophilic fibers with centrally placed nuclei (see Fig 3). There was mild type II fiber predominance seen on ATPase and NADH staining but no fiber-type grouping. Structures resembling minirods were identified on EM as well as some Z-band broadening in atrophic fibers. Whereas clearly formed nemaline rods were not seen on modified Gömöri trichrome stain or EM, mini “miliary” rods were seen in small atrophic fibers both on modified Gömöri trichrome stain and on EM.



Increased Ca^{2+} Sensitivity for Tpm3.12 ΔE218 and ΔE224 by *In Vitro* Motility Assay

Using the *in vitro* motility assay, the movement of synthetic thin filaments containing recombinant WT or mutant tropomyosin (Tpm3.12 ΔE218 and ΔE224) was compared over a range of Ca^{2+} concentrations. This assay measures two parameters that are Ca^{2+} regulated: fraction of filaments motile and sliding speed of the filaments. Ca^{2+} increased the fraction motile from 2% to 90% and the sliding speed from 1.3 to $4\mu\text{m/s}$. The EC_{50} and Hill coefficient (n_H) were determined by fitting the Hill equation to the Ca^{2+} activation curves. The mutations affected both the fraction motile and the sliding speed of the filaments in a similar way. Thin filaments reconstituted with mutant tropomyosins showed a higher Ca^{2+} sensitivity for both the fraction motile and the sliding speed; representative Ca^{2+} activation curves are shown in Figure 4. ΔE218 led to a $2^{1/2}$ -fold increase in Ca^{2+} sensitivity (EC_{50} ratio = $\Delta\text{E218}/\text{WT} = 0.40 \pm 0.07$, $p = 0.004$, $n = 4$) for the fraction motile and a 2-fold increase for the sliding speed (EC_{50} ratio = $\Delta\text{E218}/\text{WT} = 0.48 \pm 0.12$, $p = 0.02$, $n = 4$). ΔE224 also showed an increase in Ca^{2+} sensitivity in the fraction motile and the sliding speed parameter by 2.2- and 3.0-fold, respectively (EC_{50} ratio = $\Delta\text{E224}/\text{WT}$ for fraction motile = 0.46 ± 0.09 , $p = 0.01$, $n = 4$; EC_{50} ratio = $\Delta\text{E224}/\text{WT}$ for sliding speed = 0.33 ± 0.08 , $p = 0.004$, $n = 4$), indicating that both mutations cause a gain of function. There was no significant difference in the maximum fraction motile, maximum sliding speed, or Hill coefficient between either of the mutants and the WT thin filaments.

FIGURE 3: Muscle biopsy of Patient 1 (P1) at age 17 months (left) and Patient 2 (P2) at age 22 days (right). Hematoxylin & eosin (original magnification, $\times 20$) shows variability in fiber size with increased perimysial and endomysial fibrosis in P1 (A) and P2 (B). There are some rare basophilic fibers in P2 (B) with centrally placed nuclei (green arrow). Trichrome staining (original magnification, $\times 40$) does not show inclusions suggestive of nemaline rods or vacuoles in P1 (C), whereas P2 shows “miliary” rod-like inclusions in some of the atrophic fibers (D). There is variation in fiber size of both fiber types on adenosine triphosphatase (ATPase) 9 (original magnification, $\times 20$) in P1 (E) and ATPase 10.5 (original magnification, $\times 20$) in P2 (F). There is mild type 1 predominance in P1 and mild type 2 fiber predominance in P2. Nicotinamide adenine dinucleotide (original magnification, $\times 40$) in P1 (G) and P2 (H) shows an increase in staining in the periphery of some of the darker (type I) fibers, evidence of mitochondrial aggregation and contracted fibers (green arrow). (I) Electron microscopy (EM) of P1 shows Z-disk streaming (green arrow) and sarcolemmal folds (blue arrow; bar = $4\mu\text{m}$). (J) EM of P2 also shows Z-band broadening (red arrow; bar = $1.7\mu\text{m}$). There are some miliary rods (blue arrow). No clear nemaline rods were seen.

