
A FUNCTIONAL INVESTIGATION OF MUTATIONS ASSOCIATED
WITH HYPERTROPHIC AND DILATED CARDIOMYOPATHY

by

Emma Catherine Dyer

Thesis submitted for the degree of

Doctor of Philosophy
and
Diploma of Imperial College London

Department of Cardiac Medicine
National Heart and Lung Institute
Faculty of Medicine
Imperial College London

January 2008

ABSTRACT

The mutations E361G and E99K in the cardiac actin gene cause dilated and hypertrophic cardiomyopathy respectively. Transgenic mouse models have been developed that over-express these mutations in their hearts. Mutant protein was expressed at 50% of total actin. Thin filaments were reconstituted with mouse actin, human cardiac tropomyosin and troponin, and studied by *in vitro* motility assay.

Phosphorylated E361G and non-transgenic thin filaments were indistinguishable at all Ca^{2+} -concentrations (EC_{50} NTg/E361G = 1.07 ± 0.11 , $p=0.59$). E361G-actin reconstituted with dephosphorylated troponin had a lower Ca^{2+} -sensitivity than non-transgenic (EC_{50} NTg/E361G = 0.47 ± 0.09 , $p=0.01$). When we compared E361G thin filaments containing phosphorylated and dephosphorylated troponin, the Ca^{2+} -sensitivity was indistinguishable (EC_{50} E361G/E361G.dp = 1.03 ± 0.08 , $p=0.74$). This is in contrast to non-transgenic actin, where the EC_{50} of the phosphorylated thin filament was 3-times greater than dephosphorylated ($p=0.003$). The major functional change induced by the E361G mutation was therefore the abolition of the response to troponin phosphorylation. This would blunt the inotropic response *in vivo* and be the cause of the E361G DCM phenotype. The *in vitro* motility technique was extended to measure $\bar{\text{U}}$ -actinin binding affinity. The E361G mutant actin had a 5.3-fold reduced affinity for $\bar{\text{U}}$ -actinin, indicating a weaker attachment of filaments at the Z-line.

Investigation of the DCM-causing mutation TNNC1 G159D in troponin isolated from human muscle showed a higher Ca^{2+} sensitivity with native phosphorylation levels (EC_{50} NF/G159D = 4.67 ± 1.87) and upon dephosphorylation (EC_{50} NF/G159D = 1.77 ± 0.29). G159D thin filaments also blunted the change in Ca^{2+} -sensitivity when dephosphorylated (EC_{50} G159D/G159D.dp = 1.24 ± 0.17).

The E99K mutation produced a strikingly different pattern of results to DCM mutations of an increased Ca^{2+} -sensitivity and faster cross-bridge cycling rate, with phosphorylated (EC_{50} NTg/E99K = 2.54 ± 0.64 , $p=0.07$) and dephosphorylated troponin (EC_{50} NTg/E99K = 1.2). The E99K mutation also

produced a reduced but not completely suppressed response to troponin phosphorylation (EC_{50} E99K/E99K.dp = 1.14 ± 0.06 , $p=0.12$).

CONTENTS

Abstract.....	2
Contents.....	4
List of figures.....	9
List of Tables.....	13
List of Abbreviations.....	16
Acknowledgements.....	18
1. Introduction	19
1.1 Cardiac Physiology.....	19
1.2 The Cardiac Myocyte Structure.....	20
1.3 Actin: Structure and Function.....	23
1.4 Myosin Structure	26
1.5 The Mechanism of Contraction: The Cross-bridge Cycle	29
1.6 The Regulation of Cardiac Contraction	30
1.6.1 Excitation-Contraction (EC) Coupling.....	30
1.7 Contraction and Regulation by Thin Filaments.....	31
1.7.1 Ca^{2+} Regulation and Co-operativity.....	33
1.7.2 Troponin and Ca^{2+} Regulation.....	34
1.8 Adrenergic Regulation of Contractility	36
1.9 The Cardiomyopathies: Hypertrophic and Dilated.....	39
1.9.1 Dilated Cardiomyopathy	40
1.9.1.1 Disease Genes of DCM	41
1.9.1.1.1 β -Cardiac Actin Mutations	43
1.9.1.1.2 Cardiac Troponin C Mutation.....	44
1.9.2 Hypertrophic Cardiomyopathy	45
1.9.2.1 β -Cardiac Actin Mutations.....	46
1.9.3 Animal Models of DCM.....	48
1.9.4 Animal Models of HCM.....	49
1.10 Context and Objectives of this Study.....	50
2. Materials and Methods.....	52
2.1 Production of Mutant ACTC Transgenic Mice	52
2.2 Using Ear Tissue to Genotype Transgenic Mice by PCR	52
2.3 PCR Screening Transgenic Mice from Ear Tissue	53
2.4 Screening of Transgenic Mouse by 2-D Electrophoresis.....	54
2.4.1 Sample Preparation and Rehydration of Immobiline DryStrips.....	54
2.4.2 First Dimension Isoelectric Focusing (IEF)	55
2.4.3 Equilibration of Immobiline DryStrips and Second Dimension SDS- PAGE	56
2.4.4 Western Blotting.....	57
2.4.4.1 Protein Transfer	57
2.4.4.2 Immunodetection	58
2.4.4.3 Densitometric Analysis of Western Blots.....	59
2.5 Isolation of Actin from Transgenic Mouse Hearts.....	59
2.5.1 Actin isolation directly from Native Thin Filaments	59
2.6 Preparation of Proteins from Rabbit Skeletal Muscle	61
2.6.1 Preparation of F-actin from Rabbit Skeletal Muscle	61

2.6.2	Preparation of Myosin	61
2.6.3	Preparation of Rabbit Skeletal Muscle HMM	61
2.7	Collection and Storage of Non-Failing Human Myocardium	62
2.8	Preparation of Human Cardiac Troponin	63
2.8.1	Human Cardiac Troponin Preparation by Affinity Chromatography	63
2.8.1.1	Dephosphorylation of Human Cardiac Troponin	65
2.8.2	Preparation of Human Cardiac Tropomyosin	66
2.8.3	Preparation of $\bar{U}$ -Actinin	66
2.8.4	Use of Protease Inhibitors	66
2.9	Protein Analysis	66
2.9.1	Determination of Protein Concentration	66
2.9.2	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	67
2.9.2.1	Sample Preparation	67
2.9.2.2	Electrophoresis Using The Pharmacia System	67
2.9.2.3	Densitometric Analysis of SDS-PAGE Gels	68
2.9.3	Pro-Q Diamond Phosphoprotein Gel Stain	68
2.9.4	Sypro Ruby Protein Gel Stain	69
2.10	<i>In Vitro</i> Motility Assay	69
2.10.1	Fluorescent Labelling of F-Actin	70
2.10.1.1	Fluorescent labelling of Skeletal Muscle F-Actin	70
2.10.1.2	Fluorescent Labelling of Transgenic Mouse F-Actin	70
2.10.2	Pre-mixing and Incubation of Thin Filament Proteins	70
2.10.3	Motility Assay Flow Cell Preparation	70
2.10.4	Affinity Precipitation of HMM	71
2.10.5	Infusion of Proteins	71
2.10.6	Ca^{2+} /EGTA buffer Preparation	73
2.10.7	Visualisation Apparatus	74
2.10.8	Acquisition of image software	75
2.11	Statistical Analysis	76
2.12	Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to $\bar{U}$ -Actinin	76
3.	Functional Investigation of DCM Actin Mutant Glu361Gly	79
3.1	Introduction	79
3.2	Location of E361G in the Actin Polymer	79
3.3	Generation of E361G Transgenic Lines	81
3.4	Screening of E361G Transgenic Lines	82
3.5	Transgenic-E361G Mouse Phenotype	86
3.6	Isolation of F-actin from E361G Transgenic Mouse Hearts	86
3.6.1	The Acetone Powder Method: Loss of E361G	86
3.6.2	<i>In Vitro</i> Motility Analysis of Mouse F-actin	87
3.6.2.1	Length Distribution of Non-Transgenic and Transgenic Actin Filaments	88
3.6.2.2	Non-Transgenic and Transgenic Actin Filament Motility	89
3.6.2.3	Effects of Human Cardiac Tropomyosin on Actin Filament Motility	90
3.6.2.4	Effects of Non-failing Human Cardiac Troponin on Non-transgenic and Transgenic Filament Motility at High and Low Ca^{2+}	93

3.6.2.5	Comparing Ca^{2+} Concentration Curves of Non-transgenic and Transgenic Actin.....	97
3.6.2.6	Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to $\ddot{\text{U}}$ -Actinin	99
3.6.3	The Acetone Powder Method: Partial Extraction of E361G in the F-actin	101
3.6.4	<i>In Vitro</i> Motility Analysis of Mouse F-actin- ϕ	102
3.6.4.1	Actin- ϕ Filament Motility	102
3.6.4.2	Effects of Human Cardiac Tropomyosin on Actin- ϕ Filament Motility	104
3.6.4.3	Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G Filament Motility at High and Low Ca^{2+}	107
3.6.4.4	Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Actin- ϕ	111
3.6.4.5	Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin- ϕ to $\ddot{\text{U}}$ -Actinin	112
3.6.5	Isolation of F-actin from Whole Thin Filaments	113
3.6.6	<i>In Vitro</i> Motility Analysis of Mouse Thin Filament F-actin	117
3.6.6.1	E361G Thin Filament Actin Stability.....	117
3.6.6.2	Thin Filament Actin Motility	117
3.6.6.3	Effects of Cardiac Tropomyosin on Thin Filament Actin Motility ..	119
3.6.6.4	Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G Thin Filament Actin Motility at High and Low Ca^{2+} ..	123
3.6.6.5	Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Thin Filament Actin using Human Cardiac Troponin	129
3.6.6.6	Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to $\ddot{\text{U}}$ -Actinin	133
3.7	Discussion.....	135
3.7.1	Native Human Sequence Thin Filaments	135
3.7.2	Stability of E361G Mutant Actin.....	136
3.7.3	Reproducibility of the <i>In Vitro</i> Motility Assay.....	136
3.7.4	Comparisons of Transgenic Mouse and Skeletal Rabbit Actin	137
3.7.5	Comparison of Actin- ϕ and Thin Filament Actin	140
3.7.6	Comparison of Non-Transgenic Thin Filament Actin with E361G Thin Filament Actin	142
3.7.7	Altered $\ddot{\text{U}}$ -Actinin Binding	143
3.7.8	The DCM Phenotype	146
4.	The Effect of Troponin Phosphorylation on E361G Thin Filament Function.....	147
4.1	Effects of Rabbit Skeletal Muscle Troponin on Non-Transgenic and E361G Actin Motility at High and Low Ca^{2+}	147
4.1.1	Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Actin with Rabbit Skeletal Troponin	152
4.2	Effect of Cardiac Troponin Dephosphorylation on the Ca^{2+} dependence of E361G and Non-transgenic Actin Motility	155
4.3	Discussion.....	168

5.	Functional Investigation of a DCM Troponin C Mutant Gly159Asp	175
5.1	Patient History and Diagnosis	175
5.2	Location of the TNNC1 G159D Mutation.....	176
5.3	Isolation of Non-Failing and G159D Troponin	178
5.4	Phosphorylation Measurements	179
5.5	<i>In Vitro</i> Motility Analysis	181
5.5.1	Effects of Human Cardiac Tropomyosin on Actin Filament Motility	181
5.5.2	Effects of Human Cardiac Troponin on Actin Filament Motility.....	182
5.5.3	Comparing Ca^{2+} Sensitivity Curves of Non-Failing and G159D Cardiac Troponin	186
5.5.4	Effect of Troponin Dephosphorylation on the Ca^{2+} -Dependence of Motility	189
5.6	Discussion.....	198
6.	Functional Investigation of HCM Actin Mutant Glu99Lys	208
6.1	Introduction	208
6.2	Location of E99K in the Actin Molecule	208
6.3	E99K Transgenic Mouse Phenotype	210
6.4	Screening of E99K Transgenic Lines	211
6.5	Isolation of F-Actin from E99K Transgenic Mouse Hearts.....	211
6.6	Measurement of Phosphorylation Levels	214
6.7	Length Distribution of E99K and Non-Transgenic Actin	216
6.8	<i>In Vitro</i> Motility Assay	217
6.8.1	Non-Transgenic and E99K Actin Filament Motility	217
6.8.2	Effects of Human Cardiac Tropomyosin on Actin Filament Motility	218
6.8.3	Effects of Non-Failing Human Cardiac Troponin on Non-Transgenic and E99K Actin at High and Low Ca^{2+}	220
6.8.4	Comparing Ca^{2+} Concentration Curves of Non-Failing and E99K Actin	225
6.8.5	Effect of Troponin Dephosphorylation on the Ca^{2+} Dependence of Motility	228
6.8.6	Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to $\bar{\text{U}}$ -Actinin.....	233
6.9	Discussion.....	235
6.9.1	Defects of Transgenic E99K Actin.....	236
6.9.2	Comparison with Another E99K Mutation Study.....	239
6.9.3	Comparisons with Other HCM-Associated Mutations.....	240
7.	General Discussion	243
7.1	Rationale for this Study	243
7.2	Limitations of the Study	244
7.3	The <i>in vitro</i> Motility Assay.....	244
7.4	Contractile Abnormalities caused by DCM Mutations.....	245
7.4.1	E361G: Blunted Response to Troponin I Phosphorylation	246
7.4.2	Reduced $\bar{\text{U}}$ -actinin Binding	247
7.4.3	The DCM Transgenic Mouse Phenotype	247
7.4.4	The G159D Phenotype.....	247
7.5	Contractile Abnormalities caused by HCM Mutations.....	249

7.6	Future Work	250
8.	References	252

LIST OF FIGURES

Figure 1.1 Pressure and Volume Changes during a Cardiac Cycle	19
Figure 1.2 Cardiac Muscle and an Isolated Myocyte	20
Figure 1.3 Structure of the Sarcomere	21
Figure 1.4 The Length-Tension Relationship In Cardiac Muscle.....	22
Figure 1.5 Molecular Model of the Actin Monomer.....	24
Figure 1.6 Molecular Model of F-Actin	25
Figure 1.7 Structure of the Myosin Molecule.....	26
Figure 1.8 Actomyosin Interaction Model.....	27
Figure 1.9 Myosin Cross-bridge Cycle	30
Figure 1.10 Ca^{2+} Sensitivity of Isometric Force.....	31
Figure 1.11 Atomic Model of the Thin Filament.....	32
Figure 1.12 Three-State Model of the Thin Filament.....	34
Figure 1.13 Conformational Changes occurring in Troponin in response to Ca^{2+}	35
Figure 1.14 Crystal Structure of the Core Domain of Troponin in the Ca^{2+} Saturated Form.....	36
Figure 1.15 Cardiac Remodelling Patterns and Histopathology.....	40
Figure 1.16 Molecular Pathology of HCM and DCM	48
Figure 2.1. Positioning of IPG Strip on DryStrip Reswelling Tray.....	55
Figure 2.2 Positioning of IPG Strip Gels in the DryStrip aligner	56
Figure 2.3 Positioning Equilibrated IPG Strips on MultiPhor II	57
Figure 2.4. Immunodetection of E361G (A) and E99K (B) F-actin	58
Figure 2.5 SDS-PAGE of E361G Isolation Fractions	60
Figure 2.6. Affinity chromatography using the anti-TnI monoclonal antibody. ...	64
Figure 2.7 SDS-PAGE of Extracted Human Cardiac Troponin	65
Figure 2.8 Bradford Assay Calibration graph for 100 μl Sample	67
Figure 2.9. Construction of the Motility Flow Cell	71
Figure 2.10. Infusion of Proteins	72
Figure 2.11. Visualisation and recording apparatus used for the analysis of the <i>in vitro</i> motility assay.....	74
Figure 2.12. Optimum Timer intervals plotted against velocity.....	75
Figure 2.13 Theoretical Curve of Filament Competition	77
Figure 2.14. Displacement of Rabbit TRITC-Actin Filaments.....	77
Figure 2.15 Skeletal Rabbit Actin Competitive Binding Assay (A) and Fit (B)...	78
Figure 3.1. Ribbon Representation (A) and Space Filling Model (B) of the E361G Location in the Actin Polymer	80
Figure 3.2. 2-DE of E361G Transgenic Mouse Heart Myofibrils (A) and F-actin (B), Isolated via an Acetone Powder Method.....	87
Figure 3.3 Non-Transgenic and Transgenic Actin Filaments labelled with TRITC-phalloidin.....	88
Figure 3.4 Length Distribution of Non-Transgenic (13.16) and Transgenic (13.15) Actin	89
Figure 3.5 Effect of Human Cardiac Tropomyosin on Non-Transgenic and Transgenic Actin Motility: (A) Sliding Speed, and (B) % motility	91

Figure 3.6 Effect of Non-Failing Human Cardiac Troponin on Non-Transgenic and E361G Actin Motility at High and Low Ca^{2+}	93
Figure 3.7 Ca^{2+} Regulation of Thin Filaments Containing Non-Transgenic and Transgenic Actin, (A) Sliding Speed and (B) Percentage Motile (40nM Troponin)	98
Figure 3.8 $\bar{U}$ -actinin Competitive Binding Assay.....	99
Figure 3.9 $\bar{U}$ -actinin Competitive Binding Fit	100
Figure 3.10 2-DE of E361G Expression During Extraction via an Acetone Powder.....	102
Figure 3.11 Effect of Human Cardiac Tropomyosin on Non-Transgenic and Transgenic Actin- ϕ Motility: (A) Sliding Speed, and (B) % motility	104
Figure 3.12 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G filament Motility at $3.9\mu\text{M}$ Ca^{2+} and 1nM Ca^{2+}	107
Figure 3.13 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G actin- ϕ , (A) Sliding Speed and (B) Percentage Motile (40nM Troponin)	111
Figure 3.14 $\bar{U}$ -actinin Competitive Binding Assay of E361G and Non-transgenic Actin- ϕ Filaments	113
Figure 3.15 NTg (A) and E361G (B) Actin Isolation Fractions.....	114
Figure 3.16 Western Blot of E361G and Non-Transgenic Actin Isolation Fractions	115
Figure 3.17 Determination of the E361G and Non-Transgenic F-Actin Concentration	115
Figure 3.18 2-DE of E361G Expression during F-Actin Extraction.....	116
Figure 3.19 Effect of Human Cardiac Tropomyosin on Non-Transgenic and E361G Thin Filament Actin Motility: (A) Sliding Speed, and (B) % motility	119
Figure 3.20 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G filament Motility at $3.9\mu\text{M}$ Ca^{2+} and 1nM Ca^{2+}	123
Figure 3.21 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Thin Filament Actin.....	130
Figure 3.22 $\bar{U}$ -actinin Competitive Binding Assay of E361G and Non-transgenic Actin Filaments	133
Figure 3.23 Sliding Speed Comparisons of Non-transgenic Actin.....	141
Figure 3.24 Sliding Speed Comparisons of E361G Actin.....	141
Figure 3.25 Location of E361G	144
Figure 3.26 $\bar{U}$ -actinin Competitive Binding Assay of K336E	145
Figure 4.1 Effects of Rabbit Skeletal Troponin on Non-Transgenic and E361G filament Motility at $3.9\mu\text{M}$ Ca^{2+} and 1nM Ca^{2+}	148
Figure 4.2 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Thin Filament Actin with Skeletal Troponin.....	153
Figure 4.3 SDS-PAGE of Non-Failing Human Cardiac Troponin	155
Figure 4.4 Effect of Non-Transgenic Actin in Combination with Native or Dephosphorylated Troponin.....	157
Figure 4.5 Effect of Troponin Dephosphorylation on E361G Actin Motility.....	160
Figure 4.6 Effect of Troponin Dephosphorylation on Non-Transgenic and E361G Actin Motility.....	163
Figure 4.7 E361G and Non-transgenic Actin in combination with Phosphorylated and Dephosphorylated Troponin.....	165
Figure 4.9 SDS-PAGE and Ratios of E361G and Non-Transgenic Myofibrils.....	167

Figure 4.10 MRI Scanning of E361G Transgenic Mouse Hearts	171
Figure 4.11 Non-Transgenic and E361G Basal Contraction Data.....	172
Figure 4.12 Non-Transgenic and E361G Isoprenaline Response Data	172
Figure 4.13 Non-Transgenic and E361G Ca^{2+} Response Data	173
Figure 4.14 Non-Transgenic and E361G Frequency Response Data	173
Figure 5.1 Histological Analysis of Left Ventricular Myocardium.....	176
Figure 5.2 Troponin C Gly159 in the Whole Troponin Complex.....	177
Figure 5.3 SDS-PAGE Showing Extraction of Troponin from Non-Failing and G159D Heart Tissue	178
Figure 5.4 SDS-PAGE of Non-Failing and G159D Troponin.....	179
Figure 5.5 Average Phosphorylation Levels of Troponin T and Troponin I	180
Figure 5.6 Effect of Human Cardiac Tropomyosin on Rabbit Actin Motility (from data Set 1; Table 5.2 and Table 5.5).....	181
Figure 5.7 Effects of Non-failing and G159D Human Cardiac Troponin (40nM) on Actin Motility at 3.9 μM Ca^{2+} and 1nM Ca^{2+}	182
Figure 5.8 Ca^{2+} Regulation of Thin Filaments containing Non-failing or G159D Troponin.....	187
Figure 5.9 SDS-PAGE of Non-Failing and G159D Human Cardiac Troponin	190
Figure 5.10 Effect of Dephosphorylation on Non-Failing Troponin.....	191
Figure 5.11 Effect of Dephosphorylation on the Regulation by G159D Troponin	192
Figure 5.12 Effect of Dephosphorylation of Non-Failing and G159D Troponin	194
Figure 5.13 Effect of Recombinant Tropomyosin incorporated into the Non- Failing and G159D Thin Filament	196
Figure 5.14 Comparison of Phosphorylated and Dephosphorylated Non-failing and G159D Troponin	199
Figure 5.15 ESI-FTICR of 1:1 Recombinant Troponin C and G159D Troponin C Mixture	200
Figure 5.16 SELDI-MS of Recombinant Troponin C Mixtures.....	202
Figure 5.17 SELDI-MS of G159D Whole Troponin	202
Figure 5.18 Comparison of Pseudophosphorylated and Unphosphorylated Non- failing and G159D Recombinant Troponin.....	205
Figure 6.1 Location of the E99K Mutation in Actin	209
Figure 6.2 Age of Death in Male and Female E99K Transgenic Mice.....	210
Figure 6.3 SDS-PAGE of (A) E99K and (B) Non-Transgenic Actin Isolation Fractions	212
Figure 6.4 Western Blot of Actin Isolation Fractions	213
Figure 6.5 Preservation of E99K Expression	214
Figure 6.6 SDS-PAGE of E361G and Non-Transgenic Myofibrils.....	215
Figure 6.7 Non-Transgenic and E99K Actin Filaments Labelled with TRITC- phalloidin.....	216
Figure 6.8 Length Distribution of Non-Transgenic and E99K Actin	216
Figure 6.9 Effect of Tropomyosin on Sliding Speed (A) and Percentage Motility (B).....	218
Figure 6.10 Effects of Non-Failing Human Cardiac Troponin on Actin Motility at 3.9 μM Ca^{2+} and 1nM Ca^{2+}	221
Figure 6.11 Ca^{2+} Regulation of Thin Filaments Containing Non-Transgenic and E99K Actin	226
Figure 6.12 Effect of Troponin Dephosphorylation on E99K Actin Motility	230

Figure 6.13 Effect of Troponin Dephosphorylation on Non-Transgenic and E99K Actin Motility.....	233
Figure 6.14 $\bar{U}$ -Actinin Competitive Binding Assay of E99K Actin and Non-Transgenic Actin Filaments.....	234
Figure 6.15 <i>In vitro</i> Motility Assay Summary of Sliding Speed	237
Figure 6.16 <i>In vitro</i> Motility Assay Summary of Percentage Motile	237
Figure 6.17 Comparison of E99K and Non-Transgenic Actin Incorporated into Native or Dephosphorylated Thin Filaments	238

LIST OF TABLES

Table 1.1 Sarcomeric Gene Mutations in HCM and DCM.....	42
Table 2.1. Clinical details of Non-Failing Human Heart Samples.....	63
Table 2.2 Ca^{2+} Buffer Solutions	73
Table 3.1. E361G Transgenic Mouse Line 13.....	81
Table 3.2. E361G Transgenic Mouse Line 20.....	82
Table 3.3. Screening First Generation Transgenic Line 361.13	84
Table 3.4. Screening First Generation Transgenic Line 361.20	84
Table 3.5 Screening Second Generation Transgenic Line 361.13.....	85
Table 3.6 Screening Second Generation Transgenic Line 361.20.....	85
Table 3.7 Screening Third Generation Transgenic Line 361.20.....	86
Table 3.8. Comparison of Non-Transgenic and Transgenic Actin Sliding Speeds and Percentage Motile of Filaments.....	90
Table 3.9 Comparison of Non-Transgenic-Tm and Transgenic-Tm Sliding Speeds.....	92
Table 3.10 Comparison of Non-Transgenic-Tm and Transgenic-Tm Percentage Motile of Filaments.....	92
Table 3.11 Comparison of Non-transgenic and Transgenic Actin Sliding Speeds at 1nM Ca^{2+} (40nM NF Tn).....	95
Table 3.12 Comparison of Non-transgenic and Transgenic Actin Percentage Motility at 1nM Ca^{2+} (40nM NF Tn).....	95
Table 3.13 Comparison of Non-transgenic and Transgenic Actin Sliding Speeds at 3.9 μM Ca^{2+} (40nM NF Tn).....	96
Table 3.14 Comparison of Non-transgenic and Transgenic Actin Percentage Motility at 3.9 μM Ca^{2+} (40nM NF Tn)	96
Table 3.15 Comparison of Non-Transgenic and Transgenic Actin- ϕ Sliding Speeds and Percentage Motile of Filaments	103
Table 3.16 Comparison of Non-Transgenic-Tm and E361G-Tm Sliding Speeds	106
Table 3.17 Comparison of Non-Transgenic-Tm and E361G-Tm Percentage Motile of Filaments.....	106
Table 3.18 Comparison of Non-Transgenic and E361G Actin- ϕ Sliding Speeds at 1nM Ca^{2+}	109
Table 3.19 Comparison of Non-Transgenic and E361G Actin- ϕ Percentage Motility at 1nM Ca^{2+}	109
Table 3.20 Comparison of Non-Transgenic and E361G Actin- ϕ Sliding Speeds at 3.9 μM Ca^{2+}	110
Table 3.21 Comparison of Non-Transgenic and E361G Actin- ϕ Percentage Motility at 3.9 μM Ca^{2+}	110
Table 3.22 Comparison of Motility of Non-Transgenic and E361G Thin Filament Actin.....	118
Table 3.23 Comparison of Non-Transgenic.Tm and E361G.Tm Filament Sliding Speeds.....	121
Table 3.24 Comparison of Non-Transgenic.Tm and E361G.Tm Percentage Motile of Filaments.....	122
Table 3.25 Effect of Human Cardiac Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})	125
Table 3.26 Effect of Human Cardiac Troponin on Filament Percentage Motility at Low Ca^{2+} (1nM Ca^{2+}).....	126

Table 3.27 Effect of Human Cardiac Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)	127
Table 3.28 Effect of Human Cardiac Troponin on Filament Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)	128
Table 3.29 Ca^{2+} Sensitivity data for all E361G v Non-Transgenic Actin in a Non-Failing Thin Filament.....	132
Table 3.30 Comparison of Mouse Actin from Acetone Powder	137
Table 3.31 Comparison of Mouse and Rabbit Actin.....	139
Table 4.1 Effect of Rabbit Skeletal Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})	150
Table 4.2 Effect of Rabbit Skeletal Troponin on Filament Percentage Motility at Low Ca^{2+} (1nM Ca^{2+}).....	150
Table 4.3 Effect of Rabbit Skeletal Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)	151
Table 4.4 Effect of Rabbit Skeletal Troponin on Filament Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$).....	151
Table 4.5 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Actin with Skeletal Troponin.....	154
Table 4.6 Ca^{2+} Sensitivity Data for Non-Transgenic Actin Incorporated with Native or Dephosphorylated Troponin	159
Table 4.7 Ca^{2+} Sensitivity Data for E361G Actin Incorporated with Native or Dephosphorylated Troponin.....	162
Table 4.8 Ca^{2+} Sensitivity Data for Non-Transgenic and E361G Actin Incorporated with Dephosphorylated Troponin	165
Table 4.9 Pro-Q/SYPRO ratios from 5 Myofibril Experiments	168
Table 5.1 Phosphorylation Differences Between Non-Failing and G159D Troponin.....	180
Table 5.2 Effect of Human Cardiac Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})	184
Table 5.3 Effect of Human Cardiac Troponin on Filament Percentage Motility at Low Ca^{2+} (1nM Ca^{2+}).....	184
Table 5.4 Effect of Human Cardiac Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)	185
Table 5.5 Effect of Human Cardiac Troponin on Filament Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$).....	185
Table 5.6 Ca^{2+} Sensitivity Data for Non-Failing and G159D Troponin incorporated into the Native Thin Filament	188
Table 5.7 Ca^{2+} Sensitivity Data for Native and Dephosphorylated G159D Troponin.....	193
Table 5.8 Ca^{2+} sensitivity Data for Dephosphorylated Non-Failing and G159D Troponin.....	195
Table 5.9 Ca^{2+} Sensitivity Data for Non-failing and G159D Thin Filaments Incorporated with AS- $\ddot{\text{U}}$ -Tropomyosin	197
Table 5.10 Non-Failing and G159D Thin Filament Sliding Speeds at $3.9\mu\text{M Ca}^{2+}$	197
Table 6.1 Screening of E99K Transgenic Lines	211
Table 6.2 Pro-Q/SYPRO ratios from 5 Myofibril Experiments	215
Table 6.3 Comparison of Non-Transgenic and E99K Actin.....	217
Table 6.4 Effect of 40nM Human Cardiac Tropomyosin on Non-Transgenic and E99K Actin Sliding Speeds	219

Table 6.5 Effect of 40nM Human Cardiac Tropomyosin on Non-Transgenic and E99K Actin Percentage Motility.....	220
Table 6.6 Effect of 40nM Human Cardiac Troponin on Actin	223
Table 6.7 Effect of Human Cardiac Troponin on Actin	223
Table 6.8 Effect of 40nM Human Cardiac Troponin on Actin Sliding Speed at 3.9 μ M Ca ²⁺	224
Table 6.9 Effect of Human Cardiac Troponin on Actin Percentage Motility at 3.9 μ M Ca ²⁺	224
Table 6.10 Non-Transgenic and E99K Actin Sliding Speeds at 3.9 μ M Ca ²⁺ ...	228
Table 6.11 Ca ²⁺ -Sensitivity data for all Non-Transgenic and E99K Actin in a Non-Failing Thin Filament.....	228
Table 6.12 Ca ²⁺ Sensitivity Data for E99K Actin Incorporated with Native or Dephosphorylated Troponin.....	232

LIST OF ABBREVIATIONS

ϕ	TRITC-phalloidin
A.Tm	Actin.tropomyosin
ACEX	Actin Extraction Buffer
ACTC	Cardiac Actin Gene
ADP	Adenosine diphosphate
AKAP's	A-Kinase Anchoring Proteins
AoP	Aortic Pressure
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
C-terminal	Carboxyl-terminal
DCM	Dilated Cardiomyopathy
DTT	1,4-Dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane
ECL	Enhanced Chemiluminescence Reagents
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'-tetraacetic acid
F-actin	Filamentous Actin
FRET	Fluorescence Resonance Energy Transfer
G-actin	Globular Actin
HCM	Hypertrophic Cardiomyopathy
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid
HMM	Heavy meromyosin
HOCM	Hypertrophic Obstructive Cardiomyopathy
IEF	Isoelectric Focusing
LAP	Left Atrial Pressure
LVNP	Left Ventricular Non-Compaction
LVP	Left Ventricular Pressure
LVV	Left Ventricular Volume
MES	2-(N-Morpholino) ethanesulphonic acid
MHC	Myosin heavy chain

MLC	Myosin light chain
MOPS	3-(N-Morpholino) propanesulphonic acid
MRI	Magnetic Resonance Imaging
MyBP-C	Myosin binding protein-C
NF	Non-failing
N-terminal	Amino-terminal
NTg	Non-transgenic
OA	Okadaic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pCa	$-\log [\text{Ca}^{2+}]$
Pi	Inorganic Phosphate
PIPES	Piperazine-N,N-bis (2-ethanesulphonic acid)
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Phospholamban
PMSF	Phenylmethylsulfonylfluoride
RCM	Restrictive Cardiomyopathy
S1	Myosin subfragment 1
SDS	Sodium dodecyl sulphate
SERCA	Sarcoplasmic reticulum Ca^{2+} -ATPase
SR	Sarcoplasmic reticulum
Tg	Transgenic
Tm	Tropomyosin
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
Tris	Tris (hydroxymethyl) amino-methane
TRITC	Tetramethyl Rhodamine Isothiocyanate
Triton X-100	Polyethylene glycol tert-octylphenyl ether
Tween 20	Polyoxyethylenesorbitan monolaurate
WT	Wild-type

ACKNOWLEDGEMENTS

I would like to thank Professor Steven Marston for his excellent supervision and support throughout this project. I am especially grateful for his patient and critical reading of this thesis.

I express my thanks to Professor Dominic Wells and members of his laboratory for the generation and supply of transgenic mice, Dr. Adam Jacques for collecting the human G159D heart muscle sample, and to Dr. Chris Dos Remedios for supplying us with the non-failing human heart samples.

I would also like to thank the members of Professor Marston's laboratory, past and present who have contributed to a very enjoyable working environment: O'neal Copeland, Adam Jacques, Andrew Messer, Juan-Juan Feng, Weihua Song, Clare Gallon and Christopher Bayliss. I would finally like to express my gratitude to all my friends and family who have supported and encouraged me throughout my PhD.

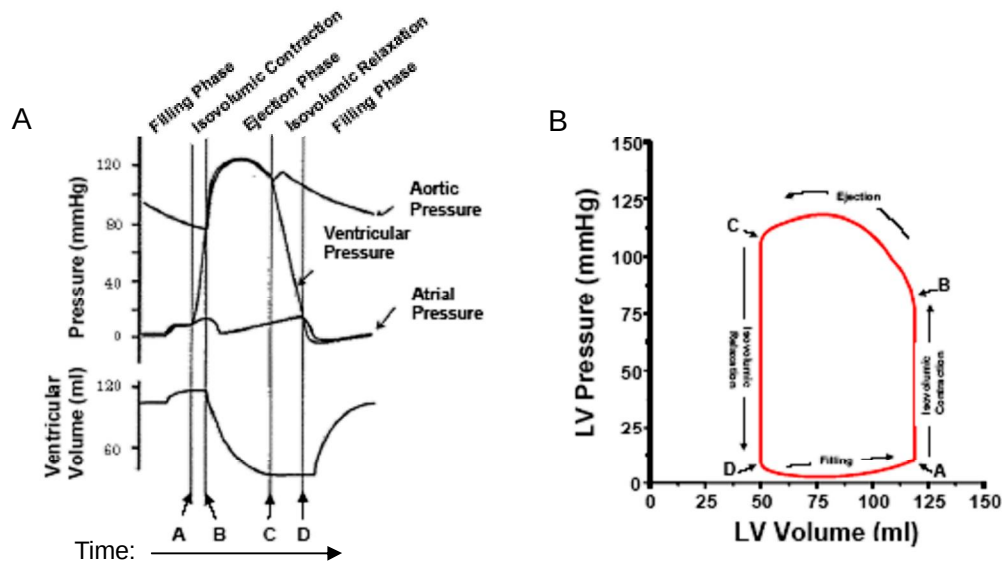
This research was funded by a studentship awarded by the British Heart Foundation.

1. INTRODUCTION

1.1 Cardiac Physiology

The cardiovascular system is composed of two major circulatory systems: the pulmonary system (pumps blood from the right side of the heart to the lungs), and the systemic system (pumps blood from the left side of the heart to the rest of the body). The heart is divided into four chambers: the right and left atria and the right and left ventricles. The upper two are the atria and the lower chambers are the ventricles. The sequence of events that occur when the heart beats is referred to as the cardiac cycle (Figure 1.1). There are two phases: diastole, where the ventricles are relaxed and fill with blood, and systole, where the ventricles contract and blood is ejected from the ventricles.

Figure 1.1 Pressure and Volume Changes during a Cardiac Cycle



(A): Plot of left ventricular volume (LVV), left ventricular pressure (LVP), left atrial pressure (LAP) and aortic pressure (AoP) as a function of time. (B): A pressure-volume loop for one cardiac cycle, the cycle proceeds in an anti-clockwise direction (Lionel, 2005).

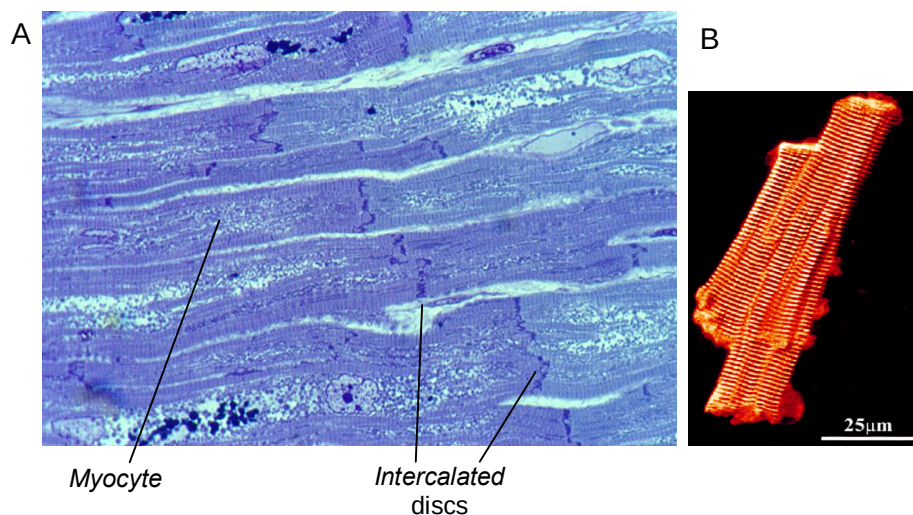
The cardiac cycle can be divided further into four phases: (a) ventricular filling, (b) isovolumetric contraction, (c) ejection (systole), (d) isovolumetric relaxation (Figure 1.1b). Point A is at the end of diastole and represents the end-diastolic pressure and maximum end-diastolic volume for the ventricle. As

the ventricle begins to contract (A - B), the LVP increases but the LV remains the same. When the LVP exceeds the aortic diastolic pressure, the ejection phase (B - C) begins. The LV volume decreases as the LVP increases at almost constant pressure and then decreases as the ventricle begins to relax (point C). When the LVP falls below the LAP (point D), the mitral valve opens and the ventricle fills to reach the maximum end-diastolic pressure at phase A (Wilcken, 2003).

1.2 The Cardiac Myocyte Structure

Cardiac myocytes are the contractile cells of the heart and constitute about 75% of the ventricular mass. They are large elongated cells, approximately 100-120 μ m long and 20-35 μ m wide (Sugden *et al.*, 2003). The close attachment of myocytes to their neighbours and also to the extra cellular matrix allows for the efficient transmission of force. Each individual myocyte makes contact with approximately 10 adjacent myocytes, through a characteristic area of the plasma membrane called the intercalated disc. Gap junctions and adherens junctions on the surface of the intercalated disc allow ions to pass through and electrical impulses to pass between myocytes (Figure 1.2 A and B).

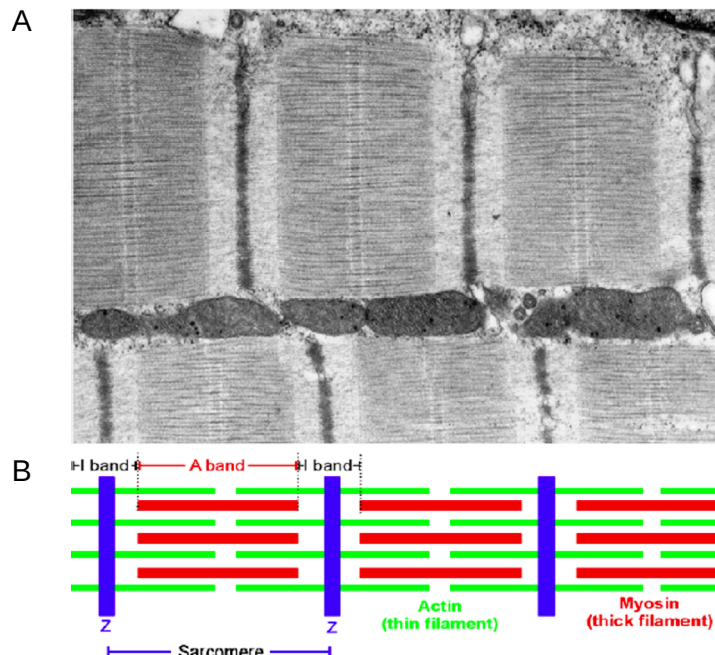
Figure 1.2 Cardiac Muscle and an Isolated Myocyte



[A] Cardiac myocytes are cut in longitudinal section showing the striated appearance. Intercalated discs can also be seen. [B] An isolated rat ventricular myocyte. The myofibrils have been visualised by immunostaining of α -actinin and viewed by confocal microscopy (Severs, 2000).

It has been known for some decades that the contractile apparatus of muscle cells are highly ordered, and contraction involves the sliding of the thick and thin filaments of the sarcomere (Huxley and Hanson, 1954). The alignment and overlap of thick and thin filaments gives the myofibril its striated appearance. Figure 1.3 illustrates the structure of the sarcomere. Thick filaments consist mainly of the protein myosin-II (named myosin for the purpose of this thesis). Thin filaments consist of a helical polymer of approximately 300 globular actin subunits, in association with the regulatory proteins tropomyosin and the troponin complex. Thin filaments are linked at the Z-line, which is mainly composed of the actin cross-linking protein α -actinin. The Z lines are associated with T-tubules and costameres. The M line lies at the centre of sarcomere and contains the protein myomesin (crosslinks thick filaments to maintain them in regular array). Titin is also present in this area, connecting the Z-line to the M-line in the sarcomere and contributing to the passive stiffness of the muscle (Severs, 2000).

Figure 1.3 Structure of the Sarcomere

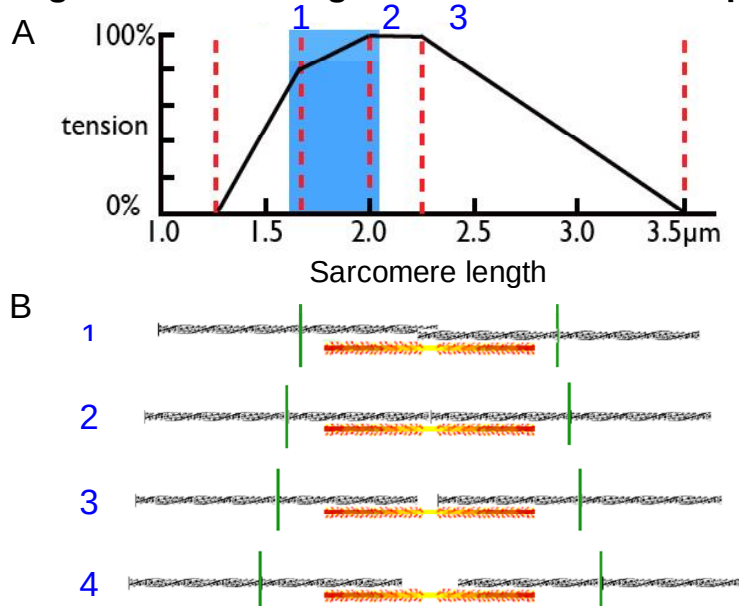


[A] Thin section electron micrograph showing the structure of cardiac muscle myofibrils. The sarcomeres of two myofibrils are shown with a row of mitochondria between. Note the greater concentration of mitochondria with cardiac muscle. [B]: Representation of the arrangement of the thick and thin filaments in relation to the striated pattern shown in [A]. Figure kindly provided by Prof. Nick Severs (Imperial College London).

Much of our understanding of the mechanism of muscle contraction has come from biochemical, mechanical and structural studies performed over several decades since the 1950s, during which methods for isolating specific muscle proteins were developed as well as the methods for measuring their specific properties (Huxley and Hanson, 1954, Huxley, 1957, Eisenberg and Moos, 1968, Weeds and Lowey, 1971, Lymn and Taylor, 1971b, Rayment *et al.*, 1993b, Kabsch *et al.*, 1990). Thick and thin filaments slide past each other due to the force generated when myosin heads interact with F-actin. This interaction is due to conformational changes in the acto-myosin cross-bridge that is coupled to ATP hydrolysis in the myosin head.

The isometric force produced by a muscle depends upon the sarcomeric length. Optimal force is produced at 2-2.25 μm , where the overlap of thick and thin filaments is such that all myosin cross-bridges can interact with actin (Figure 1.4, point 2-3). In the heart, the sarcomere is optimally stretched at 2.1 μm at the end of diastole and shortens to 1.6 μm during systole. The increase in stretch during diastole produces an increase in the force of the next contraction. In dilated cardiomyopathy (DCM), sarcomeres may be stretched beyond 2.3 μm which reduces the force of contraction (Sugden *et al.*, 2003).

Figure 1.4 The Length-Tension Relationship In Cardiac Muscle



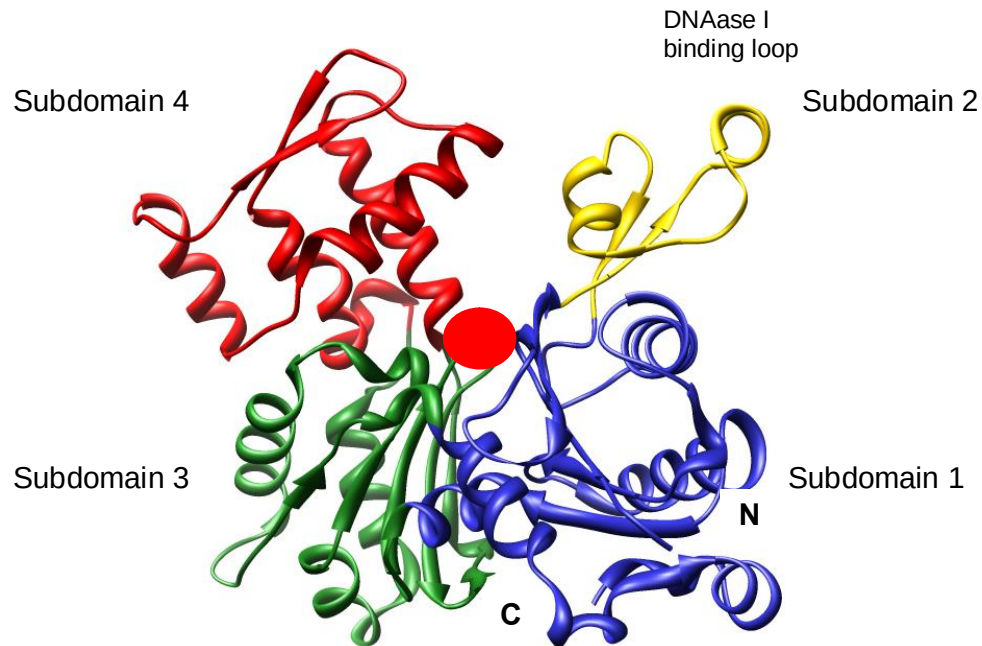
[A]: Diagram of the tension-sarcomere length relationship. [B]: Schematic representation of myofilament sliding. The range of sarcomere lengths during a normal cardiac cycle is shown in blue. Figure based on a diagram from (Gordon *et al.*, 1966).

1.3 Actin: Structure and Function

The sequence of actin is amongst the most conserved of all proteins. It was first discovered by Straub in an extract of muscle tissue (Straub, 1942) and has a variety of structural and motile functions in the cytoplasm. Monomeric actin (G-actin) polymerises under physiological salt concentrations to form F-actin. Actin performs many biological functions in the filamentous form, including muscle contraction, cell motility, morphological changes, cell division and intracellular movements (Hennessey *et al.*, 1993). Although not essential for contractile function, all actins undergo post-translational modifications, including the methylation of His73 and the modification of the N-terminus by removal of two residues followed by acetylation of the new N-terminus (Pollard and Cooper, 1986, Sheterline *et al.*, 1998).

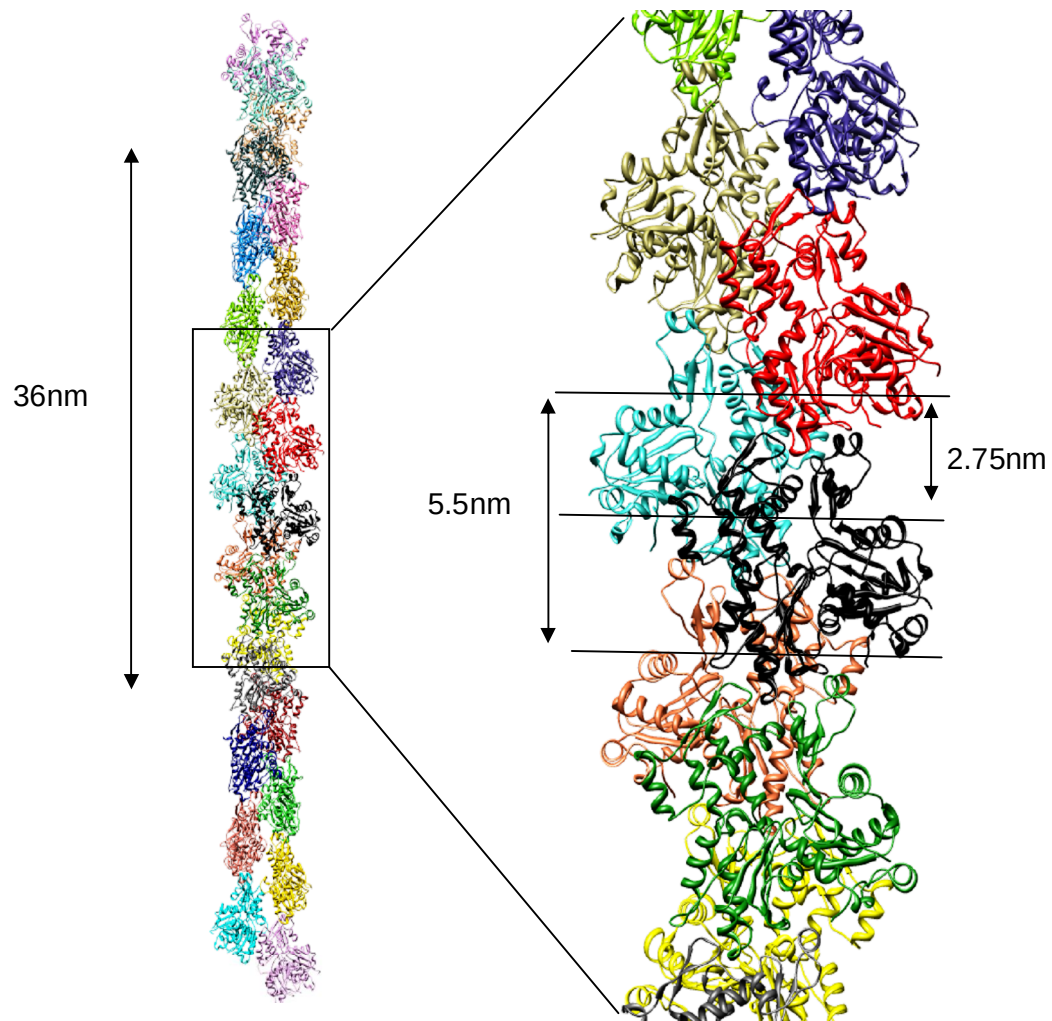
Actin filaments are anchored at the Z-line by $\bar{U}$ -actinin, which has distinct binding sites on adjacent actin monomers within residues 112-125 and 360-372 (McGough *et al.*, 1994, Broderick and Winder, 2005, Borrego-Diaz *et al.*, 2006). The anti-parallel cross-linking of actin with $\bar{U}$ -actinin forms the Z-disc and allows force to be propagated along the myofibril. The opposite orientation of actin on either side of the Z line is necessary for the sliding filaments.

The structure of G-actin was first published as a complex with DNase I in 1990 using X-ray crystallography (Kabsch *et al.*, 1990). Several structures have been reported since then in other complexes (Schutt *et al.*, 1993, McLaughlin *et al.*, 1993). Otterbein *et al.* have most recently published a crystal structure of uncomplexed actin in the ADP state (Otterbein *et al.*, 2001). The actin molecule has a maximum dimension of 9-10nm and consists of 2 domains, the smaller of which contains both N and C termini (Holmes *et al.*, 1990). The G-actin crystal structure reported by Kabsch *et al.* revealed that the actin monomer could be further divided into 4 sub domains, the inner domains 3 and 4 and outer domains 1 and 2 (Figure 1.5).

Figure 1.5 Molecular Model of the Actin Monomer

Ribbon representation of the structure of uncomplexed actin (1J6Z.pdb file). Subdomain 1 is shown in blue, subdomain 2 in gold, subdomain 3 in green, and subdomain 4 in red. ADP is shown in red. Molecular graphic image was drawn from atomic coordinates published by (Otterbein *et al.*, 2001), using the USSF Chimera package (Pettersen *et al.*, 2004).

ATP or ADP and Ca^{2+} or Mg^{2+} bind between the 2 domains of the actin monomer. The inner and outer domains are linked by two polypeptide chains (Kabsch *et al.*, 1990). ATP hydrolysis may cause a conformational change in the monomer, since slight structural differences are apparent between ADP-actin (Otterbein *et al.*, 2001) and ATP-actin (bound to DNase1) (Kabsch *et al.*, 1990). In the same year as the G-actin crystal structure was proposed, a structure of the F-actin helix at the atomic level was derived by fitting a helix of G-actin structures to low resolution X-ray fibre diffraction patterns of the filament (Holmes *et al.*, 1990). The structure was further refined by Lorenz *et al.* (Lorenz *et al.*, 1993, Lorenz *et al.*, 1995). F-actin has 13 molecules arranged as a double-helix which repeats every 36nm (Figure 1.6). The rise in subunit is 2.75nm (Geeves and Holmes, 1999). Along each of the helices, the actin monomers are spaced by 5.5nm.

Figure 1.6 Molecular Model of F-Actin

The F-actin filament, as established from the helical parameters of the standard Holmes model (Holmes *et al.*, 1990). Figure prepared using the USSF Chimera package (Pettersen *et al.*, 2004).

The structure of F-actin derived by Holmes *et al.* (Holmes *et al.*, 1990) gives a good indication of the monomers orientation within the filament. F-actin is highly flexible and each monomer can make contact with 4 other monomers in the helix (dos-Remedios and Moens, 1995). Subdomains 1 and 2 are orientated on the outside and subdomains 3 and 4 are in the centre (Rayment *et al.*, 1993a). Interactions occur both between strands and along the actin filament in each strand. The highly flexible nature of both G-actin and F-actin is

important during its interaction with myosin and subsequent force generation along the actin filament, as well as in regulation.

1.4 Myosin Structure

Myosin is probably the most important molecule that binds to actin, forming the motor essential for muscle contraction. Ventricular human cardiac thick filaments are mainly composed of β -myosin II with 5% α -myosin II. Myosin belongs to a large family of motor proteins and consists of two structurally and proteolytically defined domains (Weeds and Lowey, 1971): sub fragment 1 (S1), which is a globular domain that contains the motor domain, and the rod which is a long α -helix. The rod domains form a coiled-coil dimer that makes up the shaft of the myosin filament with the S1 domains protruding (Figure 1.7).

Figure 1.7 Structure of the Myosin Molecule

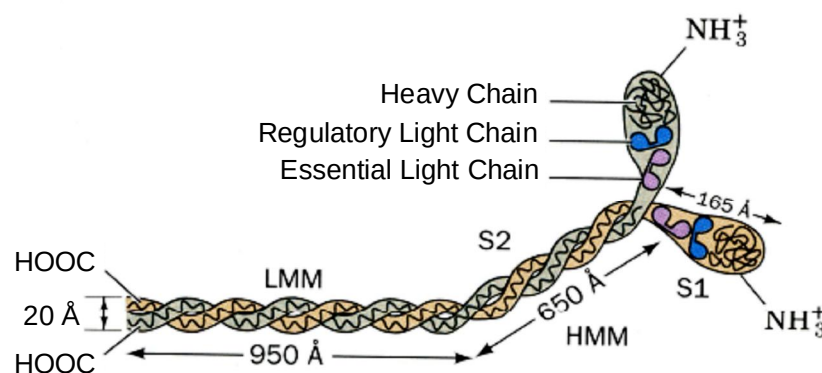


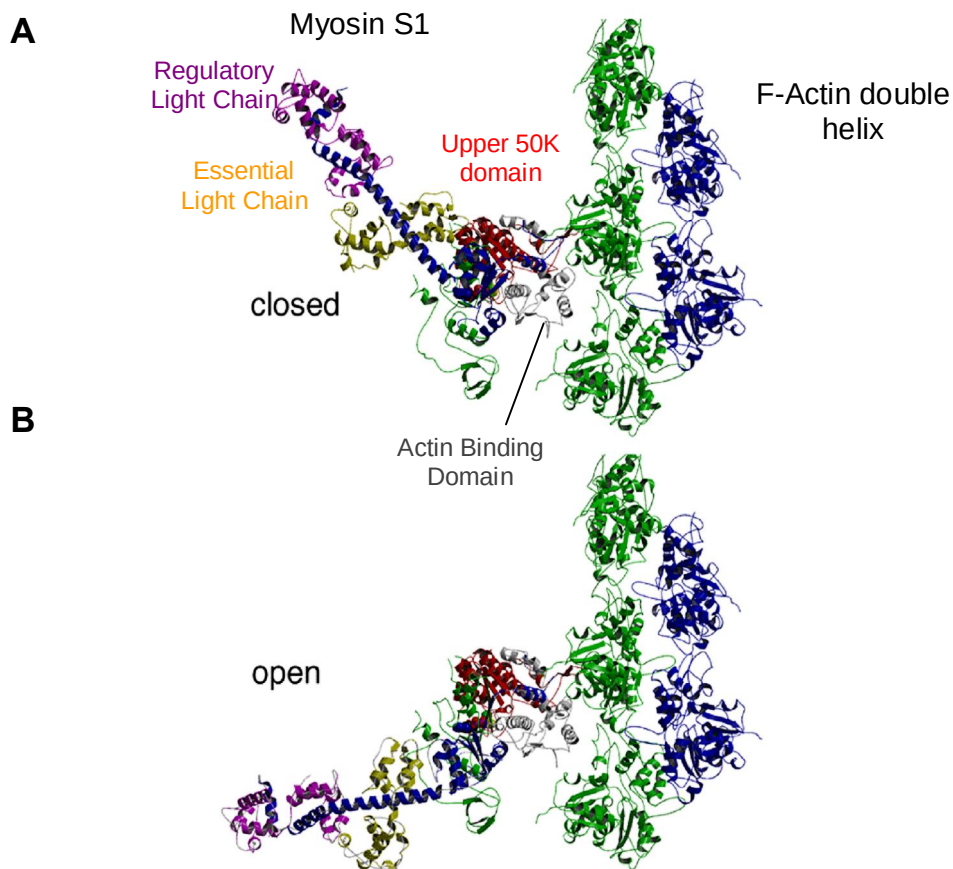
Figure obtained from (Voet *et al.*, 1995). Proteolysis can occur at the S1-S2 junction to yield S1 and the rod, or the LMM-S2 junction to yield HMM and LMM.

S1 comprises the first 843 residues of the heavy chain together with two light chains (essential and regulatory of 19kDa and 25kDa each). This domain forms the motor domain that drives muscle contraction. Tryptic cleavage has resulted in the identification of 3 sub-domains of S1. These included the N-terminal 25kDa nucleotide domain (Szilagyi *et al.*, 1979), the central actin-binding 50kDa domain, and the C-terminal 20kDa actin-binding domain (Mornet *et al.*, 1981, Sutoh, 1982). The C-terminal end of the heavy chains forms two long α -helices that are wound around together in a coiled-coil, forming the shaft

of the thick filament (S2). The S2 domain of the myosin molecule is linked to the LMM domain that forms the bipolar thick filaments (Spudich, 2001).

In the crystal structure, S1 appears as a globular domain with a long single α -helix forming a tail. The two light chains are bound to the tail, stabilising the α -helix and forming a lever arm. The current hypothesis for the mechanism of force generation by actin-myosin-ATP involves the rotation of the lever arm relative to the globular domain (Geeves and Holmes, 1999). During ATP-binding and subsequent ATP-hydrolysis, the nucleotide binding site of S1 undergoes subtle conformational changes and the lever arm is believed to swing through an angle of $\sim 90^\circ$. The rotation of the lever arm that is thought to occur in force production is illustrated in Figure 1.8.

Figure 1.8 Actomyosin Interaction Model



Three-dimensional atomic model of F-actin combined with myosin subfragment 1. From Geeves *et al.* (Geeves and Holmes, 1999). The ribbon on the left corresponds to myosin S1; an F-actin double helix consisting of 5 actin subunits is shown on the right. Figure A illustrates the lever arm's position in the pre-power-stroke state of actomyosin. Figure B illustrates the lever arm's position in the post-power-stroke state of actomyosin.

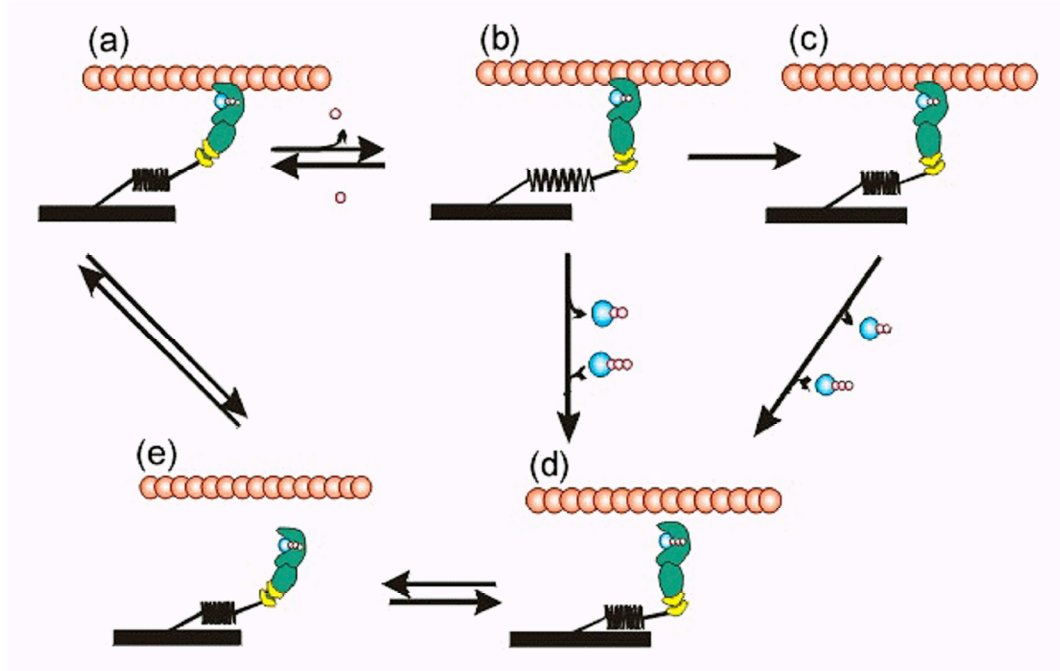
The solubility of S1 and HMM has enabled their use for *in vitro* investigations of contractile proteins. Several functional assays have been developed that recreate the basic unit of contractile muscle and allow the action of the myosin motor to be studied. The *in vitro* motility assay devised by Kron and Spudich (Kron and Spudich, 1986, Kron *et al.*, 1991) is one such valuable technique, and allows actin-myosin interactions to be studied at the molecular level. Rabbit fast skeletal muscle HMM can act as a motor on silicone-treated glass, with actin labelled with TRITC-phalloidin on the surface (Marston, 2003). The movement of actin filaments over HMM is initiated by addition of ATP, and can be visualised by epifluorescence microscopy and detected by a silicon-intensified video camera. This assay can measure the unloaded shortening velocity of muscle. The movement of actin over myosin is random but occurs at a uniform speed (Marston, 2003). Experiments that have used pure populations and mixtures of fast skeletal myosin, cardiac myosin, phosphorylated and unphosphorylated smooth muscle myosin, have shown that actin filament velocity is proportional to actin-activated ATPase activities of the myosins (Sheetz and Spudich, 1983) and the unloaded shortening velocity of muscle (Malmqvist *et al.*, 2004).

Another *in vitro* technique, the optical tweezer or optical trap, has allowed measurements of the force and step size of single myosin molecules (Sleep *et al.*, 2006). It uses the effect by which a laser beam focused by the strong objective lens of a microscope can trap microscopic objects in an energy well. A single actin filament can be stretched between two plastic beads held in optical tweezers, and the myosin can then bind to the filament allowing its working stroke to be detected from positions of the two trapped beads. It is therefore possible to recreate under the microscope the basic unit of contractile muscle and investigate how a single myosin molecule responds to an external force applied to actin via the optical trap. Measurements suggest that the displacement of actin from a single myosin molecule is approximately 10nm (Sleep *et al.*, 2006).

1.5 The Mechanism of Contraction: The Cross-bridge Cycle

Following the proposal of the sliding filament theory of muscle contraction (Huxley and Hanson, 1954), A.F Huxley devised mathematical equations for the operation of multiple independent force generations, later identified as myosin "cross-bridges" that generate the sliding forces between actin and myosin filaments (Huxley, 1957). This causes the contraction of muscle. Eisenberg and Moos studied the kinetics of activation of myosin-ATPase by actin (Eisenberg and Moos, 1968). Subsequent test-tube enzyme kinetic studies of purified actin and S1 showed the kinetic pathway of actin activation of myosin ATPase, which was interpreted as being the biochemical equivalent of the structural cycle (Lymn and Taylor, 1971a).

Our current understanding of how the actin activated enzymatic breakdown of ATP drives the cross-bridge cycle is shown in Figure 1.9. ATP binding to myosin heads either at rest (c) or when myosin heads are under a load (b), results in conformational changes occurring which cause the very rapid (500sec^{-1}) and almost irreversible dissociation of myosin from actin (Geeves and Holmes, 2005). This detachment from actin is followed by the hydrolysis of ATP to ADP and P_i , which remain tightly bound to the myosin head (e). Hydrolysis is rapid (10sec^{-1}), reversible ($K_{\text{eq}} = 10$), and is accompanied by a major conformational change (the "priming" stroke) (Geeves and Holmes, 1999). The myosin-ADP- P_i complex can bind to actin and this is followed by the powerstroke, in which the lever arm of myosin moves and P_i is released. If the muscle is placed under load, the movement satisfies an elastic element in the cross-bridge and produces force. Isometric crossbridges release ADP slowly. If the muscle is unloaded, the power stroke causes the rapid release of ADP. This results in the sliding of thin filaments past thick filaments, which causes the sarcomere to shorten and force to be generated. ATP then binds and dissociates actin from myosin.

Figure 1.9 Myosin Cross-bridge Cycle

Actin monomers are shown in orange, the myosin head in green, light chains in yellow, myosin S2 and the thick filament in black. ATP/ADP is shown in blue/pink, and Pi is pink/yellow. Figure provided by S. Weiss and M.A. Geeves, (www.kent.ac.uk/geeves/Research).

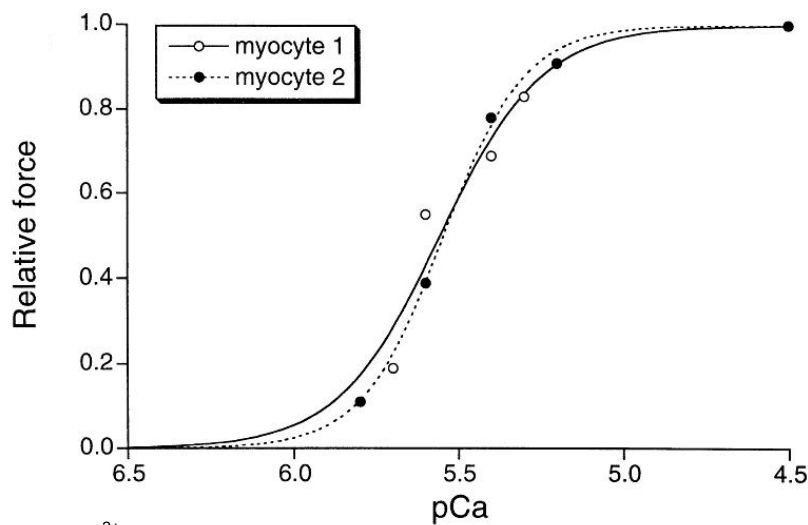
1.6 The Regulation of Cardiac Contraction

1.6.1 Excitation-Contraction (EC) Coupling

The Ca^{2+} ion is the physiological regulator of muscle contraction (Ebashi *et al.*, 1968, Yasui *et al.*, 1968, Fuchs and Briggs, 1968). EC coupling refers to the coupling of the electrical excitation of the heart to the production of contractility by Ca^{2+} ions (Sugden *et al.*, 2003). It involves the interaction of a number of proteins involved in Ca^{2+} homeostasis. Within the cell, each myofibril is surrounded by an intracellular membranous structure called the sarcoplasmic reticulum (SR). At multiple sites, swellings called junctional SR cisternae form, which press tightly against peripheral plasma membrane and invaginations of the surface called transverse tubules (T-tubules). During diastole, Ca^{2+} is sequestered by calsequestrin within the junctional SR. Depolarisation of the cell membrane causes the influx of Ca^{2+} through L-type Ca^{2+} channels of the T-tubules and plasma membrane. This triggers the opening of Ca^{2+} release channels in the junctional SR membrane (Ca^{2+} -induced Ca^{2+} -release), and

Ca^{2+} is released into the cytoplasm (Severs, 2000). The release of Ca^{2+} from the SR activates the contractile apparatus of the sarcomere (Figure 1.9). An increase in the Ca^{2+} concentration causes an increase in the force of contraction. The regulatory effect of Ca^{2+} on isometric force is shown in Figure 1.10, where contraction of demembranated cells requires only ATP as a substrate and Ca^{2+} as an activator. The Ca^{2+} switch of muscle is located in the thin filaments.

Figure 1.10 Ca^{2+} Sensitivity of Isometric Force

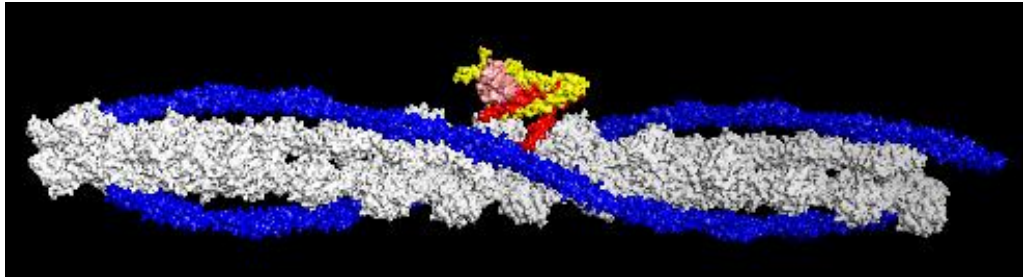


The Ca^{2+} sensitivity of isometric force, as measured in chemically skinned cardiac myocytes from human ventricular muscle (van der Velden *et al.*, 1998). Force-pCa relations have been fitted to a Hill Equation.

1.7 Contraction and Regulation by Thin Filaments

Thin filaments of the sarcomere are composed of tropomyosin and the troponin complex (troponin I, C, T) in association with F-actin (Figure 1.11). Troponin is the Ca^{2+} ion receptor that regulates contraction through interactions with tropomyosin and actin (Ebashi and Endo, 1968). Regulatory Ca^{2+} binds to troponin at the amino-terminal domain of the troponin C sub unit with an affinity of about 10^6M^{-1} (Greaser and Gergely, 1971).

Figure 1.11 Atomic Model of the Thin Filament



One repeat of the actin filament is coloured in white and tropomyosin in blue. Only a single troponin molecule is shown. There should be one on each tropomyosin strand. Troponin I is pink, troponin C is yellow, and troponin T is red. Figure prepared with MacPyMOL using the pdb files of (Pirani *et al.*, 2006).

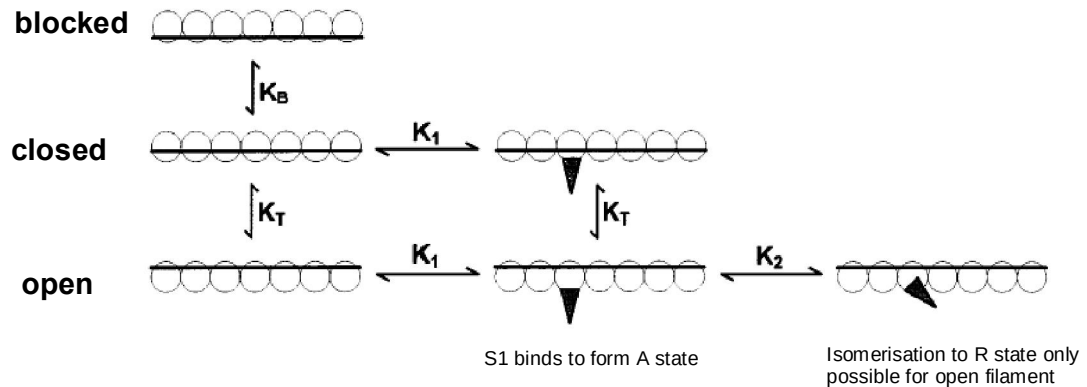
Numerous functional assays (*in vitro* motility, ATPase assays, and isometric force measurements) have enabled the thin filaments of cardiac muscle and their regulation to be studied. The *in vitro* motility assay is a particularly useful technique that allows several parameters of movement to be studied at once (Marston, 2003). It enables thin filaments to be assembled from pure proteins and the functionality of each to be assessed. This has provided useful information about the structure-function relationship of each thin filament protein. The addition of tropomyosin and troponin (at comparable concentrations), along with TRITC-labelled actin on a surface of HMM, allows the movement of actin filaments to be regulated (Fraser and Marston, 1995a, Marston, 2003). Inhibitory concentrations of troponin added to actin.tropomyosin reduce the fraction of filaments that are moving. Regulated filaments are therefore in a two-state system of movement and non-movement, and the transition depends upon troponin and the Ca^{2+} concentration. The equilibrium is entirely reversible. Troponin stops movement at pCa9 (low Ca^{2+}), but filaments move again when a high Ca^{2+} buffer (pCa5) is infused. At intermediate Ca^{2+} concentrations (pCa6.4), filaments can switch between states of moving at the same speed as at pCa5, not moving at all, and a few that stop and start. Therefore the primary role of troponin in the thin filament is in the control of the myosin cross-bridge binding to actin.tropomyosin, rather than controlling the cross-bridge turnover rate (Marston, 2003).

Measurements at 28°C into the effect of cardiac tropomyosin on the actin sliding speed have shown that movement is enhanced with increasing

concentrations, which saturated at approximately 40nM (Marston, 2003). Since actin dissociates from tropomyosin conventionally at 100-200nM, the lower concentrations needed in the *in vitro* motility assay illustrate that once tropomyosin has joined up end-to-end with actin, the rate of dissociation is slowed (Fraser and Marston, 1995a, Vilfan, 2001). Tropomyosin is composed of two α -helical chains arranged as a coiled-coil that spans 7 actin filaments (Lorenz *et al.*, 1995). This arrangement is important for the cooperative activation of the thin filament by Ca^{2+} and myosin. Information is carried along the actin filament by the lengths of repeating tropomyosin molecules. The position of tropomyosin relative to actin plays a key role in switching thin filament activity. During activation, tropomyosin moves away in a stepwise fashion, first by Ca^{2+} binding to troponin and then myosin binding to actin (Pirani *et al.*, 2005).

1.7.1 Ca^{2+} Regulation and Co-operativity

The contraction of muscle and its regulation involves co-operative and allosteric interactions between actin, myosin, tropomyosin and troponin (Lehrer and Geeves, 1998). Geeves and Halsall proposed that the binding of tropomyosin alone to actin induces two states (open and closed) (Geeves and Halsall, 1987). McKillop and Geeves subsequently proposed that in the presence of troponin and the absence of Ca^{2+} , a third state (blocked) is present (McKillop and Geeves, 1993). The three-state model of the thin filament as proposed by McKillop and Geeves is shown in Figure 1.12. Cross-bridge cycling only occurs when actomyosin is in the open state. The regulation of the equilibrium between states regulates contractility.

Figure 1.12 Three-State Model of the Thin Filament

The actin.Tm.Tn structural unit is shown as seven open circles, connected via a line representing the tropomyosin. The equilibrium between the three states is represented by the different positions of tropomyosin. The blocked state occurs when no S1 binding can occur (in the absence of Ca^{2+} when troponin is tightly bound to the filament). The closed state occurs when only weak binding can occur, and the open state occurs when isomerisation of the S1 to the rigor state is allowed. K_B is the equilibrium constant that defines the ratio between the blocked and closed states, and K_T is the equilibrium constant which defines the ratio between the closed and open states. Image adapted from (Maytum *et al.*, 1999).

The blocked state can only be detected in kinetic experiments. In steady state experiments like the *in vitro* motility assay, we only ever see evidence for the closed and open states. The blocked state may be present but it cannot be distinguished from the closed state. The correct terminology for the states measurable by steady state methods is ON (moving filaments) and OFF (non-moving filaments), with OFF corresponding to the blocked and closed state.

1.7.2 Troponin and Ca^{2+} Regulation

In vitro motility measurements have also been used to determine the distinct functional roles of the three individual subunits in whole troponin and the functional effects in native thin filaments (Bing *et al.*, 1997). The switch off at pCa9 is caused by troponin I. The interaction of troponin I and troponin C controls the inhibitory activity of troponin I and is controlled by Ca^{2+} binding to troponin C. Ca^{2+} binding to troponin C strengthens the troponin I-troponin C interaction, releases it from actin and removing the inhibition. Filament velocity is also controlled by Ca^{2+} but this is due to the presence of troponin T and its interaction with troponin C, since no such effect has been seen in the absence of troponin T (Bing *et al.*, 1997, Potter *et al.*, 1995).

In vitro motility studies correlate with biochemical studies that indicate the regulatory role of troponin T-troponin C interactions, in addition to troponin I-troponin C interactions (Potter *et al.*, 1995, Malnic *et al.*, 1998, Perry, 1998, Perry, 1999, Vinogradova *et al.*, 2005).

Figure 1.13 Conformational Changes occurring in Troponin in response to Ca^{2+}

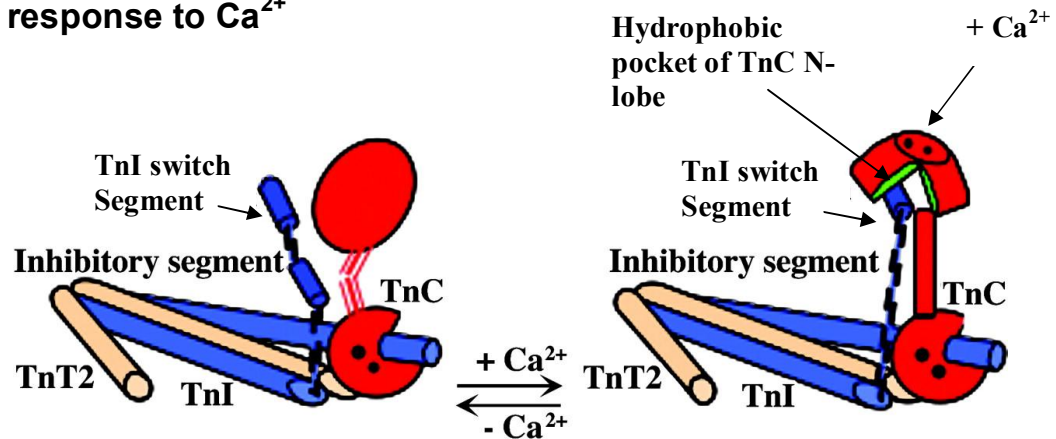
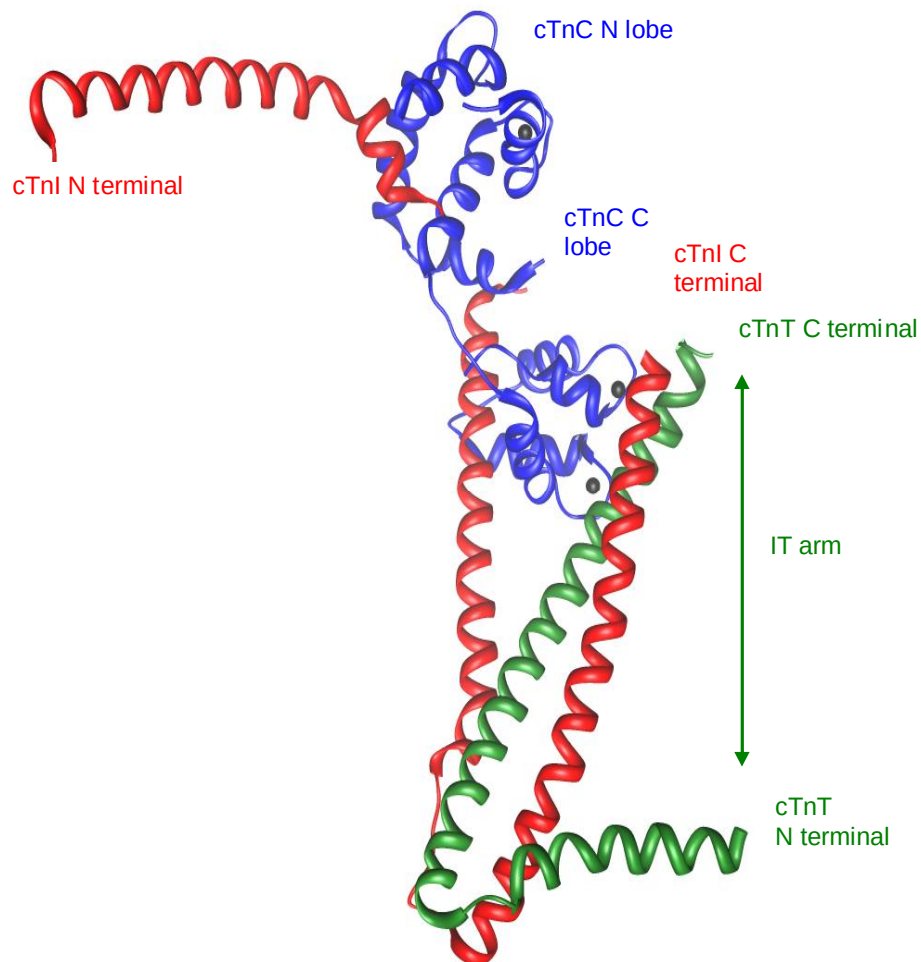


Figure adapted from (Vinogradova *et al.*, 2005). Troponin T is coloured in cream, troponin I in blue, troponin C in red. Ca^{2+} ions are shown as black circles.

Figure 1.13 illustrates the conformational changes that occur in the troponin complex in response to Ca^{2+} . In relaxed muscle, the Ca^{2+} concentration is low and the C-terminus of the inhibitory region of troponin I binds to sites on actin which prevents cross-bridge cycling. In response to Ca^{2+} , Ca^{2+} binds to troponin C and the N-terminus of troponin C binds to the C-terminal regulatory sequence of troponin I. This restores the helical conformation of troponin C and removes the troponin I inhibitory segment from actin. Troponin I modulates the Ca^{2+} binding properties of troponin C through PKA phosphorylation of the troponin I-specific N terminus (Ray and England, 1976, Solaro *et al.*, 1976). The C-terminus of troponin T binds both to troponin I (in a double β -helix) and the troponin C C-terminus and modulates the interactions of both with each other. The N-terminal half of troponin T is α -helical and binds the troponin complex to tropomyosin, anchoring troponin onto the thin filament. The publishing of the high resolution X-ray structure of the core domains of chicken skeletal muscle and human cardiac troponin has allowed the precise molecular arrangement of

the troponin complex to be visualised (Takeda *et al.*, 2003, Vinogradova *et al.*, 2005) (Figure 1.14).

Figure 1.14 Crystal Structure of the Core Domain of Troponin in the Ca^{2+} Saturated Form



Ribbon representation of the structure of the core domain, comprising troponin C (blue), troponin I (red) and troponin T (green). Note the Ca^{2+} ions are shown in black. Molecular graphic image was drawn from atomic coordinates published by (Takeda *et al.*, 2003) (pdb file 1J1E), using the USSF Chimera package (Pettersen *et al.*, 2004).

1.8 Adrenergic Regulation of Contractility

In the normal human heart, the activation of the sympathetic nervous system (e.g. through exercise, fight-or-flight response) causes an increase in cardiac output by increasing myocyte contractility (inotropy) (Houser and

Margulies, 2003). The changes that occur in ventricular inotropy are unique to cardiac muscle, and are accomplished by changes in the stroke volume (refer to cardiac cycle depicted in Figure 1.1). The inotropic response is the heart's mechanism for increasing the force generation that is independent of preload (i.e. sarcomere length). In heart muscle, inotropy is manifested as a change in V_{max} , i.e. a change in maximal fibre velocity shortening to zero afterload. An increased rate of fibre shortening leads to an increase in the rate of ventricular pressure development. The altered rate of force and pressure development by the ventricle can change the rate of ejection and the ejection fraction (refer to PV loops depicted in Figure 1.1). Increased inotropy increases the ejection fraction and decreased inotropy decreases the ejection fraction. The ejection fraction of the heart is therefore used as a clinical index for evaluating the inotropic state of the heart. In heart failure, the decrease in inotropy leads to a decreased stroke volume, an increased preload, and a decreased ejection fraction (Towbin and Bowles, 2002, Houser and Margulies, 2003).

The inotropic state of the heart is mediated by β -adrenergic stimulation. It involves the binding of adrenergic amines to β -adrenergic receptors, which activates adenylate cyclase and increases the production of cAMP. Protein kinase A (PKA) becomes activated, binds to A-kinase anchoring proteins (AKAPs), and phosphorylates numerous Ca^{2+} regulatory proteins (phospholamban, L-type Ca^{2+} channels, ryanodine receptors, myosin binding protein C and troponin) (Houser and Margulies, 2003). Additionally, PKA can affect enzymes involved in energy metabolism and can increase the production of ATP. β -adrenergic stimulation results in an increase in Ca^{2+} influx and storage by the SR. The increased Ca^{2+} release and faster kinetics increases the rate and magnitude of force generation and relaxation by the contractile apparatus. Phosphorylation of myosin binding protein C and troponin I adjusts the contractile regulatory system to do this. For the necessary background to the experiments undertaken in this thesis, only the regulation of contraction by alterations in cardiac troponin I phosphorylation by PKA will be reviewed.

Phosphorylation of cardiac troponin I occurs at residues Ser23/24 (reviewed in (Metzger and Westfall, 2004, Solaro and Rarick, 1998)), which forms part of a 27-33 amino acid N-terminal domain unique to cardiac troponin I

(Layland *et al.*, 2005). The physiological relevance of Ser23/24 phosphorylation has been extensively studied using transgenic animals, force measurements from skinned cardiac muscle preparations, as well as from biochemical studies using intact and skinned cardiac muscle preparations. Collectively, these studies have shown that the PKA phosphorylation of cardiac troponin I reduces myofilament Ca^{2+} sensitivity, increases the cross-bridge cycling rate and enhances unloaded shortening velocity (Hoh *et al.*, 1988, Saeki *et al.*, 1990, Fentzke *et al.*, 1999, Kentish *et al.*, 2001, Herron *et al.*, 2001). The reduced Ca^{2+} sensitivity is due to an increase in the rate of Ca^{2+} dissociation from troponin C, which is crucial in speeding up relaxation and allowing the heart rate to increase. However, in this tissue troponin I is already about 50% phosphorylated. PKA treated non-failing human donor cardiomyocytes show a minor decrease in the Ca^{2+} sensitivity of isometric force (van der Velden *et al.*, 2003). Similarly using non-failing human donor tissue, Messer *et al.* reported that the Ca^{2+} sensitivity of the troponin in the *in vitro* motility assay (TnI 2.1molPi/mol TnI) was increased upon dephosphorylation by phosphatase treatment (TnI 0molPi/mol TnI) (Messer *et al.*, 2006). These functional studies have provided strong evidence that myofilament properties can become modified by alterations in troponin I phosphorylation and under physiological conditions these modifications play an important role in achieving an optimal cardiac output.

The failing heart has a reduced contractile reserve and a reduced responsiveness to β -adrenergic stimulation (Houser and Margulies, 2003). The abnormal contractile properties are characterised by a decrease in the rate of maximally activated cross-bridge cycling, decreased contraction and relaxation rates and an increase in Ca^{2+} sensitivity. Collectively, these features could reduce the rate of relaxation in diastole, leading to incomplete relaxation so work output in systole may be decreased (Pagani *et al.*, 1988, Wolff *et al.*, 1996, Alpert *et al.*, 2002).

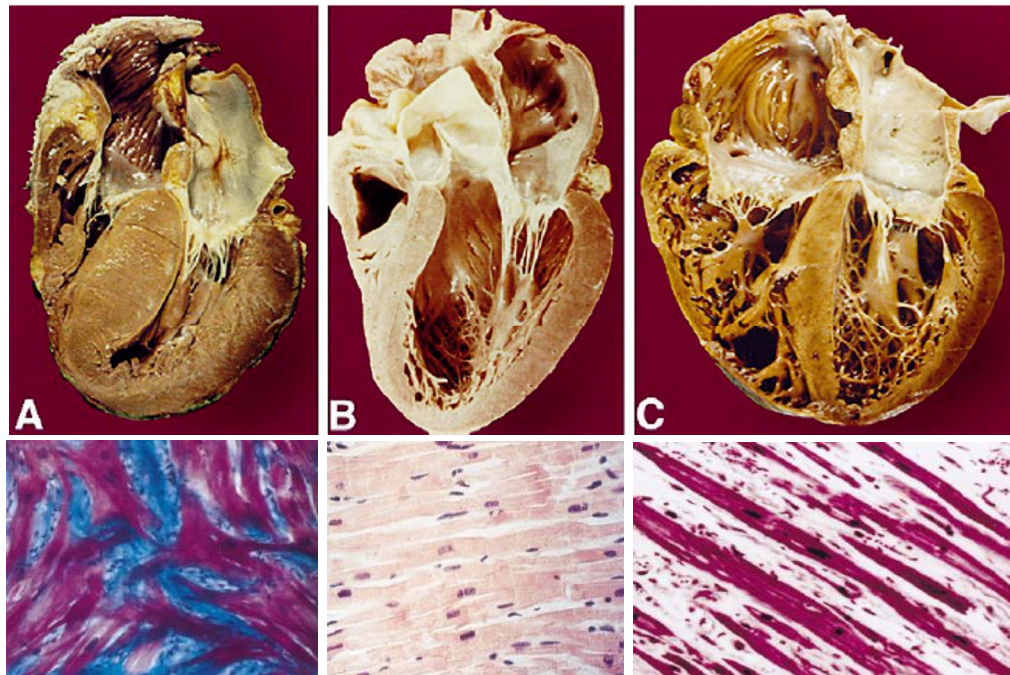
Studies of human heart muscle by *in vitro* motility assay indicate that there is a defect in the thin filaments but not the thick filaments (Purcell *et al.*, 1999, Knott *et al.*, 2002, Noguchi *et al.*, 2003). Using the *in vitro* motility assay, Messer *et al.* reported that changes in troponin function alone could account for the contractile abnormality in failing heart muscle (Messer *et al.*, 2006). The

critical difference between non-failing and failing troponin was the very low level of troponin I phosphorylation in the failing heart. In fact, the Ca^{2+} sensitivity and maximum velocity was shown to correlate with the level of troponin I phosphorylation. Van der Velden *et al.* similarly showed reductions in troponin I phosphorylation by PKA in end-stage human cardiomyocytes (van der Velden *et al.*, 2003). This was associated with an increased Ca^{2+} sensitivity. Myosin binding protein C and myosin light chain-2 are also less phosphorylated in failing heart muscle (El-Armouche *et al.*, 2007, Palmer, 2005). Reduced PKA activity and increased Ca^{2+} -PKC and phosphatase activity may work together to dephosphorylate troponin I and other regulatory proteins.