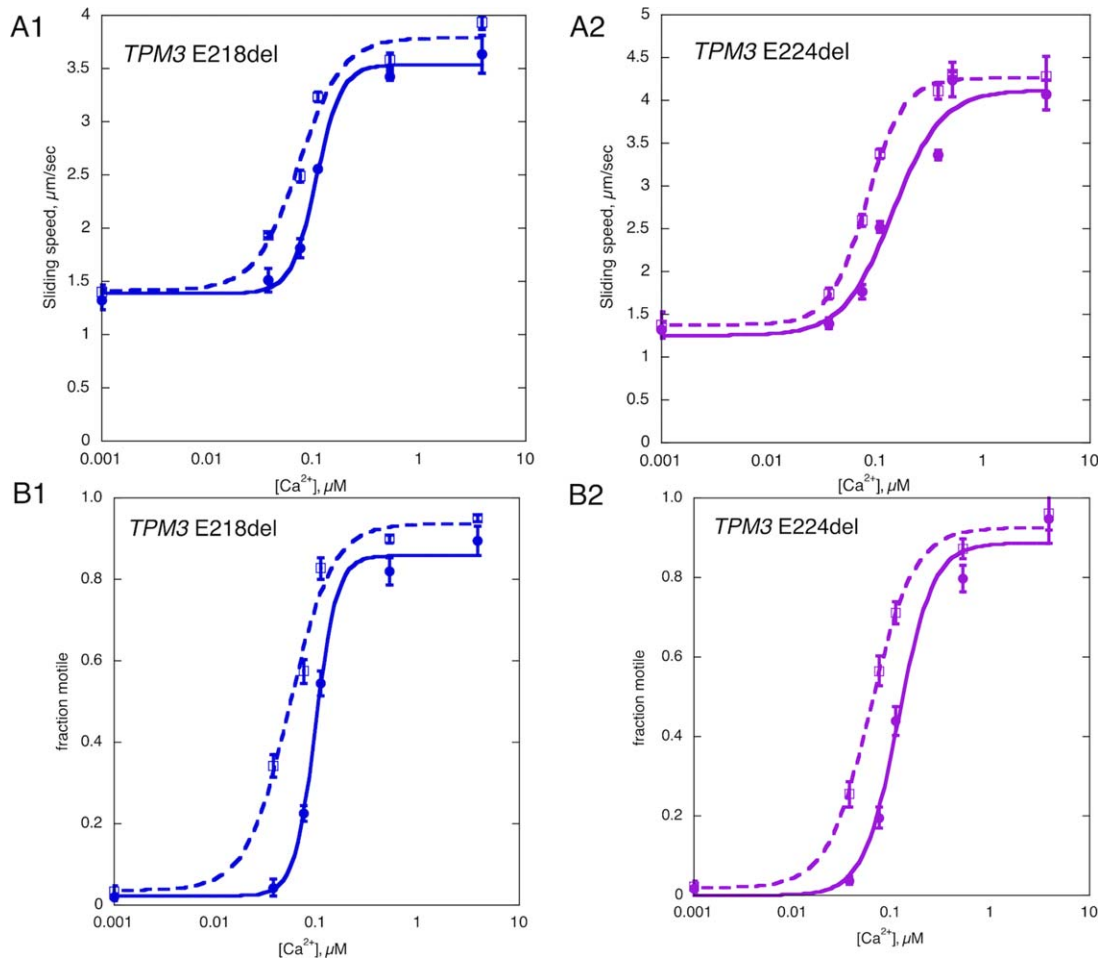


FIGURE 4: Ca^{2+} control of *in vitro* motility. Representative Ca^{2+} activation curves are shown; ΔE218 on left and ΔE224 on right are compared with wild-type control. Thin filaments were assembled using baculovirus expressed Tpm3.12, rabbit skeletal muscle actin, and rabbit skeletal muscle troponin. Thin filament movement over immobilized heavy meromyosin (HMM) was tracked and analyzed over a range of Ca^{2+} concentrations. Solid lines = control; dotted lines = mutant tropomyosin. Error bars are standard deviation for 4 measurements of motility in the same motility cell. The curves are the fits of the data to the Hill equation: sliding speed (top) and fraction of filaments motile parameters (bottom). Four separate Ca^{2+} curves were

ΔE218 and ΔE224 Permeabilized Myofibers Showed Decreased Maximum Force Generation and ΔE218 Showed Increased Ca^{2+} Sensitivity with Reduced Cross-Bridge Cycling Kinetics

Contractile properties of permeabilized myofibers isolated from biopsies from P1 and P2 (ΔE224 and ΔE218) were measured. The maximal active tension in ΔE218 ($31 \pm 5 \text{ mN/mm}^2$) and ΔE224 ($35 \pm 2 \text{ mN/mm}^2$) permeabilized myofibers showed a significant decrease in maximum force generation compared to controls ($95 \pm 14 \text{ mN/mm}^2$; Fig 5). The Ca^{2+} sensitivity of force generation in ΔE224 (5.94 ± 0.02) was increased compared to control fibers (5.81 ± 0.02). The maximum force a muscle fiber can generate is dependent on the rate of cross-bridge cycling kinetics. The rate constant of force redevelopment (K_{tr}) is a widely used parameter of cross-bridge cycling kinetics, reflecting both cross-bridge attachment and detachment. Analysis showed that cross-

bridge cycling kinetics are reduced in Tpm3.12 ΔE224 (6.1 ± 1) compared to control myofibers (9.8 ± 1), whereas maximal active tension over active stiffness, a reflection of the force per cross-bridge, was not altered between Tpm3.12 ΔE224 (2.2 ± 0.1) and control fibers (2.2 ± 0.1). Unfortunately, due to technical limitations caused by lower quality and quantity of the biopsy, we could not measure cross-bridge cycling kinetics and force per cross-bridge in Tpm3.12 ΔE218 .

As the contractile performance of myofibers is dependent on the myosin heavy chain (MHC) isoform composition, the presence of MHC isoforms was determined. No significant changes between control myofibers ($27 \pm 6\%$ slow MHC and $73 \pm 6\%$ fast MHC) and ΔE218 ($30 \pm 5\%$ slow MHC and $66 \pm 6\%$ fast MHC) or ΔE224 ($20 \pm 7\%$ slow MHC and $80 \pm 7\%$ fast MHC) were found. Therefore, the changes observed in contractile performance between control and patient