1.9 The Cardiomyopathies: Hypertrophic and Dilated

Cardiomyopathies are a group of cardiac disorders in which the structure and function of the myocardium deteriorates for any unexplained reason. They have been divided into four different diagnostic groups, based on World Health Organisation guidelines: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), and arrhythmogenic right ventricle cardiomyopathy (ARVC) (Richardson *et al.*, 1996) (Figure 1.15). Many of the conditions are hereditary (Mestroni *et al.*, 1999b, Hamid *et al.*, 2002, Richard *et al.*, 2003), and thus provide a unique resource for studying the genetic basis of disease.

Figure 1.15 Cardiac Remodelling Patterns and Histopathology



[A] HCM heart showing an increase in left ventricular wall thickness (top), with enlargement and disarray of myocytes (red) with increased interstitial fibrosis (blue) (below). **[B]** Healthy heart showing normal left ventricular wall thickness and chamber volume (top), with an orderly alignment of myocytes and minimal interstitial fibrosis in the healthy ventricular myocardium (below). **[C]** DCM showing a marked increase in left ventricular chamber volumes and atrial enlargement (top), with hypertrophy and degeneration of myocytes (dark red) without disarray and increased interstitial fibrosis (pale pink) (below). Figure adapted from (Seidman and Seidman, 2001).

1.9.1 Dilated Cardiomyopathy

DCM is characterised by unexplained left ventricular dilation, impaired systolic function and non-specific histological abnormalities (usually myocardial fibrosis) (Richardson *et al.*, 1996, Mestroni *et al.*, 1999a, Towbin and Bowles, 2002). DCM is the most common cause of heart failure and cardiac transplantation in the young, and has a prevalence of 36.5 per 100 000 in the U.S population and 38 per 100 000 in Europe (Codd *et al.*, 1989, Cohn *et al.*, 1997). Patients usually present with symptoms of cardiac failure (fatigue, breathlessness, decreased exercise tolerance), and may experience severe disease complications including arrhythmia, thrombo-embolic events and sudden death (McKenna, 2003, Ebashi and Ohtsuki, 2007).

Recent family studies have suggested that genetic factors may account for as much as 30-35% of cases (Mestroni *et al.*, 1999b, Murphy *et al.*, 2004,

Mogensen *et al.*, 2004b). Inheritance is usually autosomal dominant with incomplete penetrance, although an X-linked inheritance pattern has also been reported (Hershberger, 2005). Numerous disease genes have been identified that encode proteins of the sarcomere, nuclear envelope, cytoskeleton, Z-disc, and ion channels (Bione *et al.*, 1994, Li *et al.*, 1999, Daehmlow *et al.*, 2002, Knoll *et al.*, 2002, Schmitt *et al.*, 2003). For the purpose of this thesis, mutations in sarcomeric protein genes as a cause of familial DCM will be described.

1.9.1.1 Disease Genes of DCM

Genetic investigations of DCM patients have been carried out to evaluate the prevalence of sarcomeric gene mutations and associated disease expression in affected families (Olson *et al.*, 1998, Kamisago *et al.*, 2000, Mogensen *et al.*, 2004b). Considerable locus and allelic heterogeneity have been observed (Hershberger, 2005). To date, 25 mutations associated with DCM have been identified. Many of these mutations occur in sarcomeric proteins (Table 1.1); α -cardiac actin (Olson *et al.*, 1998), β -tropomyosin (Olson *et al.*, 2001), cardiac troponin T (Kamisago *et al.*, 2000), cardiac troponin C (Mogensen *et al.*, 2004b), β -myosin heavy chain (Kamisago *et al.*, 2000), titin (Gerull *et al.*, 2002), and muscle LIM protein (Knoll *et al.*, 2002, Mohapatra *et al.*, 2003). It is notable that mutations in the proteins of the thick and thin filaments are associated with *pure* DCM and are not accompanied by additional phenotypes (Kamisago *et al.*, 2000, Mogensen *et al.*, 2004b).

Table 1.1 Sarcomeric Gene Mutations in HCM and DCM

PROTEIN	HCM	DCM
Cardiac α -Actin	Glu99Lys, Pro164Ala, Tyr166Cys, Ala230Val, Ala295Ser, Met305Leu, Ala331Pro	Glu361Gly, Arg312His
α -Tropomyosin	Glu62Gln, Ala63Val, Lys70Thr, Val95A, Ile172Thr, Asp175Asn, Glu180Gly, Glu180Val, Leu185Arg, Glu192Lys, Met281Thr	Glu40Lys, Glu54Lys
Troponin I	Arg21Cys, Arg141Gln, Leu144Pro, Arg145Gly, Arg145Gln, Ala157Val, Arg162Trp, Arg162Gln, Arg162Pro, Ser166Phe, Lys178del, Lys183Glu, Lys183del, Arg186Gln, Ile195Met, Asp196Asn, Leu198Pro, Leu198Val, Ser199Gly, Ser199Asn, Glu202Gly, Gly203Arg, Gly203Ser, Gly203fs, Arg204Cys, Arg204His, Lys206Gln	Ala2Val
Troponin T	Phe70Leu, Phe77Leu, Ile79Asn, Glu83Lys, Val85Leu, Asp86Ala, Arg92Gln, Arg92Trp, Arg92Leu, Arg94Leu, Arg94Cys, Lys97Asn, Ala104Val, Phe110Ile, Phe110Leu, Phe110Val, Lys124Asn, Arg130Cys, Glu163Lys, Glu163del, Ser179Phe, Glu244Asp, Lys247Arg, Asn271Ile, Lys273Glu, IVS15+1G>A, Arg278Cys, Arg278Pro, Arg286His, Arg286Cys, Trp287ter,	Arg113Trp, Arg141Trp, Ala172Ser, Arg205Leu, Lys210, Asp270Asn
Troponin C	Leu29Gln	Glu159Asp

From "Sarcomere Protein Gene Mutations Database"
<http://genetics.med.harvard.edu/~seidman/cg3>

1.9.1.1.1 α -Cardiac Actin Mutations

The cardiac actin gene (ACTC) is located on chromosome 15q14. Two missense mutations in ACTC that cosegregate with DCM were identified in a candidate gene study of two small unrelated families (Olson *et al.*, 1998). DNA sequence analysis demonstrated a G-A transversion leading to Arg312His (exon 5) inherited by three individuals with DCM, and an A-G transversion leading to Glu361Gly (exon 6) inherited by two individuals with DCM. No other group has yet found any other cardiac actin mutation in DCM patients. Cardiac actin mutations are therefore a relatively rare cause of autosomal dominant DCM. Tesson *et al.* reported that the proportion of European DCM affected individuals bearing a mutation in ACTC must be less than 1.2% (Tesson *et al.*, 2000). In addition, no ACTC mutations were found in 44 probands in the USA (Li *et al.*, 1999), in 30 Japanese DCM patients, in 106 sporadic cases of DCM (Takai *et al.*, 1999) or in 11 patients belonging to 8 families and 46 sporadic cases of mostly black African origin (56%) (Mayosi *et al.*, 1999). Because of their rarity, Tesson *et al.* suggested that the two reported ACTC mutations could be incidental rare polymorphisms and not truly disease causing. However, these ACTC mutations have only been found in patients affected with DCM and not in healthy individuals.

Predictions into the pathophysiological mechanism of DCM from functional studies of these two ACTC mutations, have put forward the 'defective force transmission' hypothesis (Hennessey *et al.*, 1993, Wong *et al.*, 2002). As previously described, actin filaments transmit force between adjacent sarcomeres and neighbouring myocytes to effect the coordination of contraction. The E361G mutation is located in subdomain 1 and is within a common binding domain for α -actinin. α -actinin is found at the Z-band and intercalated disc and acts to cross-link actin filaments into regular parallel arrays. Functional studies of this actin mutation in yeast have shown it to have a reduced α -actinin binding affinity which could impair force-transduction in muscle fibres, leading to the DCM phenotype (Wong *et al.*, 2002). However, actin is a multifunctional protein and it is also possible that the contractile interaction with myosin or the regulatory interaction with tropomyosin and troponin could also be altered by the mutation. In this thesis, I have studied transgenic mice expressing E361G in their hearts. This mutation is an excellent

candidate for transgenic mouse construction since it has the same sequence in man and mouse and the mutation can also be detected by 2-D electrophoresis.

The DCM mutations in β -tropomyosin, troponin T, troponin C, and myosin heavy chain have all been previously studied, mainly by *in vitro* motility assay (Mirza *et al.*, 2005), but also in cardiac myofibrils (Chang and Potter, 2005, Debold *et al.*, 2007). Mirza *et al.* and Robinson *et al.* showed a common effect of DCM mutations; decreased Ca^{2+} sensitivity in every case, and commonly decreased cooperativity, cross-bridge turnover rate and Ca^{2+} binding affinity (Mirza *et al.*, 2005, Robinson *et al.*, 2007).

1.9.1.1.2 Cardiac Troponin C Mutation

In the study reported by Mogensen *et al.*, one family was reported with the TNNC1 mutation Gly159Asp (Mogensen *et al.*, 2004b). Penetrance was 100% and the outcome was poor, with three deaths out of eight (38%), including two from heart failure and one from sudden death. Three patients underwent transplantation (38%), and two remained stable while receiving long-term therapy. The amino acid substitution identified in TNNC1 (Glu159Asp) is localised at the C-terminal and is close to cardiac troponin I. In the *in vitro* motility assay, recombinant G159D causes a decreased Ca^{2+} sensitivity, decreased cross-bridge turnover and decreased Ca^{2+} affinity. This mutation has been intensely investigated. It has been reported that the decrease in the Ca^{2+} binding affinity of troponin C when troponin I is phosphorylated, essential in the inotropic response, is lost due to the G159D mutation (Biesiadecki *et al.*, 2007, Dong *et al.*, 2007). When the troponin is not phosphorylated, the G159D mutation did not alter the Ca^{2+} binding affinity (Biesiadecki *et al.*, 2007, Dong *et al.*, 2007). These results correlate with ATPase and tension measurements showing that the Ca^{2+} sensitivity of myofilaments containing the G159D troponin C mutation were insensitive to troponin I phosphorylation levels (Biesiadecki *et al.*, 2007, Preston *et al.*, 2007b). Alterations in the response to cardiac troponin I phosphorylation levels are therefore suggested to be important in the development of DCM. Biesiadecki *et al.* also reported that the effect of G159D to inhibit troponin I phosphorylation-induced myofilament Ca^{2+} desensitisation also increased the Ca^{2+} sensitivity. This is in contrast to *in vitro* motility and

ATPase measurements of G159D which show a decreased Ca^{2+} sensitivity (Robinson *et al.*, 2002, Mirza *et al.*, 2005).

In this thesis I have investigated troponin isolated from the heart muscle of a patient belonging to the original family with the DCM troponin C G159D mutation (Mogensen *et al.*, 2004b). This has allowed comparisons of the real disease phenotype with *in vitro* data.

1.9.2 Hypertrophic Cardiomyopathy

HCM is the most extensively studied of the four cardiomyopathies and is considered to be the commonest hereditary cardiac disease in young adults, with a prevalence of 1 in 500 (Maron *et al.*, 1995). It is an idiopathic heart muscle disorder, characterised by thickening of the left ventricle (≥ 12 -mm) in the absence of a cardiac or systemic cause (Richardson *et al.*, 1996, Seidman and Seidman, 2001). Hypertrophy in the left ventricle is usually asymmetric, involving the anterior and posterior septum and the free wall to a greater extent than the posterior wall (Seidman and Seidman, 2001). As a consequence of hypertrophy, the left ventricular chamber volumes are diminished and there is obstruction of the outflow tract, causing heart failure in some cases (Figure 1.15 A). Histopathology shows myocyte disarray is prevalent with an increase in interstitial fibrosis. Patients show a slow progression of symptoms, which include breathlessness on exercise, chest pain, sustained palpitation, syncope, gradual deterioration of left ventricular function, and a significant incidence of sudden death (McKenna, 2003). Risk factors include a previous cardiac arrest, family history of sudden death, left ventricular thickness of ≥ 30 mm, abnormal blood pressure upon exercise, and unexplained syncope (Maron *et al.*, 1996, Elliott *et al.*, 1999, Elliott *et al.*, 2000).

HCM is inherited by autosomal dominant transmission in at least 60% of cases. The disease starts to manifest itself in adolescence, although clinical presentation may occur at any age and varies between related individuals (Maron *et al.*, 1986, Niimura *et al.*, 1998, Mogensen *et al.*, 2004a). Molecular genetic studies of familial HCM have helped explain the clinical diversity, with linkage analysis identifying disease-causing mutations in eight different sarcomeric protein genes (Geisterfer-Lowrance *et al.*, 1990, Thierfelder *et al.*,

1994, Bonne *et al.*, 1995, Watkins *et al.*, 1995, Poetter *et al.*, 1996, Kimura *et al.*, 1997, Satoh *et al.*, 1999, Hoffmann *et al.*, 2001). To date, 434 mutations associated with HCM have been identified. Myosin and myosin binding protein C are the predominantly mutated proteins. HCM seems to be a disease of the sarcomere, however 40% of patients do not have mutations in these genes. Table 1.1 shows the HCM associated sarcomeric gene mutations in cardiac α -actin, β -tropomyosin, troponin I, troponin T, and troponin C.

1.9.2.1 α -Cardiac Actin Mutations

ACTC was first identified as a disease gene implicated in HCM by Mogensen *et al.* (Mogensen *et al.*, 1999). Mutation analysis revealed an Ala295Ser mutation in exon 5. Mogensen *et al.* subsequently identified two further novel mutations (Tyr166Cys and Met305Cys) in 3 out of 206 consecutive families (1.5%) with HCM (Mogensen *et al.*, 2004c). Three other mutations have also reported: a heterozygous G-to-C substitution in codon 331 (Ala331Pro), a heterozygous C-to-G substitution in codon 164 (Pro164Ala), and a G-to-A substitution in codon99 (Glu99Lys) (Olson *et al.*, 2000). The E99K mutation seems particularly interesting and has been investigated in detail in this thesis.

Olson *et al.* reported that in one small family, the E99K mutation was inherited by six individuals with HCM and one with isolated trabeculation of the left ventricular apex. They reported that mutations in ACTC appeared to be a rare cause of HCM ($\geq 1\%$), but ACTC mutations were disproportionately high in apical HCM patients (50%) (Olson *et al.*, 2000). Subsequent genetic analyses of DNA from a cohort of 15 probands with apical HCM reported the presence of the E99K mutation in 16 individuals from two families with apical HCM (Arad *et al.*, 2005b). A shared haplotype was identified in the two families, providing odds of >100 to 1 that the E99K mutation caused apical HCM in both families. However, the E99K mutation identified in the small family reported by Olson *et al.* was shown to have arisen independently and be genetically unrelated to the two families reported by Arad *et al.* This is strong evidence that the E99K mutation is associated with apical hypertrophy. A later large clinical study screened for the E99K mutation in 247 families with HCM, DCM, or LVNC (left ventricular non-compaction) (Monserrat *et al.*, 2007). This mutation was found

in 23 patients with LVNC, in addition to 43 with HCM and 22 with apical HCM. Atrial and ventricular septal defects were found in nine E99K mutation carriers from the four families.

Bookwalter and Trybus have reported functional studies on the E99K mutation after expression of high yields of E99K and wild type actin in the baculovirus/insect cell expression system (Bookwalter and Trybus, 2006). *In vitro* motility studies showed that E99K actin had a reduced velocity and supported a 30% lower force than wild type actin when $\bar{U}$ -actinin was used as a load. Bookwalter and Trybus proposed that the slower motility, reduced average force, and a weakened interaction with myosin in the presence of ATP might be sufficient to trigger the HCM phenotype by the E99K mutation. However, this phenotype is unlike any other HCM mutation that has been studied. In this thesis, the functional analysis of actin from transgenic mice expressing the E99K mutation in their hearts is reported.

The HCM mutations in $\bar{U}$ -tropomyosin, troponin T, and troponin I have previously been functionally studied, mainly using the *in vitro* motility assay and ATPase measurements (Mirza *et al.*, 2005, Robinson *et al.*, 2002). A common pattern is reported of an increased Ca^{2+} sensitivity and an increased maximum cross-bridge cycling rate. Either together or alone, these various alterations in myofilament function caused by HCM associated mutations are predicted to impair relaxation, reduce diastolic compliance, reduce contractile reserve and preserve systolic function under baseline conditions (Knollmann and Potter, 2001), leading to the HCM phenotype. According to current published data, heart failure, DCM and HCM have clearly distinct phenotypes (Marston and Hodgkinson, 2001, Knollmann and Potter, 2001, Robinson *et al.*, 2002, Marston, 2003, Chang *et al.*, 2005, Mirza *et al.*, 2005) (Figure 1.16).

Figure 1.16 Molecular Pathology of HCM and DCM

	Maximum Crossbridge Cycling rate	Relaxation	Ca ²⁺ Sensitivity
Genetic DCM			
Genetic HCM			
Acquired DCM			
RCM			

Acquired and genetic DCM show opposite trends in Ca²⁺ sensitivity upon function analysis. HCM mutations are hypercontractile, whilst DCM mutations are hypocontractile.

1.9.3 Animal Models of DCM

Several transgenic mouse models that carry contractile protein mutations associated with DCM have been generated to explore the molecular mechanism of this disease. The most recent of these models has been the troponin T mutation Δ Lys210 (Morimoto *et al.*, 2006). The mutant mice developed enlarged hearts and showed a high incidence of sudden death. Histological examination showed a significant dilation of both ventricles and fibrosis. Echocardiography showed systolic dysfunction of the left ventricle, although the cardiac output was unchanged. This suggests systolic dysfunction in the mutant mice was fully compensated through the enlarged heart, possibly through a compensatory increase in the Ca²⁺ transient. Skinned cardiac muscle fibres from Δ Lys210 mice show a decreased Ca²⁺ sensitivity of force generation, an increase in the intracellular Ca²⁺ transient, but no difference in the maximum isometric force. This suggests that the Ca²⁺ transient was increased to compensate for the decrease in Ca²⁺ sensitivity (Du *et al.*, 2007). These findings correlate with previous results from directly exchanging recombinant human Δ Lys210 into skinned rabbit cardiac muscle fibres

(Morimoto *et al.*, 2002), and *in vitro* motility assay and ATPase results (Mirza *et al.*, 2005, Robinson *et al.*, 2002).

The establishment of two transgenic mouse lines carrying the β -tropomyosin Glu54Lys (E54K) mutation has allowed the molecular mechanism of DCM to be further studied (Rajan *et al.*, 2007). Histological analysis showed that the mice expressing high levels of the transgene die by one month, due to severe dilation of both right and left ventricles. Low copy number mice survive for more than six months, although they show altered systolic and diastolic function. The time to peak pressure and half time to relaxation were both increased. The E54K mutation has shown a number of anomalies *in vitro* and may not conform to the general principles of DCM mutations (Mirza *et al.*, 2007).

1.9.4 Animal Models of HCM

Many models have been generated that contain sarcomeric gene mutations known to cause HCM, including β -myosin heavy chain, myosin binding protein C, β -tropomyosin, cardiac troponin T and cardiac troponin I. The first HCM mouse model generated used a gene targeting strategy and contained the β -myosin heavy chain mutation Arg403Gln (Fatkin *et al.*, 1999). This mutation has a high penetrance in humans, presenting at 90% in HCM patients by 20 years old (Doolan *et al.*, 2004). Homozygous animals died shortly after birth. Secondary heterozygotes survived but showed considerable myocyte disarray, hypertrophy, and fibrosis which increased with age (Fatkin *et al.*, 1999). Functional studies showed an increase in actin-activated cycling and an increased rate of pressure rise and force development (Fatkin *et al.*, 1999, Dalloz *et al.*, 2001).

The role for cardiac troponin T, troponin I and myosin binding protein C mutations in HCM has been investigated through transgenesis (Oberst *et al.*, 1998, Yang *et al.*, 1998, Tardiff *et al.*, 1999, James *et al.*, 2000). The troponin I mutation R145E showed an increase in the Ca^{2+} sensitivity of skinned muscle fibres and at the whole organ level there was hypercontractility with diastolic dysfunction (James *et al.*, 2000). Oberst *et al.* studied the Arg92Gln troponin T mutation, which lead to myocyte disarray, substantial fibrosis and diastolic

dysfunction, all common features of human HCM. Yang *et al.* generated transgenic mice with varying amounts of a mutated myosin binding protein C (lacking the myosin and titin binding domains), and showed an increased Ca^{2+} sensitivity and a decreased power output, resulting in myocyte disarray. Similarly, Miller *et al.* and Chandra *et al.* reported that transgenic mice expressing the Ile79Asn and Arg92Gln troponin T mutations exhibited an increase in the Ca^{2+} sensitivity of force production in skinned muscle fibres (Miller *et al.*, 2001, Chandra *et al.*, 2001). Knollman *et al.* subsequently examined the effect of the Ile79Asn mutation on cardiac performance and electrophysiology of the whole heart, *in vitro*, and *in vivo* (Knollmann *et al.*, 2001). The higher Ca^{2+} sensitivity enhanced the base-line contractility but lead to cardiac dysfunction during inotropic stimulation with isoproterenol. This lead the authors to hypothesise that the effect of a mutation can be dependent upon the inotropic state of the heart.

In many cases, transgenic animals have provided the opportunity to examine the functional consequences of sarcomeric gene mutations in an *in vivo* system, thereby removing many of the limitations of the *in vitro* assays. Transgenic models seemed to have validated the hypothesis that HCM and DCM are diseases of the sarcomere (Doolan *et al.*, 2004).

1.10 Context and Objectives of this Study

Investigations into how HCM and DCM-associated sarcomeric gene mutations affect the *in vitro* contractile protein function and cause the development of the disease state are widely reported, but there is a certain amount of variability in the genotype-phenotype correlations that have been made. Previous work in our laboratory has used the *in vitro* motility assay to study the functional effects of HCM and DCM mutations in troponin I, troponin C, troponin T and β -tropomyosin (Marston, 2003, Mirza *et al.*, 2005, Mirza *et al.*, 2007). However, these functional investigations used recombinant proteins where the troponin was not phosphorylated, hence created an unphysiological thin filament. The main objective of this thesis has been to establish whether there are alternative or additional mechanisms by which HCM and DCM mutations might cause cardiac muscle dysfunction, using an experimental

system that is as close as possible to native human cardiac thin filaments. In particular it was desirable to test whether the mutations interfere with the inotropic response since attenuation of this response is a characteristic of failing hearts in general (Messer *et al.*, 2006).

This thesis represents the first investigation to test the effect of actin HCM and DCM linked mutations generated in transgenic mice. The highly conserved and abundant nature of actin in the heart has allowed this protein to be a prime candidate to functionally investigate. Human and mouse actins have identical amino acid sequences and also identical adult isoforms distribution in their hearts (20% skeletal, 80% cardiac). Actin cannot be expressed *in vitro* in *Escherichia.coli* due to its complex folding mechanism and posttranslational modifications (Sheterline *et al.*, 1998). The expression of mutant actin has however been possible in yeast, drosophila, and Baculovirus/Sf9 cell systems but with limited success. It was hypothesised that the generation of transgenic models with disease causing ACTC mutations expressed at ratios characteristic of the human phenotype will provide a reliable preparation for investigating actins interaction with its many partner proteins and its altered functioning in disease. It was intended that the reconstitution of mouse actin (containing the human ACTC sequence with E361G and E99K sequence substitutions) along with human cardiac troponin and tropomyosin will allow the physiological conditions of the human thin filament to be closely reproduced. The E361G and E99K ACTC mutations are well-defined mutations in DCM and HCM patients respectively (Olson *et al.*, 1998, Arad *et al.*, 2005b, Monserrat *et al.*, 2007), although few clearly defined functional studies have been reported (Wong *et al.*, 2002, Bookwalter and Trybus, 2006). This thesis investigates the transgenic mice at the molecular level, but the mouse models ought to be suitable for investigations of the physiological consequences of these mutations in both the intact heart and whole animal. The fortuitous availability of a human muscle sample from a patient with the troponin C mutation G159D (Mogensen *et al.*, 2004b) allowed the study of another DCM linked mutation with the physiological post translational modifications and mutant to wild type ratios characteristic of the human disease phenotype.

2. MATERIALS AND METHODS

2.1 Production of Mutant ACTC Transgenic Mice

The two mutant cardiac actin cDNA constructs (Glu361Gly and Glu99Lys) were provided by Dr. Kristen Nowak (University of Western Australia) in a pBSII KS vector. Mutations were inserted into the human cardiac actin sequence by Dr. Charles Redwood (University of Oxford). The transgene expression vector was first cut with Hind III then partially filled in with two nucleotides (AG) using Klenow fragment. After purification the vector was then digested with Sal1. The mutant cardiac actin cDNA was removed from the pBSII KS vector by first digesting with Spe I, then filling the 3' end with two nucleotides (C,T) using Klenow fragment. After purification the vector was digested with Sal1. This allowed directional cloning of the mutant actin construct into the transgene expression vector using a Sal1 site at the 5' end and a Spe1/Hind III hybrid at the 3' end.

Transgene expression was restricted to the heart using an alpha-myosin heavy chain promoter construct kindly provided by Dr. Mike Gollob and originally produced in the laboratory of Dr. Jeffrey Robbins (Subramaniam *et al.*, 1991). In this construct, the promoter region of alpha-myosin heavy chain started with the 3' untranslated region of the beta-myosin heavy chain and the first three exons of alpha-myosin heavy chain that were non-coding.

Transgenic mice were generated by pronuclear microinjection of gel purified transgenic constructs (after removal of the plasmid backbone with Not I) into the pronucleus of fertilised mouse eggs on a C57BL10xCBA/Ca hybrid background (Wells *et al.*, 1995, Akbar *et al.*, 2003, Antoniv *et al.*, 2005, Davies *et al.*, 2006, Mollica *et al.*, 2006). Treated embryos were returned to a pseudopregnant CD-1 foster mother, generated by mating with a vasectomised male mouse, and the resultant pups were identified and genotyped by PCR from ear notch samples.

2.2 Using Ear Tissue to Genotype Transgenic Mice by PCR

PCR was performed in the laboratory of Prof. Dominic Wells (Gene Targeting Unit, Charing Cross Hospital). One average sized ear notch (1.5-3

mm² of tissue) provided approximately 5µg of DNA, which was ample for PCR. A 10x stock of PCR homogenisation buffer was made up (500mM KCl, 100mM Tris-HCl pH 8.3, 0.1mg/ml Gelatin, 0.45% v/v Tween 20, 0.45% v/v Nonidet P40). After autoclaving this solution, 5mg/ml Proteinase K was added and mixed well. Aliquots of 5ml were made of 1x buffer and kept frozen. Each ear notch was placed into individual eppendorf tubes and 50µl of 1x PCR homogenisation buffer was added to each one. They were placed in a water bath set at 55°C overnight (or until all tissue had fully digested, which took only a few hours). Once digested, the samples were heated to 100°C for 10 minutes to de-activate the proteinase K in the buffer. Once cooled, the PCR was performed directly on this solution.

2.3 PCR Screening Transgenic Mice from Ear Tissue

Prior to performing PCR, all pipettes were dismantled, washed with warm water, and allowed to dry in an oven. They were then irradiated with U.V and reassembled. The Qiagen kit, which contained all ingredients for the PCR mix was prepared to cover the total number of samples that were being made. To achieve good PCR results from ear tissue, the PCR was optimised using ear DNA. Usually 1µl of neat ear solution was added to the PCR mix, although it was sometimes necessary to dilute the solution 1:10 or 1:25 before adding it to the rest of the PCR mix. The Qiagen mix recipe for one reaction consisted of 2.5µl 10x buffer containing 1.5mM MgCl₂, 10µl 5x Q solution, 4µl dNTP, 2µl forward primer, 2µl reverse primer, 1µl template (i.e. plasmid or sample DNA). It was made up to 25µl with water. The forward primer was located in the promoter element of the transgene (MHC-F Sequence (5'→3') *TGACAGACAGATCCCTCCTATCTCC* and the reverse primer was located in the mutant actin (ACTC-R) (5'→3') *GGCATCTTAGAAGCATTGCGG*. Each primer was diluted to a 10µM concentration with water before use. The same number of reactions worth of enzyme were prepared. The enzyme recipe for one reaction consisted of 2.5µl 10x buffer, 0.25µl Taq DNA polymerase, 22.25µl water. 25µl of the Qiagen mix was aliquotted into the correct number of labelled PCR tubes. Samples were loaded into the PCR and run for the following cycling programme:

- 1) 5 minutes at 94°C (Hot start PCR)
- 2) Hold at 94°C for addition of enzyme

Steps 1 and 2 were linked to run one after the other automatically, as were steps 3, 4, and 5. When only 1 ½ minutes were left on the first step, the tube of enzyme was heated. After the first 5 minute cycling step, 25µl of enzyme was pipetted into the bottom of each tube. Steps 3, 4, and 5 were then started.

- 3) Cycle (at optimised conditions)
- 4) Hold at 72°C for 2.5 minutes
- 5) Cool to 4°C for storage

The PCR cycling step took 2 ½ hours to complete, and samples were stored in the freezer if required.

2.4 Screening of Transgenic Mouse by 2-D Electrophoresis

The charge change of the E361G and E99K mutations (+1 and + 2 respectively) allowed the identity and quantity of mutant cardiac actin in the transgenic mouse cardiac tissue to be separated by 2-D Electrophoresis, using the method described by Ilkovski *et al.* (Ilkovski *et al.*, 2004). This accurate screening method of mutant protein in muscle was essential for the efficient generation of the transgenic lines.

2.4.1 Sample Preparation and Rehydration of Immobiline

DryStrips

15µg tissue (wet weight) was homogenised in 150µl wash buffer (20mM KPO₄, 2mM MgCl₂, 1mM DTT, 1mM NaN₃, 50% v/v glycerol, 0.5% v/v Triton X-100, pH7.0). Tissue homogenate or the pure F-actin was solubilised in lysis buffer (8M urea, 2% Triton X-100, 36mM DTT) to a quantity of 15µg/ml tissue or 4µg/ml actin. Samples were made up to a volume of 340µl with rehydration buffer (lysis buffer, 2% IPG buffer from Amersham Biosciences, 1µg/ml protease inhibitors E-64, chymostatin, leupeptin and pepstatin which were added just prior to use) at 0.15µg/340µl tissue or 0.04µg/340µl actin. The protein solutions were loaded slowly into separate slots of the Immobiline DryStrip Reswelling Tray. Individual dehydrated 18cm pH4-7 IPG strips (Amersham Biosciences) were placed over the protein solutions in the prepared

slots (Figure 2.1), and overlayed with 2-3ml DryStrip Cover Fluid to prevent evaporation and urea crystallisation. Up to six IPG strips were prepared and rehydrated at one time. The Immobiline DryStrip Reswelling Tray was left overnight at room temperature.

Figure 2.1. Positioning of IPG Strip on DryStrip Reswelling Tray

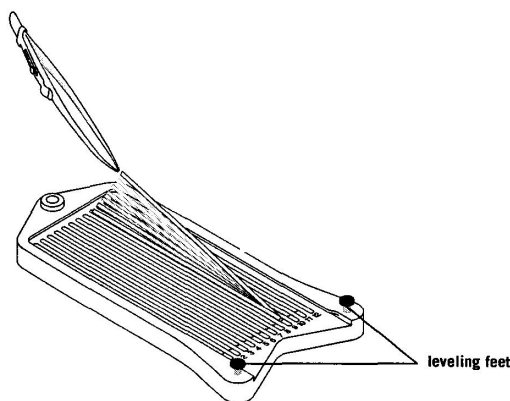


Image obtained from Amersham Biosciences *2-D Electrophoresis Principles and Methods Handbook* (juang.bst.ntu.edu.tw/Protein/proteomics/2D%20Electrophoresis.pdf)

2.4.2 First Dimension Isoelectric Focusing (IEF)

The first dimension run separated proteins according to their isoelectric points. The Immobiline DryStrip tray was placed onto the Multiphor II electrophoresis unit cooling plate (20°C) that had been covered with approximately 3-4 ml of DryStrip Cover Fluid. This served as conducting fluid to ensure good contact between cooling plate and the tray. The anodic electrode of the tray was positioned at the top of the plate near the cooling tubes, and electrode leads connected to the Multiphor II unit.

The Drystrip tray was covered with 10ml DryStrip Cover Fluid. This fluid protected the strips from evaporation and the samples from oxidation. The rehydrated strips were placed into an Immobiline strip aligner, and positioned onto the tray (Figure 2.2). Once the electrodes were correctly positioned over moistened IEF electrode strips at the cathode and anode, 70-80 ml DryStrip Cover Fluid was poured into the tray to completely cover the strips.

Figure 2.2 Positioning of IPG Strip Gels in the DryStrip aligner

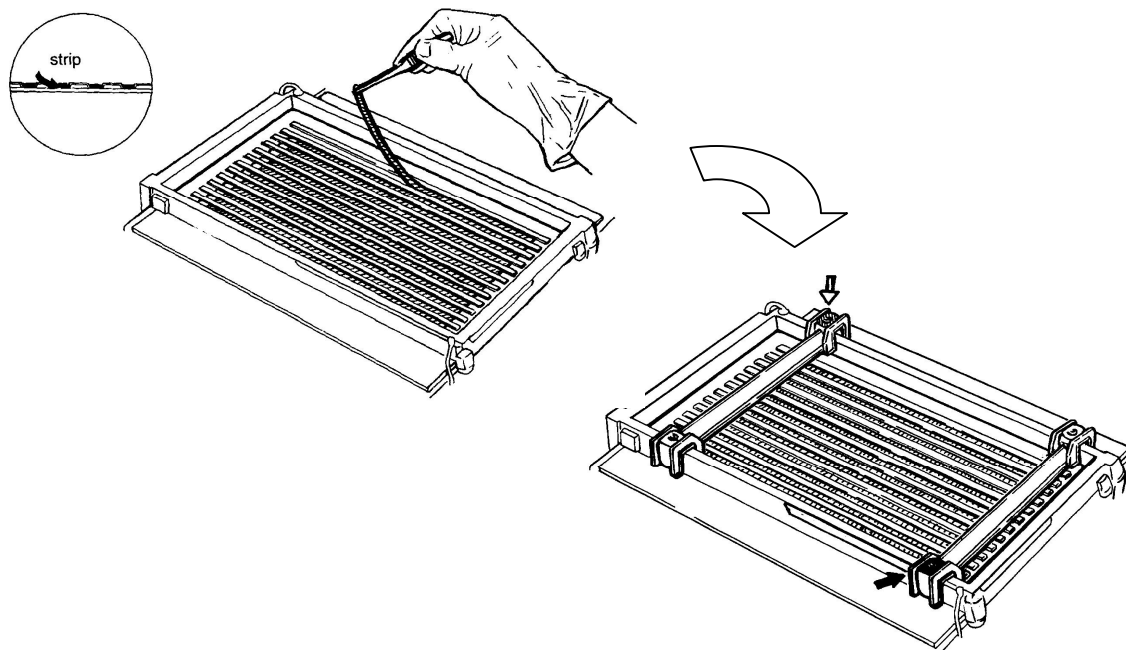


Image obtained from Amersham Biosciences *2-D Electrophoresis Principles and Methods Handbook* (juang.bst.ntu.edu.tw/Protein/proteomics/2D%20Electrophoresis.pdf)

Isoelectric focusing was performed in two phases. The first phase ran at 500V, 1mA, and 5W for 4 hours. The voltage was then increased to 3500V and run for 12.5 hours. If more than four strips had been run in the first dimension, the unused strips could be wrapped in cling film and stored at -80°C for later use. The strips were stored prior to equilibration.

2.4.3 Equilibration of Immobiline DryStrips and Second Dimension SDS-PAGE

Equilibration involved the incubation of each DryStrip with 20ml equilibration solution No.1 (375mM Tris-HCl pH8.0, 6M urea, 2% SDS, 20% glycerol, 65mM DTT) for 10 minutes. Equilibration solution no.2 (solution no.1, 243mM iodoacetamide, and a few grains of Bromophenol Blue) replaced the first solution for an additional 10 minutes. Each 18 cm DryStrip was cut down to 6 cm by removing 6 cm from the anodic and cathodic ends, thus enabling two strips to be run in second dimension on a 15 cm gel. The remaining 6 cm

central part of the DryStrip, which contained the focused actin (Pi 5.7), was placed onto a half size (15cm) ExcelGel gradient 8-18% and run in three phases. The first phase ran at 600V, 10mA, and 15W for 30 minutes, after which the IPG strip gel was removed. The current was then increased to 25mA and run for 5 minutes. The cathodic buffer strip was moved forward by 1-2 mm and the position of the cathodic electrode adjusted. The final phase ran at 600V, 25mA, and 15 W for 70 minutes.

Figure 2.3 Positioning Equilibrated IPG Strips on MultiPhor II

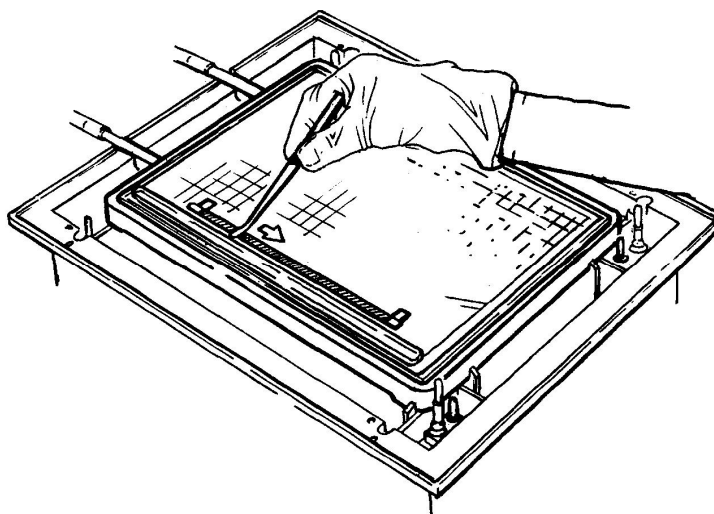


Image obtained from Amersham Biosciences *2-D Electrophoresis Principles and Methods Handbook* (juang.bst.ntu.edu.tw/Protein/proteomics/2D%20Electrophoresis.pdf)

2.4.4 Western Blotting

2.4.4.1 Protein Transfer

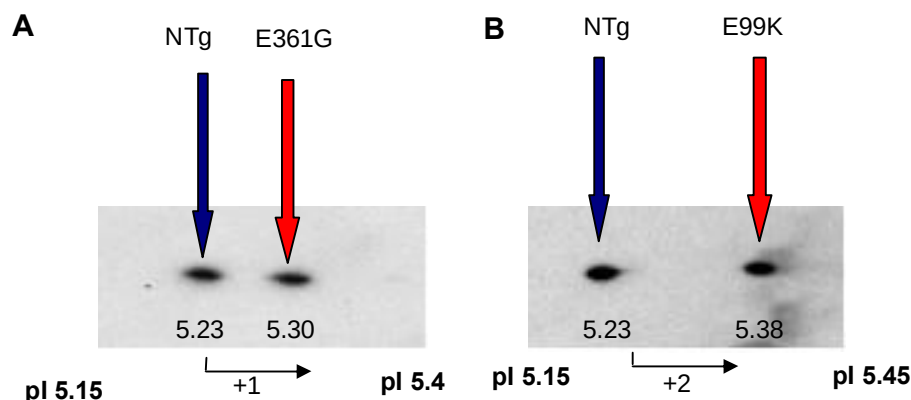
A nitrocellulose membrane (0.45 μ m pore size, Amersham Biosciences, RPN 303D) and 2 pieces of BIO-RAD extra thick filter paper (Cat No.1703969, Protean XL, 18.5x19cm) were cut to the size of the gel (10x13cm) and pre-soaked in blotting buffer (20% methanol, 48mM Tris, 39mM glycine, 1.3mM SDS) for 15 minutes. After SDS-PAGE, the gel was stripped from its plastic backing. One sheet of filter paper, the nitrocellulose membrane, the gel and a further sheet of filter paper were arranged to form a sandwich on the semi-dry transblotter cell (BIO-RAD 221BR). The separated proteins were electrophoretically transferred onto the nitrocellulose membrane at 350mA and

25volts for 1 hour at room temperature. The nitrocellulose membrane was blocked by immersing the membrane for 1 hour at room temperature (alternatively overnight at 4°C) in blocking buffer consisting of 1% dried milk powder, 0.1% Tween 20 and 1 x phosphate buffered saline (PBS consisting of 137 mM NaCl, 27 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3).

2.4.4.2 Immunodetection

The membrane was incubated with primary antibody (Sigma, 5C5 monoclonal mouse anti-actin, A2172) in blocking buffer at 1/1000 dilution for 1 hour at 4°C. The membrane underwent 3 x 5 minute washes in PBS with 0.1% Tween 20 before incubation with sheep anti-mouse IgG coupled with horseradish peroxidase (Amersham Biosciences, NXA931), (1/1000 dilution in blocking buffer), for 1 hour at 4°C. Following a further 3 x 5 minute PBS washes, the membrane was activated by soaking with enhanced chemiluminescence reagents (ECL, Amersham Biosciences, RPN2109) for one minute and exposed to ECL Hyperfilm (Hyperfilm, Amersham Biosciences, RPN1674K). Films were exposed for approximately 1 minute and then developed (Figure 2.4).

Figure 2.4. Immunodetection of E361G (A) and E99K (B) F-actin



Non-transgenic (NTg) actin has a pI value of 5.23 (obtained from UniProtKB Swiss-Prot database). E361G actin has a pI of 5.30 and E99K of 5.38. The two mutant actins ran at the predicted pI. E361G and E99K transgenics express mutant cardiac actin at approximately 50% of the total actin.

2.4.4.3 Densitometric Analysis of Western Blots

Protein bands detected by western blotting were measured by scanning densitometry. The ECL films were digitised using a HP Scanjet 4800 (HP Invent) desktop flatbed scanner with transilluminator, saved as TIFF files, then analysed with GeneTools 3.07 software run on an IBM PC (SynGene). Upgrades to the system subsequently allowed chemifluorescence directly in a G:Box Chemi HR 16 system. TIFF files were analysed with GeneTools 3.07 software run on GeneSnap 6.08 (SynGene).

2.5 Isolation of Actin from Transgenic Mouse Hearts

F-actin was isolated simultaneously from 5-7 week-old non-transgenic and mutant (E361G and E99K) mouse hearts. E361G F-actin was deficient in the mutation when extracted via an acetone powder method (Razzaq *et al.*, 1999). This indicated the monomeric form was unstable and aggregated. A method to isolate pure actin filaments directly from native thin filaments was developed. This preserved the 50% mutant actin content (Tobacman and Sawyer, 1990). This actin contained a mixed population of transgenic cardiac actin, non-transgenic cardiac actin, and α -skeletal actin.

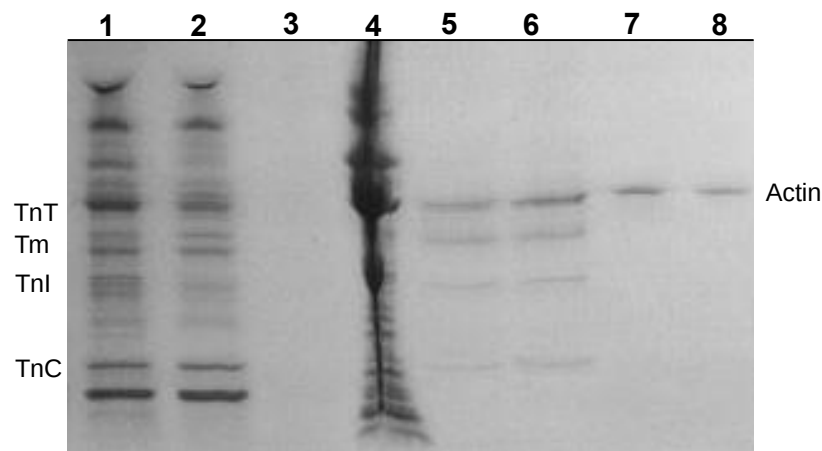
2.5.1 Actin isolation directly from Native Thin Filaments

One heart (50-100mg) was pulverised in a liquid nitrogen cooled percussion mortar and homogenised in 10 x volume of wash buffer containing 20mM Pi, 0.1M NaCl, 5mM MgCl₂, 0.5 mM EGTA, 5mM DTT with 2 μ g/ml each of the protease inhibitors E-64, chymostatin, leupeptin and pepstatin A, pH 7.0. Samples were spun at 16,662xg (13,400rpm, eppendorf centrifuge 5417R, F-45-24-11 rotor) for 3 minutes at 4°C. This extraction step was repeated 4 times and the pellet was then extracted twice in 1.5 volume of thin filament extraction buffer (5mM ATP/100ml wash buffer) and centrifuged at 16,662xg for 3 minutes at 4°C. The two supernatants were combined and centrifuged at 111,700xg (50,000rpm, Sorvall RCM120SE, S100-AT6 rotor) for 5 minutes at 4°C. The supernatant was further spun at 446,800xg (100,000rpm, Sorvall RCM120SE, S100-AT6 rotor) for 20 minutes at 4°C. The pellet containing thin filaments was dialysed in 1.5 volume of a buffer containing 20mM Pi, 0.1M NaCl, 5mM MgCl₂,

and 1mM ATP, pH6.0 for 3 hours at 4°C. A 2-minute spin at 16,662xg (13,000rpm, eppendorf centrifuge 5417R, F-45-24-11 rotor) at 4°C removed the remaining myosin. 3M KCl stock was added to the supernatant to a final concentration of 0.8M to dissociate actin from troponin-tropomyosin. 150nM TRITC-phalloidin was also added to the supernatant. The dissociated thin filaments were spun at 337,000xg, (100,000rpm Sorvall RCM120SE, S100-AT6 rotor) for 20 minutes at 4°C, and the pellet containing the F-actin was resuspended in 100µl ACEX (actin extraction buffer) (2mM Tris-HCl, 0.2mM CaCl₂, 0.2mM ATP, 1mM DTT, pH8.0) overnight at 4°C.

Western blotting analysis determined the F-actin concentration to be approximately 0.5 mg/ml and the yield of actin approximately 1µg per 1mg of tissue. The F-actin was labelled with TRITC-phalloidin, and remained functional for use in the *in vitro* motility assay for 7-10 days. Various sample fractions were taken throughout the isolation process for SDS-PAGE and western blot analysis to investigate the mutant actin properties (Figure 2.5).

Figure 2.5 SDS-PAGE of E361G Isolation Fractions



SDS-PAGE stained with Coomassie Blue of E361G actin isolation fractions: (1) whole muscle homogenate, (2) first wash supernatant, (3) last wash supernatant, (4) pellet after two extractions in thin filament extraction buffer, (5) KCl supernatant* - 150nM TRITC-phalloidin, (6) KCl supernatant* + 150nM TRITC-phalloidin, (7) f-actin pellet - TRITC-phalloidin, (8) f-actin + TRITC-phalloidin. *Native thin filaments were mixed with 0.8M KCl and f-actin was sedimented by centrifugation for 20-min at 100,000rpm.

2.6 Preparation of Proteins from Rabbit Skeletal Muscle

Skeletal muscle actin and myosin were extracted from rabbit back muscle for use in *in vitro* studies. The muscle was excised immediately after sacrifice and cooled on ice.

2.6.1 Preparation of F-actin from Rabbit Skeletal Muscle

The preparation of skeletal muscle F-actin was carried out by O'Neal Copeland in the laboratory of Professor Steven Marston, following the method of Spudich *et al.* (Spudich and Watt, 1971). An additional step involving the purification of the G-actin through a Sephacryl S300 gel filtration column was included (Alahyan and EL-Mezgueldi, 2005, Alahyan *et al.*, 2006). A volume of 2ml F-actin was routinely obtained, with the concentration ranging between 5-20 mg/ml. The F-actin was fluorescently labelled with TRITC-phalloidin (section 2.10.1) or prepared for use in the affinity precipitation of HMM (section 2.10.4). It remained functional for up to 3 weeks after preparation.

2.6.2 Preparation of Myosin

The preparation of skeletal muscle myosin was carried out by O'Neal Copeland in the laboratory of Professor Steven Marston, following the method of Margossian and Lowry (Margossian and Lowey, 1982). The purified myosin was rapidly frozen as a concentrated solution (20-40 mg/ml) in liquid nitrogen. The myosin could be stored for up to 1 year in 50 µl aliquots under liquid nitrogen.

2.6.3 Preparation of Rabbit Skeletal Muscle HMM

The method of rabbit skeletal muscle HMM preparation was based on that of Okamoto (Okamoto and Sekine, 1985, Fraser and Marston, 1995a), and was prepared for use as the immobilised motor in the *in vitro* motility assay. A 100µl aliquot of rabbit skeletal myosin of approximately 30 mg/ml was thawed from liquid nitrogen on ice and diluted 10-fold with HMM buffer A (0.1mM NaHCO₃, 0.1mM EGTA pH 7.4, 1mM DTT). The homogenate was incubated on ice for 20 minutes. The myosin was pelleted by centrifugation at 25,000xg

(Eppendorf centrifuge 5417R with the F-45-24-11 rotor at 16,400rpm for 10 minutes at 4°C).

The myosin was resuspended in 100µl of HMM buffer B (20mM Imidazole-HCl pH 7.0, 1M KCl, 4mM MgCl₂, 2mM DTT) and incubated for 10 minutes on ice. The solution was made up to 200µl with HMM Buffer A to a concentration of approximately 15mg/ml. It was then incubated for 10 minutes in an eppendorf thermomixer at 25°C. 200µl of the myosin solution was digested using TLCK treated α -chymotrypsin (Sigma type VII, C-3142) at 15µg/ml concentration (7.5µl of a 0.4mg/ml solution) and incubated for exactly 8 minutes at 25°C. The digestion was stopped by adding 2700µl of ice cold HMM buffer A containing 3mM MgCl₂ and 0.1mM PMSF. The mixture was incubated on ice for 60 minutes and then centrifuged at 337,000xg (100,000rpm, Sorvall MC-120SE, S100-AT6 rotor) for 15 minutes at 4°C to remove any undigested myosin. The supernatant contained pure HMM at 0.3-0.7mg/ml and was suitable for use in the motility assay, after affinity precipitation (section 2.10.4) for 3-4 days.

2.7 Collection and Storage of Non-Failing Human Myocardium

Four non-failing (donor) human heart muscle samples were used as a source of human cardiac troponin. Clinical details are shown in Table 2.1. Samples were supplied by Professor C. Dos Remedios, University of Sydney, Australia. Ethical approval was obtained from The Brompton, Harefield & NHLI, London and St Vincent's Hospital, Sydney. The investigation conformed to the principles outlined in the Declaration of Helsinki (World Medical Association, 1996). The non-failing adult myocardium was obtained from subjects who were assessed as potential heart donors but were subsequently rejected on technical grounds or where no suitable transplant recipient was found. The donors had no history of cardiac disease, a normal cardiac examination, a normal admission ECG, and normal ventricular function on transthoracic echocardiography performed within 24 hours of heart explantation. These criteria were set to exclude any significant myocardial contractile dysfunction. Immediately following explantation, the ventricular wall was cut up into 1g segments, frozen in liquid nitrogen and stored for later analysis.

Table 2.1. Clinical details of Non-Failing Human Heart Samples

Sample	Diagnosis	Operation	Gender	Age
NF 9	Trauma	Donor	M	54
NF 10	Grade V SAM	Donor	F	48
NF 11	Excess inotropes; grade IV SAH	Donor	M	51
NF 12	LA/RA/RV	Donor	F	59

Donor i heart transplant after brain stem death, SAM - Systolic Anterior Motion, SAH i Subarachnoid Haemorrhage

2.8 Preparation of Human Cardiac Troponin

Human cardiac troponin was isolated from the non-failing human donor hearts by affinity chromatography.

2.8.1 Human Cardiac Troponin Preparation by Affinity Chromatography

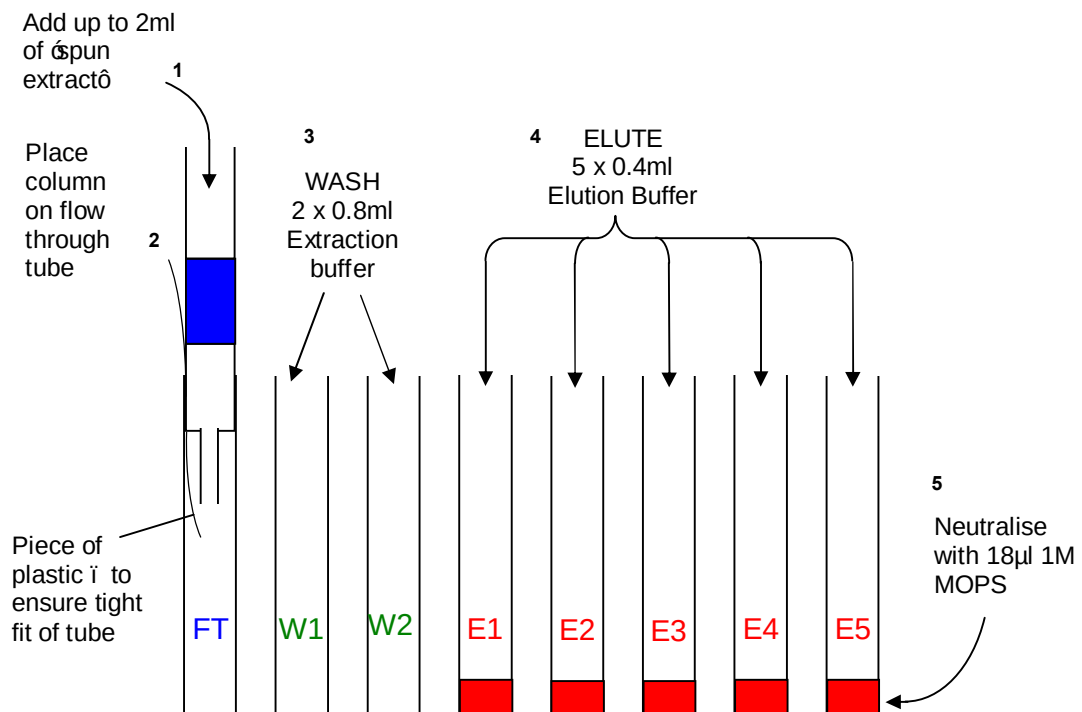
An affinity separation method was developed using an anti-cardiac Troponin I monoclonal antibody (16A11 HyTest, Finland) conjugated to CNBr Sepharose CL-4B. Professor N. Gusev and G. Filatov from Moscow State University provided the immobilised antibodies. All steps were carried out at 4°C (Filatov *et al.*, 1998, Katrukha *et al.*, 1995, Messer *et al.*, 2006).

100-150 mg of frozen non-failing heart muscle was pulverised in a liquid nitrogen cooled percussion mortar. Pulverised human heart muscle was manually homogenised in 1.5ml of a wash buffer containing 5mM NaH₂PO₄, 5mM Na₂HPO₄ pH 7.0, 0.1M NaCl, 5mM MgCl₂, 0.5mM EGTA, 0.1% Triton X-100 and 5mM DTT with 2µg/ml each of the protease inhibitors E-64, chymostatin, leupeptin and pepstatin A. The homogenate was centrifuged at 13,400rpm (Eppendorf centrifuge 5417R, F-45-24-11 rotor) for 3 minutes and the supernatant discarded. This step was repeated 4 times. Cardiac proteins were extracted by homogenisation in an extraction buffer (0.7M LiCl, 25mM Tris pH 7.5, 5mM EGTA, 0.1mM CaCl₂, 5mM DTT, protease inhibitors). The first extraction was for 25 minutes on a Dynal sample mixer followed by

centrifugation for 3 minutes at 13,400rpm (Eppendorf centrifuge 5417R, F-45-24-11 rotor). The supernatant was retained and the pellet extracted three more times for 20,15 and 10 minutes. The combined supernatants were pooled and centrifuged at 337,000xg for 20 minutes (100,000rpm Sorvall MC-120SE, S100-AT6 rotor). A maximum volume of 2ml of the centrifuged extract was placed on the affinity column with 0.6ml of the affinity matrix and mixed for 15 minutes at 4°C on the Dynal sample mixer to ensure complete binding to the antibody.

The column was placed on the flow through (FT) tube (Figure 2.6) and unbound proteins such as actin, myosin and tropomyosin run off. 0.8ml of the extraction buffer was passed through the column twice to make sure all unbound proteins had been eluted. Troponin was eluted from the column by the addition of 0.1M glycine (pH 2) in five 0.4ml aliquots. This was immediately neutralised by 1M MOPS (pH 8), avoiding any damage to the troponin structure.

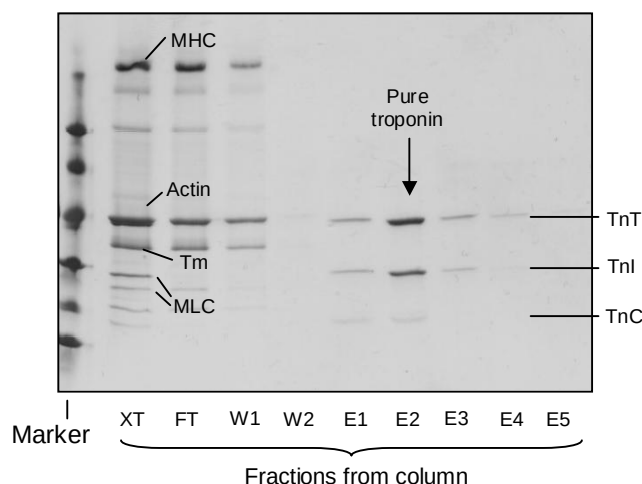
Figure 2.6. Affinity chromatography using the anti-TnI monoclonal antibody.



W - wash, E - Elute fraction. Troponin routinely eluted in the E2 fraction.

The fractions were analysed by SDS gel electrophoresis to locate the troponin elution fraction (Figure 2.7). Fractions containing pure troponin were dialysed overnight against a buffer consisting of 1M KCl, 10mM MES pH 6.0, 0.1mM CaCl₂, 5mM DTT and 2µg/ml protease inhibitors. The dialysate was centrifuged at 16,000rpm, Eppendorf centrifuge 5417R, F-45-24-11 rotor for 3 minutes to remove any insoluble material. This solution contained pure troponin, which was used in functional analysis for a maximum of five days. The yield was normally 600µl of 7µM troponin from 100mg of tissue.

Figure 2.7 SDS-PAGE of Extracted Human Cardiac Troponin



XT ï Extract, FT ï Flow through, W ï Wash, E ï Elute fraction, MHC ï Myosin heavy chain, MLC ï Myosin light chain, Tm ï Tropomyosin, Tn ï Troponin.

2.8.1.1 Dephosphorylation of Human Cardiac Troponin

Before the overnight dialysis step, troponin could be dephosphorylated by incubating with 1.4 U/ml acid phosphatase (Sigma, P-0157) (Messer *et al.*, 2006). The extracted troponin was mixed with the phosphatase and then dialysed overnight at 4°C against the troponin dialysis buffer. The dialysate was centrifuged at 16,000rpm, Eppendorf centrifuge 5417R, F-45-24-11 rotor for 3 minutes to remove any insoluble material. The supernatant contained dephosphorylated troponin.

2.8.2 Preparation of Human Cardiac Tropomyosin

Human cardiac tropomyosin was prepared from an ethanol-ether powder, based on the method of Potter *et al.* (Potter, 1982) from 300g of human cardiac muscle. The preparation was carried out by O'Neal Copeland in the laboratory of Professor Steven Marston. All steps were carried out at 4°C.

2.8.3 Preparation of α -Actinin

α -actinin was prepared by Professor Steven Marston from chicken gizzard based on the method of Craig *et al.* (Craig *et al.*, 1982).

2.8.4 Use of Protease Inhibitors

All buffers used during protein extraction contained a mixture of protease inhibitors at a concentration of 2 μ g/ml from 20mg/ml (Marston and Smith, 1984), to prevent proteolytic degradation. The protease inhibitors used were chymostatin, leupeptin, pepstatin A and E-64.

2.9 Protein Analysis

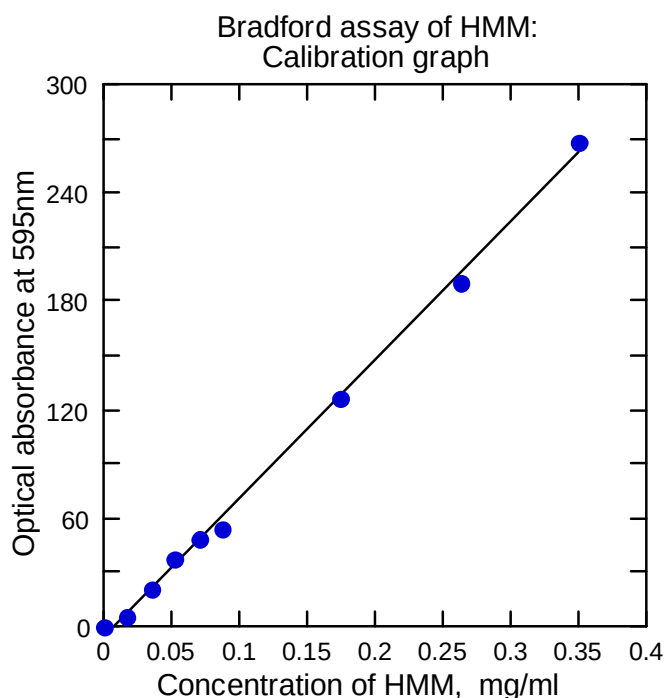
2.9.1 Determination of Protein Concentration

Protein concentrations were determined by the method of Lowry *et al.* (Lowry *et al.*, 1951). Aliquots of protein, typically 10-100 μ g were taken and made up to 500 μ l with 1M NaOH. 5ml of freshly prepared LOWRY solution A (1 part 0.5% CuSO₄ + 1 part 1% NaK Tartrate + 48 parts 2% Na₂CO₃) was added, tubes vortexed and then incubated for 10 minutes at room temperature. After addition of 500 μ l of solution B (1 part Folin and Ciocalteu's reagent + 1 part dH₂O) the solution was mixed thoroughly and incubated for 30 minutes at room temperature. The absorbance of the solution was measured at 700nm and the concentration of protein determined using a calibration chart constructed by measuring the total nitrogen content of the actin and myosin.

The Bradford Assay was used for the rapid measurement of HMM concentrations. 100 μ l of HMM or myosin was added to 1ml of Bradford Reagent, mixed thoroughly and incubated at room temperature for 5 minutes. The absorbance of the solution was measured at 595nm and the protein

concentrations derived from a calibration curve of known HMM concentrations, see Figure 2.8.

Figure 2.8 Bradford Assay Calibration graph for 100 μ l Sample



2.9.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.9.2.1 Sample Preparation

Protein solutions were mixed 1:1 (vol:vol) with SDS sample buffer (20mM Tris-HCl pH 8.0, 2.5% SDS, 5% 2-mercaptoethanol, PAGE Blue) and heated for 5 minutes at 95-100°C. Dilute proteins were mixed 5:1 (vol:vol) with 3xSDS sample buffer (20mM Tris-HCl pH 8.0, 7.5% SDS, 15% 2-mercaptoethanol, PAGE blue) so as to maximise the concentration of the sample loaded.

2.9.2.2 Electrophoresis Using The Pharmacia System

Precast gels on plastic backings (Excelgel-SDS 8-18% gradient, Pharmacia LKB) were orientated on the precooled horizontal plate of a Multiphor II electrophoresis system. Polyacrylamide anodic and cathodic buffer strips were placed at each end. Loading strips consisting of 5mm x 10mm size

pieces of filter paper were spaced approximately 5mm apart adjacent to the cathodic buffer strip. Prepared samples of up to 20µl total volume, 1-10µg pure protein, were pipetted onto the filter papers. Full size 24cm gels were run at 50mA/600V/30W maximum (25mA current for 12cm sized gels) for 80 minutes at 10°C.

Upon completion of electrophoresis, the gels were immersed in a fixing solution (50% methanol, 10% acetic acid) for 20 minutes with gentle agitation. The protein bands were visualised by soaking the gel for approximately 20 minutes in Coomassie Blue staining solution (PhastGel tablets, 17-0518-01), then destaining in 25% methanol, 8% acetic acid, both steps with gentle agitation. The gel was preserved under cellophane by incubating in destain containing 10% glycerol for 30 minutes. The gel was removed and allowed to dry overnight at room temperature.

2.9.2.3 Densitometric Analysis of SDS-PAGE Gels

Protein bands detected by SDS-PAGE gels were quantified by scanning densitometry.

2.9.3 Pro-Q Diamond Phosphoprotein Gel Stain

The Pro-Q Diamond phosphoprotein stain (Molecular probes, P33300) is a fluorescent method for detecting phosphoproteins in gels. This highly sensitive phosphoprotein stain specifically detects phosphate moieties on threonine, serine and tyrosine residues (Steinberg *et al.*, 2003, Schulenberg *et al.*, 2003) and enables the analysis of the phosphorylation of a single protein.

After running a standard SDS-PAGE, the gel was immersed in 100ml of fixing solution (50% methanol, 10% acetic acid) and incubated overnight with gentle agitation at room temperature. The gel was washed twice in 100ml of dH₂O with gentle agitation for 10 minutes and then incubated in the dark in 50ml of Pro-Q Diamond phosphoprotein gel stain with gentle agitation for 75 ÷ 120 minutes. The gel was destained by being incubated twice for 60 minutes each in the dark with 80ml of Destain Solution (undisclosed formula).

The gel was removed from its plastic backing to prevent any background fluorescence, and viewed on a U.V transilluminator. Digital images were

obtained using the GeneGenius apparatus (Syngene) with GeneSnap programme and images were saved as TIFF files. The gel was stained with Coomassie Blue (2.9.2.2) without agitation.

2.9.4 Sypro Ruby Protein Gel Stain

Sypro Ruby protein gel stain (Molecular Probes, S12000) is a ready-to-use, ultrasensitive, luminescent stain for the detection of proteins separated by polyacrylamide gel electrophoresis. After running a standard SDS-PAGE, the gel was placed in a clean container with 100ml of fix solution (50% methanol, 7% acetic acid) and gently agitated for 30 minutes. This step was repeated once more with fresh fix solution. 60ml of Sypro Ruby gel stain was added and incubated overnight at room temperature. The gel was washed in a clean container with 100ml of wash solution (10% methanol, 7% acetic acid) for 30 minutes. This step helps to minimise the background staining irregularities and stain speckles on the gel. Before imaging, the gel was rinsed twice in ultrapure water for 5 minutes to prevent possible corrosive damage to the imager.

Sypro Ruby protein gel stain has two excitation maxima, one at approximately 280nm and one at approximately 450nm, and has an emission maximum near 610nm. The gel was removed from its plastic backing and viewed on a 300nm U.V transilluminator. Digital images were obtained using the GeneGenius apparatus (Syngene) with GeneSnap programme and images were saved as TIFF files.

2.10 *In Vitro* Motility Assay

The *In Vitro* Motility Assay technique, devised by Kron and Spudich (Kron and Spudich, 1986), was used to make a detailed quantitative analysis of human cardiac troponin-tropomyosin control of actin filament movement under immobilised HMM. Several modifications have been applied to the original technique (Fraser and Marston, 1995a, Fraser and Marston, 1995b, Bing *et al.*, 1997, Purcell *et al.*, 1999, Bing *et al.*, 2000).

2.10.1 Fluorescent Labelling of F-Actin

F-actin was labelled with TRITC-phalloidin (ϕ), for visualisation under a fluorescence microscope.

2.10.1.1 Fluorescent labelling of Skeletal Muscle F-Actin

36 μ l of 33 μ M TRITC-phalloidin in methanol (Sigma P1951), was added to 438 μ l of motility buffer A and mixed thoroughly by vortexing. 20 μ l of 1mg/ml fresh F-actin was added and mixed gently to give a final concentration of 1 μ M. The eppendorf tube was wrapped in aluminium foil to prevent photo-degradation of the label and incubated at 4°C for 16-18 hours for full labelling of the F-actin by the rhodamine-phalloidin. This labelled actin provided a stock of actin- ϕ , which could be used for motility studies for 5-10 days.

2.10.1.2 Fluorescent Labelling of Transgenic Mouse F-Actin

139 μ l of buffer A was thoroughly mixed with 18 μ l of 33 μ M TRITC-phalloidin. 90 μ l F-actin, isolated from transgenic mouse cardiac muscle was added and gently mixed. This stock of actin- ϕ was incubated for 16-18 hours at 4°C in a covered eppendorf and remained functional in the *in vitro* motility assay for 5-10 days.

2.10.2 Pre-mixing and Incubation of Thin Filament Proteins

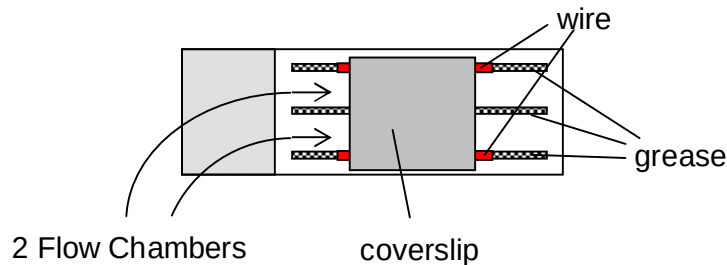
F-Actin- ϕ -tropomyosin and F-Actin- ϕ -tropomyosin-troponin complexes were formed at 10x assay concentration. 100nM F-Actin- ϕ , 100nM human cardiac tropomyosin, and 0-80nM human cardiac troponin were mixed in motility buffer A (50mM KCl, 25mM Imidazole, 4mM MgCl₂, 1mM EDTA, 5mM dithiothreitol, pH 7.4), and incubated for 10 minutes on ice to ensure complete binding. The mixtures were subsequently diluted 10-fold in motility buffer A immediately prior to infusion into the motility cell.

2.10.3 Motility Assay Flow Cell Preparation

All *in vitro* motility assay experiments were carried out with the preparation of a motility flow cell as described by Kron *et al.* (Kron and Spudich, 1986), (Figure 2.9). The flow cell was constructed from a microscope slide

(Sigma, S8400), and a freshly siliconised coverslip prepared by soaking in 0.2% dichloromethylsilane (Fluka 39909) in chloroform.

Figure 2.9. Construction of the Motility Flow Cell



Dual chambers removed variation and allowed simultaneous comparisons of different samples under identical conditions. Any coverslip showing adherent liquid after immersion in the solution was discarded, as this indicated incomplete silconisation (Sundberg *et al.*, 2003).

2.10.4 Affinity Precipitation of HMM

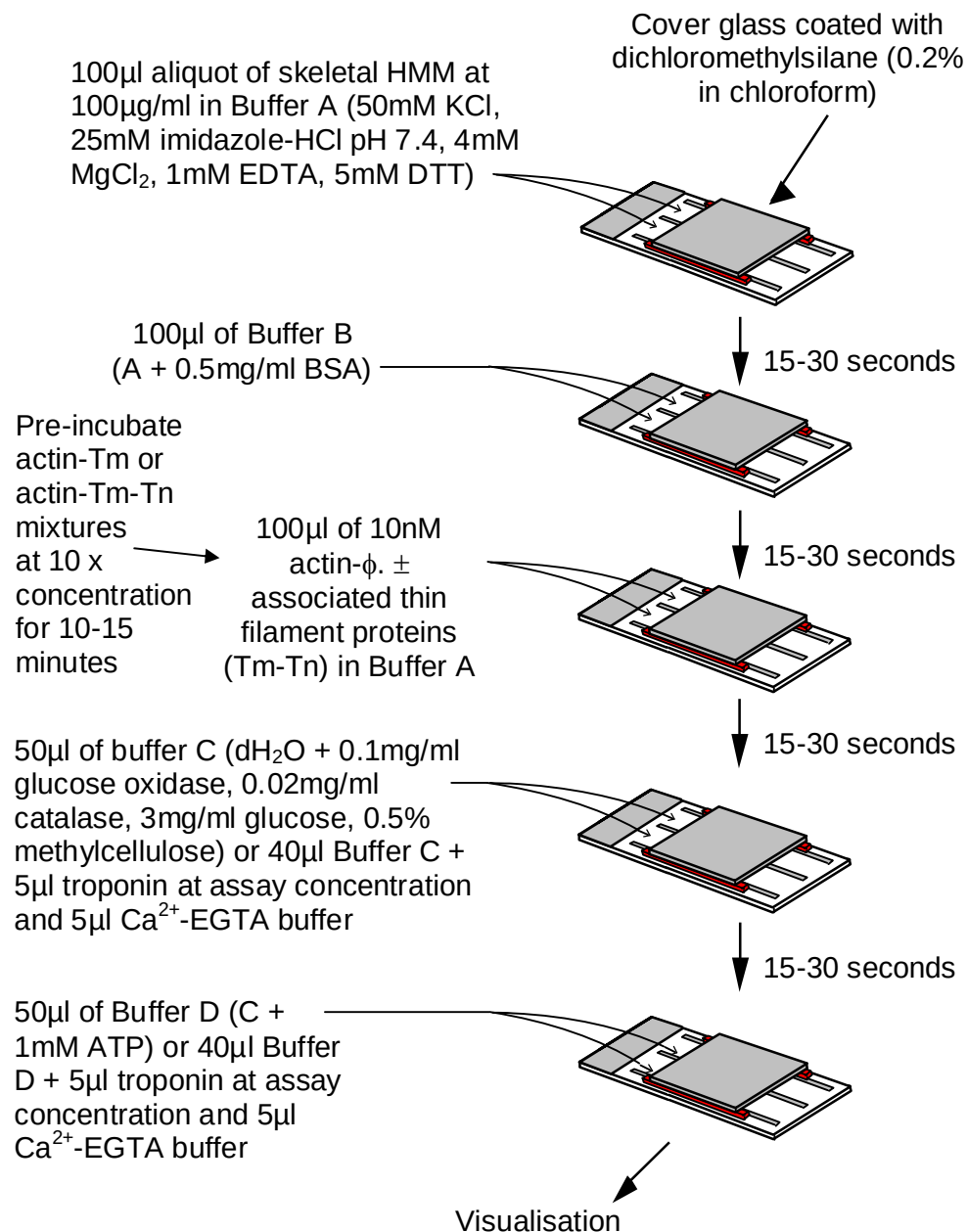
Filament movement was inhibited by the presence of rigor heads which show ATP insensitive binding to actin filaments. This effect was minimised by the affinity precipitation of HMM preparations. HMM, F-Actin, and ATP were mixed to final concentrations of 200 $\mu\text{m}/\text{ml}$, 150 $\mu\text{g}/\text{ml}$, and 1mM respectively in buffer A, and incubated on ice for 10 minutes. The mixture was centrifugated at 100,000rpm (Sorvall RCM120SE, S100-AT6 rotor) for 15 minutes at 4°C to pellet F-actin and associated myosin rigor heads. HMM remained in the supernatant and was suitable for use in the *in vitro* motility assay for up to three hours.

2.10.5 Infusion of Proteins

Protein components and buffers were infused into the motility flow cell at 30-second intervals. A 100 μl aliquot of HMM at 100 $\mu\text{g}/\text{ml}$ was infused down first, providing the base of immobilised HMM on the coverslip. This was followed by 100 μl of buffer B (A + 0.5mg/ml bovine serum albumin) and then 100 μl of 10nM actin- ϕ $\pm$ associated tropomyosin-troponin in buffer A. 50 μl of buffer C (B

± 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose, 0.5% methylcellulose, $\pm$ troponin at assay concentration), and 50 μ l of buffer D (C + 1mM ATP) were then infused.

Figure 2.10. Infusion of Proteins



2.10.6 Ca^{2+} /EGTA buffer Preparation

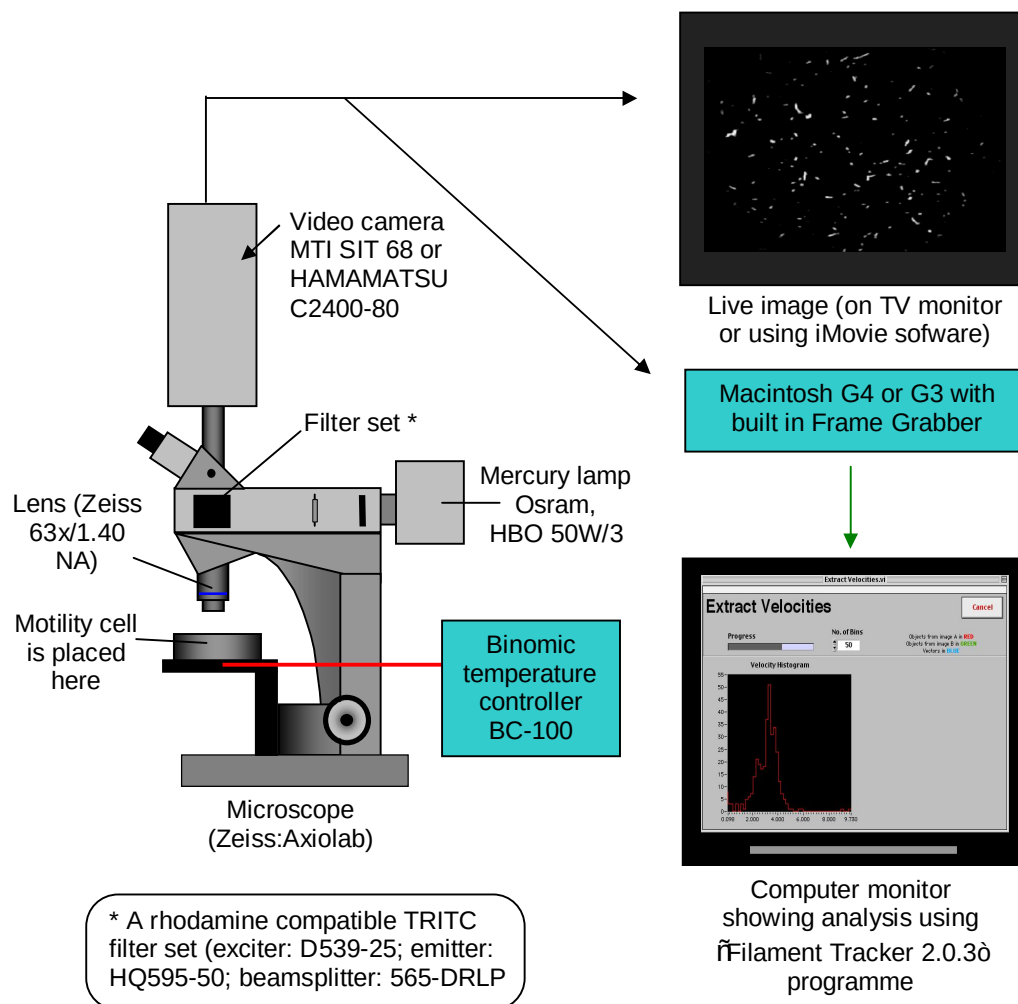
Ca^{2+} -EGTA buffers were prepared by adapting the method of McGuigan, (McGuigan *et al.* 1991). Buffers of different $[\text{Ca}^{2+}]$ were made by mixing an EGTA buffer (50mM KCl, 25mM Imidazole pH 7.4, 50mM EGTA (free acid), 1mM EDTA, 4mM MgCl_2 , 95mM KOH, 1.43mM HCl, 8mM NaCl with the pH adjusted using concentrated HCl) and a Ca^{2+} -EGTA buffer (50mM KCl, 25mM Imidazole pH 7.4, 50mM EGTA, 50mM CaCl_2 , 1mM EDTA, 4mM MgCl_2 , 8mM NaCl with the pH adjusted using 1M KOH) using appropriate ratios to obtain Ca^{2+} -EGTA buffers ranging from pCa9 to pCa5.4, shown in table Table 2.2. The pCa values were determined using the Maxchelator computer programme (Bers *et al.* 1994).

Table 2.2 Ca^{2+} Buffer Solutions

Solution	Volume of 50mM EGTA buffer added (ml)	Volume of 50mM Ca /EGTA buffer added (ml)	Ratio	pCa	Free $[\text{Ca}^{2+}]$
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	3.72×10^{-6}

2.10.7 Visualisation Apparatus

Figure 2.11. Visualisation and recording apparatus used for the analysis of the *in vitro* motility assay.



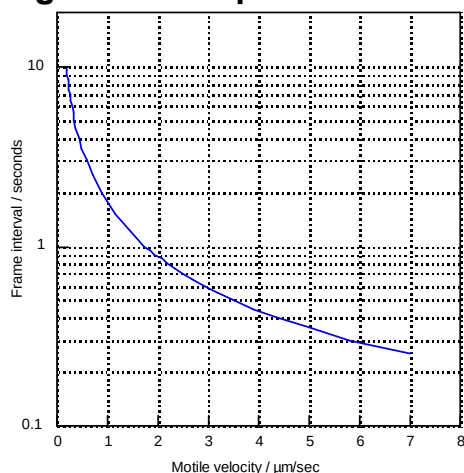
Two different visualisation setups were used during this research. Both setups used a Zeiss AxioLab microscope fitted with an epifluorescence illuminator (Zeiss 39 26 42-9901). One microscope was connected to a DAGW MTI SIT 68 video camera, which was linked to a TV monitor. Image digitisation and filament movement was performed on a single hardware unit containing integrated software. Computing hardware was a Macintosh G4 computer with a built in PCI Neotech frame grabber. The other setup used a Hamamatsu C2400-80 camera head. Analogue signals from the time base corrector (DataVideo TCB-1000) were digitised by a Formac Studio DV converter

attached to a Macintosh G4 computer. The live images were viewed and recorded using iMovie software. A Macintosh G3 computer was used with a built in PCI Neotech frame grabber to perform image digitisation and analysis of filament movement

2.10.8 Acquisition of image software

Filament movement was analysed directly from the image viewed via the camera by using a Filament Tracker program (Marston *et al.*, 1996, Marston.S.B. *et al.*, 1998, Marston, 2003). If successive pairs of frames were compared the program searched for and detected the new position of every filament and showed its vector on screen. Velocity was calculated and shown as a frequency histogram. Normally 10-15 images were acquired to obtain 250-500 filament vectors, which ensured minimum random errors. In order to give the maximum ratio between random filament oscillation, pixel size and distance of filament movement between images, the timer interval was set to allow filament movement of approximately 1.6-1.8 μ m between captured images (Figure 2.12). These timer intervals were fixed for any single experiment. The algorithm could not deal with filaments that met, crossed or divided. Direct comparisons show that the data produced by automatic tracking was indistinguishable from manual tracking. For any given experiment once the conditions were optimum for image acquisition, images were captured from four separate areas chosen at random to obtain data that was representative of the whole flow cell, without experimental bias.

Figure 2.12. Optimum Timer intervals plotted against velocity



2.11 Statistical Analysis

The *in vitro* motility experiments consisted of direct comparisons of non-transgenic and transgenic thin filaments in the same motility cell to give minimal errors. The mean sliding speed and percentage motile were calculated and mean values and standard errors were recorded. Results are expressed as mean $\pm$ standard error unless otherwise indicated. Data was compared for statistical significance using the Student's t-test where $p < 0.05$ was classed as significant. Ca^{2+} -activation curves were fitted to the Hill equation.

$$\text{Hill Equation: } X = a + \frac{b_n [\text{Ca}^{2+}]^n}{(k + [\text{Ca}^{2+}]^n)}$$

a = min value
 b_n = max - min
 k = EC_{50}
 n = hill co-efficient

2.12 Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to α -Actinin

The *in vitro* motility technique was extended to measure the relative affinity of $\tilde{\text{U}}$ -actinin binding by competition between TRITC-phalloidin labelled actin and non-fluorescent phalloidin labelled actin. The principle was to coat the flow cell cover glass with 100 μl $\tilde{\text{U}}$ -actinin at low concentration (5 $\mu\text{g}/\text{ml}$ in Buffer A), followed by 100 μl of buffer B (A + 0.5 mg/ml bovine serum albumin). Actin was bound to the surface by infusing a 100 μl mixture of TRITC actin- ϕ and non-fluorescent actin in a range of ratios at fixed 10 nM concentrations. 50 μl buffer C ($B \pm 0.1$ mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose) was added without methylcellulose to prevent non-specific binding. Up to 10 filament fields were recorded per cell and the number of actin filaments was determined using the Tracker software.

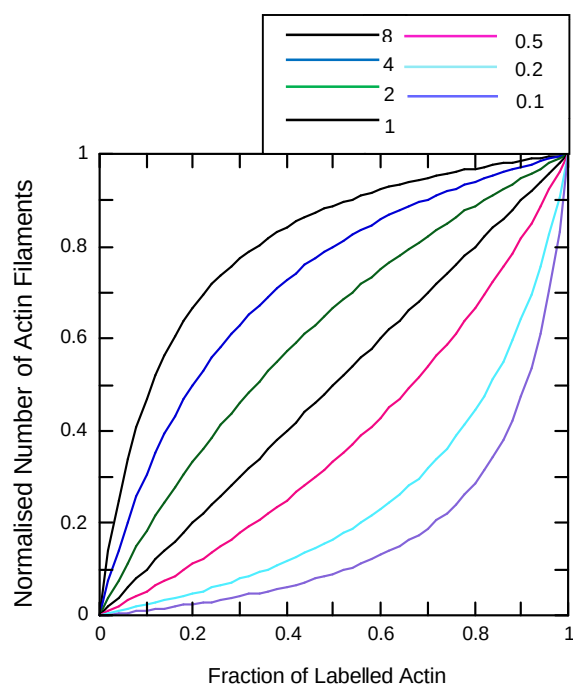
Since actin is in great excess over $\tilde{\text{U}}$ -actinin, TRITC actin- ϕ competes with non-fluorescent actin to bind to the $\tilde{\text{U}}$ -actinin. The amount of fluorescent relative to non-fluorescent actin filaments bound depends upon the ratio of the two species to their relative affinity, defined by the equation:

$$y = F.K / (F.K + (1-F))$$

F= fraction of fluorescently labelled filaments
K= ratio of binding constant

Figure 2.13 illustrates how the number of labelled actin filaments varies with the fraction of labelled actin for various binding constant values.

Figure 2.13 Theoretical Curve of Filament Competition



The images in Figure 2.14 show the displacement of fluorescent skeletal rabbit actin filaments by increasing concentrations of non-fluorescent rabbit actin. There is an equal affinity of fluorescent and non-fluorescent rabbit actin for the $\bar{U}$ -actinin. The number of rabbit actin filaments can be plotted against the fraction that are labelled, producing a K value of approximately 1 (Figure 2.15).

Figure 2.14. Displacement of Rabbit TRITC-Actin Filaments

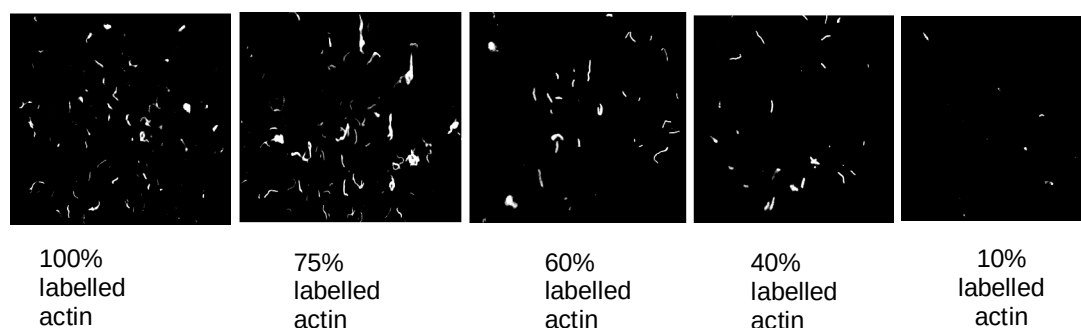
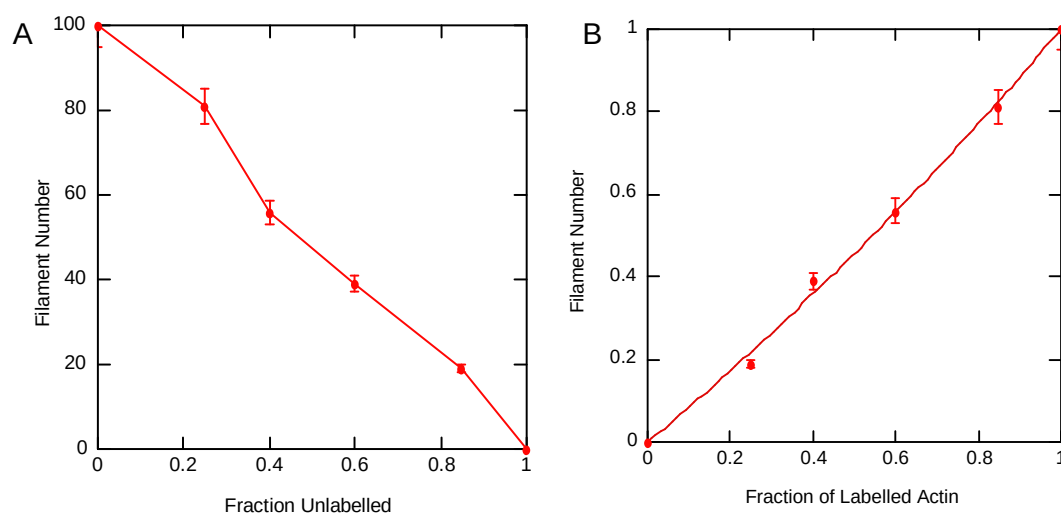


Figure 2.15 Skeletal Rabbit Actin Competitive Binding Assay (A) and Fit (B)



The K value for rabbit actin is 0.843 ± 0.042 .

3. FUNCTIONAL INVESTIGATION OF DCM ACTIN MUTANT GLU361GLY

3.1 Introduction

Mutations that cause DCM have been found in genes that code for most of the proteins in the cardiac muscle contractile apparatus, including the cardiac actin gene (ACTC). This chapter will describe the functional investigation of the cardiac actin DCM mutation Glu361Gly (E361G) (Olson *et al.*, 1998) by the well-established *in vitro* motility assay.

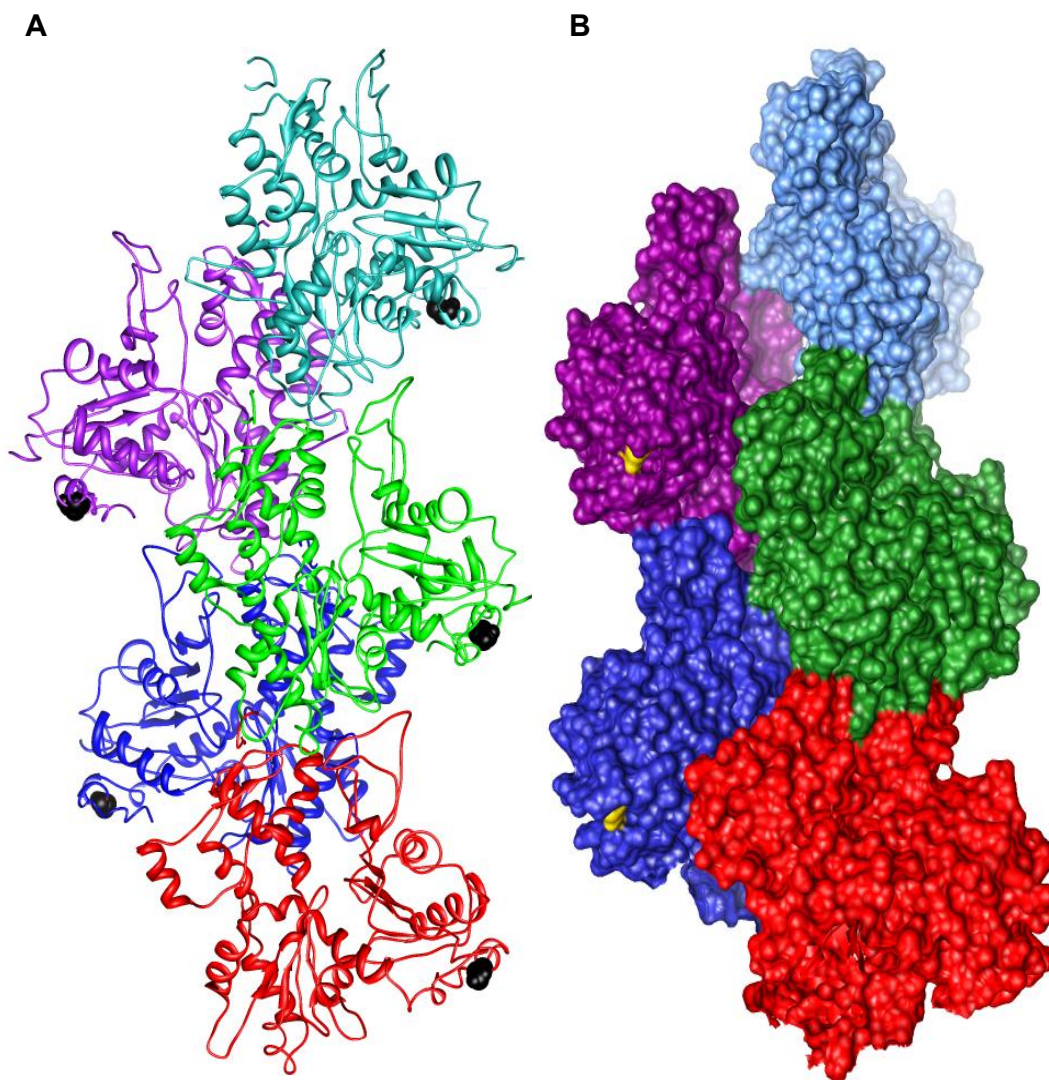
The transgenic mice that over expressed the E361G actin in their hearts were supplied by Professor Dominic Wells, Gene Targeting Unit, Charing Cross Hospital. This has been the first functional study on cardiac actin extracted from transgenic mouse hearts that carried the E361G mutation. It has allowed the E361G mutation to be studied in an experimental system that reproduced the conditions in the muscle of patients with familial DCM more accurately than previous experiments.