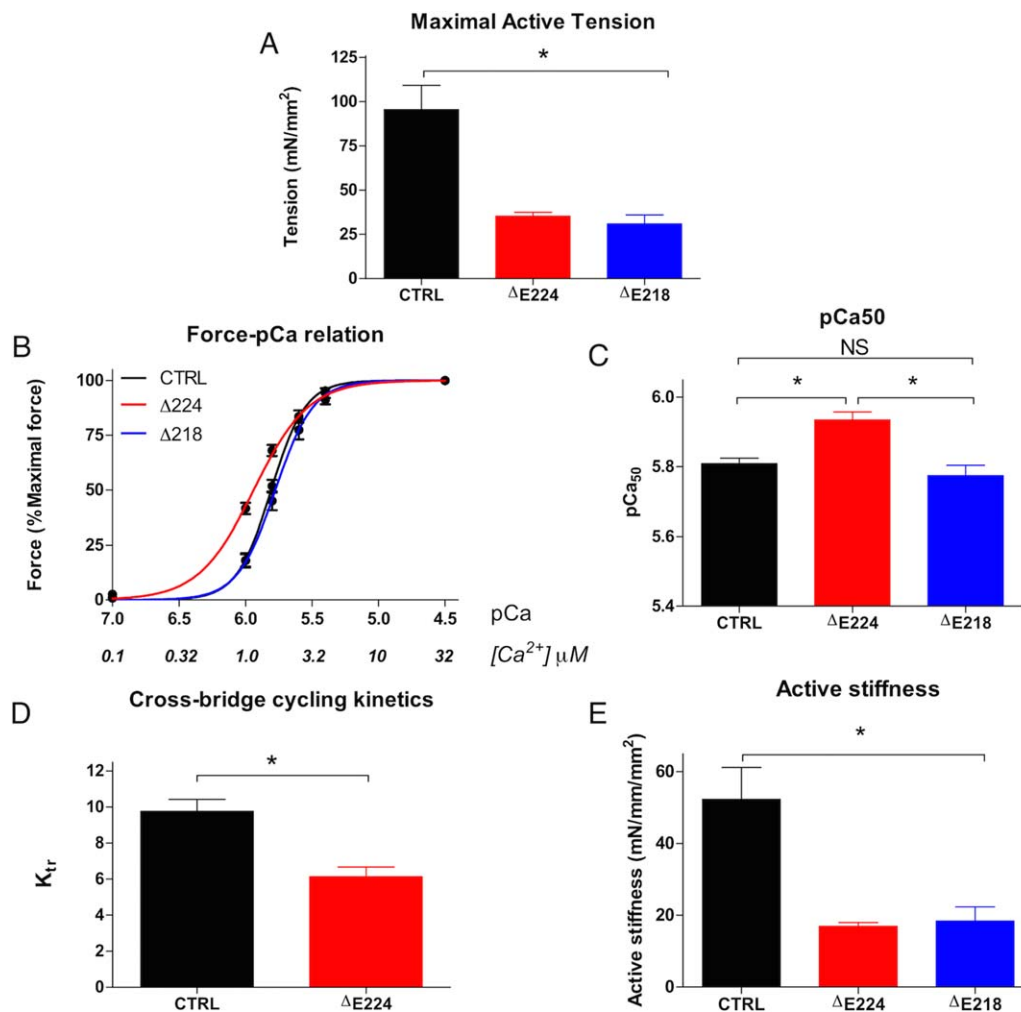


FIGURE 5: (A) A significant decrease in maximum force generation was seen in single fiber myofibers from Tpm3.12 ΔE224 and ΔE218 compared to controls. (B, C) The Ca²⁺ sensitivity of force generation was increased in ΔE224 compared to controls and ΔE218. Additionally, there is reduced cross-bridge cycling kinetics in Tpm3.12 ΔE224 (D), whereas the active stiffness was not altered between Tpm3.12 ΔE224 and control (E). NS = not significant. * = $P < 0.05$. pCa = $-\log(10)$ of the calcium concentration. pCa₅₀ = Ca²⁺ concentration required to generate 50% of maximal force. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

myofibers were not influenced by changes in myosin heavy isoform composition, and are more likely due to the specific mutations.

Discussion

Muscle fiber-based stiffness, in the absence of clinically detectable weakness, is a rare manifestation of congenital muscle disease. It was originally reported in Canadian aboriginal patients with a fatal condition characterized by progressive muscle stiffness and respiratory insufficiency¹⁵ due to recessive mutations in *CRYAB*. *CRYAB* codes for the small chaperone $\alpha\beta$ -crystallin; however, the mechanism responsible for the muscle stiffness remains unclear. Additionally, a specific *de novo* mutation in *ACTA1* was found in a newborn with congenital episodic stiffness, hypertrophic muscles, and nemaline rod muscle pathol-

ogy.¹⁴ Our report is the first to demonstrate specific *TPM3* deletions as the cause of a novel phenotypic manifestation of marked and generalized congenital muscle stiffness. This phenotype is notable for an absence of muscle weakness in combination with muscle stiffness, which is so severe that it can result in early ventilatory failure, likely due to an incapacity for respiratory muscle relaxation, as evidenced by the high inspiratory pressures required during mechanical ventilation in P2.

The muscle thin filament is a cooperative/allosteric system. Its basic unit consists of a backbone of actin monomers, the troponin-tropomyosin complex, and nebulin.²⁴ Muscle contraction is regulated by Ca²⁺ via its release and reuptake from the sarcoplasmic reticulum. Ca²⁺ binding to troponin C on the thin filaments of muscle results in switching contraction from the “off” to