3.2 Location of E361G in the Actin Polymer

The location of the E361G mutation is shown in Figure 3.1 A and B. It occupies subdomain 1 of the actin monomer, and is located at an important $\bar{U}$ -actinin binding site. The E361G substitution may therefore affect the anchoring of thin filaments to the Z-bands and intercalated discs in the sarcomere (Olson *et al.*, 1998). It was reported by Wong *et al.* (Wong *et al.*, 2002) that E361G yeast mutant actin had a threefold reduction in $\bar{U}$ -actinin binding.

E361G is a charge change mutation. The substitution of glycine for glutamic acid leads to a loss in the surface charge and a hole in the hydrophobic core. Only a small proportion of the glutamic acid side chain is actually located on the surface of the actin molecule (Figure 3.1 B), with the majority of the mutation embedded in the core. The position of the mutation is unlikely to be involved directly with actin polymerisation.

Figure 3.1. Ribbon Representation (A) and Space Filling Model (B) of the E361G Location in the Actin Polymer



The position of the E361G mutation on the actin surface is shown as black spheres in figure A. Figure B shows the position of E361G in yellow. The space filling molecular model illustrates the proportion of the mutation that is exposed on the surface of the actin molecule. Much is hidden in the core of the actin subunit. These molecular graphics images were produced using the USSF Chimera package (Pettersen *et al.*, 2004).

3.3 Generation of E361G Transgenic Lines

Early stages of the study established two transgenic E361G lines, line 13 and line 20 (Table 3.1, Table 3.2). PCR analysis determined the presence or absence of the mutant transgene in individuals of each line. The PCR was performed at the Gene Targeting Unit, Charing Cross Hospital by Dr. Ke Liu.

Table 3.1. E361G Transgenic Mouse Line 13

Line 13	Sample	PCR	Gender	Age (days)	Body weight (g)	Heart weight (g)
1st generation	361.13.14	+	N/A	N/A	18.20	0.08
	361.13.15	+	N/A	N/A	16.06	0.05
	361.13.16	+	N/A	N/A	15.91	0.06
	361.13.17	-	N/A	N/A	14.82	0.05
	361.13.18	+	N/A	N/A	13.86	0.05
	361.13.19	+	N/A	N/A	17.10	0.07
	361.13.20	-	N/A	N/A	16.43	0.08
2nd generation	361.13.2.7	-	M	46	34.65	0.14
	361.13.2.8	+	M	46	34.55	0.14
	361.13.2.10	+	F	46	26.91	0.13
	361.13.2.11	+	F	46	23.84	0.11
	361.13.2.12		F	46	25.85	0.13

Table 3.2. E361G Transgenic Mouse Line 20

Line 20	Sample	PCR	Gender	Age (Days)	Body Weight (g)	Heart Weight (g)
1st generation	361.20.3	+	N/A	N/A	N/A	N/A
	361.20.4	-	N/A	N/A	N/A	N/A
	361.20.6	-	N/A	N/A	20.28	0.08
	361.20.7	-	N/A	N/A	19.47	0.09
	361.20.8	+	N/A	N/A	17.79	0.07
	361.20.9	+	N/A	N/A	18.06	0.07
	361.20.12	+	N/A	N/A	16.76	0.08
2nd generation	361.20.1.6	+	F	48	27.00	0.15
	361.20.1.7	+	F	48	24.30	0.10
	361.20.1.8	-	F	48	35.30	0.11
	361.20.1.9	+	M	35	29.85	0.12
	361.20.1.10	+	M	35	29.67	0.13
	361.20.1.11	-	M	35	26.42	0.11
	361.20.1.12	+	M	35	27.29	0.12
	361.20.1.13	-	M	35	28.12	0.10
	361.20.1.15	+	F	35	N/A	N/A
	361.20.1.16	+	F	35	23.60	0.09
	361.20.1.17	-	F	35	22.59	0.11
	361.20.1.20	+	F	N/A	N/A	N/A
	361.20.1.22	+	F	N/A	N/A	N/A
	361.20.55.26	+	F	N/A	N/A	N/A
	361.20.1.20.1	+	F	N/A	N/A	N/A
3rd generation						

Two generations of transgenic mouse line 361.13 were successfully bred. Line 361.20 was bred into the third generation. The hearts were excised from 4-7 week old mice. The heart-weight to body-weight ratios did not indicate cardiac dilation in transgenic-E361G mice.

3.4 Screening of E361G Transgenic Lines

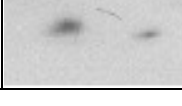
The 2-D Electrophoresis technique (Ilkovski *et al.*, 2004) was developed to test for the presence of the E361G mutant actin protein. Some of the first generation of PCR positive mice in lines 361.13 and 361.20 showed expression of mutant actin in their hearts that amounted to 50% of the total actin (Table 3.3,

Table 3.4). There were at least 50% false positives in the first generation (Table 3.3 and Table 3.4, starred individuals^{*}). These were detected as PCR positive but no expression was detected upon 2-D Electrophoresis. The PCR cycling program was modified at this stage to be more specific (performed in the laboratory of Prof Dominic Wells). The modifications involved an improved hot start - putting the enzyme into the master mix, warming up the heat block to 94°C and then quickly adding the tubes. Additionally, the ramp speed of the temperature change was slowed. The original set of primers remained in use.

Table 3.3. Screening First Generation Transgenic Line 361.13

	Sample	PCR Result	2-DE	% E361G actin
361.13 1st generation	13.14 *	+		0
	13.15	+		41
	13.16 *	+		0
	13.17	-		0
	13.19 *	+		0

Table 3.4. Screening First Generation Transgenic Line 361.20

	Sample	PCR Result	2-DE	% E361G actin
361.20 1st generation	20.3	+		48
	20.8 *	+		7.5
	20.9 *	+		0
	20.12	+		22

After improvements to the PCR technique, the transmission of the transgene into the second generation of line 13 was still only successful in 50% of the PCR positive hearts (Table 3.5). Transgenic line 361.13 was abandoned after the second generation due to the low and inconsistent transgene transmission rates.

Table 3.5 Screening Second Generation Transgenic Line 361.13

	Sample	PCR Result	2-DE	% E361G actin
361.13 2nd generation	13.2.8	+		48
	13.2.10	+		0
	13.2.11	+		0
	13.2.14	+		49

All second-generation PCR positive mice in line 361.20 were stably expressing E361G cardiac actin at 50% (Table 3.6).

Table 3.6 Screening Second Generation Transgenic Line 361.20

	Sample	PCR Result	2-DE	% E361G actin
361.20 2nd generation	20.1.6	+		52
	20.1.7	+		48
	20.1.9	+		57
	20.1.10	+		44
	20.1.12	+		49

A third generation of mice were bred from line 361.20 (Table 3.7). Expression levels remained consistent, amounting to approximately 50% of the total actin. Having shown that PCR correctly predicted transgenic mice, most of the third generation mice were not tested by 2-D electrophoresis.

Table 3.7 Screening Third Generation Transgenic Line 361.20

	Sample	PCR Result	2-DE	% E361G actin
361.20 3rd generation	361.20.1.20.1	+		52

3.5 Transgenic-E361G Mouse Phenotype

E361G-transgenic mice exhibited no overt phenotype at rest. Their heart weights and cardiac morphology appeared normal when measured by Magnetic Resonance Imaging (MRI), and the mice had a normal lifespan. However, the effects of increasing their cardiac output through exercise or through drugs such as the beta-1 adrenergic agonist dobutamine were not studied.

3.6 Isolation of F-actin from E361G Transgenic Mouse Hearts

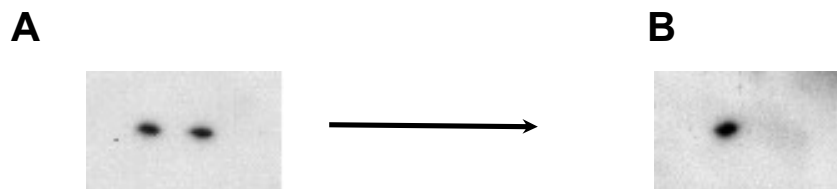
Initially actin was isolated from mouse hearts by a modification of the methods of Bing *et al.* and Razzaq *et al.*, developed for the preparation of actin from a small quantity of starting material (Bing *et al.*, 1998, Razzaq *et al.*, 1999). This involved acetone dehydration followed by extraction of actin in the monomeric form. The mutation was not incorporated into the F-actin by this method unless 150nM TRITC- phalloidin was added during the polymerisation step. This lead to approximately 25-40% of E361G actin being retained in the F-actin. In order to obtain F-actin containing E361G G-actin at higher levels, native thin filaments were extracted. This successfully preserved the 50% mutation expression in the F-actin. The results of these three modifications to the F-actin isolation procedure and the *in vitro* motility assay results are outlined below.

3.6.1 The Acetone Powder Method: Loss of E361G

F-actin was originally isolated via an acetone powder method, based on the method of Razzaq *et al.* (Razzaq *et al.*, 1999). 2-D Electrophoresis of the E361G transgenic mouse heart myofibrils showed mutant actin was expressed in the heart at 50%. *In vitro* motility analysis was carried on the F-actin isolated

from these mouse hearts (3.6.2) but no significant differences from non-transgenic were ever observed. The 2-D Electrophoresis technique was subsequently used at this stage to assess the expression of E361G mutant cardiac actin in the F-actin. It showed the pure F-actin to be entirely deficient in the E361G mutation (Figure 3.2).

Figure 3.2. 2-DE of E361G Transgenic Mouse Heart Myofibrils (A) and F-actin (B), Isolated via an Acetone Powder Method



A single spot of non-transgenic F-actin (probed with 5C5 anti actin antibody) indicated the loss of mutant cardiac actin when extracted via the acetone powder method.

3.6.2 *In Vitro* Motility Analysis of Mouse F-actin

The *in vitro* motility assay is a versatile method of studying thin filament regulation. Several parameters can be measured at one time, including the percentage motility, sliding speed, and the number of filaments attached onto the myosin-covered surface. Comparative experiments are run in split motility cells where the actin is the only variable.

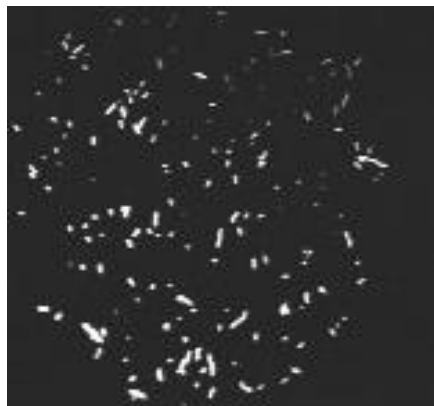
A large amount of *in vitro* motility analysis was carried out on actin from transgenic mice before discovering the final F-actin had lost the mutant E361G actin. However, the results of these experiments are still useful. These experiments have demonstrated the contractile and regulatory properties of mouse actin, and have allowed comparisons to be made with rabbit actin. The reproducibility of the actin preparation and its measurement has also been demonstrated. The finding that two actin preparations can give identical results was an important control. It has meant that any differences that were seen were due to the mutation and likely to be real. The actin isolated from PCR positive transgenic mice which contained no transgene will be referred to as α G-NTG ϕ in the following sections.

The basic experiments included measuring the sliding speed of transgenic and non-transgenic actin over skeletal muscle HMM, the titration of troponin at $3.9\mu\text{M}$ and 1nM Ca^{2+} , and measuring the Ca^{2+} -dependence of motility at saturating troponin concentrations.

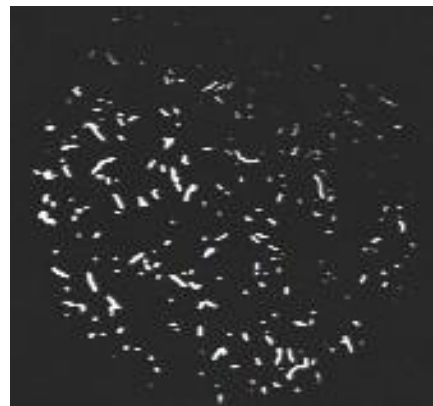
3.6.2.1 Length Distribution of Non-Transgenic and TG-NTG Actin Filaments

Non-transgenic and TG-NTG actin filaments had similar appearances when labelled with TRITC-phalloidin (Figure 3.3). Measurements using the DIAS 3.2 programme showed TG-NTG actin and non-transgenic actin had similar filament length distributions (Figure 3.4). The mean length of non-transgenic actin was $9.362\mu\text{m} \pm 0.502$ and TG-NTG actin was $9.042\mu\text{m} \pm 0.545$.

Figure 3.3 Non-Transgenic and TG-NTG Actin Filaments labelled with TRITC-phalloidin

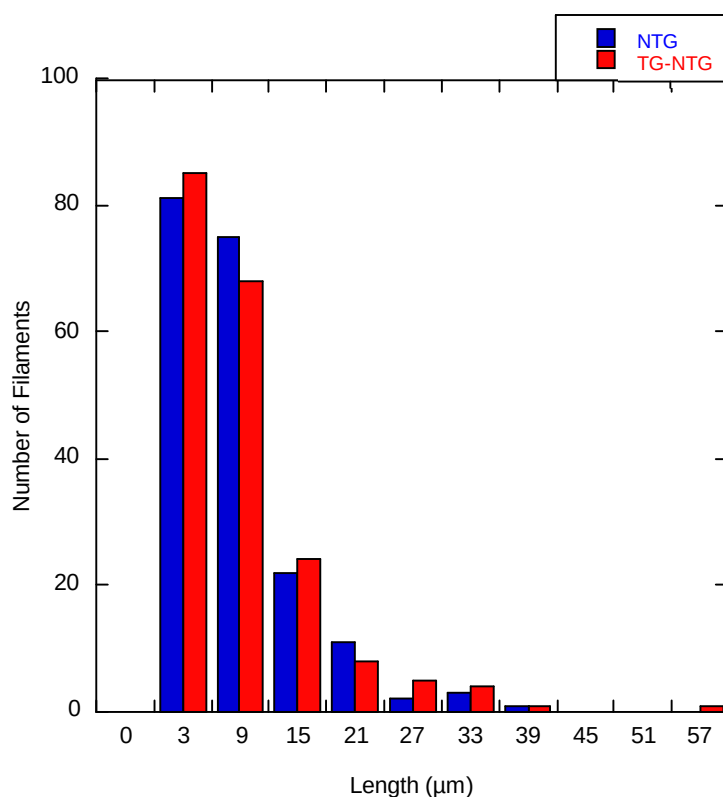


Non-Transgenic
Actin
(sample 13.16)



TG-NTG Actin
(sample 13.15)

Figure 3.4 Length Distribution of Non-Transgenic (13.16) and TG-NTG (13.15) Actin



Non-transgenic (13.16) and TG-NTG (13.15) are the individual mice identification numbers.

3.6.2.2 Non-Transgenic and TG-NTG Actin Filament Motility

The sliding speed and percentage motility of non-transgenic and TG-NTG actin over HMM was compared (Table 3.8). Actin sliding speeds and the percentage motile of filaments were of a high standard and comparable to values that can be obtained with rabbit actin. Good consistencies of actin sliding speeds were established. There was no significant difference between the sliding speeds of the two actins (paired t-test, $p = 0.78$, $n = 18$). The percentage motile of filaments was also not significantly different for non-transgenic and transgenic actin (paired t-test, $p = 1.0$).

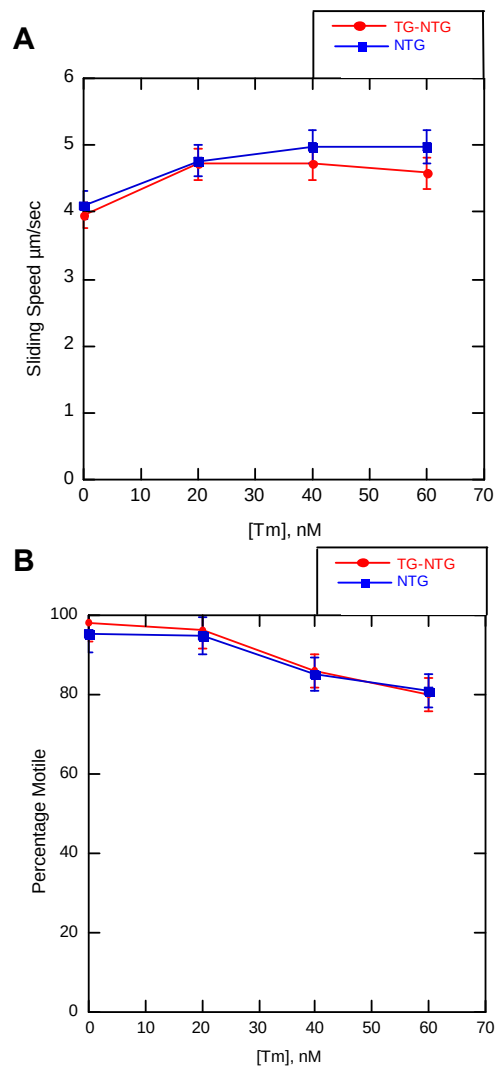
Table 3.8. Comparison of Non-Transgenic and TG-NTG Actin Sliding Speeds and Percentage Motile of Filaments

Mouse Line		Filament Sliding Speed ($\mu\text{m}/\text{sec}$)		Filament Percentage Motile	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.17	20.3	3.3 ± 0.05 3.2 ± 0.05 3.6 ± 0.07	3.2 ± 0.03 3.6 ± 0.06 3.7 ± 0.05	95 ± 1.49 97 ± 0.73 94 ± 1.26	96 ± 2.01 96 ± 1.24 97 ± 0.73
13.16	13.15	3.5 ± 0.16 3.2 ± 0.10 4.1 ± 0.04 2.3 ± 0.19 2.8 ± 0.12 3.2 ± 0.30 3.0 ± 0.04 3.7 ± 0.12 2.8 ± 0.07 3.1 ± 0.16 3.3 ± 0.20 3.8 ± 0.27 3.7 ± 0.28 4.0 ± 0.12 4.2 ± 0.34	3.7 ± 0.01 4.0 ± 0.09 4.0 ± 0.28 2.1 ± 0.12 2.6 ± 0.12 2.8 ± 0.17 3.2 ± 0.05 3.0 ± 0.07 3.5 ± 0.04 3.0 ± 0.02 3.4 ± 0.11 4.2 ± 0.11 4.0 ± 0.29 3.6 ± 0.14 3.7 ± 0.02	98 ± 1.10 95 ± 1.25 96 ± 0.87 99 ± 0.29 99 ± 0.29 98 ± 0.75 98 ± 2.63 99 ± 0.58 98 ± 0.91 91 ± 6.37 99 ± 0.58 88 ± 12.1 91 ± 1.15 94 ± 2.08 83 ± 3.70	95 ± 2.45 94 ± 4.37 98 ± 0.85 91 ± 2.80 99 ± 0.63 91 ± 2.35 92 ± 3.50 96 ± 1.29 99 ± 0.75 91 ± 1.37 99 ± 0.95 95 ± 5.40 91 ± 2.67 93 ± 1.93 99 ± 0.25
Mean $\pm$ SE (n = 18)		3.38 ± 0.12	3.41 ± 0.13	95.11 ± 1.04	95.11 ± 0.72
T Test P Value		Paired = 0.78 Unpaired = 0.87		Paired = 1.0 Unpaired = 1.0	

3.6.2.3 Effects of Human Cardiac Tropomyosin on Actin Filament Motility

The functional effect of human cardiac tropomyosin was measured by titrating tropomyosin against 10nM non-transgenic and transgenic actin filaments. An optimal increase was seen at 40 nM tropomyosin provided that actin and tropomyosin were mixed at a 10 times concentration and then diluted to the working concentration. 6 measurements were taken from one set of paired non-transgenic and TG-NTG mouse hearts and the averages are shown in Table 3.9 and Table 3.10.

Figure 3.5 Effect of Human Cardiac Tropomyosin on Non-Transgenic and TG-NTG Actin Motility: (A) Sliding Speed, and (B) % motility



Non-transgenic and TG-NTG actin sliding speeds increased when tropomyosin was added. Non-transgenic actin increased by 23%. This was a significant increase in the paired t-test ($P = 0.01$). TG-NTG actin increased by 15%, which was also a significant increase in the paired t-test, ($p = 0.002$). Overall, TG-NTG actin.tropomyosin had a 7% slower sliding speed compared to non-transgenic actin, and this was significant in the paired t-test ($p = 0.001$).

The addition of tropomyosin caused a decrease in the percentage motile of filaments (Table 3.10). Non-transgenic actin-tm decreased by 12% and TG-NTG actin-tm decreased by 7%. However, there was no significant difference between the TG-NTG and non-transgenic actin.tropomyosin in the paired t-test ($p=0.61$). This can be compared with the percentage motility of rabbit actin filaments, which increased by 13% (Messer, 2007 PhD Thesis).

Table 3.9 Comparison of Non-Transgenic Actin-Tm and TG-NTG Actin-Tm Sliding Speeds

Filament Sliding Speed (μm/sec)					
Mouse Line		Actin		Actin.Tm	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.16	13.15	4.1 ± 0.04	4.0 ± 0.28	5.0 ± 0.67	4.7 ± 0.09
		2.3 ± 0.19	2.1 ± 0.12	2.8 ± 0.12	2.6 ± 0.12
		2.8 ± 0.12	2.6 ± 0.12	3.1 ± 0.09	2.8 ± 0.12
		3.2 ± 0.30	2.8 ± 0.17	3.4 ± 0.13	3.3 ± 0.12
		3.0 ± 0.04	3.2 ± 0.05	4.0 ± 0.12	3.6 ± 0.14
		2.8 ± 0.07	3.5 ± 0.04	4.1 ± 0.13	3.8 ± 0.15
		Mean ±SE (n = 6)		3.03 ± 0.25	3.03 ± 0.27
T Test P Value		Paired =1.0 Unpaired =1.0		Paired =0.001 Unpaired =0.57	
T-test of Actin: Actin.Tm				Paired=0.01 Unpaired=0.12	Paired=0.002 Unpaired=0.32

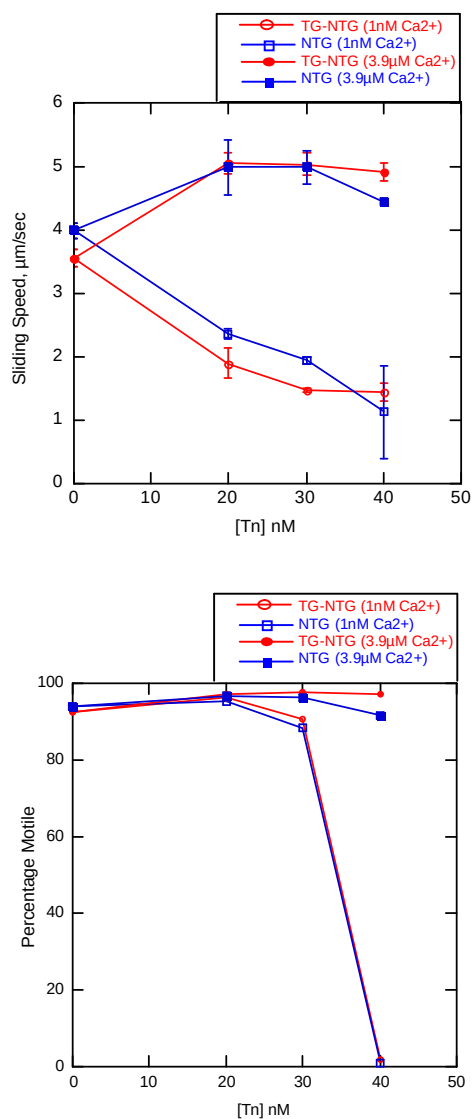
Table 3.10 Comparison of Non-Transgenic Actin-Tm and TG-NTG Actin-Tm Percentage Motile of Filaments

Percentage Motile of Filaments					
Mouse Line		Actin		Actin.Tm	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.16	13.15	96 ± 0.87	98 ± 0.85	85 ± 0.82	86 ± 2.64
		98 ± 0.75	91 ± 2.35	88 ± 12.1	95 ± 5.40
		99 ± 0.29	91 ± 2.80	69 ± 0.57	53 ± 2.52
		99 ± 0.29	99 ± 0.63	91 ± 6.80	97 ± 1.30
		98 ± 2.63	92 ± 3.50	99 ± 1.41	99 ± 1.50
		98 ± 0.91	98 ± 0.75	83 ± 1.30	99 ± 6.80
		Mean ± SE (n = 6)		98.00 ± 0.45	94.83 ± 1.58
T Test		Paired = 0.13		Paired = 0.61	
P Value		Unpaired = 0.08		Unpaired = 0.79	
T-test of Actin: Actin.Tm				Paired =0.03	Paired=0.37
				Unpaired=0.01	Unpaired=0.39

3.6.2.4 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and TG-NTG Thin Filament Motility at High and Low Ca^{2+}

Thin filaments were assembled using TG-NTG or non-transgenic actin, human cardiac tropomyosin and non-failing human cardiac troponin. Troponin at increasing concentrations (20-40nM) was titrated with 10nM actin and 60nM human cardiac tropomyosin.

Figure 3.6 Effect of Non-Failing Human Cardiac Troponin on Non-Transgenic and TG-NTG Actin Motility at High and Low Ca^{2+}



An initial titration was carried out at 1nM Ca^{2+} to obtain a concentration where troponin produced a maximum switch-off. Filament motility for both non-transgenic and TG-NTG actin was switched off by 40nM troponin with the percentage motility at 3-4% and the sliding speed reduced by 46% for TG-NTG actin and 52% for non-transgenic actin. Troponin I is responsible for this 'switch-off' effect (Bing *et al.*, 1997). There were no significant differences between non-transgenic and TG-NTG actin sliding speeds at low Ca^{2+} (paired t-test, $p = 0.56$). The percentage motile of non-transgenic and TG-NTG actin filaments was also not significantly different in the paired t-test ($p = 0.33$). The motility of both non-transgenic and TG-NTG actin were inhibited to a similar degree at low Ca^{2+} .

At high Ca^{2+} , actin filament sliding speeds and the percentage motile of filaments reach a maximum value with increasing troponin concentrations and then level off or fall below the maximum value. This 'switch-on' effect is mediated by troponin T and troponin C. At high Ca^{2+} , 40nM troponin produced a maximum increase in the actin sliding speed and the percentage motile of actin filaments. Non-transgenic actin sliding speed increased 3% over actin.tropomyosin. In comparison, TG-NTG actin produced a much larger increase in sliding speed of 13% over actin.tropomyosin. This larger increase in sliding speed of the TG-NTG actin caused the significant difference between non-transgenic and TG-NTG actin.tropomyosin to be lost. At high Ca^{2+} , there was no significant difference between non-transgenic and TG-NTG actin sliding speeds (paired t-test, $p = 0.67$). The percentage motile of non-transgenic and TG-NTG actin filaments was also not significantly different in the paired t-test ($p = 0.72$). The collective data from 6 experiments are shown in Table 3.11 to Table 3.15.

Table 3.11 Comparison of Non-transgenic and TG-NTG Actin Sliding Speeds at 1nM Ca²⁺ (40nM NF Tn)

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (1nM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTG	TG- NTG	NTG	TG-NTG	NTG	TG-NTG	NTG	TG- NTG
13.16	13.15	5.0±0.67	4.7±0.09	3.8±0.03	4.1±0.03	0.8	0.9
		2.8±0.12	2.6±0.12	1.3±0.13	1.5±0.46	0.5	0.6
		3.1±0.09	2.8±0.12	1.7±0.13	1.6±0.52	0.6	0.6
		3.4±0.13	3.3±0.12	1.4±0.15	1.6±0.67	0.4	0.5
		4.0±0.12	3.6±0.14	1.1±0.09	1.5±0.16	0.3	0.4
		4.1±0.13	3.8±0.15	1.5±0.25	1.0±0.10	0.4	0.3
Mean ± SE (n = 6)		3.73 ± 0.33	3.47 ±0.31	1.80 ±0.41	1.88 ±0.45	0.50 ± 0.07	0.55 ±0.08
T-test		Paired = 0.001		Paired = 0.56		Paired = 0.20	
P Value		Unpaired = 0.57		Unpaired = 0.89		Unpaired = 0.66	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=0.001 Unpaired=0.004	Paired=0.004 Unpaired=0.016		

Table 3.12 Comparison of Non-transgenic and TG-NTG Actin Percentage Motility at 1nM Ca²⁺ (40nM NF Tn)

Mouse Line		% Motile Actin.Tm		% Motile Actin.Tm.Tn (1nM Ca ²⁺)		Relative % motility of Actin.Tm.Tn: Actin.Tm	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.16	13.15	85 ± 0.82	86 ± 2.64	1 ± 0.20	3 ± 1.42	0.01	0.03
		88 ± 12.1	95 ± 5.40	9 ± 0.63	9 ± 0.98	0.10	0.10
		69 ± 0.57	53 ± 2.52	4 ± 1.45	9 ± 5.42	0.06	0.17
		91 ± 6.80	97 ± 1.30	3 ± 4.52	2 ± 1.46	0.03	0.02
		99 ± 1.41	99 ± 1.50	1 ± 6.23	2 ± 5.23	0.01	0.02
		83 ± 1.30	99 ± 6.80	2 ± 7.21	1 ± 2.31	0.02	0.01
Mean ± SE (n = 6)		85.83 ± 4.07	88.17 ± 7.3	3.33 ±1.23	4.33 ±1.50	0.04 ±0.01	0.06 ±0.03
T-test P Value		Paired = 0.61 Unpaired = 0.79		Paired = 0.33 Unpaired = 0.62		Paired = 0.33 Unpaired = 0.52	
T-test of Actin.Tm: Actin.Tm.Tn			Paired=<.0001 Unpaired=<.0001		Paired=0.0002 Unpaired=<.0001		

Table 3.13 Comparison of Non-transgenic and TG-NTG Actin Sliding Speeds at 3.9 μ M Ca²⁺ (40nM NF Tn)

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.16	13.15	5.0±0.67	4.7±0.09	4.1±0.28	4.8±0.14	0.8	1.0
		2.8±0.12	2.6±0.12	2.7±0.14	2.8±0.07	0.9	1.1
		3.1±0.09	2.8±0.12	3.2±0.21	2.6±0.19	1.1	0.9
		3.4±0.13	3.3±0.12	3.9±0.18	3.9±0.10	1.2	1.2
		4.0±0.12	3.6±0.14	4.5±0.04	4.9±0.14	1.1	1.4
		4.1±0.13	3.8±0.15	4.7±0.76	4.6±0.09	1.2	1.2
		Mean ± SE (n = 6)		3.73 ± 0.33	3.47 ± 0.31	3.85 ± 0.31	3.93 ± 0.42
T-test P Value		Paired = 0.001 Unpaired = 0.57		Paired = 0.67 Unpaired = 0.88		Paired = 0.32 Unpaired = 0.42	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=0.64 Unpaired=0.80	Paired=0.09 Unpaired=0.39		

Table 3.14 Comparison of Non-transgenic and TG-NTG Actin Percentage Motility at 3.9 μ M Ca²⁺ (40nM NF Tn)

Mouse Line		% Motile Actin.Tm		% Motile Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative % motility of Actin.Tm.Tn: Actin.Tm	
NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG	NTG	TG-NTG
13.16	13.15	85 ± 0.82	86 ± 2.64	90±2.62	83±1.50	1.1	1.0
		88 ± 12.1	95 ± 5.40	99±6.23	98±5.60	1.1	1.0
		69 ± 0.57	53 ± 2.52	63±1.65	66±4.20	0.9	1.2
		91 ± 6.80	97 ± 1.30	87±1.59	90±1.36	0.9	0.9
		99 ± 1.41	99 ± 1.50	92±2.36	97±1.87	0.9	1.0
		83 ± 1.30	99 ± 6.80	97±4.26	98±4.63	1.2	1.0
Mean ± SE (n = 6)		85.83 ± 4.07	88.17 ± 7.3	88.00 ± 5.32	88.67 ± 5.14	1.02 ± 0.05	1.02 ± 0.04
T-test P Value		Paired = 0.61 Unpaired = 0.79		Paired = 0.72 Unpaired = 0.93		Paired = 1.0 Unpaired = 1.0	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=0.59 Unpaired=0.75	Paired=0.87 Unpaired=0.96		

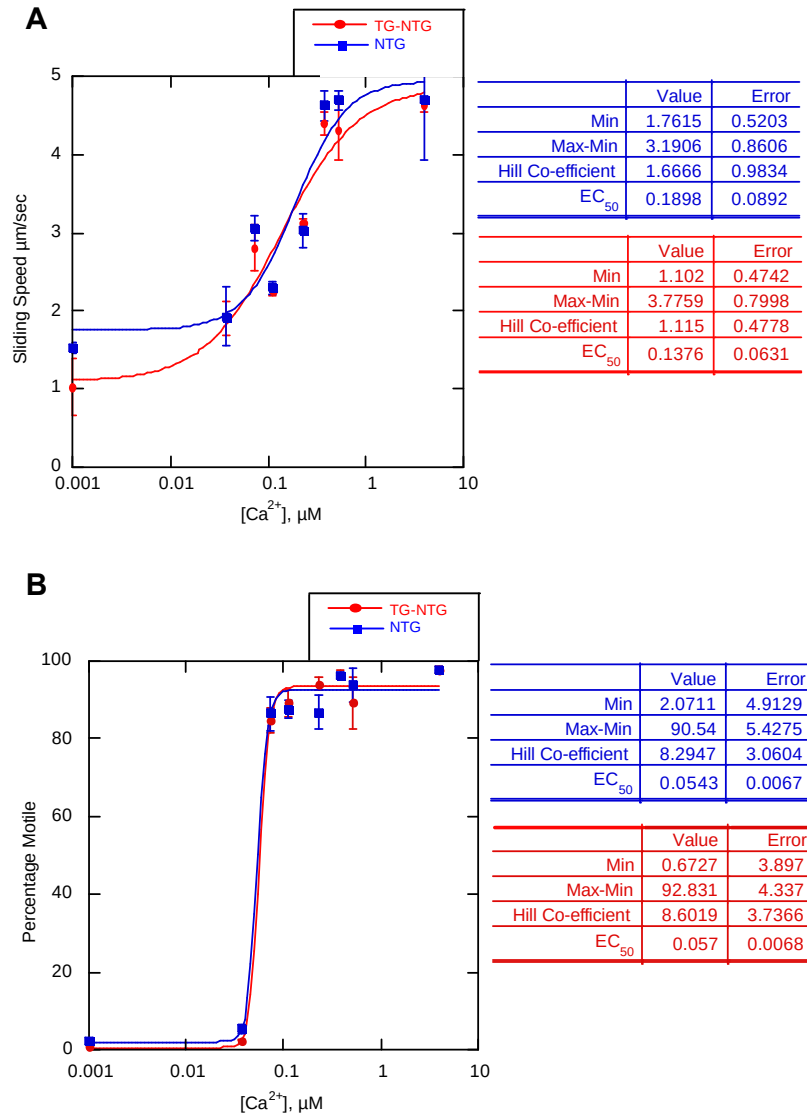
3.6.2.5 Comparing Ca^{2+} Concentration Curves of Non-transgenic and TG-NTG Actin

The minimum concentration of troponin that gave a good switch off at low Ca^{2+} and a good switch on at high Ca^{2+} was used for the Ca^{2+} concentration curves. 10nM non-transgenic or transgenic actin was titrated against 40-60nM human cardiac troponin and 60nM tropomyosin to determine the optimum troponin concentration. Thin filaments were then compared simultaneously over a range of Ca^{2+} concentrations from 1nM to 3.9 μM Ca^{2+} . All experiments were carried out at 28°C. The percentage motile of filaments and sliding speed parameters were fitted to a 4-parameter Hill Equation using KaleidaGraph.

$$\text{Hill Equation: } X = a + \frac{bn [\text{Ca}^{2+}]^n}{(k + [\text{Ca}^{2+}]^n)}$$

a = min value
 bn = max - min
 k = EC_{50}
 n = hill co-efficient

Figure 3.7 Ca^{2+} Regulation of Thin Filaments Containing Non-Transgenic and TG-NTG Actin, (A) Sliding Speed and (B) Percentage Motile (40nM Troponin)



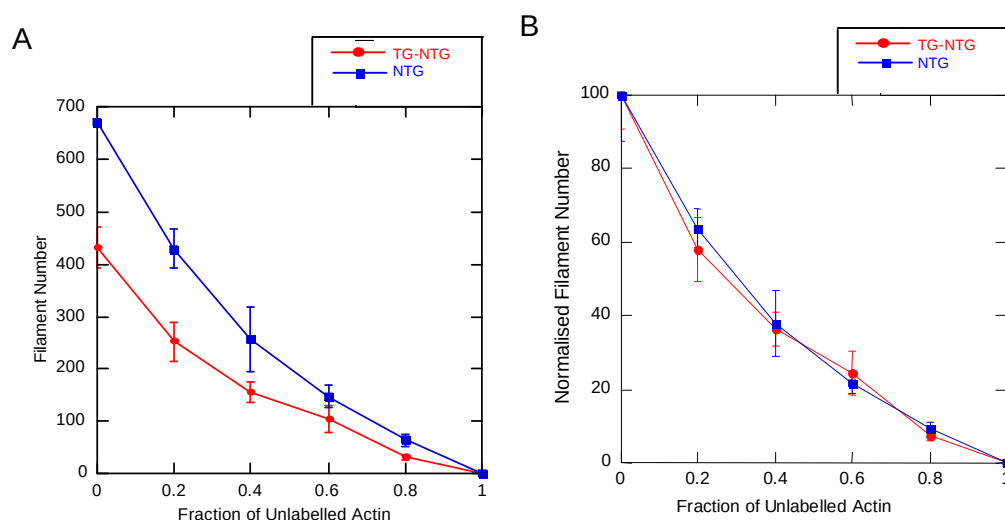
A single paired experiment was completed and is shown above. Thin filaments containing TG-NTG and non-transgenic actin were not significantly different. The EC_{50} ratio of non-transgenic to TG-NTG actin sliding speeds was 1.4, and 0.95 for the percentage motility. The sliding speed of non-transgenic actin increased by $3.2\mu\text{m}/\text{sec}$ from low to high Ca^{2+} , and TG-NTG actin

increased by 3.8 $\mu\text{m}/\text{sec}$. The percentage motile of non-transgenic filaments increased 98% from low to high Ca^{2+} , and TG-NTG actin increased 99%.

3.6.2.6 Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to α -Actinin

The E361G substitution in actin is located within the $\bar{U}$ -actinin binding site and may affect the anchoring of thin filaments to the Z-bands and intercalated discs in the sarcomere (Olson *et al.*, 1998). Because of the possibility that the E361G mutation may disrupt important contacts between actin and $\bar{U}$ -actinin, the actin- $\bar{U}$ -actinin binding affinity in a competitive binding assay was investigated. TRITC- phalloidin labelled TG-NTG and non-transgenic actin filaments were mixed in a range of ratios with non-fluorescent TG-NTG or non-transgenic actin.

Figure 3.8 α -actinin Competitive Binding Assay



Both graphs show how the number of fluorescent filaments varies with the fraction of unlabelled TG-NTG and non-transgenic actin. Graphs A and B were produced from the same assay. The actual numbers of fluorescent actin filaments are plotted in graph A. In graph B, the numbers of both TG-NTG and non-transgenic fluorescent actin filaments have been normalised to start at 100 filaments. The remaining numbers have then been plotted as a ratio to this original number.

When normalised, there was no difference in the binding of TG-NTG and non-transgenic actin for $\bar{U}$ -actinin. Since actin was in great excess over $\bar{U}$ -

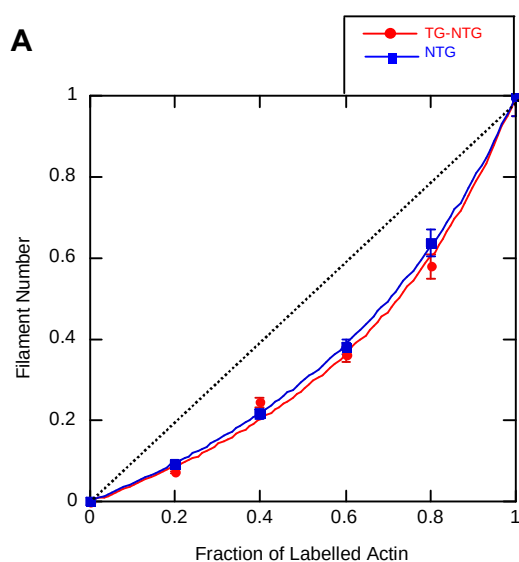
actinin, TRITC actin- ϕ competed with non-fluorescent actin to bind to the $\bar{U}$ -actinin. The amount of fluorescent relative to non-fluorescent actin filaments that bound depended upon the ratio of the two species to their relative affinity, defined by the equation:

$$y = \frac{F \cdot K}{F \cdot K + (1 - F)}$$

y = Filament number
F = Fraction of fluorescently labelled filaments
K = Ratio of binding constant

Figure 3.9 shows the fraction of labelled actin plotted against the number of filaments normalised to 1 for 100% labelled actin. It has been fitted to the $\bar{U}$ -actinin binding equation and shows there is no difference in the affinity of TG-NTG actin for $\bar{U}$ -actinin compared to non-transgenic actin for $\bar{U}$ -actinin. TG-NTG actin had K value of 0.381 ± 0.002 , and non-transgenic actin had a K value of 0.423 ± 0.006 . Both TG-NTG and non-transgenic actin had an affinity for $\bar{U}$ -actinin of less than rabbit actin ($K < 1$).

Figure 3.9 α -actinin Competitive Binding Fit

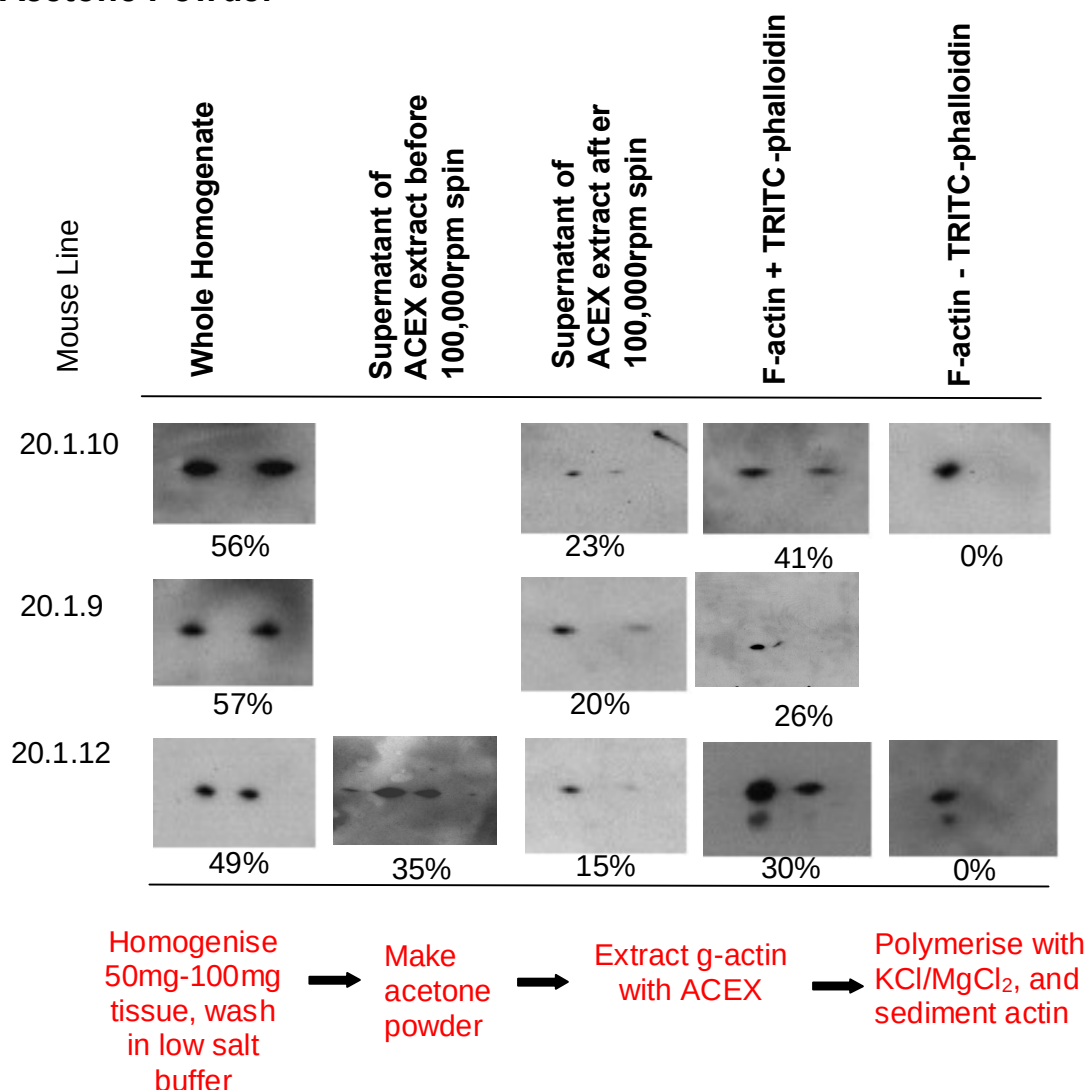


3.6.3 The Acetone Powder Method: Partial Extraction of E361G in the F-actin

Since the E361G mutation was not incorporated into the F-actin when actin was isolated from an acetone powder (Bing *et al.*, 1998, Razzaq *et al.*, 1999), the extraction process was modified. The extraction involved acetone dehydration, the extraction of actin in its monomeric form, and polymerisation by MgCl_2 and KCl. The addition of 150nM TRITC-rhodamine phalloidin during the polymerisation step allowed E361G transgenic actin to be partially extracted from the acetone powder. The actin extracted via this method and described in this section will be referred to as actin- ϕ .