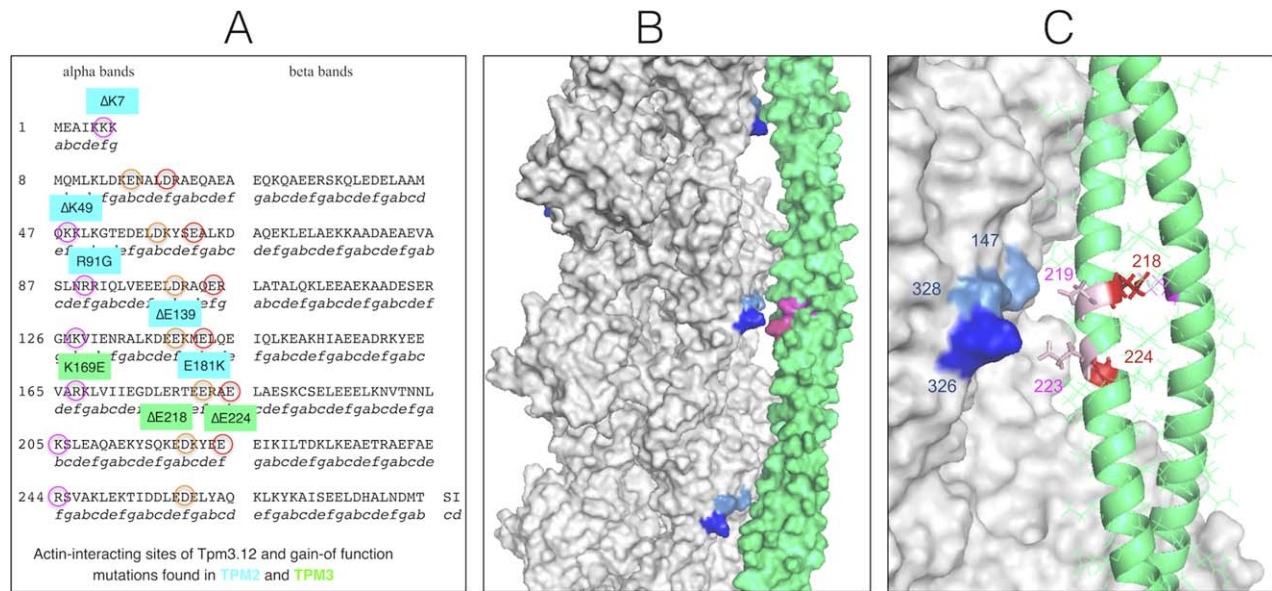


FIGURE 6: (A) Location of gain-of-function mutations in tropomyosin isoforms Tpm2.2 and Tpm3.12. The amino acids predicted to interact with actin are circled. (B) Structure of the F-actin-tropomyosin complex determined by Li et al.³² Surface rendering was made using PyMol. Actin K326, K328, and R147 are shown in different shades of blue, and tropomyosin 218, 219, 223, and 224 are shown in magenta. (C) The interface of tropomyosin period 6 with actin. Actin K326, K328, and R147 are shown in different shades of blue, E218 and E224 are shown in red, and D219 and E223 are shown in pink. Actin is represented by surface rendering; the secondary structure of tropomyosin backbone (coiled-coil alpha helix) is shown with the side chains in line notation.

the “on” state by causing a sideward shift of the actin-binding tropomyosin dimer, exposing the myosin binding site on the actin filament.^{30–32} Tropomyosins thus play a vital role in transmitting the Ca^{2+} signal from the troponin to the actin myosin binding site. Mutations in *TPM3* (coding for Tpm3.12) are an established molecular cause of a clinically and histopathologically heterogeneous group of myopathies, characterized by progressive weakness and by variable but characteristic findings on muscle pathology including nemaline rods, CFTD, or cap-like structures. The phenotype associated with Tpm3.12 ΔE218 and ΔE224 , with its congenital stiffness resulting from marked muscle hypercontractility, is significantly different from these known manifestation of *TPM3*-related disease. Muscle biopsy analysis did not reveal the typical muscle pathology seen in tropomyosin-related disease, including clear nemaline rods, although small rod-like structures (“miliary rods”) were seen in atrophic fibers of P2. We also found evidence of myofiber damage histologically, likely due to the permanently hypercontracted state of the muscle fibers, consistent with increased CK values. Six *TPM2* and 1 *TPM3* mutation that result in increased Ca^{2+} sensitivity have been reported in patients with only moderately progressive contractures and myopathic weakness.^{11,12,33} In contrast, our patients presented with a distinctive and considerably more severe congenital hypercontractile phenotype with

generalized muscle stiffness leading to severe respiratory failure in the neonatal period in one patient which represents a novel phenotype for tropomyosin-related disease.

Mutations in tropomyosin or actin that produce a hypercontractile phenotype may destabilize the “switched off” state, shifting the equilibrium in favor of the “switched on” state of actin-tropomyosin, which allows for myosin binding, thereby increasing Ca^{2+} sensitivity and, in one case, increasing maximum force generated and cross-bridge cycling rate.^{11,14,34,35} The finding that the Tpm3.12 ΔE218 and ΔE224 mutations introduced into recombinantly expressed tropomyosin resulted in increased Ca^{2+} sensitivity in the *in vitro* motility assay (see Fig 4), and that the Tpm3.12 ΔE224 biopsy has increased Ca^{2+} sensitivity of force production (see Fig 5), is compatible with this mechanism as the basis of the hypercontractile phenotype.

The hypercontractile phenotype associated with these Tpm3.12 mutations also conforms to the predictions made by Marston et al²² on biophysical grounds (Fig 6), which we now confirm clinically as well as *in vitro*. The atomic resolution structure of tropomyosin-bound actin shows that tropomyosin makes contact with actin at only two points: with actin Asp25 and with a cluster of basic amino acids actin K326, K328, and R147.³⁶ Tropomyosin has a seven-fold repeated structure corresponding to seven actin-binding interactions. Three

Tpm2.2 mutations and the Tpm3.12 mutation K169E were shown to destabilize the interaction with actin Asp25, based on energy landscape calculations, and increase Ca^{2+} sensitivity in *in vitro* motility assay, consistent with gain of contractile function.^{22,37} More importantly, two Tpm2.2 mutations, ΔE139 and E181K, destabilize the interaction with the actin K326, K328, and R147 cluster. All of these mutations involve a charge loss or reversal in a motif of two basic or acidic amino acids. There are nine such motifs in all tropomyosin isoforms. Mutations have been described in myopathy patients with increased *in vitro* Ca^{2+} sensitivity for six of the nine motifs (five in Tpm2.2 and one in Tpm3.12, 3 of which were associated with a phenotype of arthrogryposis or some signs of stiffness or contractures in addition to weakness). The remaining three motifs are at 218–219 (ED), 223–224 (EE), and 257–258 (ED) in either tropomyosin.²² Thus, the “stiff” patients’ mutations reported here are in the first two of these three remaining domains, which were predicted to result in a hypercontractile state. It is striking that the hypercontractile consequences in our cases are clinically more pronounced compared to the manifestations of other tropomyosin mutations previously reported. Notably, most of the mutations tested *in vitro* and found to display increased Ca^{2+} sensitivity are located adjacent to the tropomyosin amino acid that interacts with actin rather than at the site itself.²² This also applies to Tpm3.12 E218 and E224, which do not interact with actin, whereas neighboring Tpm3.12 E219 and E223 interact directly with the actin K326, K328, and R147 cluster (see Fig 6). In light of the structural analysis modeling of these physical interactions, it is significant that the actin K326N mutation at the cognate site in actin itself was the first “stiff” patient mutation reported in a thin filament protein.^{14,22,38} The degree of stiffness seen in that patient appeared similar to our observation.¹⁴