2D-electrophoresis showed that E361G transgenic actin- ϕ was extracted less than the non-transgenic actin- ϕ , with only 25-40% of E361G actin being expressed in the F-actin (Figure 3.10). The E361G actin could not co-polymerise with the non-transgenic actin if phalloidin was not present. 2D-electrophoresis results showed a considerable amount of the mutant cardiac actin was lost during its extraction from the acetone powder using ACEX. This highlights the unstable nature of the mutant G-actin. Mutant actin expression in the ACEX extraction supernatant after the high-speed spin was reduced to approximately 20%, since the instability of the mutant G-actin caused it to aggregate.

Figure 3.10 2-DE of E361G Expression During Extraction via an Acetone Powder



3.6.4 *In Vitro* Motility Analysis of Mouse F-actin- ϕ

3.6.4.1 Actin- ϕ Filament Motility

27 separate measurements were taken using 5 pairs of hearts and the averages are shown in Table 3.15. E361G actin- ϕ sliding speeds were slower than non-transgenic actin- ϕ . The difference was significant in the paired t-test ($p = <0.001$). The sliding speed of the E361G actin- ϕ was 0.95 x the sliding speed of the non-transgenic actin- ϕ , which was a 5% drop. E361G also had a

significantly lower percentage motile of filaments than non-transgenic actin- ϕ (paired t-test, $p = 0.006$). The percentage motile of E361G actin- ϕ filaments was 3% lower.

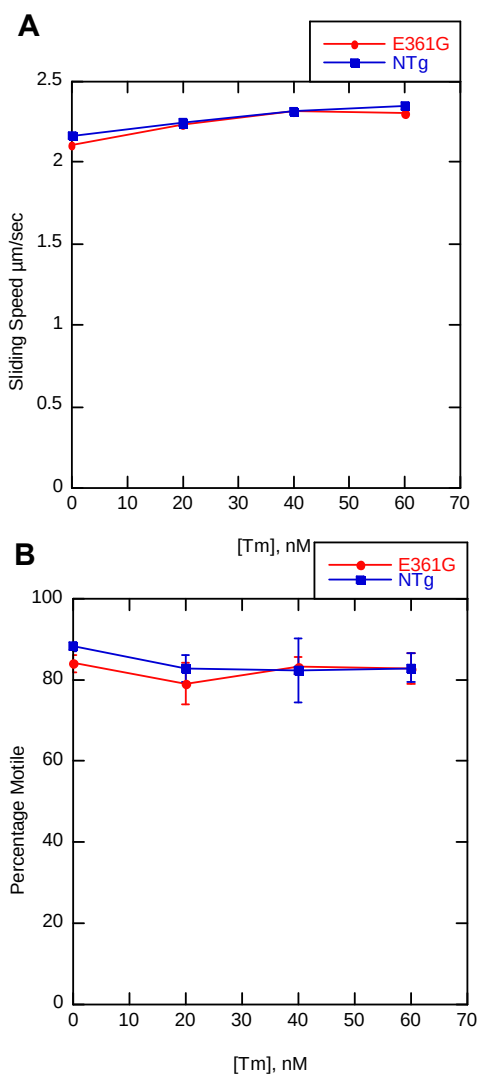
Table 3.15 Comparison of Non-Transgenic and Transgenic Actin- ϕ Sliding Speeds and Percentage Motile of Filaments

Mouse Line		Filament Sliding Speed ($\mu\text{m}/\text{sec}$)		Filament Percentage Motile	
NTg	E361G	NTg	E361G	NTg	E361G
20.1.17	20.1.10	2.3 $\pm$ 0.06 2.1 $\pm$ 0.03 2.2 $\pm$ 0.07 2.3 $\pm$ 0.03 2.5 $\pm$ 0.11 2.5 $\pm$ 0.06 2.8 $\pm$ 0.03 2.9 $\pm$ 0.03 3.1 $\pm$ 0.03 3.5 $\pm$ 0.05 3.1 $\pm$ 0.15 3.3 $\pm$ 0.04 3.4 $\pm$ 0.08 3.3 $\pm$ 0.04	2.3 $\pm$ 0.45 2.0 $\pm$ 0.03 2.1 $\pm$ 0.03 2.2 $\pm$ 0.05 2.3 $\pm$ 0.06 2.4 $\pm$ 0.07 2.7 $\pm$ 0.08 2.8 $\pm$ 0.05 3.0 $\pm$ 0.12 3.3 $\pm$ 0.05 3.2 $\pm$ 0.07 3.2 $\pm$ 0.08 3.2 $\pm$ 0.06 3.2 $\pm$ 0.09	99 $\pm$ 0.67 91 $\pm$ 3.49 85 $\pm$ 4.6 99 $\pm$ 0.75 95 $\pm$ 1.25 96 $\pm$ 0.87 97 $\pm$ 1.69 97 $\pm$ 1.26 98 $\pm$ 0.97 98 $\pm$ 0.68 97 $\pm$ 2.76 99 $\pm$ 0.40 99 $\pm$ 0.51 97 $\pm$ 1.22	96 $\pm$ 2.33 94 $\pm$ 0.50 79 $\pm$ 6.09 87 $\pm$ 7.64 94 $\pm$ 4.37 98 $\pm$ 0.85 88 $\pm$ 3.20 92 $\pm$ 3.37 96 $\pm$ 2.91 97 $\pm$ 2.27 99 $\pm$ 0.55 98 $\pm$ 0.60 98 $\pm$ 1.11 93 $\pm$ 2.63
20.1.13	20.1.9	2.7 $\pm$ 0.08 3.0 $\pm$ 0.02 3.1 $\pm$ 0.05 2.9 $\pm$ 0.06	2.5 $\pm$ 0.13 2.8 $\pm$ 0.04 2.9 $\pm$ 0.08 2.9 $\pm$ 0.05	95 $\pm$ 1.49 97 $\pm$ 0.73 96 $\pm$ 1.21 97 $\pm$ 1.6	96 $\pm$ 2.01 96 $\pm$ 1.24 94 $\pm$ 1.26 99 $\pm$ 0.50
20.1.8	20.1.7	2.2 $\pm$ 0.04 2.2 $\pm$ 0.04	2.1 $\pm$ 0.01 2.2 $\pm$ 0.01	88 $\pm$ 1.20 85 $\pm$ 4.19	84 $\pm$ 1.96 86 $\pm$ 4.76
13.2.13	20.1.12	2.7 $\pm$ 0.04 2.4 $\pm$ 0.02	2.6 $\pm$ 0.002 2.5 $\pm$ 0.07	97 $\pm$ 0.29 84 $\pm$ 7.88	96 $\pm$ 1.19 93 $\pm$ 2.01
13.2.12	20.1.16	3.0 $\pm$ 0.04 2.9 $\pm$ 0.04 3.0 $\pm$ 0.02 3.0 $\pm$ 0.09 2.6 $\pm$ 0.04	2.8 $\pm$ 0.11 2.6 $\pm$ 0.13 2.6 $\pm$ 0.02 2.7 $\pm$ 0.04 2.5 $\pm$ 0.03	86 $\pm$ 4.10 78 $\pm$ 4.16 86 $\pm$ 4.50 89 $\pm$ 3.00 75 $\pm$ 2.56	76 $\pm$ 6.40 68 $\pm$ 11.4 72 $\pm$ 2.32 81 $\pm$ 1.15 67 $\pm$ 4.91
Mean $\pm$ SE (n = 27)		2.78 $\pm$ 0.08	2.65 $\pm$ 0.07	92.59 $\pm$ 1.32	89.56 $\pm$ 1.87
T Test P Value		Paired = <0.001 Unpaired = 0.24		Paired = 0.006 Unpaired = 0.19	

3.6.4.2 Effects of Human Cardiac Tropomyosin on Actin- ϕ Filament Motility

Cardiac tropomyosin was titrated against 10nM non-transgenic and E361G actin- ϕ . The data for a single paired non-transgenic and E361G comparison is shown in Figure 3.11. An optimal increase was seen at 60nM tropomyosin. Data was collected from 12 separate plots (5 paired mouse hearts) and the averages are shown in Table 3.16 and Table 3.17.

Figure 3.11 Effect of Human Cardiac Tropomyosin on Non-Transgenic and Transgenic Actin- ϕ Motility: (A) Sliding Speed, and (B) % motility



After 12 separate measurements, the sliding speed of pure E361G actin- ϕ was 0.94 x slower than non-transgenic actin- ϕ . This was a 6% drop and the difference was significant ($p = 0.002$), as previously found. At optimum tropomyosin concentrations, the sliding speeds of E361G increased by 7% and non-transgenic actin- ϕ increased by 2%. The addition of tropomyosin therefore removed the difference seen between the sliding speeds of pure non-transgenic and E361G actin- ϕ ($p = 0.57$).

The percentage motile of E361G actin- ϕ filaments was 0.94 x lower than the percentage of non-transgenic actin- ϕ filaments. This is a 6% drop, which is significant ($p = 0.02$). The addition of tropomyosin removed the difference in the percentage of non-transgenic and E361G motile actin- ϕ filaments ($p = 0.75$).

Table 3.16 Comparison of Non-Transgenic-Tm and E361G-Tm Sliding Speeds

Filament Sliding Speed (μm/sec)					
Mouse Line		Actin-φ		Actin.Tm	
NTg	Tg	Non-Transgenic	Transgenic	Non-Transgenic	Transgenic
20.1.17	20.1.10	2.3 ± 0.06	2.3 ± 0.45	2.4 ± 0.04	2.5 ± 0.02
		2.1 ± 0.03	2.0 ± 0.03	2.5 ± 0.03	2.5 ± 0.08
		2.2 ± 0.07	2.1 ± 0.03	2.6 ± 0.06	2.7 ± 0.05
20.1.13	20.1.9	3.0 ± 0.02	2.8 ± 0.04	3.2 ± 0.03	2.9 ± 0.23
		2.7 ± 0.08	2.5 ± 0.13	2.9 ± 0.04	2.8 ± 0.09
20.1.8	20.1.7	2.2 ± 0.04	2.1 ± 0.01	2.4 ± 0.03	2.3 ± 0.02
13.2.13	20.1.12	2.7 ± 0.04	2.6 ± 0.002	2.6 ± 0.07	3.0 ± 0.11
		2.4 ± 0.02	2.5 ± 0.07	2.5 ± 0.05	2.6 ± 0.02
13.2.12	20.1.16	3.0 ± 0.04	2.8 ± 0.11	2.7 ± 0.03	2.5 ± 0.07
		2.9 ± 0.04	2.6 ± 0.13	2.9 ± 0.09	2.6 ± 0.08
		3.0 ± 0.02	2.6 ± 0.02	2.8 ± 0.009	2.7 ± 0.08
		3.0 ± 0.09	2.7 ± 0.04	2.6 ± 0.02	2.6 ± 0.02
Mean ± SE (n = 12)		2.63 ± 0.10	2.47 ± 0.08	2.68 ± 0.07	2.64 ± 0.06
T Test P Value		Paired = 0.002 Unpaired = 0.24		Paired = 0.57 Unpaired = 0.71	
T-test of Actin-φ: Actin.Tm				Paired=0.52 Unpaired=0.69	Paired= 0.04 Unpaired=0.09

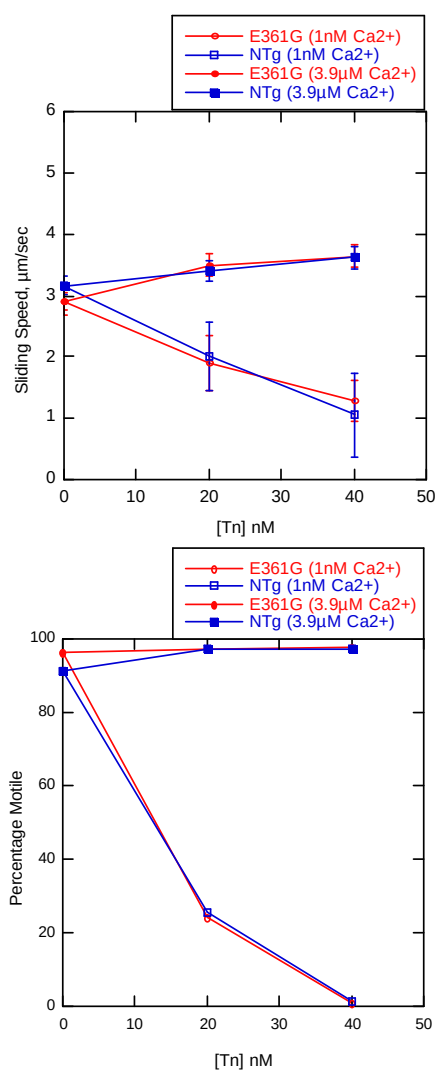
Table 3.17 Comparison of Non-Transgenic-Tm and E361G-Tm Percentage Motile of Filaments

Percentage Motile of Filaments					
Mouse Line		Actin- ϕ		Actin.Tm	
NTg	Tg	Non-Transgenic	Transgenic	Non-Transgenic	Transgenic
20.1.17	20.1.10	85 $\pm$ 4.60	79 $\pm$ 6.09	86 $\pm$ 2.12	93 $\pm$ 1.70
		99 $\pm$ 0.75	87 $\pm$ 7.64	87 $\pm$ 2.16	96 $\pm$ 3.57
		97 $\pm$ 1.69	88 $\pm$ 3.20	99 $\pm$ 0.29	91 $\pm$ 2.80
20.1.13	20.1.9	95 $\pm$ 1.49	96 $\pm$ 2.01	91 $\pm$ 3.14	96 $\pm$ 1.88
		97 $\pm$ 0.73	96 $\pm$ 1.24	100 $\pm$ 0.29	94 $\pm$ 1.44
20.1.8	20.1.7	88 $\pm$ 1.20	84 $\pm$ 1.96	83 $\pm$ 3.81	83 $\pm$ 3.43
13.2.13	20.1.12	97 $\pm$ 0.29	96 $\pm$ 1.19	93 $\pm$ 2.25	92 $\pm$ 1.11
		84 $\pm$ 7.88	93 $\pm$ 2.01	81 $\pm$ 1.73	74 $\pm$ 5.70
13.2.12	20.1.16	86 $\pm$ 4.10	76 $\pm$ 6.4	72 $\pm$ 9.33	63 $\pm$ 6.35
		78 $\pm$ 4.16	68 $\pm$ 11.4	74 $\pm$ 6.59	72 $\pm$ 5.34
		86 $\pm$ 4.50	72 $\pm$ 2.32	86 $\pm$ 1.85	86 $\pm$ 4.49
		75 $\pm$ 2.56	67 $\pm$ 4.91	76 $\pm$ 9.61	81 $\pm$ 2.53
Average $\pm$ SE (n = 12)		88.92 $\pm$ 2.31	83.50 $\pm$ 3.15	85.67 $\pm$ 2.63	85.08 $\pm$ 3.10
T Test		Paired = 0.02		Paired = 0.75	
P Value		Unpaired = 0.18		Unpaired = 0.89	
T-test of Actin- ϕ : Actin.Tm				Paired =0.06 Unpaired=0.36	Paired=0.61 Unpaired=0.72

3.6.4.3 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G Filament Motility at High and Low Ca^{2+}

Troponin at increasing concentrations (20-40nM) was titrated with 10nM actin- ϕ and 60nM human cardiac tropomyosin. Sliding speeds and the percentage motile of actin- ϕ filaments were measured at high Ca^{2+} concentration ($3.9\mu\text{M Ca}^{2+}$) and low Ca^{2+} concentration (1nM Ca^{2+}).

Figure 3.12 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G filament Motility at $3.9\mu\text{M Ca}^{2+}$ and 1nM Ca^{2+}



Actin- ϕ filament motility for both non-transgenic and E361G actin- ϕ was switched off by 40nM troponin with the percentage motility at less than 3% and the sliding speed reduced by 50%. There were no significant differences between non-transgenic and transgenic actin- ϕ sliding speeds at low Ca^{2+} (paired t-test, $p = 0.36$). The percentage motile of non-transgenic and transgenic actin- ϕ filaments was also not significantly different in the paired t-test ($p = 0.45$) (Table 3.18 and Table 3.19).

At high Ca^{2+} , 40nM troponin produced a maximum increase in the actin- ϕ sliding speed and the percentage motile of actin- ϕ filaments. Non-transgenic actin sliding speed increased 11% over actin.tropomyosin, and transgenic actin- ϕ increased 9% over actin.tropomyosin. Troponin increased the percentage motile of actin- ϕ filaments an average of 1% for non-transgenic actin- ϕ and 4% for E361G actin- ϕ . Over 9 assays, the percentage motile of E361G actin- ϕ filaments was not significantly different from non-transgenic actin- ϕ (paired t-test, $p = 0.61$) (Table 3.20 and Table 3.21).

At high Ca^{2+} , the increase in the sliding speed and the percentage motile of non-transgenic and E361G actin- ϕ filaments was small. This is a characteristic of mouse actin- ϕ . Larger increases are produced with rabbit actin- ϕ . At high Ca^{2+} , 40nM non-failing troponin increased rabbit actin- ϕ sliding speed by 26% over actin-tropomyosin thin filaments and the percentage motility was increased by approximately 5% (Messer, PhD Thesis, 2007).

Table 3.18 Comparison of Non-Transgenic and E361G Actin- ϕ Sliding Speeds at 1nM Ca²⁺

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (1nM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
Tg	NTg	NTg	E361G	NTg	E361G	NTg	E361G
20.1.17	20.1.10	2.4 ± 0.04	2.5 ± 0.02	1.7 ± 0.21	1.0 ± 0.01	0.7	0.4
		2.5 ± 0.03	2.5 ± 0.08	1.7 ± 0.34	1.6 ± 0.12	0.7	0.6
		2.6 ± 0.06	2.7 ± 0.05	1.4 ± 0.20	1.5 ± 0.20	0.5	0.6
20.1.13	20.1.9	3.2 ± 0.03	2.9 ± 0.23	1.1 ± 0.69	1.3 ± 0.33	0.3	0.4
		2.9 ± 0.04	2.8 ± 0.09	0.6 ± 0.11	1.0 ± 0.13	0.2	0.4
20.1.8	20.1.7	2.4 ± 0.03	2.3 ± 0.02	1.0 ± 0.14	0.5 ± 0.25	0.4	0.2
13.2.13	20.1.12	2.6 ± 0.07	3.0 ± 0.11	1.5 ± 0.19	0.8 ± 0.49	0.6	0.3
		2.5 ± 0.05	2.6 ± 0.02	1.6 ± 0.24	1.8 ± 0.11	0.6	0.7
13.2.12	20.1.16	2.6 ± 0.02	2.6 ± 0.02	1.2 ± 0.05	1.1 ± 0.37	0.5	0.4
Mean ± SE n = 9		2.63 ± 0.09	2.66 ± 0.07	1.31 ± 0.12	1.18 ± 0.14	0.50 ± 0.06	0.44 ± 0.05
T-test P Value		Paired = 0.74 Unpaired = 0.85		Paired = 0.36 Unpaired = 0.48		Paired = 0.40 Unpaired = 0.49	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=<.0001 Unpaired=<.0001	Paired=<.0001 Unpaired=<.0001		

Table 3.19 Comparison of Non-Transgenic and E361G Actin- ϕ Percentage Motility at 1nM Ca²⁺

Mouse Line		% Motile Actin.Tm		% Motile Actin.Tm.Tn (1nM Ca ²⁺)		Relative % Motility of Actin.Tm.Tn: Actin.Tm	
Tg	NTg	NTg	E361G	NTg	E361G	NTg	E361G
20.1.17	20.1.10	86 ± 2.12	93 ± 1.7	1 ± 0.75	1 ± 0.	0.01	0.01
		87 ± 2.16	96 ± 3.57	6 ± 1.65	7 ± 1.48	0.10	0.07
		99 ± 0.29	91 ± 2.8	3 ± 0.30	3 ± 1.08	0.02	0.03
20.1.13	20.1.9	91 ± 3.14	96 ± 1.88	1 ± 0.58	1 ± 0.20	0.01	0.01
		100 ± 0.29	94 ± 1.44	1 ± 0.28	1 ± 0.48	0.01	0.01
20.1.8	20.1.7	83 ± 3.81	83 ± 3.43	4 ± 1.25	4 ± 1.85	0.05	0.04
13.2.13	20.1.12	93 ± 2.25	92 ± 1.11	8 ± 2.39	6 ± 1.49	0.08	0.06
		81 ± 1.73	74 ± 5.70	2 ± 0.25	1 ± 0.41	0.03	0.01
13.2.12	20.1.16	72 ± 9.33	63 ± 6.35	1 ±0.10	1 ± 0.47	0.01	0.02
Mean ± SE n = 9		88.00 ± 2.97	86.89 ± 3.85	3.00 ± 0.85	2.78 ± 0.80	0.04 ± 0.01	0.03 ± 0.008
T-test P Value		Paired = 0.64 Unpaired = 0.82		Paired = 0.45 Unpaired = 0.85		Paired = 0.20 Unpaired = 0.63	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=<.0001 Unpaired=<.0001		Paired=<.0001 Unpaired=<.0001	

Table 3.20 Comparison of Non-Transgenic and E361G Actin- ϕ Sliding Speeds at 3.9 μ M Ca²⁺

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
Tg	NTg	NTg	E361G	NTg	E361G	NTg	E361G
20.1.1 7	20.1.1 0	2.4 ± 0.04	2.5 ± 0.02	3.2 ± 0.08	3.5 ± 0.09	1.3	1.4
		2.5 ± 0.03	2.5 ± 0.08	2.8 ± 0.03	2.4 ± 0.05	1.1	1.0
		2.6 ± 0.06	2.7 ± 0.05	2.3 ± 0.05	2.2 ± 0.04	0.9	0.8
20.1.1 3	20.1.9	3.2 ± 0.03	2.9 ± 0.23	3.6 ± 0.03	3.6 ± 0.04	1.2	1.3
		2.9 ± 0.04	2.8 ± 0.09	2.8 ± 0.03	2.7 ± 0.04	0.9	0.9
20.1.8	20.1.7	2.4 ± 0.03	2.3 ± 0.02	3.5 ± 0.15	3.8 ± 0.06	1.5	1.6
13.2.1 3	20.1.1 2	2.6 ± 0.07	3.0 ± 0.11	2.6 ± 0.18	2.9 ± 0.04	1.0	0.9
		2.5 ± 0.05	2.6 ± 0.02	2.8 ± 0.02	2.4 ± 0.03	1.1	0.9
13.2.1 2	20.1.1 6	2.6 ± 0.02	2.6 ± 0.02	2.7 ± 0.08	2.7 ± 0.04	1.0	1.1
Mean ± SE n = 9		2.63 ± 0.09	2.66 ± 0.07	2.92 ± 0.14	2.91 ± 0.19	1.11 ± 0.06	1.10 ± 0.09
T-test P Value		Paired = 0.74 Unpaired = 0.85		Paired = 0.91 Unpaired = 0.96		Paired = 0.78 Unpaired = 0.92	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=0.08 Unpaired=0.10	Paired=0.27 Unpaired=0.24		

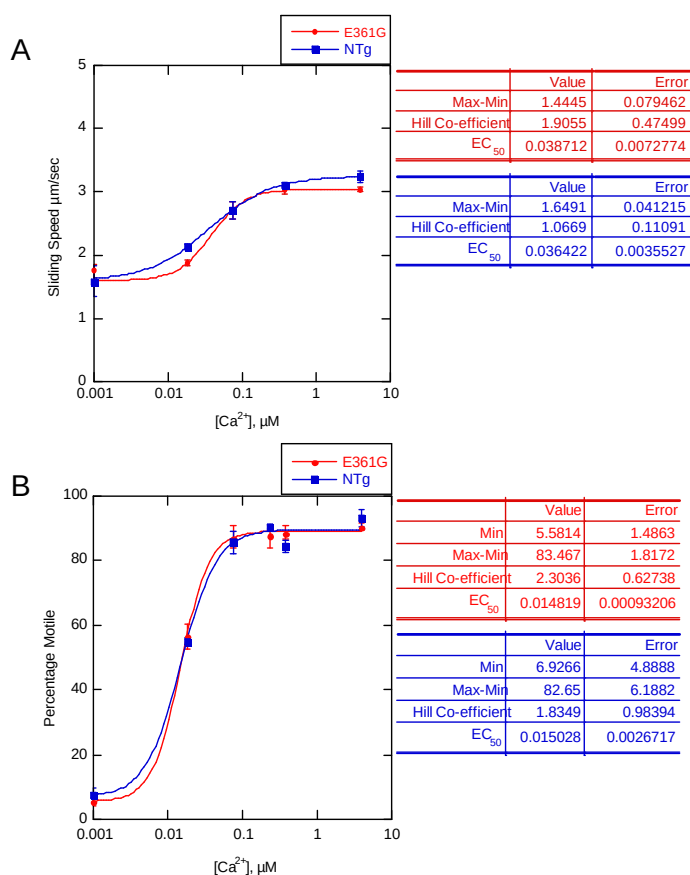
Table 3.21 Comparison of Non-Transgenic and E361G Actin- ϕ Percentage Motility at 3.9 μ M Ca²⁺

Mouse Line		% Motile Actin.Tm		% Motile Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative % motility of Actin.Tm.Tn: Actin.Tm	
Tg	NTg	NTg	Tg	NTg	Tg	NTg	Tg
20.1.1 7	20.1.1 0	86 ± 2.12	93 ± 1.7	99 ± 0.95	97 ± 0.48	1.2	1.1
		87 ± 2.16	96 ± 3.57	82 ± 4.29	81 ± 3.49	0.9	0.8
		99 ± 0.29	91 ± 2.8	87 ± 2.81	81 ± 3.71	0.9	0.9
20.1.1 3	20.1.9	91 ± 3.14	96 ± 1.88	97 ± 1.07	98 ± 0.37	1.1	1.0
		100 ± 0.29	94 ± 1.5	98 ± 0.6	91 ± 3.08	1.0	1.0
20.1.8	20.1.7	83 ± 3.81	83 ± 3.43	82 ± 1.65	95 ± 1.08	1.0	1.1
13.2.1 3	20.1.1 2	93 ± 2.25	92 ± 1.11	89 ± 0.33	92 ± 1.25	1.0	1.0
		81 ± 1.73	74 ± 5.70	97 ± 0.35	96 ± 0.45	1.2	1.3
13.2.1 2	20.1.1 6	72 ± 9.33	63 ± 6.35	72 ± 2.89	83 ± 2.47	1.0	1.3
Mean ± SE n = 9		88.00 ± 2.97	86.89 ± 3.85	89.20 ± 3.11	90.40 ± 2.32	1.03 ± 0.04	1.07 ± 0.06
T-test P Value		Paired = 0.64 Unpaired = 0.82		Paired = 0.61 Unpaired = 0.76		Paired = 0.50 Unpaired = 0.63	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=0.69 Unpaired=0.78	Paired=0.42 Unpaired=0.44		

3.6.4.4 Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Actin- ϕ

The Ca^{2+} dependence of motility was measured across a range of Ca^{2+} concentrations from 1nM to 3.9 μM Ca^{2+} . 10nM actin- ϕ , 60nM tropomyosin and 40nM non-failing troponin were incubated. This troponin concentration produced an optimal switch on and switch off of sliding speed and percentage motility.

Figure 3.13 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G actin- ϕ , (A) Sliding Speed and (B) Percentage Motile (40nM Troponin)



In graph A, the minimum sliding speed values of both non-transgenic and E361G actin- ϕ have been set at 1.6 $\mu\text{m}/\text{sec}$. This produces an accurate fit of both curves. Graph B shows the percentage motile of filaments fitted to the 4-parameter Hill equation.

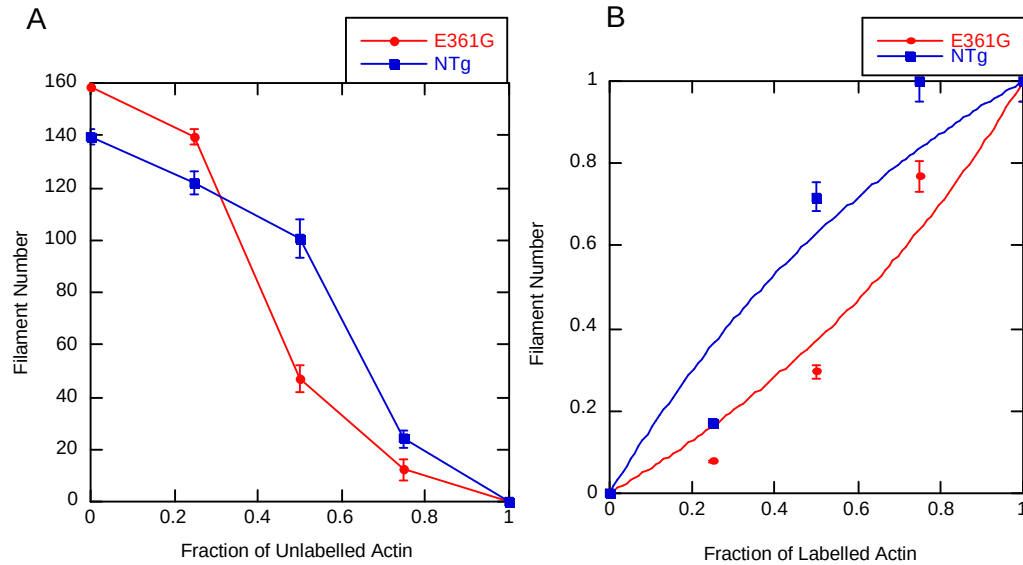
Measurement of the Ca^{2+} dependence of motility showed that thin filaments containing E361G and non-transgenic actin- ϕ were not significantly different. The EC_{50} ratio of non-transgenic to transgenic actin- ϕ sliding speeds was 0.93, and 1.01 for the percentage motility. The sliding speed of non-transgenic actin- ϕ increased by $1.6 \mu\text{m}/\text{sec}$ from low to high Ca^{2+} , and transgenic actin- ϕ increased $1.4 \mu\text{m}/\text{sec}$. The percentage motile of non-transgenic filaments increased 92% from low to high Ca^{2+} , and transgenic actin- ϕ increased 94%.

Numerous Ca^{2+} concentration curves were carried out but only one suitable curve was produced. This is specific to actin- ϕ and may have been due to the method used to extract the mouse actin- ϕ , which involved acetone dehydration followed by the extraction of actin- ϕ in the monomeric form. Subsequent experiments on actin extracted from native thin filaments produced suitable Ca^{2+} concentration curves from the start.

3.6.4.5 Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin- ϕ to α -Actinin

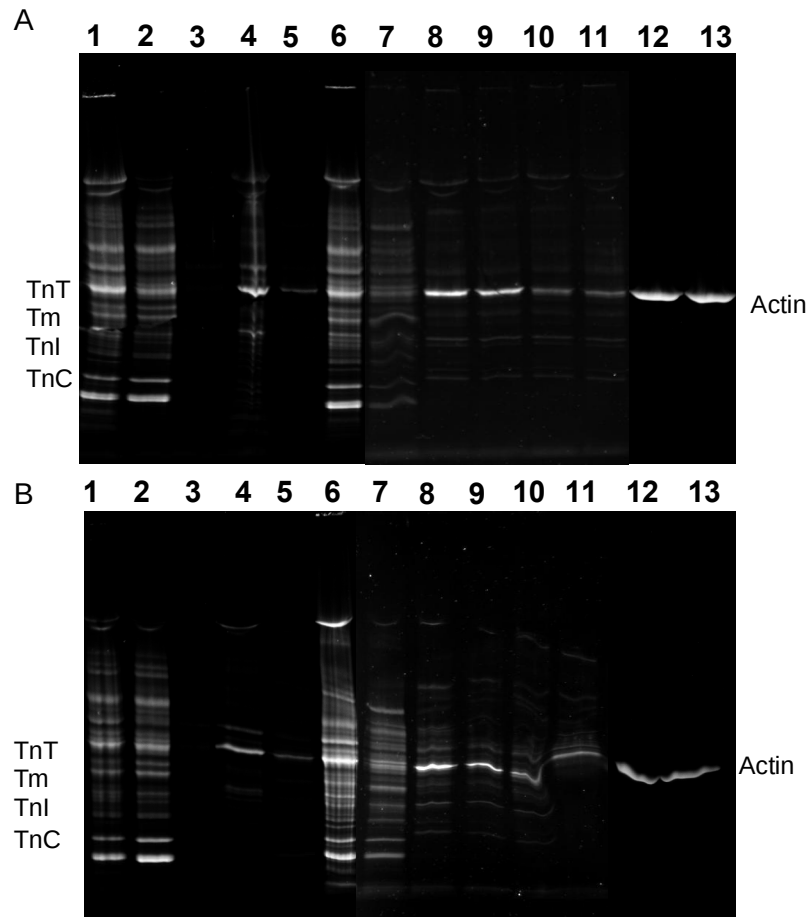
E361G actin- ϕ was displaced from $\tilde{\text{U}}$ -actinin by lower concentrations of non-fluorescent actin- ϕ compared to the non-transgenic actin- ϕ . This indicates the E361G actin- ϕ filaments have a weaker binding affinity for $\tilde{\text{U}}$ -actinin and may have a weaker attachment at the Z-line. Graph A shows the actual number of filaments and graph B shows these numbers fitted to the $\tilde{\text{U}}$ -actinin binding equation. There was 2.88-fold less affinity of E361G actin- ϕ for $\tilde{\text{U}}$ -actinin compared to non-transgenic actin- ϕ . Transgenic actin- ϕ had a K value of 0.59 ± 0.14 , and non-transgenic actin- ϕ had a K value of 1.70 ± 0.64 . The affinity of E361G actin- ϕ for $\tilde{\text{U}}$ -actinin was less than rabbit actin, whilst the affinity of the non-transgenic actin- ϕ is greater than rabbit actin.

Figure 3.14 α -actinin Competitive Binding Assay of E361G and Non-transgenic Actin- ϕ Filaments

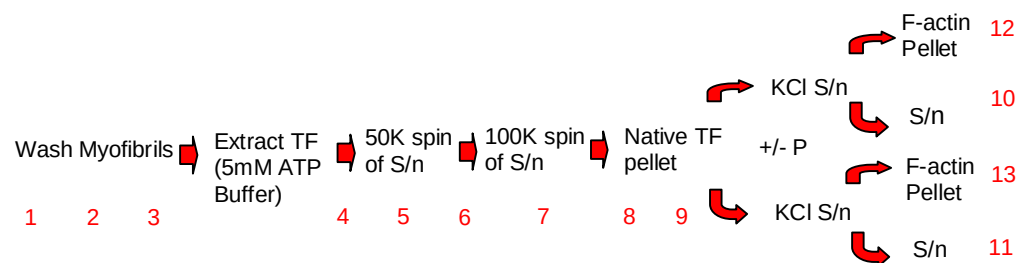


3.6.5 Isolation of F-actin from Whole Thin Filaments

Since monomeric E361G actin extracted by the acetone powder route was unstable, a method was tried that did not involve the polymerisation of actin. Actin was isolated directly from whole thin filaments. This preserved the 50% expression of E361G in the F-actin. The E361G content was therefore the same as in the whole tissue. The properties of E361G cardiac actin were assessed by SDS-PAGE (stained with Sypro Ruby stain) (Figure 3.15) and western blotting (Figure 3.16).

Figure 3.15 NTg (A) and E361G (B) Actin Isolation Fractions

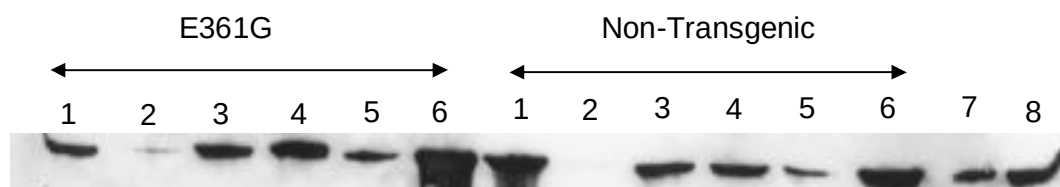
(1) Whole muscle homogenate, (2) first wash s/n, (3) last wash s/n, (4) S/n after 2 extractions in thin filament extraction buffer, (5) S/n after 50K spin, (6) Pellet after 50K spin, (7) S/n after 100K spin, (8) Resuspended pellet of 100K spin before dialysis, (9) S/n after dialysis, (10) KCl s/n + 150nM phalloidin, (11) KCl s/n i 150nM phalloidin, (12) F-actin (+ phalloidin), (13) F-actin (- phalloidin).



The whole thin filaments were pelleted after the 100K spin of the thin filament extract supernatants (fraction 8). The pellet was resuspended in an ATP buffer and dialysed for 3 hours. After dialysis (fraction 9), the thin filaments were mixed with 0.8M KCl, with and without phalloidin (fraction 10 and 11), and

the F-actin was pelleted (fraction 12 and 13). No differences were detected during the isolation of non-transgenic and E361G actin. The final F-actin pellet was pure for both non-transgenic and E361G actin.

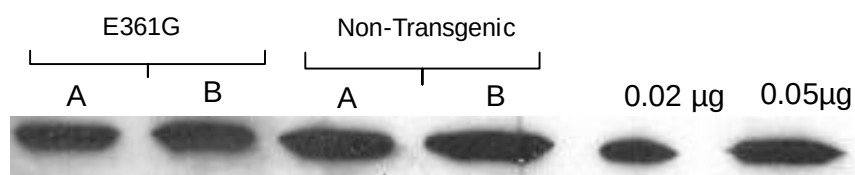
Figure 3.16 Western Blot of E361G and Non-Transgenic Actin Isolation Fractions



(1) whole homogenate, (2) first wash, (3) KCl supernatant - 150nM TRITC-phalloidin, (4) KCl supernatant + 150nM TRITC-phalloidin, (5) f-actin pellet - TRITC-phalloidin, (6) f-actin + TRITC-phalloidin, (7) 0.02µg actin marker, (8) 0.05µg marker.

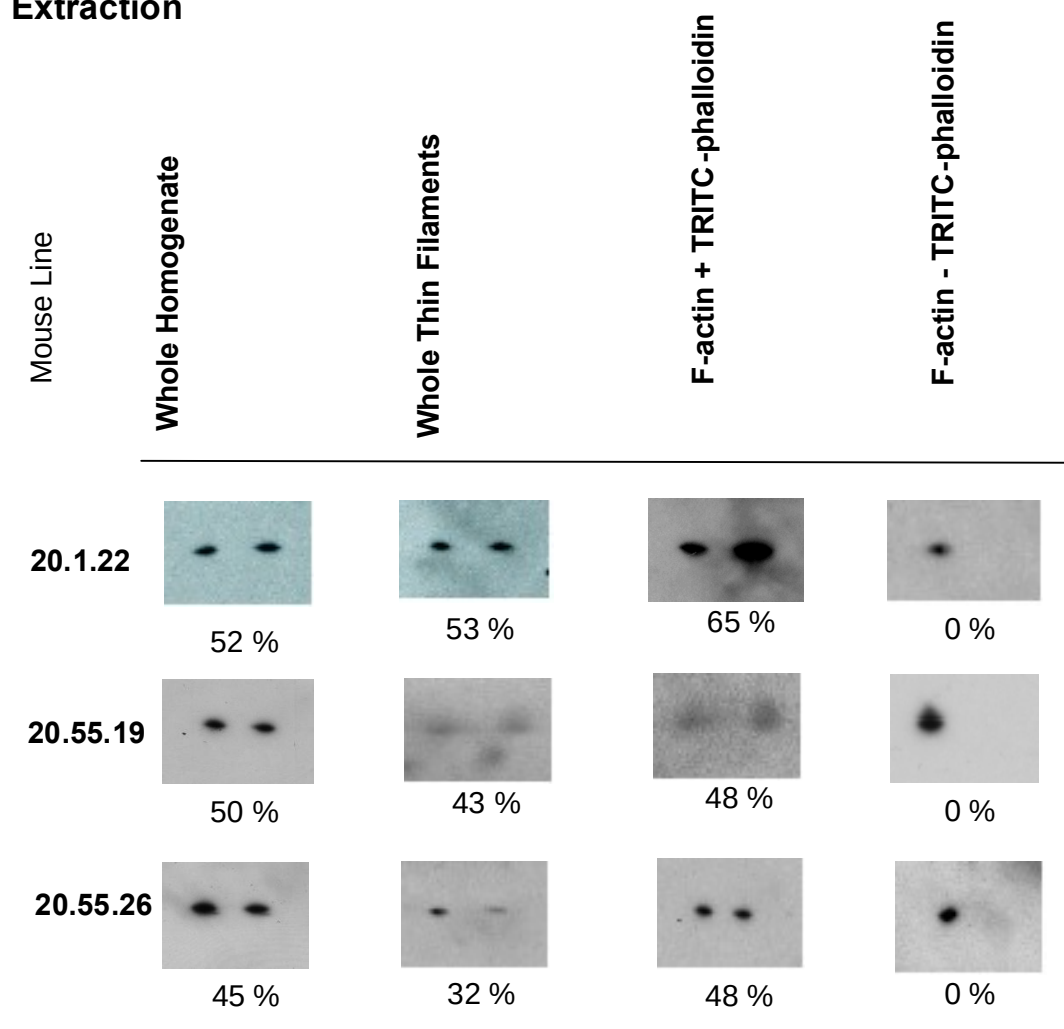
The E361G F-actin yield was optimal when TRITC-phalloidin was added (fraction 6). Western blotting analysis determined the F-actin concentration to be approximately 0.5 mg/ml and the yield of actin approximately 1µg per 1mg of tissue (Figure 3.17).

Figure 3.17 Determination of the E361G and Non-Transgenic F-Actin Concentration



The actins in lane A had 150nM TRITC-phalloidin added to the KCl supernatant. Lane B had no TRITC-phalloidin added.

Figure 3.18 2-DE of E361G Expression during F-Actin Extraction



2-D Electrophoresis was carried out on the whole muscle homogenate, whole thin filaments, and F-actin from three separate actin preparations. Extracting actin from whole thin filaments preserved the E361G actin expression at approximately 50% in the final F-actin. The expression was the same as in the whole tissue. E361G actin could only be extracted when TRITC-phalloidin was added to the supernatant containing the thin filaments along with 0.8M KCl.

3.6.6 *In Vitro* Motility Analysis of Mouse Thin Filament F-actin

3.6.6.1 E361G Thin Filament Actin Stability

E361G thin filament actin remained stable for use in the *in vitro* motility assay for up to 10 days from its isolation. The brightness and contrast of the filaments were retained and the properties of the thin filament actin in the *in vitro* motility assay remained consistent.

3.6.6.2 Thin Filament Actin Motility

E361G actin filaments moved 7% slower than non-transgenic filaments. This was statistically significant over 34 measurements made from 7 separate paired non-transgenic and E361G mouse hearts ($p=0.003$). There was no difference in the percentage of E361G and non-transgenic filaments that were motile ($p=0.58$) (Table 3.22).