The *in vitro* sliding data obtained with mutant protein provide the link crucial to understanding the clinical hypercontractile phenotype; however, the consequences of the mutation on the entire thin filament and contractile fiber show additional complexities. Notably, the skinned fiber preparation had decreased maximal active tension resulting in a decrease in maximal force generation, whereas the force per cross-bridge was not altered. This raises the possibility that the cycling of cross-bridges back into the “off” state is impaired. The observed reduction in rate constant of force redevelopment, which indicates a reduced effectiveness of cross-bridge cycling, could explain the impaired force generation capacity of the muscle. Muscle fibers from P1 (ΔE224) generated a higher relative force at the same Ca^{2+} concentration, which may there-

fore compensate for the loss of maximal force-generating capacity. Because there was no evidence of weakness in the patients (at the time of examination), we surmise that the hypercontractility at submaximal calcium concentrations is so overwhelming that any decrease in maximal force does not come to bear on the phenotype. The continuous cellular stress of the hypercontractility of the muscle may cause future muscle damage and breakdown in our patients, possibly resulting in weakness and findings of tropomyosin muscle pathology over time. This assumption is supported by the CK elevations and also by the markedly abnormal muscle ultrasound findings in P2.

The question remains as to why the stiffness phenotype in our patients is significantly more severe and generalized compared to the best characterized gain of function mutation in Tpm2.2, the ΔK7 deletion, identified in patients with progressive, early onset contractures without clear muscle weakness in childhood but with some stiffness noted in one 14-year-old boy.¹¹ Tpm2.2 is expressed in both fast- and slow-twitch fibers. Increased Ca^{2+} sensitivity of force generation has also been observed in patients with distal arthrogryposis caused by mutations in troponin I (*TNNI2*) and troponin T3 (*TNNT3*), encoding for contractile apparatus proteins specific to fast-twitch fibers.³⁹ Contractures due to distal muscle involvement, which are predominantly fast-twitch fibers, typically develop prenatally but have little to no progression over time, with relatively normal proximal muscle strength and histology.⁴⁰ The generalized and more severe congenital stiffness observed in the Tpm3.12 ΔE218 and ΔE224 patients may thus be partially related to the expression pattern, with Tpm3.12 being the most relevant tropomyosin for all slow-twitch fibers.

Finally, we considered how the molecular mechanisms reported here could inform a rational therapeutic approach to these patients. P1 showed improvement with botulinum toxin injections, leading to increased range of motion, improved gait, and increased jaw mobility with enhanced speech production. Decreased neuromuscular transmission from the botulinum application would lead to less activation of excitation–contraction coupling, likely taking the edge off the baseline hypercontractility associated with the underlying *TPM3* mutation. Given the growing amount of literature supporting changed Ca^{2+} sensitivity of the actin–tropomyosin complex as a mechanism for muscle disease, future therapeutic strategies may involve reducing the release of Ca^{2+} at the sarcomere. An alternative approach would be to desensitize the thin filament to Ca^{2+} . In cases of decreased Ca^{2+} sensitivity, compounds that act as Ca^{2+} sensitizers, which are under active development in the neuromuscular and heart failure field, may be utilized.^{39,40} The latter type of

approach would likely be detrimental in the patients reported here.

In conclusion, in this study we identified two patients with a marked congenital hypercontractile phenotype caused by dominantly acting *de novo* mutations in Tpm3.12, ΔE218 and ΔE224, representing the most severe tropomyosin-related hypercontractile phenotype recognized to date. Our experiments confirm a mechanism of increased Ca²⁺ sensitivity by which Tpm3.12 mutant muscle fibers contract at lower Ca²⁺ concentrations compared to WT fibers. Our data lend *in vivo* and *in vitro* support to the conclusion that the increased Ca²⁺ sensitivity observed for both ΔE218 and ΔE224 results from a destabilization of the “off” state of the thin filament, resulting in excessively sensitized excitation–contraction coupling of the contractile apparatus, as previously predicted for ΔE218 on theoretical biophysical grounds. Mutations in tropomyosin, specifically in *TPM3*, should therefore be considered when evaluating a patient with congenital-onset muscle stiffness.

Acknowledgment

This study was supported by an Australian National Health and Medical Research Council fellowship (APP1002147, N.G.L.) and project grant (APP1022707, E.M., R.O.); an Australian Research Council Future Fellowship (FT100100734, K.J.N.); and grants from the British Heart Foundation (RG/11/20/29266, FS/12/24/29568, S.B.M., M.K.).

We thank the NIH Intramural Sequencing Center for performing the exome sequencing; the Analytic and Translational Genetics Unit at Massachusetts General Hospital and Genomes Management Application at University of Miami Miller School of Medicine for their help in the exome analysis; the Exome Aggregation Consortium; and the groups that provided exome variant data for comparison.

Authorship

Conception, design, and coordination of the study: S.D., N.G.L., N.F.C., C.A.C.O., S.B.M., C.G.B.; acquisition and analysis of clinical and biopsy data: J.K., M.L.Y., M.A.G., M.E.L., A.R., Y.H., J.D., M.K., E.P.W., A.R.F.; genetic analysis and interpretation: S.D., M.B.N., V.B.; permeabilized myofiber contractile performance: J.M.d.W., C.A.C.O.; *in vitro* motility assay: M.P., S.B.M.; synthesis of tropomyosin in *Sg* insect cells: K.J.N., E.M., R.O.; writing of the final manuscript: S.D., V.B., A.R.F., C.A.C.O., S.B.M., C.G.B.; contributed equally: M.P., J.M.d.W., M.B.N.