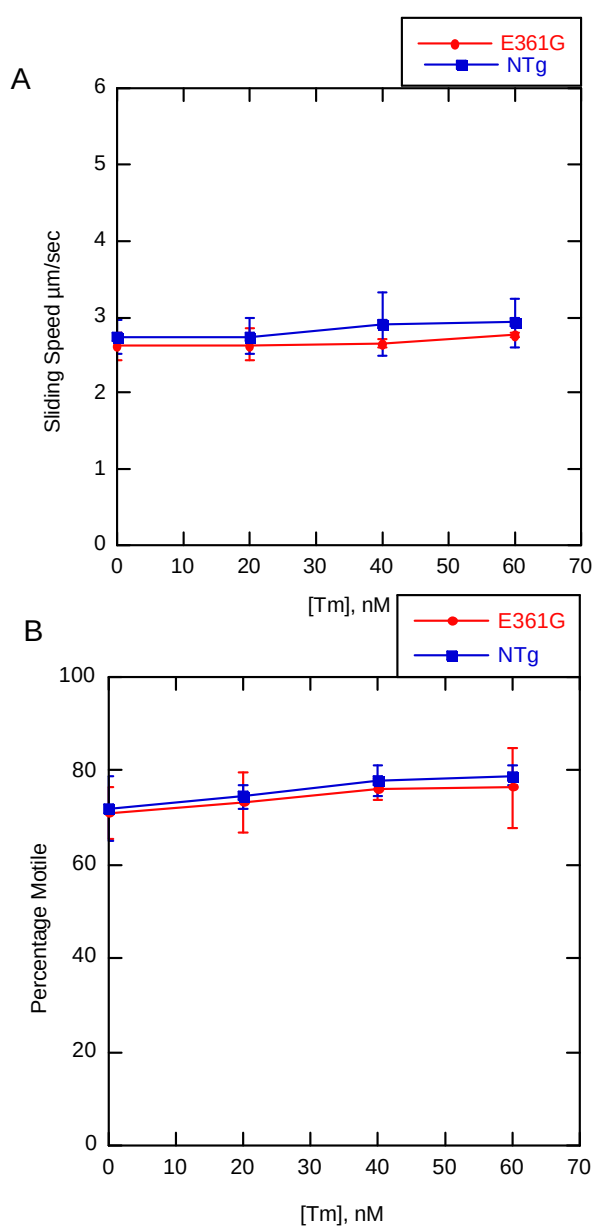
Table 3.22 Comparison of Motility of Non-Transgenic and E361G Thin Filament Actin

Mouse Line		Filament Sliding Speed ($\mu\text{m}/\text{sec}$)		Filaments Percentage Motile	
NTg	E361G	NTg	E361G	NTg	E361G
13.29-	20.1.18	3.2 $\pm$ 0.09	3.4 $\pm$ 0.14	55 $\pm$ 3.60	64 $\pm$ 3.66
		2.9 $\pm$ 0.13	3.9 $\pm$ 0.07	49 $\pm$ 2.60	54 $\pm$ 2.03
		2.7 $\pm$ 0.22	2.8 $\pm$ 0.11	50 $\pm$ 4.05	61 $\pm$ 4.33
		4.1 $\pm$ 0.26	3.1 $\pm$ 0.24	69 $\pm$ 5.78	64 $\pm$ 2.65
		3.3 $\pm$ 0.24	2.7 $\pm$ 0.39	66 $\pm$ 11.5	67 $\pm$ 5.84
		2.6 $\pm$ 0.39	2.8 $\pm$ 0.14	62 $\pm$ 8.00	68 $\pm$ 7.23
13.30	20.1.17	3.0 $\pm$ 0.27	2.4 $\pm$ 0.20	79 $\pm$ 5.03	68 $\pm$ 3.61
		3.5 $\pm$ 0.40	3.5 $\pm$ 0.16	72 $\pm$ 1.00	78 $\pm$ 3.76
		2.4 $\pm$ 0.11	2.1 $\pm$ 0.15	72 $\pm$ 7.02	71 $\pm$ 5.51
		2.7 $\pm$ 0.22	2.6 $\pm$ 0.18	62 $\pm$ 3.71	72 $\pm$ 3.21
		2.7 $\pm$ 0.02	2.8 $\pm$ 0.12	67 $\pm$ 4.55	57 $\pm$ 10.3
20.1.19	20.1.15	2.3 $\pm$ 0.06	2.0 $\pm$ 0.04	88 $\pm$ 1.37	72 $\pm$ 4.24
		2.5 $\pm$ 0.11	1.9 $\pm$ 0.05	89 $\pm$ 1.45	55 $\pm$ 3.19
		2.9 $\pm$ 0.18	2.7 $\pm$ 0.09	87 $\pm$ 4.98	84 $\pm$ 8.29
		2.1 $\pm$ 0.04	2.0 $\pm$ 0.10	59 $\pm$ 4.10	53 $\pm$ 5.61
		1.9 $\pm$ 0.07	1.9 $\pm$ 0.03	58 $\pm$ 2.35	58 $\pm$ 6.99
		2.0 $\pm$ 0.18	2.2 $\pm$ 0.12	65 $\pm$ 12.06	73 $\pm$ 11.08
20.1.14	20.1.22	2.3 $\pm$ 0.09	2.4 $\pm$ 0.07	64 $\pm$ 3.94	77 $\pm$ 2.42
		2.7 $\pm$ 0.34	2.6 $\pm$ 0.13	74 $\pm$ 9.28	89 $\pm$ 3.28
		3.6 $\pm$ 0.11	3.1 $\pm$ 0.03	83 $\pm$ 2.72	81 $\pm$ 0.29
20.1.20	20.1.20.1	3.0 $\pm$ 0.06	2.7 $\pm$ 0.08	91 $\pm$ 0.91	88 $\pm$ 0.96
		3.6 $\pm$ 0.32	2.9 $\pm$ 0.02	74 $\pm$ 6.49	66 $\pm$ 2.90
		1.9 $\pm$ 0.09	1.6 $\pm$ 0.02	67 $\pm$ 6.92	77 $\pm$ 1.80
20.1.16	20.55.19	3.3 $\pm$ 0.04	2.8 $\pm$ 0.03	76 $\pm$ 3.42	71 $\pm$ 2.12
		3.6 $\pm$ 0.34	2.9 $\pm$ 0.05	76 $\pm$ 13.9	61 $\pm$ 2.91
		2.5 $\pm$ 0.06	2.4 $\pm$ 0.09	70 $\pm$ 0.58	64 $\pm$ 9.3
		3.2 $\pm$ 0.07	3.2 $\pm$ 0.05	88 $\pm$ 3.68	93 $\pm$ 2.01
		3.4 $\pm$ 0.12	3.3 $\pm$ 0.08	94 $\pm$ 1.29	92 $\pm$ 1.63
20.55.16	20.55.26	2.1 $\pm$ 0.03	1.9 $\pm$ 0.05	79 $\pm$ 4.53	77 $\pm$ 2.04
		2.5 $\pm$ 0.06	2.5 $\pm$ 0.05	80 $\pm$ 2.17	84 $\pm$ 2.92
		3.5 $\pm$ 0.07	3.1 $\pm$ 0.24	95 $\pm$ 1.31	99 $\pm$ 0.25
		2.3 $\pm$ 0.03	2.3 $\pm$ 0.09	85 $\pm$ 2.17	78 $\pm$ 1.80
		2.9 $\pm$ 0.06	2.2 $\pm$ 0.07	80 $\pm$ 2.99	83 $\pm$ 2.72
		3.9 $\pm$ 0.09	3.3 $\pm$ 0.24	94 $\pm$ 2.50	88 $\pm$ 7.97
Mean $\pm$ SE n = 34		2.86 $\pm$ 0.10	2.65 $\pm$ 0.09	74.09 $\pm$ 2.51	73.1 $\pm$ 2.09
T Test P Value		Paired =0.003 Unpaired =0.13		Paired =0.58 Unpaired =0.76	

3.6.6.3 Effects of Cardiac Tropomyosin on Thin Filament Actin Motility

20-60nM human cardiac tropomyosin was titrated against 10nM non-transgenic and E361G thin filament actin. 30 measurements were taken from 7 separate paired non-transgenic and E361G mouse hearts (Table 3.23).

Figure 3.19 Effect of Human Cardiac Tropomyosin on Non-Transgenic and E361G Thin Filament Actin Motility: (A) Sliding Speed, and (B) % motility



An optimal increase in non-transgenic and E361G thin filament actin sliding speeds was seen at 60nM tropomyosin. The sliding speed of non-transgenic thin filament actin increased by 8% and E361G thin filament actin increased by 7%. The increase in non-transgenic thin filament actin sliding speeds with tropomyosin was significant in the paired t-test ($p = 0.03$). The increase with E361G thin filament actin was not significant ($p = 0.07$). The significant difference in the sliding speeds of pure non-transgenic and E361G thin filament actin was maintained at optimum tropomyosin concentrations. E361G thin filament actin moved 7% slower than non-transgenic thin filament actin ($p = 0.001$).

The percentage motility of plain non-transgenic and E361G thin filament thin filament actin was not significantly different over 30 measurements ($p = 0.65$). An optimal increase occurred at 60nM tropomyosin. The percentage motile of non-transgenic actin.tropomyosin increased 5% and E361G actin.tropomyosin increased 3%. The increase in the percent motility of non-transgenic thin filament actin with 60nM tropomyosin was significantly different ($p = 0.03$). The percent motility of E361G thin filament actin was not significantly different ($p = 0.40$). Over 30 measurements, the percent motility of E361G thin filament actin was 0.97 x that of non-transgenic actin (3% drop). This was significant ($p = 0.05$).

Table 3.23 Comparison of Non-Transgenic.Tm and E361G.Tm Filament Sliding Speeds

		Filament Sliding Speed (μm/sec)			
Mouse Line		Actin		Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	3.2 ± 0.09	3.4 ± 0.14	3.8 ± 0.06	3.2 ± 0.03
		2.9 ± 0.13	3.9 ± 0.07	3.5 ± 0.14	3.3 ± 0.06
		2.7 ± 0.22	2.8 ± 0.11	3.5 ± 0.40	2.9 ± 0.13
		4.1 ± 0.26	3.1 ± 0.24	3.5 ± 0.14	3.9 ± 0.22
		2.6 ± 0.39	2.8 ± 0.14	2.8 ± 0.29	2.2 ± 0.12
13.30	20.1.17	3.0 ± 0.27	2.4 ± 0.20	3.4 ± 0.40	2.3 ± 0.09
		2.4 ± 0.11	2.1 ± 0.15	3.0 ± 0.22	2.8 ± 0.08
		2.7 ± 0.22	2.6 ± 0.18	2.9 ± 0.33	2.8 ± 0.03
		2.7 ± 0.02	2.8 ± 0.12	3.2 ± 0.13	3.0 ± 0.23
20.1.19	20.1.15	2.3 ± 0.06	2.0 ± 0.04	2.6 ± 0.06	2.5 ± 0.04
		2.5 ± 0.11	1.9 ± 0.05	2.9 ± 0.33	2.3 ± 0.27
		2.9 ± 0.18	2.7 ± 0.09	2.1 ± 0.02	2.1 ± 0.11
		2.1 ± 0.04	2.0 ± 0.10	3.0 ± 0.07	2.8 ± 0.07
		1.9 ± 0.07	1.9 ± 0.03	2.6 ± 0.08	2.1 ± 0.19
		2.0 ± 0.18	2.2 ± 0.12	2.4 ± 0.08	2.4 ± 0.28
20.1.14	20.1.22	2.3 ± 0.09	2.4 ± 0.07	2.9 ± 0.10	2.6 ± 0.21
		2.7 ± 0.34	2.6 ± 0.13	2.9 ± 0.17	3.0 ± 0.04
20.1.20	20.1.20.1	3.0 ± 0.06	2.7 ± 0.08	3.4 ± 0.07	3.6 ± 0.02
		3.6 ± 0.32	2.9 ± 0.02	2.5 ± 0.01	2.3 ± 0.04
		1.9 ± 0.09	1.6 ± 0.02	1.9 ± 0.06	2.3 ± 0.06
20.1.16	20.55.19	3.3 ± 0.04	2.8 ± 0.03	3.2 ± 0.08	2.9 ± 0.15
		2.5 ± 0.06	2.4 ± 0.09	2.8 ± 0.19	2.4 ± 0.09
		3.2 ± 0.07	3.2 ± 0.05	2.0 ± 0.15	2.3 ± 0.02
		3.4 ± 0.12	3.3 ± 0.08	3.8 ± 0.04	3.3 ± 0.07
20.55.16	20.55.26	2.1 ± 0.03	1.9 ± 0.05	2.2 ± 0.30	1.6 ± 0.10
		2.5 ± 0.06	2.5 ± 0.05	3.1 ± 0.07	2.9 ± 0.11
		3.5 ± 0.07	3.1 ± 0.24	3.7 ± 0.16	3.7 ± 0.08
		2.3 ± 0.03	2.3 ± 0.09	2.6 ± 0.05	2.5 ± 0.10
		2.9 ± 0.06	2.2 ± 0.07	3.6 ± 0.09	3.3 ± 0.03
		3.9 ± 0.09	3.3 ± 0.24	4.0 ± 0.04	3.7 ± 0.06
Mean ± SE n = 30		2.77 ±0.10	2.59 ±0.10	2.99 ±0.10	2.77 ±0.10
T Test P Value		Paired =0.02 Unpaired =0.22		Paired =0.001 Unpaired =0.12	
T-test of Actin: Actin.Tm			Paired= 0.03 Unpaired= 0.13		Paired=0.07 Unpaired=0.23

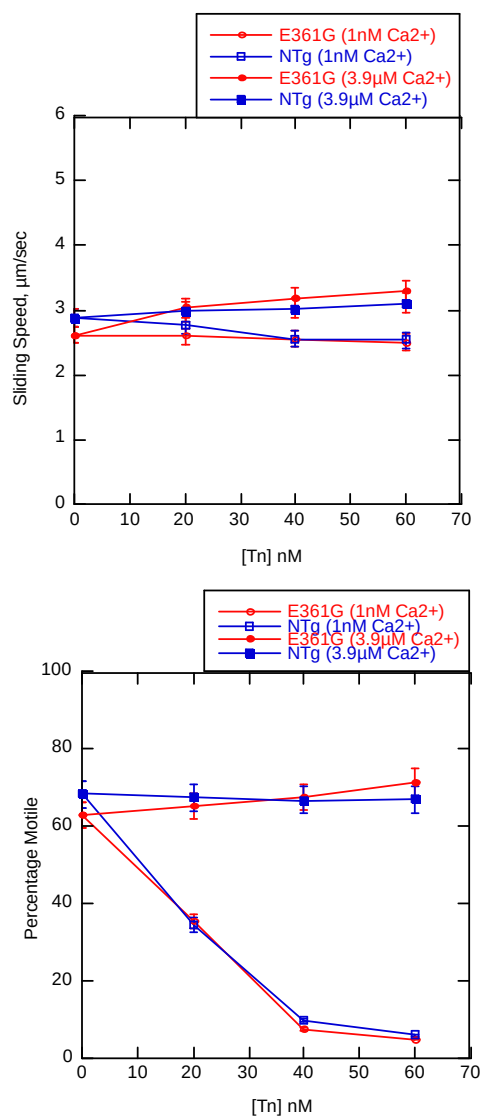
**Table 3.24 Comparison of Non-Transgenic.Tm and E361G.Tm
Percentage Motile of Filaments**

Mouse Line		Filaments Percentage Motile			
		Actin		Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	55 ± 3.60	64 ± 3.66	71 ± 4.73	71 ± 4.00
		49 ± 2.60	54 ± 2.03	56 ± 6.08	69 ± 6.11
		50 ± 4.05	61 ± 4.33	72 ± 1.41	66 ± 1.45
		69 ± 5.78	64 ± 2.65	81 ± 1.41	80 ± 4.91
		66 ± 11.5	67 ± 5.84	53 ± 7.50	32 ± 7.78
13.30	20.1.17	79 ± 5.03	68 ± 3.61	82 ± 15.40	73 ± 6.89
		62 ± 3.71	72 ± 3.21	76 ± 5.17	79 ± 14.5
		72 ± 7.02	71 ± 5.51	79 ± 2.00	77 ± 8.54
		67 ± 4.55	57 ± 10.3	75 ± 5.64	83 ± 0.48
20.1.19	20.1.15	87 ± 1.37	72 ± 4.24	85 ± 2.53	82 ± 1.03
		89 ± 1.45	55 ± 3.19	87 ± 5.60	84 ± 4.19
		87 ± 4.98	84 ± 8.29	64 ± 2.29	67 ± 4.07
		59 ± 4.10	53 ± 5.61	68 ± 0.75	65 ± 5.05
		58 ± 2.35	58 ± 6.99	58 ± 0.65	49 ± 2.0
		64 ± 12.06	73±11.08	70 ± 0.88	58 ± 5.69
20.1.14	20.1.22	64 ± 3.94	77 ± 2.42	68 ± 2.87	63 ± 3.09
		74 ± 9.28	89 ± 3.28	78 ± 2.17	78 ± 2.33
20.1.20	20.1.20.1	91 ± 0.91	88 ± 0.96	94 ± 0.48	91 ± 0.96
		74 ± 6.49	66 ± 2.90	75 ± 4.26	69 ± 4.19
		67 ± 6.92	77 ± 1.80	75 ± 4.26	59 ± 2.93
20.1.16	20.55.19	76 ± 3.42	71 ± 2.12	94 ± 4.36	93 ± 3.12
		70 ± 0.58	64 ± 9.3	79 ± 2.0	77 ± 3.54
		88 ± 3.68	93 ± 2.01	82 ± 3.33	87 ± 4.33
		94 ± 1.29	92 ± 1.63	95 ± 0.84	92 ± 1.83
20.55.16	20.55.26	79 ± 4.53	77 ± 2.04	74 ± 6.51	78 ± 5.23
		80 ± 2.17	84 ± 2.92	86 ± 2.45	89 ± 1.83
		95 ± 1.31	99 ± 0.25	94 ± 4.36	93 ± 3.12
		85 ± 2.17	78 ± 1.80	96 ± 2.63	93 ± 1.04
		80 ± 2.99	83 ± 2.72	85 ± 1.75	85 ± 1.11
		94 ± 2.50	88 ± 7.97	89 ± 4.10	83 ± 1.20
Mean ± SE n = 30		74.13 ± 2.43	73.30 ± 2.31	78.03 ± 2.11	75.50 ± 2.59
T Test P Value		Paired =0.65 Unpaired =0.80		Paired =0.05 Unpaired =0.45	
T-test of Actin: Actin.Tm			Paired=0.03 Unpaired=0.23		Paired=0.40 Unpaired=0.53

3.6.6.4 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G Thin Filament Actin Motility at High and Low Ca^{2+}

20-60nM human cardiac troponin was titrated with 10nM actin and 60nM tropomyosin at high Ca^{2+} (3.9 μM) and low Ca^{2+} (1nM). 30 measurements were taken from 7 separate paired non-transgenic and E361G mouse hearts (Table 3.25 - Table 3.28).

Figure 3.20 Effects of Non-failing Human Cardiac Troponin on Non-transgenic and E361G filament Motility at 3.9 μM Ca^{2+} and 1nM Ca^{2+}



In these titration experiments, between 40-60nM troponin produced an optimum switch-off and switch-on in the percentage motility and the sliding speeds of thin filament actin. At low Ca^{2+} the sliding speed was reduced by 32% for non-transgenic actin and 34% for E361G actin, compared to actin.tropomyosin. The decrease in the sliding speed for both actins was significant (<0.0001). At optimum troponin concentrations, the sliding speed of E361G actin was 10% slower than non-transgenic actin, although this is not a significant difference in the paired t-test ($p = 0.06$). The reduced sliding speeds of mouse actin at low Ca^{2+} were not as large as those seen with rabbit actin, which had a 58% reduction. However, these differences could be due to technical reasons as the mouse and rabbit contractile systems incorporated different human cardiac troponin and tropomyosin preparations.

The percentage motility was reduced by 89% for both non-transgenic and E361G thin filament actin to an average of 9% motility at low Ca^{2+} . These reductions are slightly less than were seen with rabbit actin, which reduced by 96% motility at low Ca^{2+} . At optimum troponin concentrations, there was no significant difference in the percentage motility of non-transgenic and E361G thin filament actin ($p = 0.85$).

At high Ca^{2+} , non-transgenic thin filament actin sliding speed increased 2% over actin.tropomyosin and E361G thin filament actin increased 14% over actin.tropomyosin. The increase of E361G was significant ($p = 0.005$). The change in sliding speed at high Ca^{2+} was very small. This appears to be a characteristic of mouse thin filament actin and is different from the changes seen with rabbit actin, which increased sliding speed at high Ca^{2+} by 26%. A significant observation has been the π crossing over of non-transgenic and E361G thin filament actin when comparing their sliding speeds at high Ca^{2+} . E361G thin filament actin had a 7% slower sliding speed when incorporated with tropomyosin compared to non-transgenic thin filament actin with tropomyosin ($p = 0.001$). At high Ca^{2+} , the sliding speed of E361G thin filament actin increases to have a slightly faster sliding speed than non-transgenic actin, although this is not a significant difference ($p = 0.14$). The percent motility of thin filament actin did not significantly change at high Ca^{2+} and there was no difference between non-transgenic and E361G thin filament actin ($p = 0.42$). Thus, non-transgenic and E361G thin filament actin were indistinguishable

when incorporated into thin filaments.

Table 3.25 Effect of Human Cardiac Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (1nM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	3.8 ± 0.06	3.2 ± 0.03	2.6 ± 0.46	2.6 ± 0.36	0.7	0.8
		3.5 ± 0.14	3.3 ± 0.06	2.8 ± 0.05	1.9 ± 0.44	0.8	0.6
		3.5 ± 0.40	2.9 ± 0.13	2.9 ± 0.31	2.1 ± 0.15	0.8	0.7
		3.5 ± 0.14	3.9 ± 0.22	3.1 ± 0.57	2.1 ± 0.47	0.9	0.5
		2.8 ± 0.29	2.2 ± 0.12	1.1 ± 0.33	1.4 ± 0.18	0.3	0.6
13.30	20.1.17	3.4 ± 0.4	2.3 ± 0.09	2.3 ± 0.13	2.2 ± 0.42	0.7	1.0
		3.0 ± 0.22	2.8 ± 0.08	1.7 ± 0.18	1.8 ± 0.33	0.6	0.6
		2.9 ± 0.33	2.8 ± 0.03	0.8 ± 0.47	1.9 ± 0.10	0.3	0.7
		3.2 ± 0.13	3.0 ± 0.23	2.4 ± 0.15	1.6 ± 0.22	0.7	0.5
20.1.19	20.1.15	2.6 ± 0.06	2.5 ± 0.04	2.5 ± 0.13	2.4 ± 0.06	0.9	0.9
		2.9 ± 0.33	2.3 ± 0.27	1.4 ± 0.75	2.1 ± 0.19	0.5	0.9
		2.1 ± 0.02	2.1 ± 0.11	1.9 ± 0.06	1.7 ± 0.13	0.9	0.8
		3.0 ± 0.07	2.8 ± 0.07	1.6 ± 0.09	1.8 ± 0.21	0.5	0.6
		2.6 ± 0.08	2.1 ± 0.19	2.1 ± 0.35	1.3 ± 0.45	0.8	0.6
		2.4 ± 0.08	2.4 ± 0.28	1.5 ± 0.10	1.4 ± 0.18	0.7	0.6
20.1.14	20.1.22	2.9 ± 0.10	2.6 ± 0.21	2.5 ± 0.24	2.5 ± 0.20	0.9	1.0
		2.9 ± 0.17	3.0 ± 0.04	1.8 ± 0.20	2.1 ± 0.21	0.6	0.7
20.1.20	20.1.20.1	3.4 ± 0.07	3.6 ± 0.02	2.4 ± 0.21	1.7 ± 0.20	0.7	0.5
		2.5 ± 0.01	2.3 ± 0.04	2.5 ± 0.36	2.1 ± 0.22	1.0	0.9
		1.9 ± 0.06	2.3 ± 0.06	2.1 ± 0.35	1.3 ± 0.45	1.1	0.6
20.1.16	20.55.19	3.2 ± 0.08	2.9 ± 0.15	2.6 ± 0.46	2.6 ± 0.36	0.8	0.9
		2.8 ± 0.19	2.4 ± 0.09	1.8 ± 0.08	0.3 ± 0.14	0.6	0.1
		2.0 ± 0.15	2.3 ± 0.02	1.3 ± 0.11	1.4 ± 0.79	0.7	0.6
		3.8 ± 0.04	3.3 ± 0.07	1.5 ± 0.08	1.9 ± 0.43	0.4	0.6
20.55.16	20.55.26	2.2 ± 0.30	1.6 ± 0.10	2.1 ± 0.47	2.5 ± 0.27	1.0	1.6
		3.1 ± 0.07	2.9 ± 0.11	1.9 ± 0.19	1.5 ± 0.17	0.6	0.5
		3.7 ± 0.16	3.7 ± 0.08	2.0 ± 0.06	2.0 ± 0.21	0.5	0.6
		2.6 ± 0.05	2.5 ± 0.10	2.5 ± 0.24	2.5 ± 0.20	1.0	1.0
		3.6 ± 0.09	3.3 ± 0.03	2.1 ± 0.09	1.2 ± 0.42	0.6	0.4
		4.0 ± 0.04	3.7 ± 0.06	1.5 ± 0.20	1.3 ± 0.13	0.4	0.3
Mean ± SE n = 30		2.99 ±0.10	2.77 ±0.10	2.04±0.10	1.84±0.09	0.70±0.04	0.69±0.05
T-test P Value		Paired =0.001 Unpaired =0.12		Paired = 0.06 Unpaired = 0.15		Paired = 0.83 Unpaired =0.87	
T-test of Actin.Tm: Actin.Tm.Tn			Paired=Q0001 Unpaired=Q0001		Paired=Q0001 Unpaired=Q0001		

**Table 3.26 Effect of Human Cardiac Troponin on Filament
Percentage Motility at Low Ca^{2+} (1nM Ca^{2+})**

Mouse Line		% Motility Actin.Tm		% Motility Actin.Tm.Tn (1nM Ca ²⁺)		Relative % Motility of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	71 ± 4.73	71 ± 4.00	5 ± 1.73	7 ± 2.96	0.1	0.1
		56 ± 6.08	69 ± 6.11	4 ± 5.66	14 ± 14.1	0.1	0.2
		72 ± 1.41	66 ± 1.45	10 ± 1.15	13 ± 2.03	0.1	0.2
		81 ± 1.41	80 ± 4.91	10 ± 2.40	8 ±2.96	0.1	0.1
		53 ± 7.5	32 ± 7.78	6 ± 0.14	3 ± 0.08	0.1	0.1
13.30	20.1.17	82 ± 15.4	73 ± 6.89	19 ± 2.60	19 ± 2.03	0.2	0.3
		76 ± 5.17	79 ± 14.50	10 ± 3.86	7 ± 3.28	0.1	0.1
		79 ± 2.0	77 ± 8.54	6 ± 3.40	6 ± 0.95	0.1	0.1
		75 ± 5.64	83 ± 0.48	10 ± 1.67	3 ± 1.76	0.1	0.03
20.1.19	20.1.15	85 ± 2.53	82 ± 1.03	19 ± 1.78	14 ± 0.41	0.2	0.2
		87 ± 5.6	84 ± 4.19	9 ± 6.17	5 ± 1.35	0.1	0.1
		64 ± 2.29	67 ± 4.07	6 ± 2.65	5 ± 2.50	0.01	0.1
		68 ± 0.75	65 ± 5.05	4 ± 0.96	5 ± 1.93	0.1	0.1
		58 ± 0.65	49 ± 2.0	6 ± 1.93	5 ± 2.35	0.1	0.1
		70 ± 0.88	58 ± 5.69	5 ± 3.5	4 ± 2.38	0.1	0.1
20.1.14	20.1.22	68 ± 2.87	63 ± 3.09	7 ± 1.00	14 ± 1.67	0.1	0.2
		78 ± 2.17	78 ± 2.33	6 ± 1.76	3 ± 0.88	0.1	0.04
20.1.20	20.1.20.1	94 ± 0.48	91 ± 0.96	10 ± 2.10	3 ± 1.11	0.1	0.04
		75 ± 4.26	69 ± 4.19	13 ± 1.85	7 ± 1.65	0.2	0.1
		75 ± 4.26	59 ± 2.93	6 ± 1.93	5 ± 2.35	0.1	0.1
20.1.16	20.55.19	94 ± 4.36	93 ± 3.12	5 ± 1.73	7 ± 2.96	0.1	0.1
		79 ± 2.0	77 ± 3.54	7 ± 3.28	6 ± 0.39	0.1	0.1
		82 ± 3.33	87 ± 4.33	8 ± 1.04	7 ± 1.25	0.1	0.1
		95 ± 0.84	92 ± 1.83	6 ± 2.33	12 ± 4.57	0.1	0.1
20.55.16	20.55.26	74 ± 6.51	78 ± 5.23	16 ± 9.70	17 ± 7.05	0.2	0.2
		86 ± 2.45	89 ± 1.83	5 ± 2.14	6 ± 0.65	0.1	0.1
		94 ± 4.36	93 ± 3.12	21 ± 0.87	32 ± 1.53	0.2	0.4
		96 ± 2.63	93 ± 1.04	7 ± 1.00	14 ± 1.67	0.1	0.2
		85 ± 1.75	85 ± 1.11	14 ± 1.70	5 ± 2.56	0.2	0.1
		89 ± 4.10	83 ± 1.20	5 ± 1.80	4 ± 1.04	0.1	0.04
Mean ± SE n = 30		78.03 ± 2.11	75.50 ± 2.59	8.83 ±0.86	8.67 ±1.15	0.12 ±0.01	0.13 ±0.01
T-test P Value		Paired =0.05 Unpaired =0.45		Paired =0.85 Unpaired =0.91		Paired =0.36 Unpaired =0.50	
T-test of Actin.Tm: Actin.Tm.Tn				Paired=Q0001 Unpaired=Q0001		Paired=Q0001 Unpaired=Q0001	

Table 3.27 Effect of Human Cardiac Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	3.8 ± 0.06	3.2 ± 0.03	3.4 ± 0.05	3.4 ± 0.02	0.9	1.2
		3.5 ± 0.14	3.3 ± 0.06	3.7 ± 0.47	3.5 ± 0.20	1.1	1.1
		3.5 ± 0.40	2.9 ± 0.13	3.6 ± 0.13	3.8 ± 0.04	1.0	1.3
		3.5 ± 0.14	3.9 ± 0.22	4.6 ± 0.10	4.8 ± 0.07	1.3	1.2
		2.8 ± 0.29	2.2 ± 0.12	2.5 ± 0.18	3.2 ± 0.01	0.9	1.5
13.30	20.1.17	3.4 ± 0.4	2.3 ± 0.09	3.4 ± 0.11	3.2 ± 0.09	1.0	1.4
		3.0 ± 0.22	2.8 ± 0.08	4.6 ± 0.02	4.9 ± 0.22	1.6	1.8
		2.9 ± 0.33	2.8 ± 0.03	3.0 ± 0.12	3.0 ± 0.04	1.0	1.1
		3.2 ± 0.13	3.0 ± 0.23	3.1 ± 0.38	2.9 ± 0.25	1.0	1.0
20.1.19	20.1.15	2.6 ± 0.06	2.5 ± 0.04	2.9 ± 0.14	3.4 ± 0.27	1.1	1.4
		2.9 ± 0.33	2.3 ± 0.27	3.0 ± 0.11	3.0 ± 0.04	1.0	1.3
		2.1 ± 0.02	2.1 ± 0.11	3.2 ± 0.03	3.2 ± 0.26	1.5	1.5
		3.0 ± 0.07	2.8 ± 0.07	2.8 ± 0.05	3.0 ± 0.035	0.9	1.1
		2.6 ± 0.08	2.1 ± 0.19	2.3 ± 0.06	2.2 ± 0.06	0.9	1.1
		2.4 ± 0.08	2.4 ± 0.28	2.1 ± 0.12	2.2 ± 0.04	0.9	0.9
20.1.14	20.1.22	2.9 ± 0.10	2.6 ± 0.21	3.1 ± 0.15	3.3 ± 0.07	1.1	1.3
		2.9 ± 0.17	3.0 ± 0.04	2.9 ± 0.02	2.9 ± 0.07	1.0	1.0
20.1.20	20.1.20.1	3.4 ± 0.07	3.6 ± 0.02	3.3 ± 0.08	3.4 ± 0.05	1.0	1.0
		2.5 ± 0.01	2.3 ± 0.04	2.7 ± 0.10	2.5 ± 0.04	1.1	1.1
		1.9 ± 0.06	2.3 ± 0.06	2.6 ± 0.31	2.8 ± 0.04	1.4	1.2
20.1.16	20.55.19	3.2 ± 0.08	2.9 ± 0.15	3.1 ± 0.03	2.8 ± 0.11	1.0	1.0
		2.8 ± 0.19	2.4 ± 0.09	2.8 ± 0.06	2.8 ± 0.06	1.0	1.2
		2.0 ± 0.15	2.3 ± 0.02	2.4 ± 0.04	2.7 ± 0.38	1.2	1.2
		3.8 ± 0.04	3.3 ± 0.07	2.9 ± 0.07	3.3 ± 0.12	0.8	1.0
20.55.16	20.55.26	2.2 ± 0.30	1.6 ± 0.10	2.5 ± 0.08	3.3 ± 0.04	1.2	2.1
		3.1 ± 0.07	2.9 ± 0.11	2.9 ± 0.11	3.1 ± 0.08	0.9	1.0
		3.7 ± 0.16	3.7 ± 0.08	4.1 ± 0.24	3.3 ± 0.14	1.1	0.9
		2.6 ± 0.05	2.5 ± 0.10	3.0 ± 0.05	3.3 ± 0.09	1.2	1.4
		3.6 ± 0.09	3.3 ± 0.03	3.2 ± 0.05	3.0 ± 0.04	0.9	0.9
		4.0 ± 0.04	3.7 ± 0.06	2.1 ± 0.12	2.2 ± 0.04	0.5	0.6
Mean ± SE n = 30		2.99 ±0.10	2.77 ±0.10	3.06 ± 0.11	3.15 ± 0.11	1.05 ± 0.04	1.19 ± 0.05
T-test P Value		Paired =0.001 Unpaired =0.12		Paired = 0.14 Unpaired = 0.58		Paired = 0.002 Unpaired = 0.03	
T-test of Actin.Tm: Actin.Tm.Tn			Paired=0.56 Unpaired=0.66		Paired=0.005 Unpaired=0.01		

**Table 3.28 Effect of Human Cardiac Troponin on Filament
Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)**

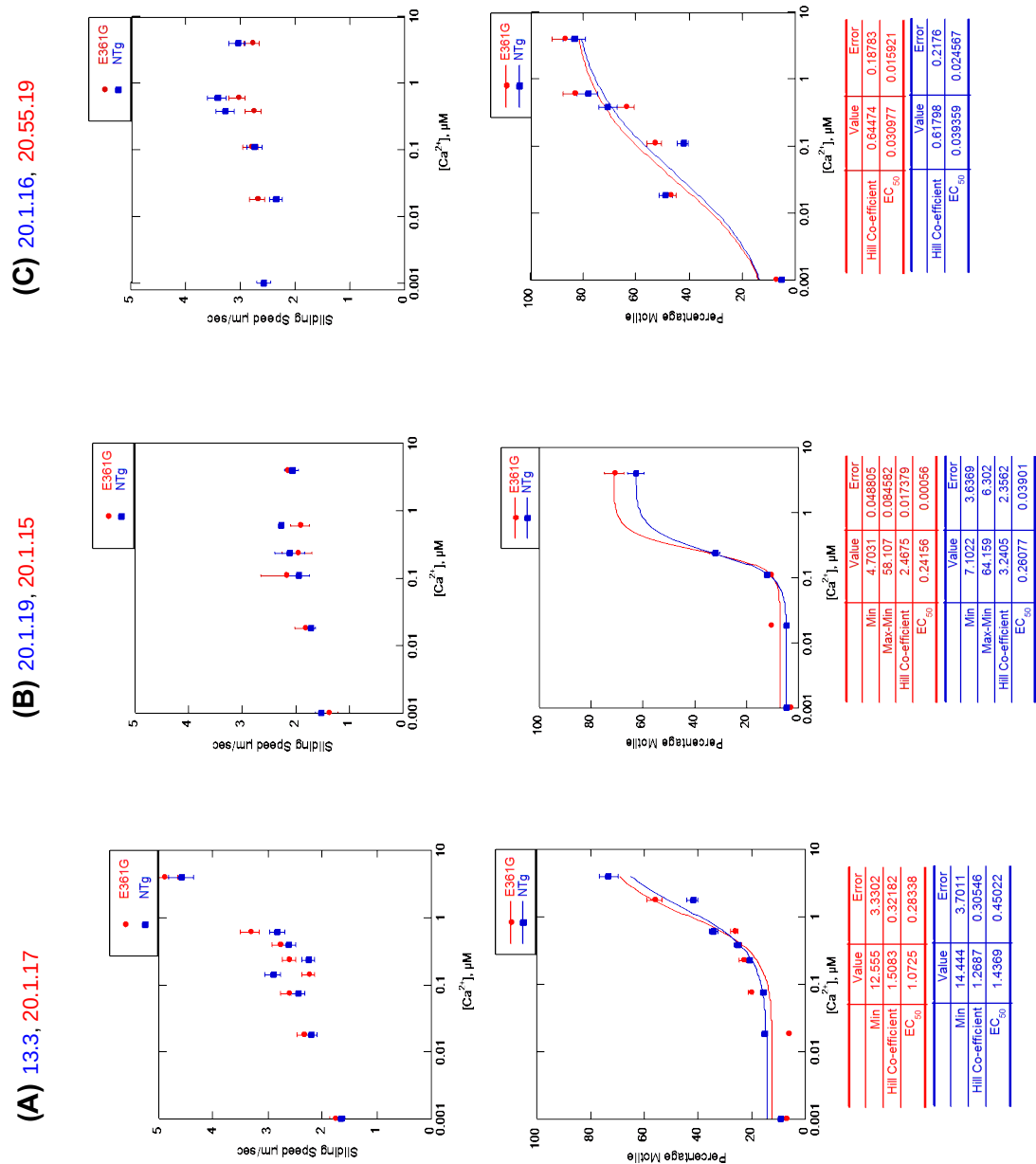
Mouse Line		% Motility Actin.Tm		% Motility Actin.Tm.Tn ($3.9\mu\text{M Ca}^{2+}$)		Relative % Motility of Actin.Tm.Tn: Actin. Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
13.29	20.1.18	71 $\pm$ 4.73 56 $\pm$ 6.08 72 $\pm$ 1.41 81 $\pm$ 1.41 53 $\pm$ 7.5	71 $\pm$ 4.00 69 $\pm$ 6.11 66 $\pm$ 1.45 80 $\pm$ 4.91 32 $\pm$ 7.78	90 $\pm$ 1.33 80 $\pm$ 7.42 82 $\pm$ 1.86 86 $\pm$ 1.53 56 $\pm$ 2.19	95 $\pm$ 0.65 60 $\pm$ 3.48 63 $\pm$ 5.03 90 $\pm$ 4.33 79 $\pm$ 4.41	1.3 1.4 1.1 1.1 1.1	1.3 0.9 1.0 1.1 2.5
13.30	20.1.17	82 $\pm$ 15.4 76 $\pm$ 5.17 79 $\pm$ 2.0 75 $\pm$ 5.64	73 $\pm$ 6.89 79 $\pm$ 14.50 77 $\pm$ 8.54 83 $\pm$ 0.48	83 $\pm$ 1.20 73 $\pm$ 4.30 83 $\pm$ 2.70 79 $\pm$ 3.28	89 $\pm$ 4.10 73 $\pm$ 5.36 81 $\pm$ 0.29 73 $\pm$ 4.16	1.0 1.0 1.2 1.1	1.2 0.9 1.4 0.9
20.1.19	20.1.15	85 $\pm$ 2.53 87 $\pm$ 5.6 64 $\pm$ 2.29 68 $\pm$ 0.75 58 $\pm$ 0.65 70 $\pm$ 0.88	82 $\pm$ 1.03 84 $\pm$ 4.19 67 $\pm$ 4.07 65 $\pm$ 5.05 49 $\pm$ 2.0 58 $\pm$ 5.69	77 $\pm$ 8.33 72 $\pm$ 2.85 67 $\pm$ 5.68 70 $\pm$ 2.31 46 $\pm$ 2.67 63 $\pm$ 1.50	87 $\pm$ 11.5 75 $\pm$ 1.25 71 $\pm$ 3.83 76 $\pm$ 1.00 58 $\pm$ 6.50 71 $\pm$ 3.09	0.9 0.8 1.0 1.0 0.8 0.8	1.1 0.9 1.1 1.2 1.2 0.9
20.1.14	20.1.22	68 $\pm$ 2.87 78 $\pm$ 2.17	63 $\pm$ 3.09 78 $\pm$ 2.33	52 $\pm$ 5.33 85 $\pm$ 3.57	51 $\pm$ 1.86 82 $\pm$ 2.48	0.8 1.1	0.8 1.1
20.1.20	20.1.20.1	94 $\pm$ 0.48 75 $\pm$ 4.26 75 $\pm$ 4.26	91 $\pm$ 0.96 69 $\pm$ 4.19 59 $\pm$ 2.93	79 $\pm$ 6.57 78 $\pm$ 3.67 58 $\pm$ 4.00	83 $\pm$ 4.50 79 $\pm$ 1.85 80 $\pm$ 3.81	0.9 1.0 0.8	0.9 0.8 1.1
20.1.16	20.55.19	94 $\pm$ 4.36 79 $\pm$ 2.00 82 $\pm$ 3.33 95 $\pm$ 0.84	93 $\pm$ 3.12 77 $\pm$ 3.54 87 $\pm$ 4.33 92 $\pm$ 1.83	84 $\pm$ 4.19 76 $\pm$ 2.20 77 $\pm$ 1.90 70 $\pm$ 1.30	87 $\pm$ 5.60 65 $\pm$ 2.00 81 $\pm$ 1.65 79 $\pm$ 3.93	0.9 1.0 0.9 0.7	0.9 1.2 1.4 0.9
20.55.1 6	20.55.26	74 $\pm$ 6.51 86 $\pm$ 2.45 94 $\pm$ 4.36 96 $\pm$ 2.63 85 $\pm$ 1.75 89 $\pm$ 4.10	78 $\pm$ 5.23 89 $\pm$ 1.83 93 $\pm$ 3.12 93 $\pm$ 1.04 85 $\pm$ 1.11 83 $\pm$ 1.20	79 $\pm$ 6.10 68 $\pm$ 1.00 87 $\pm$ 3.18 64 $\pm$ 2.89 81 $\pm$ 3.10 71 $\pm$ 3.10	89 $\pm$ 7.8 57 $\pm$ 8.00 79 $\pm$ 2.72 59 $\pm$ 3.38 85 $\pm$ 3.32 63 $\pm$ 1.50	1.0 0.8 0.9 0.7 0.9 0.8	0.9 0.9 0.9 1.1 0.6 0.8
Mean $\pm$ SE n = 30		78.03 $\pm$ 2.11	75.50 $\pm$ 2.59	73.87 $\pm$ 1.9 8	75.33 $\pm$ 2.08	0.96 $\pm$ 0.03	1.06 $\pm$ 0.06
T-test P Value		Paired =0.05 Unpaired =0.45		Paired =0.42 Unpaired =0.61		Paired = 0.09 Unpaired =0.13	
T-test of Actin.Tm: Actin.Tm.Tn			Paired=0.08 Unpaired=0.16		Paired=0.96 Unpaired=0.96		

3.6.6.5 Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Thin Filament Actin using Human Cardiac Troponin

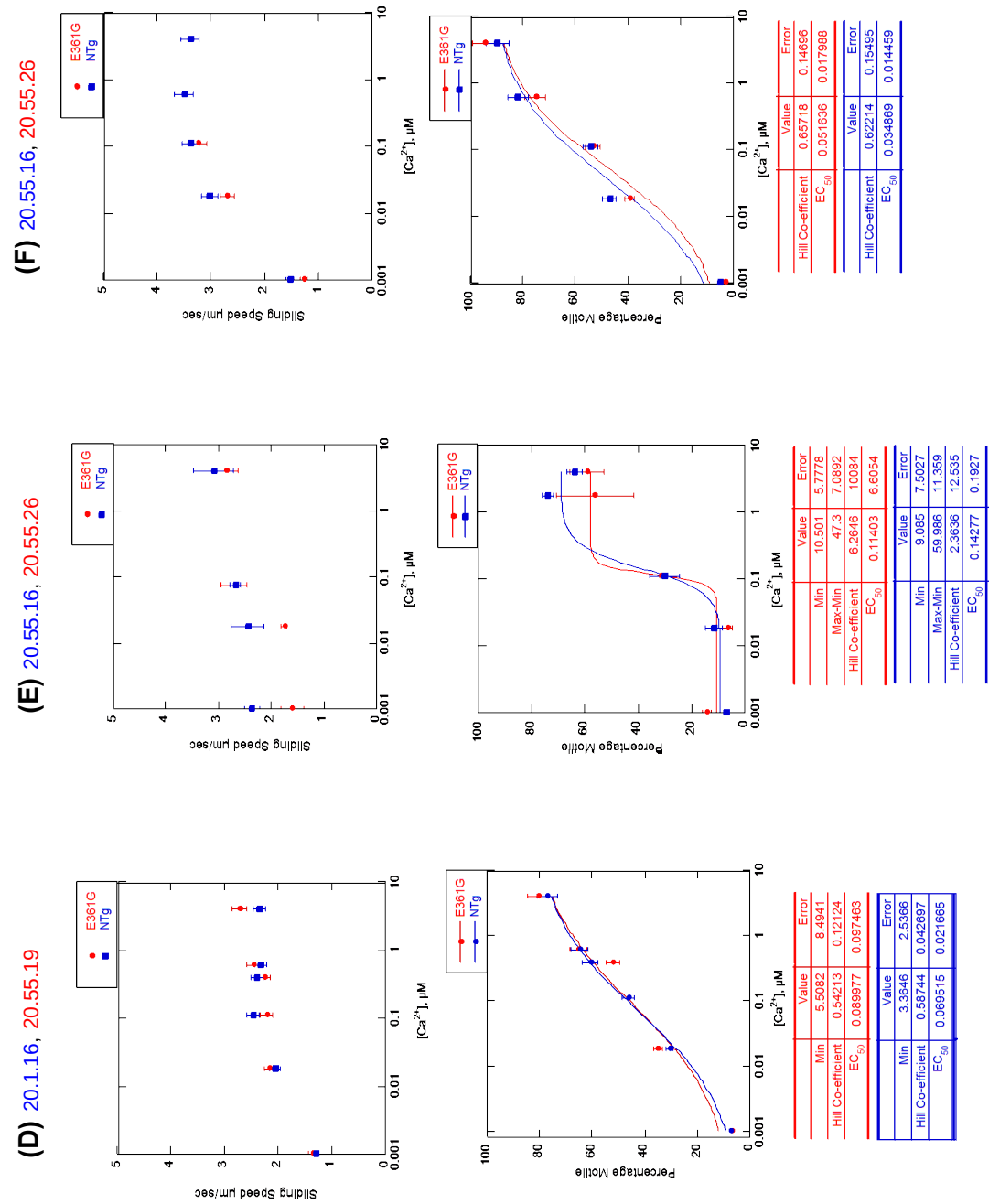
Initial experiments tested the effect of E361G thin filament actin in a non-failing thin filament. Thin filaments were reconstituted with E361G or non-transgenic thin filament actin, human cardiac tropomyosin, and cardiac troponin that was purified from non-failing human heart muscle (troponin I phosphorylation 2.1 mols/mol). The Ca^{2+} sensitivity of sliding and the percentage motile of thin filament actin were measured with actin from 6 paired non-transgenic and E361G mouse hearts (Figure 3.21 A-F, Table 3.29). The individual mouse heart sample numbers for non-transgenic and mutant actin are highlighted in blue and red respectively.

Tracking of E361G and non-transgenic actin sliding speeds produced large variability in the Ca^{2+} sensitivity plots. No accurate fit has been possible of the sliding speeds, and the points have simply been plotted.

Figure 3.21 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Thin Filament Actin



The percentage motility curves have had the following parameters fixed to obtain an accurate sigmoidal curve: Graph A (Max value fixed at 65%), Graph C (Min and Max values fixed at 6% and 79%).



Graph D (Max value fixed at 79%), Graph F (Min and Max values fixed at 3% and 89%).

Table 3.29 Ca^{2+} Sensitivity data for all E361G v Non-Transgenic Actin in a Non-Failing Thin Filament.