A full list of contributing groups can be found at <http://exac.broadinstitute.org/about>.

Potential Conflicts of Interest

Nothing to report.

References

1. Perry SV. Vertebrate tropomyosin: distribution, properties and function. *J Muscle Res Cell Motil* 2001;22:5–49.
2. Murakami K, Stewart M, Nozawa K, et al. Structural basis for tropomyosin overlap in thin (actin) filaments and the generation of a molecular swivel by troponin-T. *Proc Natl Acad Sci U S A* 2008;105:7200–7205.
3. De Paula AM, Franques J, Fernandez C, et al. A TPM3 mutation causing cap myopathy. *Neuromuscul Disord* 2009;19:685–688.
4. Ohlsson M, Fidzianska A, Tajsharghi H, Oldfors A. TPM3 mutation in one of the original cases of cap disease. *Neurology* 2009;72:1961–1963.
5. Schreckenbach T, Schröder JM, Voit T, et al. Novel TPM3 mutation in a family with cap myopathy and review of the literature. *Neuromuscul Disord* 2014;24:117–124.
6. Clarke NF, Kolski H, Dye DE, et al. Mutations in TPM3 are a common cause of congenital fiber type disproportion. *Ann Neurol* 2008;63:329–337.
7. Laing NG, Wilton SD, Akkari PA, et al. A mutation in the alpha tropomyosin gene TPM3 associated with autosomal dominant nemaline myopathy NEM1. *Nat Genet* 1995;10:249.
8. Tan P, Briner J, Boltshauser E, et al. Homozygosity for a nonsense mutation in the alpha-tropomyosin slow gene TPM3 in a patient with severe infantile nemaline myopathy. *Neuromuscul Disord* 1999;9:573–579.
9. Wattanasirichaigoon D, Swoboda KJ, Takada F, et al. Mutations of the slow muscle alpha-tropomyosin gene, TPM3, are a rare cause of nemaline myopathy. *Neurology* 2002;59:613–617.
10. Lehtokari V-L, Pelin K, Donner K, et al. Identification of a founder mutation in TPM3 in nemaline myopathy patients of Turkish origin. *Eur J Hum Genet* 2008;16:1055–1061.
11. Mokbel N, Ilkovski B, Kreissl M, et al. K7del is a common TPM2 gene mutation associated with nemaline myopathy and raised myofibre calcium sensitivity. *Brain* 2013;136(pt 2):494–507.
12. Davidson AE, Siddiqui FM, Lopez MA, et al. Novel deletion of lysine 7 expands the clinical, histopathological and genetic spectrum of TPM2-related myopathies. *Brain* 2013;136(pt 2):508–521.
13. Ottenheijm CAC, Lawlor MW, Stienen GJM, et al. Changes in cross-bridge cycling underlie muscle weakness in patients with tropomyosin 3-based myopathy. *Hum Mol Genet* 2011;20:2015–2025.
14. Jain RK, Jayawant S, Squier W, et al. Nemaline myopathy with stiffness and hypertonia associated with an ACTA1 mutation. *Neurology* 2012;78:1100–1103.
15. Del Bigio MR, Chudley AE, Sarnat HB, et al. Infantile muscular dystrophy in Canadian aboriginals is an αB-crystallinopathy. *Ann Neurol* 2011;69:866–871.
16. Gonzalez MA, Lebricio RFA, Van Booven D, et al. GENomes Management Application (GEM.app): a new software tool for large-scale collaborative genome analysis. *Hum Mutat* 2013;34:842–846.
17. Akkari PA, Song Y, Hitchcock-DeGregori S, et al. Expression and biological activity of Baculovirus generated wild-type human slow alpha tropomyosin and the Met9Arg mutant responsible for a

- dominant form of nemaline myopathy. *Biochem Biophys Res Commun* 2002;296:300–304.
18. Kron SJ, Toyoshima YY, Uyeda TQ, Spudich JA. Assays for actin sliding movement over myosin-coated surfaces. *Methods Enzymol* 1991;196:399–416.
19. Marston SB, Fraser ID, Bing W, Roper G. A simple method for automatic tracking of actin filaments in the motility assay. *J Muscle Res Cell Motil* 1996;17:497–506.
20. Messer AE, Jacques AM, Marston SB. Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. *J Mol Cell Cardiol* 2007;42:247–259.
21. Bayliss CR, Jacques AM, Leung M-C, et al. Myofibrillar Ca(2+) sensitivity is uncoupled from troponin I phosphorylation in hypertrophic obstructive cardiomyopathy due to abnormal troponin T. *Cardiovasc Res* 2013;97:500–508.
22. Marston S, Memo M, Messer A, et al. Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients. *Hum Mol Genet* 2013;22:4978–4987.
23. Memo M, Leung M-C, Ward DG, et al. Familial dilated cardiomyopathy mutations uncouple troponin I phosphorylation from changes in myofibrillar Ca2+ sensitivity. *Cardiovasc Res* 2013;99:65–73.
24. Fraser ID, Marston SB. In vitro motility analysis of actin-tropomyosin regulation by troponin and calcium. The thin filament is switched as a single cooperative unit. *J Biol Chem* 1995;270:7836–7841.
25. Ottenheijm CAC, Witt CC, Stienen GJ, et al. Thin filament length dysregulation contributes to muscle weakness in nemaline myopathy patients with nebulin deficiency. *Hum Mol Genet* 2009;18:2359–2369.
26. Ottenheijm CAC, Hooijman P, DeChene ET, et al. Altered myofilament function depresses force generation in patients with nebulin-based nemaline myopathy (NEM2). *J Struct Biol* 2010;170:334–343.
27. Malfatti E, Lehtokari V-L, Böhm J, et al. Muscle histopathology in nebulin-related nemaline myopathy: ultrastructural findings correlated to disease severity and genotype. *Acta Neuropathol Commun* 2014;2:44.
28. Caremani M, Dantzig J, Goldman YE, et al. Effect of inorganic phosphate on the force and number of myosin cross-bridges during the isometric contraction of permeabilized muscle fibers from rabbit psoas. *Biophys J* 2008;95:5798–5808.
29. Manders E, Bogaard H-J, Handoko ML, et al. Contractile dysfunction of left ventricular cardiomyocytes in patients with pulmonary arterial hypertension. *J Am Coll Cardiol* 2014;64:28–37.
30. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev* 2000;80:853–924.
31. Behrmann E, Müller M, Penczek PA, et al. Structure of the rigor actin-tropomyosin-myosin complex. *Cell* 2012;150:327–338.
32. Li XE, Tobacman LS, Mun JY, et al. Tropomyosin position on F-actin revealed by EM reconstruction and computational chemistry. *Biophys J* 2011;100:1005–1013.
33. Marttila M, Lehtokari VL, Marston S, et al. Mutation update and genotype-phenotype correlations of novel and previously described mutations in TPM2 and TPM3 causing congenital myopathies. *Hum Mutat* 2014;35:779–790.
34. Ochala J, Gokhin DS, Pénisson-Besnier I, et al. Congenital myopathy-causing tropomyosin mutations induce thin filament dysfunction via distinct physiological mechanisms. *Hum Mol Genet* 2012;21:4473–4485.
35. Memo M, Marston S. Skeletal muscle myopathy mutations at the actin tropomyosin interface that cause gain- or loss-of-function. *J Muscle Res Cell Motil* 2013;34:165–169.
36. Orzechowski M, Li XE, Fischer S, Lehman W. An atomic model of the tropomyosin cable on F-actin. *Biophys J* 2014;107:694–699.
37. Orzechowski M, Fischer S, Moore JR, et al. Energy landscapes reveal the myopathic effects of tropomyosin mutations. *Arch Biochem Biophys* 2014;564:89–99.
38. Orzechowski M, Moore JR, Fischer S, Lehman W. Tropomyosin movement on F-actin during muscle activation explained by energy landscapes. *Arch Biochem Biophys* 2014;545:63–68.
39. Perrone SV, Kaplinsky EJ. Calcium sensitizer agents: a new class of inotropic agents in the treatment of decompensated heart failure. *Int J Cardiol* 2005;103:248–255.
40. De Winter JM, Buck D, Hidalgo C, et al. Troponin activator augments muscle force in nemaline myopathy patients with nebulin mutations. *J Med Genet* 2013;50:383–392.

(De-)sensitisation versus uncoupling: what drives cardiomyopathies in the thin filament? The known unknowns

Steven Marston, Andrew Messer, Maria Papadaki

NHLI, Imperial College London, London, W12 0NN, UK

Accepted Manuscript

Dr Hwang's recent letter concerning our paper raises some very interesting points about the molecular mechanism of familial cardiomyopathy (HCM and DCM) due to mutations in the proteins of the thin filament and the key role of troponin.

In principle such mutations could cause defects in force generation or force transmission but so far all the mutations in thin filament proteins that have been investigated in detail indicate abnormal force production linked to abnormal Ca^{2+} -regulation. Troponin, in concert with tropomyosin and actin comprises the Ca^{2+} -dependent switch of muscle and the properties of this switch are modulated by PKA phosphorylating TnI, therefore troponin abnormalities are likely to play a central role in cardiomyopathies. The case has been made that HCM-causing mutations increase myofilament Ca^{2+} -sensitivity and this is sufficient to trigger the symptoms of HCM. On the other hand DCM-causing mutations uncouple the normal relationship between Ca^{2+} -sensitivity and PKA-dependent phosphorylation of troponin I (TnI) and this has been shown to blunt responses to adrenergic stimulation and predispose the heart to systolic dysfunction under stress ^{1,2}.

Our recently published work highlights the importance of troponin still more, since we have demonstrated that small molecule Ca^{2+} -sensitisers (EMD57033 and Bepridil) can mimic the uncoupling effect of mutations, whilst the Ca^{2+} -desensitiser epigallocatechin-3-gallate (EGCG) can restore the modulation of Ca^{2+} -sensitivity by TnI phosphorylation to thin filaments containing DCM or HCM mutations that uncouple this relationship ³. All of these reagents act by binding to troponin; thus understanding how Ca^{2+} and phosphorylation control

contractility through troponin is the key to determining how thin filament cardiomyopathies are initiated

There are severe practical problems in linking troponin structure with function because the most important parts of the complex are intrinsically disordered and cannot be defined in terms of a static structure. These include the cardiac-specific N-terminus of cTnI (amino acids 1-30) that contains the PKA phosphorylation sites (Serines 22 and 23), the so-called switch peptide of cTnI (148-164), that plays a key role in the Ca^{2+} -dependent switching on and off of the thin filament, and the C-terminus of troponin T (cTnT₃ 280-298) whose function is not known although it is likely to be located close to the regulatory region of troponin and may interact with the other two peptides. Protein nuclear magnetic resonance has been used to determine the structure of the missing peptides and their binary complexes with troponin C (TnC) in several states ⁴⁻⁹. When TnI is unphosphorylated there is a weak ionic bond between the N terminal peptide and the regulatory Ca^{2+} -binding EF hand of TnC. When Ser 22 and 23 are phosphorylated the binding is further weakened. We have proposed that the unphosphorylated state can also be disrupted by mutations or other alterations in any component of the thin filament or by small molecules resulting in uncoupling. However, whilst it can account for all our experimental observations, we do not believe that this hypothesis can be tested until the structure and dynamics of the whole troponin structure is known. This question is now being addressed by molecular dynamics simulations, but many practical and technical problems need to be solved before a believable structural and dynamic description of the regulatory elements of troponin becomes possible.

In his letter to the editors Dr Hwang presents a model based on his extensive experience of investigating troponin by protein nuclear magnetic resonance protocols ⁶ that is proposed to be able to explain the structural basis of the results we obtained. In this model an alternative mechanism of Ca^{2+} -dependent activation is proposed in addition to the current consensus mechanism that accounts for Ca^{2+} -switching of muscle and its modulation by cTnI phosphorylation (see for instance Solaro et al. 2008¹⁰). It is proposed that the helical segment of cTnI (39-60), downstream of the phosphorylatable peptide, that normally interacts strongly with the C-terminal domain of TnC can become dissociated, thus allowing a different interaction between the two domains of TnC that would have a higher Ca^{2+} -sensitivity and not involve cTnI 1-30, therefore it would be uncoupled. The rationale for proposing this is the observation that we measure uncoupling and the effects of EMD57033 and EGCG in the *in vitro* motility assay, where the Ca^{2+} -sensitivity of filament activation is very high (EC_{50} 0.1-0.3 μM) whereas the Ca^{2+} -sensitivity of contractility in skinned cardiac muscle in the phosphorylated and unphosphorylated states is about ten times lower.

However, there seems no justification for a two-mechanism model. As described in our papers, Ca^{2+} -regulation of thin filaments occurs normally when measured by the *in vitro* motility assay and all the effects of phosphorylation, mutants, Ca^{2+} sensitizers and desensitizers and, especially uncoupling and re-coupling, on Ca^{2+} -sensitivity were consistently observed using the IVMA technique and, in the case of the small molecules, effects were shown to be fully reversible. In these studies Ca^{2+} -sensitivity ranged between 0.04 and 0.2 μM so there is no reason to invoke two mechanisms

based on different Ca^{2+} -sensitivity ranges. In fact it would be very hard indeed to explain how drugs such as EGCG could reverse uncoupling if they were acting through a mechanism separate from the one that modulates Ca^{2+} -sensitivity by phosphorylation or mutations. The issue of absolute Ca^{2+} -sensitivity, and even the experimental system studied is irrelevant since we have clearly shown that exactly the same modulation of Ca^{2+} -sensitivity of contraction by phosphorylation, drugs and mutations and the recoupling due to EGCG can be demonstrated in myofibrils, where the Ca^{2+} -sensitivity is in the 0.4-2 μM range^{3, 11}

What we undoubtedly agree upon is the central role of troponin abnormalities in cardiomyopathy and the need to know much more about the structure and dynamics of the intrinsically disordered regulatory segments of the molecule. Not only is this necessary to provide a molecular mechanism for the physiological effects of TnI phosphorylation and mutations in thin filaments that cause cardiomyopathy, it is also essential in order to understand how small molecules can interact with this system.¹⁴ This is clinically relevant since Ca^{2+} -sensitisers acting on TnC have been investigated as inotropic drugs and our discovery of the ability of EGCG and similar compounds to reverse the uncoupling effect of DCM-causing mutations (and some HCM-causing mutations) points to potential therapies for familial cardiomyopathies.

REFERENCES

1. Messer A, Marston S. Investigating the role of uncoupling of Troponin I phosphorylation from changes in myofibrillar Ca^{2+} -sensitivity in the pathogenesis of Cardiomyopathy. *Front Physiol* 2014;5:315.
2. Marston SB. How Do Mutations in Contractile Proteins Cause the Primary Familial Cardiomyopathies? *J Cardiovasc Transl Res* 2011;4:245-255.
3. Papadaki M, Vikhorev P, Marston S, Messer A. Epigallocatechin-3-Gallate Reverses the Defects in Modulation of Ca^{2+} -Sensitivity by Troponin I Phosphorylation Caused by Hypertrophic and Dilated Cardiomyopathy Mutations in Cardiac Muscle. *Biophys J* 2015;108:361a-362a.
4. Howarth JW, Meller J, Solaro RJ, Trewhella J, Rosevear PR. Phosphorylation-dependent conformational transition of the cardiac specific N-extension of troponin I in cardiac troponin. *J Mol Biol* 2007;373:706-722.
5. Ward DG, Brewer SM, Calvert MJ, Gallon CE, Gao Y, Trayer IP. Characterization of the interaction between the N-terminal extension of human cardiac troponin I and troponin C. *Biochemistry* 2004;43:4020-4027.

6. Hwang PM, Cai F, Pineda-Sanabria SE, Corson DC, Sykes BD. The cardiac-specific N-terminal region of troponin I positions the regulatory domain of troponin C. *Proc Natl Acad Sci USA* 2014;111:14412-14417.
7. Baryshnikova OK, Li MX, Sykes BD. Modulation of cardiac troponin C function by the cardiac-specific N-terminus of troponin I: influence of PKA phosphorylation and involvement in cardiomyopathies. *J Mol Biol* 2008;375:735-751.
8. Robertson IM, Li MX, Sykes BD. Solution structure of human cardiac troponin C in complex with the green tea polyphenol, (-)-epigallocatechin 3-gallate. *J Biol Chem* 2009;284:23012-23023.
9. Wang X, Li MX, Spyropoulos L, Beier N, Chandra M, Solaro RJ, Sykes BD. Structure of the C-domain of human cardiac troponin C in complex with the Ca²⁺ sensitizing drug EMD 57033. *J Biol Chem* 2001;276:25456-25466.
10. Solaro RJ, Rosevear P, Kobayashi T. The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. *Biochem Biophys Res Commun* 2008;369:82-87.
11. Vikhorev PG, Song W, Wilkinson R, Copeland O, Messer AE, Ferenczi MA, Marston SB. The dilated cardiomyopathy-causing mutation ACTC E361G in cardiac muscle myofibrils specifically abolishes modulation of Ca(2+) regulation by phosphorylation of troponin I. *Biophys J* 2014;107:2369-2380.