Mouse Line		EC ₅₀ Percentage Motility, μM		$\frac{\text{EC}_{50} \text{ NTg}}{\text{EC}_{50} \text{ E361G}}$
Non-Transgenic	E361G	Non-Transgenic	E361G	
13.3	20.1.17	1.437 ± 0.450	1.073 ± 0.283	1.34
20.1.19	20.1.15	0.261 ± 0.039	0.242 ± 0.001	1.08
20.1.16	20.55.19	0.039 ± 0.025	0.031 ± 0.016	1.27
20.1.16	20.55.19	0.070 ± 0.022	0.090 ± 0.098	0.77
20.55.16	20.55.26	0.143 ± 0.193	0.114 ± 6.606	1.25
20.55.16	20.55.26	0.035 ± 0.014	0.052 ± 0.018	0.68
Mean: ± SE (n = 6)		0.330 ± 0.224	0.267 ± 0.164	1.065 ± 0.114
Student's t-test (paired)		p = 0.34		single group, p = 0.592 (compared with 1)
(Unpaired)		p = 0.82		

The table shows the actual EC₅₀ values and the difference in EC₅₀ values expressed as a ratio of NTg to E361G for the percentage motility.

The Ca^{2+} sensitivity of E361G thin filament actin was the same as non-transgenic actin in both the sliding speed and percentage motile parameters. The thin filament actin sliding speeds were variable across a range of Ca^{2+} concentrations. Only the percentage motility could be accurately fitted to the Hill Equation. There was no significant difference between the percentage motility EC₅₀ values of non-transgenic and E361G actin (p = 0.34), and the ratio of EC₅₀ values was not significantly different from 1 (p = 0.59).

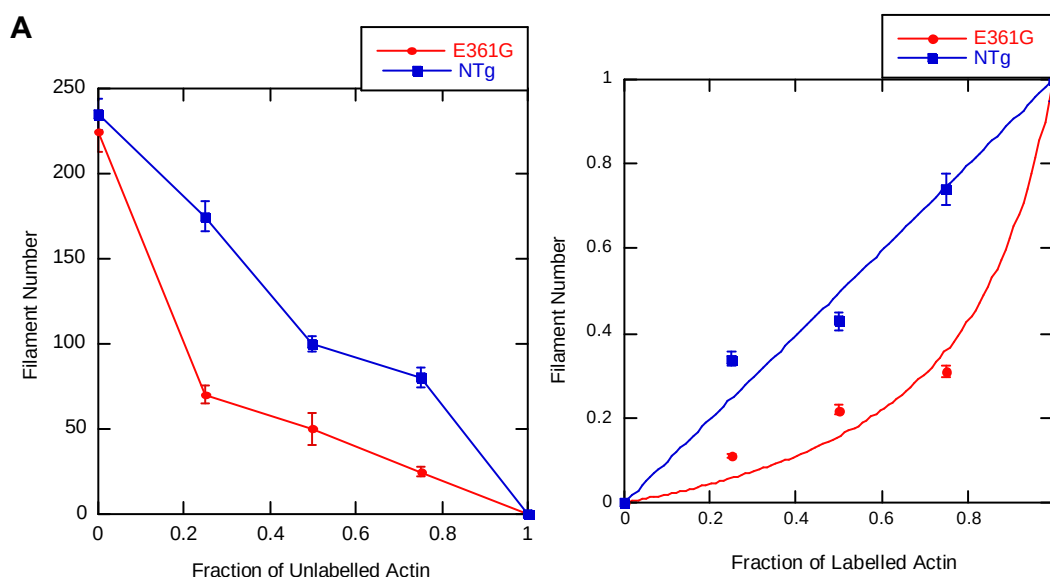
The change in sliding speed from low to high Ca^{2+} was minimal. The sliding speed of non-transgenic thin filament actin increased by 1.2 $\mu\text{m}/\text{sec}$ from low to high Ca^{2+} , and transgenic thin filament actin increased by 1.5 $\mu\text{m}/\text{sec}$. This is in contrast to the large change obtained when the mouse actin was extracted from an acetone powder (Figure 3.7), which produced an increase of 3.8 $\mu\text{m}/\text{sec}$. The percentage motile of non-transgenic and E361G thin filament actin both increased between 90-95% from low to high Ca^{2+} .

Considerable variation between experiments was observed in both the non-transgenic and E361G EC_{50} values. This variability between experiments was thought to be a characteristic of the mouse actin, and was different from the reproducible EC_{50} values that can be obtained between experiments using rabbit actin.

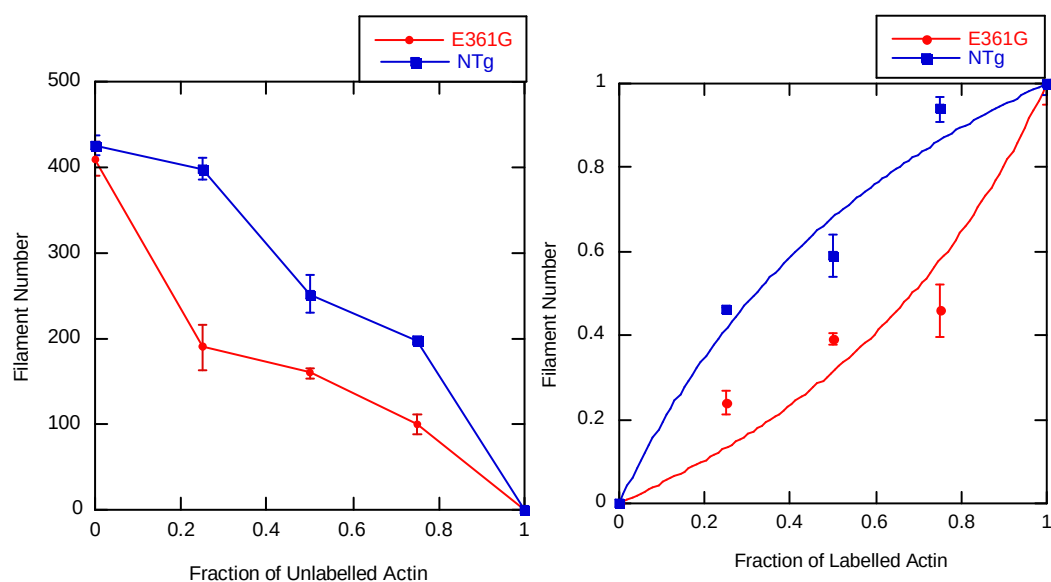
3.6.6.6 Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to α -Actinin

Altering the method of actin isolation did not change the $\ddot{U}$ -actinin competitive binding assay result. E361G thin filament actin was displaced from $\ddot{U}$ -actinin by lower concentrations of non-fluorescent actin compared to the non-transgenic actin. The assay has been repeated 3 times to produce reduced binding affinity of E361G actin by an average of 5.3-fold. The results are shown in Figure 3.22 A, B, and C. Fitting the number of filaments to the $\ddot{U}$ -actinin binding equation produces the graph on the right.

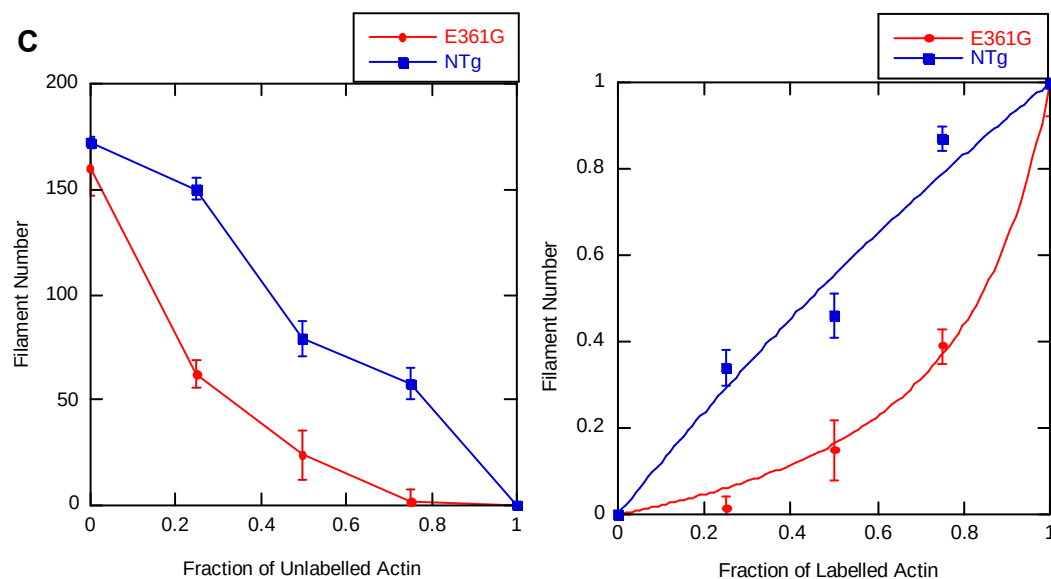
Figure 3.22 α -actinin Competitive Binding Assay of E361G and Non-transgenic Actin Filaments



There is 5.2-fold less affinity of E361G actin for $\ddot{U}$ -actinin compared to non-transgenic actin. Transgenic actin has a K value of 0.19 ± 0.03 , and non-transgenic actin has a K value of 0.98 ± 0.15 .

B

There is 4.6-fold less affinity of E361G actin for $\bar{U}$ -actinin compared to non-transgenic actin. Transgenic actin has a binding constant of 0.46 ± 0.12 , and non-transgenic actin has a binding constant of 2.13 ± 0.39 .

C

There is 6.2-fold less affinity of E361G actin for $\bar{U}$ -actinin compared to non-transgenic actin. Transgenic actin has a binding constant of 0.20 ± 0.08 , and non-transgenic actin has a binding constant of 1.24 ± 0.23 .

3.7 Discussion

Transgenic mice were generated to over express E361G mutant cardiac actin in their hearts. The mutant cardiac actin cDNA construct was provided by Dr. Kristen Nowak at the University of Western Australia, and inserted into the human cardiac actin sequence by Dr. Charles Redwood at the University of Oxford. The transgenic expression was restricted to the heart using an alpha-myosin heavy chain promoter construct kindly provided by Dr. Mike Gollob and originally produced in the laboratory of Dr. Jeffrey Robbins. The transgenic mice were generated by Professor Dominic Wells by pronuclear injection of gel purified transgenic constructs into the pronucleus of fertilised mouse eggs on a C57BL10 x CBA/Ca hybrid background. Successive screening by PCR followed by 2-D Electrophoresis showed one stably expressing transgenic line (361.20) had been generated, which expressed E361G at 50% of the total actin in the hearts of the transgenic mice. Extracting actin directly from native thin filaments preserved the 50% mutant actin content in the F-actin.

3.7.1 Native Human Sequence Thin Filaments

Actin is the most highly conserved contractile protein. An important reason for choosing this DCM mutation in actin was that the amino acid sequence of cardiac actin in man and mouse is identical, and the distribution of cardiac and skeletal actin isoforms in the heart (ratio of 1:4) is reported to be the same in most species (Vandekerckhove *et al.*, 1986, Bergen *et al.*, 2003). Thin filaments were reconstituted from purified polymeric actin from the mouse hearts, along with human cardiac tropomyosin and troponin isolated from non-failing human heart muscle. The amino acid sequences, post-translational modifications and the phosphorylation levels of the non-transgenic thin filaments should have been the same as in non-failing human heart muscle, and the E361G thin filament should represent the conditions of the muscle of familial DCM patients. Line 20 expressed 50% mutation, which is the expected ratio in a dominant mutation and has been found with mutations in myosin (Fananapazir *et al.*, 1993) and tropomyosin (Bottinelli *et al.*, 1998).

3.7.2 Stability of E361G Mutant Actin

The E361G actin was largely present in an insoluble state when it was extracted from a mouse-muscle acetone powder, indicating that monomeric E361G actin tended to aggregate. The small proportion of the E361G actin monomer present did not polymerise unless phalloidin was present. This property has also been observed in other actin mutations, including the nemaline myopathy mutations M132V (Marston *et al.*, 2004) and the EGFP-tagged D286G actin (J-J.Feng, PhD Thesis 2007). This instability in laboratory conditions causes problems for experimentation, but does not necessarily affect the mutant mouse *in vivo*, since 50% mutant was clearly incorporated into myofibrils of line 20 mice.

3.7.3 Reproducibility of the *In Vitro* Motility Assay

The F-actin extracted initially from E361G transgenic mouse hearts contained no mutation. These comparative experiments with non-transgenic actin have provided a measure of the reproducibility of simultaneous actin isolations as well as the functional measurements by the *in vitro* motility assay (Table 3.30). The finding that actin extracted from two hearts can produce functionally identical actin is strong evidence that any later differences seen between thin filament transgenic E361G-actin and non-transgenic actin are genuine and caused by the mutation.

Table 3.30 Comparison of Mouse Actin from Acetone Powder

	Non-Transgenic Actin	Transgenic Actin
Actin Sliding Speed ($\mu\text{m}/\text{sec}$)	3.03 ± 0.25	3.03 ± 0.27
Paired = 1.0, Unpaired = 1.0 (n = 6)		
Actin % Motility	98 ± 0.45	95 ± 1.58
Paired = 0.13, Unpaired = 0.08 (n = 6)		
Actin.Tm sliding speed ($\mu\text{m}/\text{sec}$)	3.73 ± 0.33	3.47 ± 0.31
Paired = 0.001, Unpaired = 0.57 (n = 6)		
Actin.Tm % motility	86 ± 4.07	88 ± 7.30
Paired = 0.61, Unpaired = 0.79 (n = 6)		
Actin.Tm.Tn sliding speed + Ca^{2+}	3.85 ± 0.31	3.93 ± 0.42
Paired = 0.67, Unpaired = 0.88 (n = 6)		
Actin.Tm.Tn sliding speed - Ca^{2+}	1.8 ± 0.41	1.88 ± 0.45
Paired = 0.56, Unpaired = 0.89 (n = 6)		
Actin.Tm.Tn % motility + Ca^{2+}	88 ± 5.32	89 ± 5.14
Paired = 0.72, Unpaired = 0.93 (n = 6)		
Actin.Tm.Tn % motility - Ca^{2+}	3.33 ± 1.23	4.33 ± 1.50
Paired = 0.33, Unpaired = 0.62 (n = 6)		
Ca^{2+} sensitivity of sliding EC_{50} value (n = 1)	0.190 ± 0.09	0.138 ± 0.06
Ca^{2+} sensitivity of % motile EC_{50} value (n = 1)	0.054 ± 0.007	0.057 ± 0.007
$\bar{\text{U}}$ -actinin binding (K value) (n = 1)	0.423 ± 0.006	0.381 ± 0.002

3.7.4 Comparisons of Transgenic Mouse and Skeletal Rabbit Actin

Skeletal rabbit actin is regularly used in our laboratory and hence motor and regulatory comparisons can be made with the non-transgenic mouse actin (Table 3.31). Although it is interesting to draw these comparisons, it is important to note that the two actins are not entirely identical. Rabbit F-actin has been

depolymerised and the G-actin purified on sephacryl300 before a second polymerisation, giving it a higher purity than the mouse actin. The rabbit actin *in vitro* motility data was provided from studies by A. Messer (PhD Thesis, 2007). Thin filaments were reconstituted using skeletal rabbit actin, human cardiac tropomyosin, and troponin extracted from donor non-failing human heart muscle. All other conditions of the *in vitro* motility assay were identical.

The motor properties of mouse actin proved comparable with the rabbit actin. There was no significant difference between the sliding speeds ($p = 0.52$), although the percentage motility of rabbit actin was significantly less ($p = <0.0001$). The effects of human cardiac tropomyosin on mouse and rabbit actin sliding speed were not significantly different ($p = 0.40$). Both mouse and rabbit actin sliding speed increased by 10%. The regulatory properties were compared by reconstituting thin filaments with mouse or rabbit actin, along with human cardiac tropomyosin and troponin. Measurements of actin sliding speeds and percentage motility were made at high and low Ca^{2+} . Mouse and rabbits actins were switched on and off optimally within similar ranges of human cardiac troponin (40-60nM). At high Ca^{2+} , the rabbit actin exhibited greater increases in its sliding speed (26%) compared to mouse actin (3%). However, the percentage motility increases over actin.tropomyosin were not different for rabbit (5%) or mouse actin (3%). At low Ca^{2+} , the sliding speeds of both actins were switched off to a similar degree, and the percentage motility of both actins was reduced to approximately 3%. Only one Ca^{2+} activation curve was assayed with non-transgenic mouse actin (Figure 3.7). The pCa_{50} and the hill co-efficient were in the range of those obtained with rabbit actin, as was the range of velocity and percentage motility between high and low Ca^{2+} . Finally, the $\bar{U}$ -actinin binding experiments indicated that mouse actin affinity was not different from rabbit, and both had a K value of 0.8.

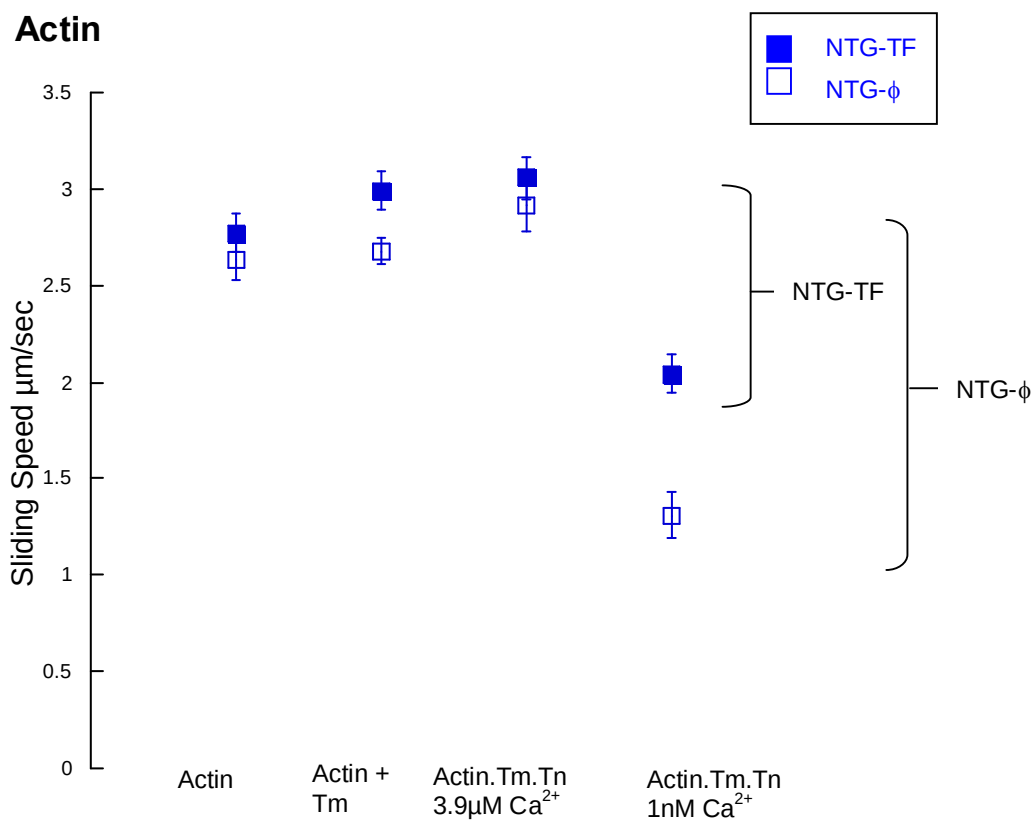
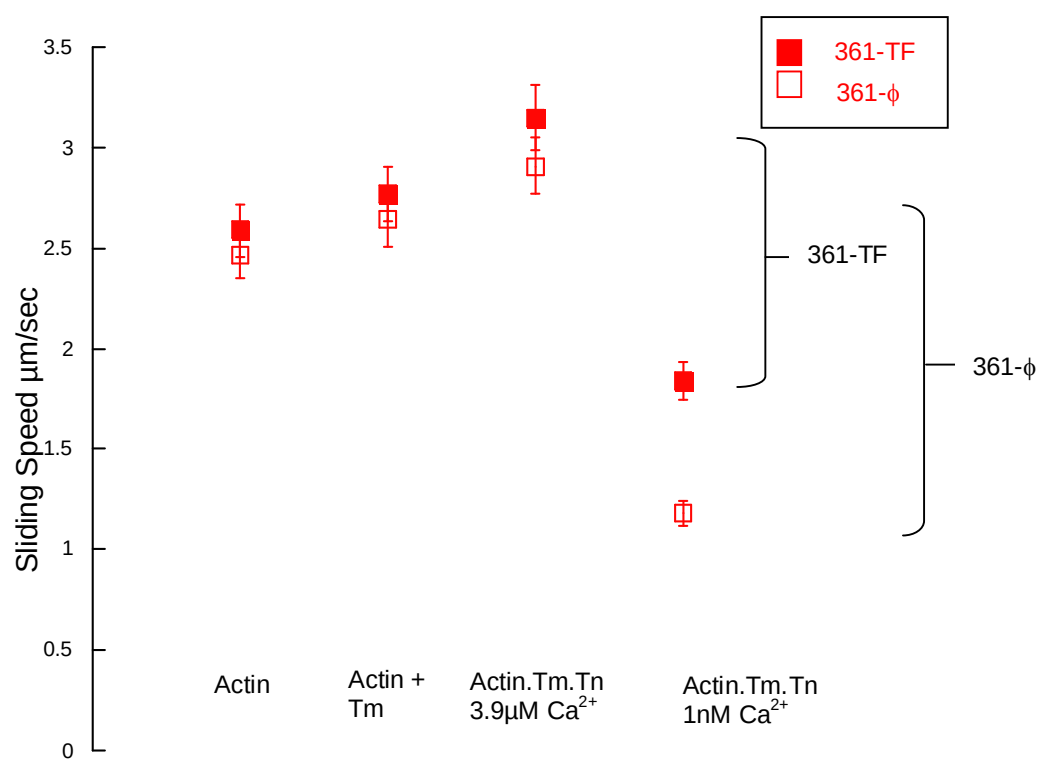
In conclusion, the function of non-transgenic mouse actin in the *in vitro* motility assay was very similar to the well-studied rabbit actin. This is to be expected, as they have the same amino acid sequence and were prepared by similar methods.

Table 3.31 Comparison of Mouse and Rabbit Actin

	Non-Transgenic Actin	Rabbit Actin
Actin Sliding Speed ($\mu\text{m}/\text{sec}$)	3.03 ± 0.25 (n = 6)	3.23 ± 0.18 (n = 16)
Unpaired = 0.52		
Actin % Motility	98 ± 0.45 (n = 6)	75 ± 2.24 (n = 16)
Unpaired = <0.0001		
Actin.Tm sliding speed ($\mu\text{m}/\text{sec}$)	3.73 ± 0.33 (n = 6)	3.47 ± 0.143 (n = 16)
Unpaired = 0.40		
Actin.Tm % motility	86 ± 4.07 (n = 6)	85 ± 1.91 (n = 16)
Unpaired = 0.87		
Actin.Tm.Tn sliding speed + Ca^{2+}	3.85 ± 0.31 (n = 6)	4.36 ± 0.23 (n = 16)
Unpaired = 0.25		
Actin.Tm.Tn sliding speed - Ca^{2+}	1.8 ± 0.41 (n = 6)	1.47 ± 0.04 (n = 16)
Unpaired = 0.20		
Actin.Tm.Tn % motility + Ca^{2+}	88 ± 5.32 (n = 6)	89.23 ± 1.90 (n = 16)
Unpaired = 0.78		
Actin.Tm.Tn % motility - Ca^{2+}	3.33 ± 1.23 (n = 6)	3.10 ± 0.56 (n = 16)
Unpaired = 0.84		
Ca^{2+} sensitivity of sliding EC_{50} value	0.190 ± 0.09	0.195 ± 0.026
Hill Co-efficient	1.67 ± 0.98	2.21 ± 0.48
Ca^{2+} sensitivity of % motile EC_{50} value	0.054 ± 0.007	0.08 ± 0.07
Hill Co-efficient	8.30 ± 3.06	2.17 ± 0.41
$\bar{\text{U}}$ -actinin binding (K value)	0.833 ± 0.433	0.843 ± 0.042

3.7.5 Comparison of Actin- ϕ and Thin Filament Actin

Preparing native thin filaments and then removing troponin and tropomyosin successfully isolated pure mouse actin. This avoided the unstable monomeric state of the E361G actin. The thin filament actin contained 50% mutant actin, which was the same as the whole muscle it was derived from. However, the functional properties of thin filament actin were not identical to actin- ϕ (Figure 3.23). The sliding speed of non-transgenic thin filament actin was 5% faster than non-transgenic actin- ϕ , and this difference was increased to 10% upon titration with human cardiac tropomyosin. Non-transgenic thin filament actin had a sliding speed of 2.99 $\mu\text{m}/\text{sec}$ with 60nM tropomyosin compared to 2.68 $\mu\text{m}/\text{sec}$ with actin- ϕ .tropomyosin. Comparing the actin regulation at high and low Ca^{2+} has shown that at optimum troponin concentrations, the acetone powder extracted non-transgenic actin- ϕ showed a greater increase in its sliding speed (9%) than thin filament actin (2%), and a greater switch off in its sliding speed (51%) compared to thin filament actin (32%). A similar trend was shown for E361G thin filament actin compared to E361G- ϕ (Figure 3.24). The sliding speed of E361G thin filament actin was 5% faster than E361G- ϕ , and 3% faster than E361G- ϕ actin upon titration with human cardiac tropomyosin. At high Ca^{2+} , E361G- ϕ showed a lower increase in its sliding speed (9%) compared to thin filament actin (14%), although the switch off was greater for E361G- ϕ (56%) compared to thin filament actin (34%).

Figure 3.23 Sliding Speed Comparisons of Non-transgenic**Figure 3.24 Sliding Speed Comparisons of E361G Actin**

Overall, a greater range in sliding speeds from high to low Ca^{2+} was shown for actin- ϕ compared to thin filament actin (1.6 $\mu\text{m}/\text{sec}$ and 0.7 $\mu\text{m}/\text{sec}$ respectively). The inability of thin filament extracted mouse actin to produce adequate Ca^{2+} curves based on the actin sliding speeds may have been due to this small net increase. In contrast, the change in percentage motility from high to low Ca^{2+} for the thin filament actin (90%) was comparable to that of actin- ϕ (96%), and suitable Ca^{2+} curves could be plotted for thin filament mouse actin.

Variability was also observed in the percentage motility EC_{50} values for mouse thin filament extracted actin. This variability was encountered between repeated experiments but not within a paired assay. Non-transgenic thin filament actin had a 5.5-fold range in EC_{50} percentage motility values, and E361G actin had a 4.4-fold range. Such large variability was not shown for actin- ϕ (Figure 3.13) or for rabbit actin, which only had a 2-fold range over 6 measurements. The variability of the EC_{50} values may simply be due to the nature of the thin filament mouse actin, since mouse actin- ϕ and rabbit actin thin filaments were similarly incorporated with non-failing human cardiac tropomyosin and troponin. However, the troponin could potentially have been the cause of the variability. Mouse actin thin filaments were reconstituted with troponin extracted from the more recently received non-failing human heart samples N10-N12. Mouse actin- ϕ and rabbit thin filaments were reconstituted with troponin extracted from five different heart samples (N5-N9). Although all these heart samples were all from non-failing human donors there was the potential for a difference.

3.7.6 Comparison of Non-Transgenic Thin Filament Actin with E361G Thin Filament Actin

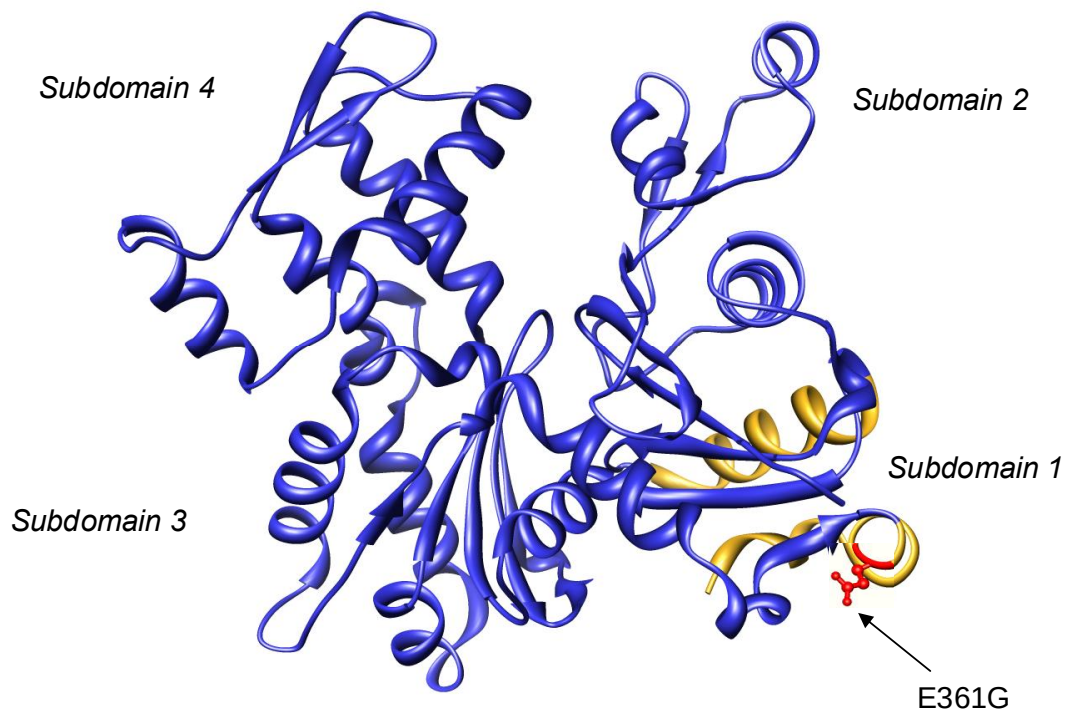
E361G thin filament actin moved 7% more slowly than non-transgenic thin filament actin, and this was a significant difference ($p = 0.003$). The slower sliding speed of E361G thin filament actin was maintained upon titration with human cardiac tropomyosin ($p = 0.001$). The percentage motility of E361G thin filament actin was not statistically different to non-transgenic thin filament actin ($p = 0.58$), and the addition of tropomyosin did not alter this trend. The slower sliding speed of E361G actin is in contrast to the findings of Wong *et al.* They

reported no impact on actomyosin function on studies of E361G expressed in yeast and purified for *in vitro* motility functional studies (Wong *et al.*, 2002). Similarly, Johara *et al.* found that mutations in this region of actin did not affect the sliding speed in the *in vitro* motility assay (Johara *et al.*, 1993).

E361G and non-transgenic thin filament actin regulation of sliding speed was not statistically different at high Ca^{2+} ($p = 0.14$) or low Ca^{2+} ($p = 0.06$), nor was the percentage motility statistically different at high Ca^{2+} ($p = 0.42$) or low Ca^{2+} ($p = 0.85$). The Ca^{2+} sensitivity of E361G and non-transgenic thin filament actin across a range of Ca^{2+} concentrations showed no differences in the sliding speeds or the EC_{50} values of percentage motility ($p = 0.34$). The ratio of percentage motility EC_{50} values was also not significantly different from 1 ($p = 0.59$).

3.7.7 Altered α -Actinin Binding

Two identical subunits of $\tilde{\text{U}}$ -actinin ($M_r = 100,000$) bind to actin within residues 112-125 and 360-372 (Lebart *et al.*, 1993). Due to the position of the E361G mutation in this universally conserved region of actin that binds to $\tilde{\text{U}}$ -actinin (Figure 3.25), the mutation has been postulated to cause a defective force transmission in cardiac myocytes (Olson *et al.*, 1998). Indeed, the studies reported in this thesis support this idea, as the E361G thin filament actin had a 5.3-fold reduction in $\tilde{\text{U}}$ -actinin binding compared to non-transgenic actin (Figure 3.22). Similarly, Wong *et al.* showed a 3-fold reduction in E361G actin expressed in yeast (Wong *et al.*, 2002). Since the E361G mutation showed only moderate changes in actomyosin function, the reduction in binding of E361G actin for $\tilde{\text{U}}$ -actinin and the resulting impairment of cardiac muscle function may be enough alone to cause the DCM phenotype.

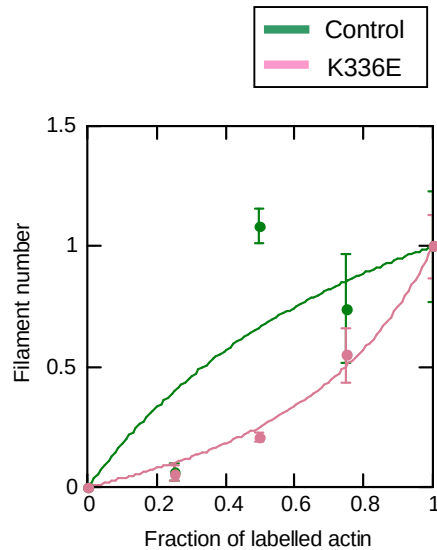
Figure 3.25 Location of E361G

Location of E361G (red) on the actin molecule in relation to the ̢-actinin binding sites (residues 112-125 and 360-372, shown in yellow). Molecular graphic image was drawn from atomic coordinates published by (Otterbein *et al.*, 2001) (pdb file 1J6Z), using the USSF Chimera package (Pettersen *et al.*, 2004).

This hypothesis is further supported by data from two other actin mutations (K336E and R372H), which have been shown to have a reduced binding affinity for ̢-actinin. Both are also located close to the ̢-actinin binding site. K336E is a lysine to glutamate substitution and is the first report of HCM associated with nemaline myopathy and a mutation in ACTA1 (D'Amico *et al.*, 2006). Nemaline myopathy is characterised by general muscle weakness and the presence of nemaline bodies (Sanoudou and Beggs, 2001). More than 100 ACTC1 mutations are known to cause the disease (Costa *et al.*, 2004). The ̢-actinin competitive binding assay was carried out on a skeletal muscle biopsy sample from a 6-month-old patient with this mutation (J-J.Feng, PhD Thesis 2007), and K336E actin bound to ̢-actinin with a 7-fold lower affinity than control actin (Figure 3.26). This weaker binding of actin to ̢-actinin could impair the force transmission in both skeletal and cardiac muscle, which was

compatible with the patient's symptoms of muscle weakness and cardiomyopathy.

Figure 3.26 α -actinin Competitive Binding Assay of K336E



Data supplied by J-J Feng (PhD Thesis 2007). Three K336E α -actinin plots were carried out. One average result, defined by the α -actinin binding equation is shown above. Over three separate experiments, the control actin had a k value of 3.5 ± 1.86 and the K336E actin had a K value of 0.5 ± 0.18 , indicating a 7-fold difference in affinity for α -actinin.

Additionally, drosophila studies on the ACTA1 nemaline myopathy mutation R372H have suggested that the primary defect caused by this mutation is reduced α -actinin-actin binding (Sevdali and Sparrow, 2007). Transgenic flies heterologous for the R372H mutation complete the normal IFM myogenesis and hatching flies can fly. However, they become flightless by day 7 and the *Drosophila* show the equivalent of human nemaline myopathy. Immunofluorescence and electron microscopy showed severe loss of muscle structure resulting from the R372H mutation. From the known α -actinin binding sites on the actin monomer, the mutation is thought to decrease the strength of the F-actin/ α -actinin binding, leading to muscle damage and the breakdown of the muscle structure.

3.7.8 The DCM Phenotype

Previous *in vitro* studies on cardiac muscle thin filament mutations associated with DCM, have reported that there are features common to all DCM mutations (Robinson *et al.*, 2002, Mirza *et al.*, 2005). These include a slower sliding speed, diminished ATPase rates and a decreased Ca^{2+} sensitivity (1.3-2.0 fold increase in EC_{50}). DCM has therefore been described as having a hypocontractile phenotype (Marston, 2003). Collectively, these findings were made from five troponin T mutants (R131W, R141W, R205L, Δ Lys-210, D270N), two β -tropomyosin mutants (E40K and E54K), and one troponin C mutation (G159D). Human cardiac troponin was reconstituted using recombinant troponin T, I and C subunits. Wild type troponin and tropomyosin containing either troponin T or troponin C DCM mutants were compared in thin filaments reconstituted with human Ala-Ser β -tropomyosin (for ATPase assays) or native human cardiac tropomyosin (for *in vitro* motility analysis). Tropomyosin mutations were studied using thin filaments reconstituted using recombinant troponin (ATPase assays) or native human troponin (*in vitro* motility).

These common functional properties reported for DCM-causing mutations are however in contrast to the E361G actin *in vitro* motility results covered in this chapter. The cross bridge turnover difference was small (E361G actin 7% slower) and the Ca^{2+} sensitivity was indistinguishable from actin of non-transgenic littermates (EC_{50} NTg/E361G = 1.1, $p = 0.59$). However, one important difference to note is that the studies reported in this thesis tested E361G actin in combination with non-failing human cardiac troponin that had native phosphorylation levels of 3.1 mols Pi/mol TnT and 2.1 mol/Pi/mol TnI (Messer *et al.*, 2006). Previous studies used recombinant troponin that was not phosphorylated (Robinson *et al.*, 2002, Mirza *et al.*, 2005). The phosphorylation state of the troponin incorporated into the thin filament may have uncovered the genuine functional effect caused by the E361G mutation. *In vitro* motility results of the E361G actin in combination with altered levels of troponin phosphorylation are covered in the next chapter.

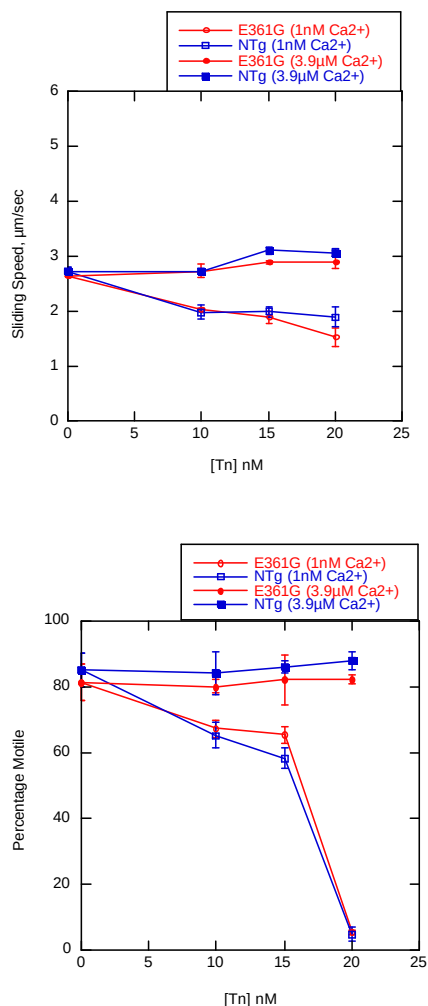
4. THE EFFECT OF TROPONIN PHOSPHORYLATION ON E361G THIN FILAMENT FUNCTION

Chapter 3 outlined the *in vitro* motility results of E361G actin when incorporated with human cardiac tropomyosin and native human cardiac troponin. In contrast to previous DCM mutations investigated by *in vitro* motility assay (Robinson *et al.*, 2002, Mirza *et al.*, 2005), E361G actin did not cause any significant difference in Ca^{2+} regulation. However, it has previously been reported that the type of troponin incorporated into the thin filament could reveal different effects of mutant proteins (Bing *et al.*, 2000). The effect of non-transgenic and E361G actin in combination with rabbit skeletal troponin (in which troponin I is not phosphorylated) was therefore investigated.

4.1 Effects of Rabbit Skeletal Muscle Troponin on Non-Transgenic and E361G Actin Motility at High and Low Ca^{2+}

The regulation of thin filaments containing E361G and non-transgenic actin with human cardiac tropomyosin and rabbit skeletal muscle troponin was investigated. 10-20nM rabbit skeletal troponin was titrated with 10nM actin and 60nM tropomyosin at high Ca^{2+} (3.9 μM) and low Ca^{2+} (1nM). 20nM troponin produced an optimum switch-off and switch-on of the percentage motility and the sliding speeds of actin filaments (Figure 4.1). 8 measurements from 2 paired non-transgenic and E361G heart hearts are tabulated in Table 4.1 to Table 4.4.

Figure 4.1 Effects of Rabbit Skeletal Troponin on Non-Transgenic and E361G filament Motility at $3.9\mu\text{M Ca}^{2+}$ and 1nM Ca^{2+}



At low Ca^{2+} , the sliding speed was reduced by 26% for non-transgenic actin and 27% for E361G actin. The decrease in the sliding speed for both actins was significant (<0.0001 and 0.005 respectively). At optimum troponin concentrations, the sliding speed of E361G actin was 10% slower than non-transgenic actin, although this was not a significant difference in the paired t-test ($p = 0.22$). The percentage motility at low Ca^{2+} was reduced by 86% for both non-transgenic and E361G actin to 11% and 10% respectively. These values were similar to the conditions seen with rabbit actin, which was reduced by 96% motility at low Ca^{2+} . At optimum troponin concentrations, there was no

significant difference in the percentage motility of non-transgenic and E361G actin ($p = 0.67$).

At high Ca^{2+} , non-transgenic actin sliding speed increased 13% over actin.tropomyosin and E361G actin increased 19% over actin.tropomyosin. These increases were larger than when human cardiac troponin was used (2% and 14% respectively). At high Ca^{2+} , no π crossing over π of the sliding speeds was observed. E361G actin had a 16% slower sliding speed when incorporated with tropomyosin compared to non-transgenic actin with tropomyosin ($p = 0.03$), and at high Ca^{2+} the sliding speed of E361G was still slower by 9%. This difference at high Ca^{2+} was significant ($p = 0.03$). The percent motility of E361G actin filaments was 6% less than non-transgenic actin at high Ca^{2+} . This difference is not significant however ($p = 0.14$).

Table 4.1 Effect of Rabbit Skeletal Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (1nM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
20.1.19	20.1.15	2.6 ± 0.02	2.4 ± 0.04	1.9 ± 0.1	2.0 ± 0.17	0.73	0.83
		3.3 ± 0.05	3.0 ± 0.14	2.5 ± 0.13	2.4 ± 0.06	0.76	0.80
		2.7 ± 0.28	2.3 ± 0.06	1.9 ± 0.06	1.7 ± 0.13	0.70	0.74
		3.1 ± 0.12	2.8 ± 0.11	2.1 ± 0.47	2.5 ± 0.27	0.68	0.89
13.31	20.1	2.1 ± 0.06	1.9 ± 0.23	1.7 ± 0.15	1.7 ± 0.05	0.81	0.89
		3.2 ± 0.06	1.9 ± 0.36	1.9 ± 0.43	1.5 ± 0.08	0.59	0.79
		2.9 ± 0.03	2.7 ± 0.13	2.1 ± 0.09	1.2 ± 0.42	0.72	0.44
		2.7 ± 0.09	2.6 ± 0.03	1.9 ± 0.19	1.5 ± 0.17	0.70	0.58
Mean ± SE n = 8		2.83 ± 0.14	2.45 ± 0.14	2.00 ± 0.08	1.81 ± 0.16	0.71 ± 0.02	0.75 ± 0.06
T-test P Value		Paired = 0.03 Unpaired = 0.08		Paired = 0.22 Unpaired = 0.32		Paired = 0.58 Unpaired = 0.58	
T-test of Actin.Tm: Actin.Tm.Tn			Paired= <.0001 Unpaired=0.0002		Paired= 0.005 Unpaired= 0.001		

Table 4.2 Effect of Rabbit Skeletal Troponin on Filament Percentage Motility at Low Ca^{2+} (1nM Ca^{2+})

Mouse Line		% Motility Actin.Tm		% Motility Actin.Tm.Tn (1nM Ca ²⁺)		Relative % Motility of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NT	E361G
20.1.19	20.1.15	83 ± 1.41	80 ± 8.26	12 ± 2.22	16 ± 3.50	0.14	0.20
		78 ± 6.51	75 ± 5.32	19 ± 1.78	14 ± 0.41	0.24	0.19
		65 ± 2.65	63 ± 6.23	6 ± 0.65	5 ± 2.50	0.09	0.08
		87 ± 4.26	86 ± 1.56	16 ± 9.7	17 ± 7.05	0.18	0.20
13.31	20.1	89 ± 2.16	85 ± 1.32	5 ± 1.73	13 ± 2.00	0.06	0.15
		75 ± 3.16	77 ± 2.63	12 ± 4.57	6 ± 2.33	0.16	0.08
		75 ± 2.36	61 ± 2.31	14 ± 1.70	5 ± 2.56	0.19	0.08
		85 ± 5.12	81 ± 5.63	5 ± 2.14	6 ± 0.65	0.06	0.07
Mean ± SE n = 8		79.63 ± 2.81	76.00 ± 3.32	11.13 ± 1.88	10.25 ± 1.85	0.14± 0.02	0.13 ± 0.02
T-test P Value		Paired = 0.06 Unpaired= 0.42		Paired = 0.67 Unpaired= 0.74		Paired= 0.73 Unpaired= 0.78	
T-test of Actin.Tm: Actin.Tm.Tn				Paired= <.0001 Unpaired=<.0001		Paired=<.0001 Unpaired= <.0001	

Table 4.3 Effect of Rabbit Skeletal Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)

Mouse Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NT	E361G
20.1.19	20.1.15	2.6 ± 0.02	2.4 ± 0.04	2.5 ± 0.06	2.1 ± 0.08	0.96	0.88
		3.3 ± 0.05	3.0 ± 0.14	3.4 ± 0.27	2.9 ± 0.14	1.03	1.03
		2.7 ± 0.28	2.3 ± 0.06	3.2 ± 0.03	3.2 ± 0.26	1.19	1.39
		3.1 ± 0.12	2.8 ± 0.11	3.3 ± 0.04	2.5 ± 0.08	1.06	0.89
13.31	20.1	2.1 ± 0.06	1.9 ± 0.23	3.7 ± 0.09	3.7 ± 0.03	1.76	1.95
		3.2 ± 0.06	1.9 ± 0.36	3.3 ± 0.12	2.9 ± 0.07	1.03	1.53
		2.9 ± 0.03	2.7 ± 0.13	3.1 ± 0.06	3.1 ± 0.07	1.07	1.14
		2.7 ± 0.09	2.6 ± 0.03	3.1 ± 0.08	2.9 ± 0.11	1.15	1.12
Mean ± SE n = 8		2.83 ± 0.14	2.45 ± 0.14	3.20 ± 0.12	2.91 ± 0.17	1.16 ± 0.09	1.24 ± 0.13
T-test P Value		Paired = 0.03 Unpaired = 0.08		Paired = 0.03 Unpaired = 0.19		Paired = 0.29 Unpaired = 0.60	
T-test of Actin.Tm: Actin.Tm.Tn			Paired= 0.08 Unpaired= 0.06		Paired= 0.12 Unpaired= 0.05		

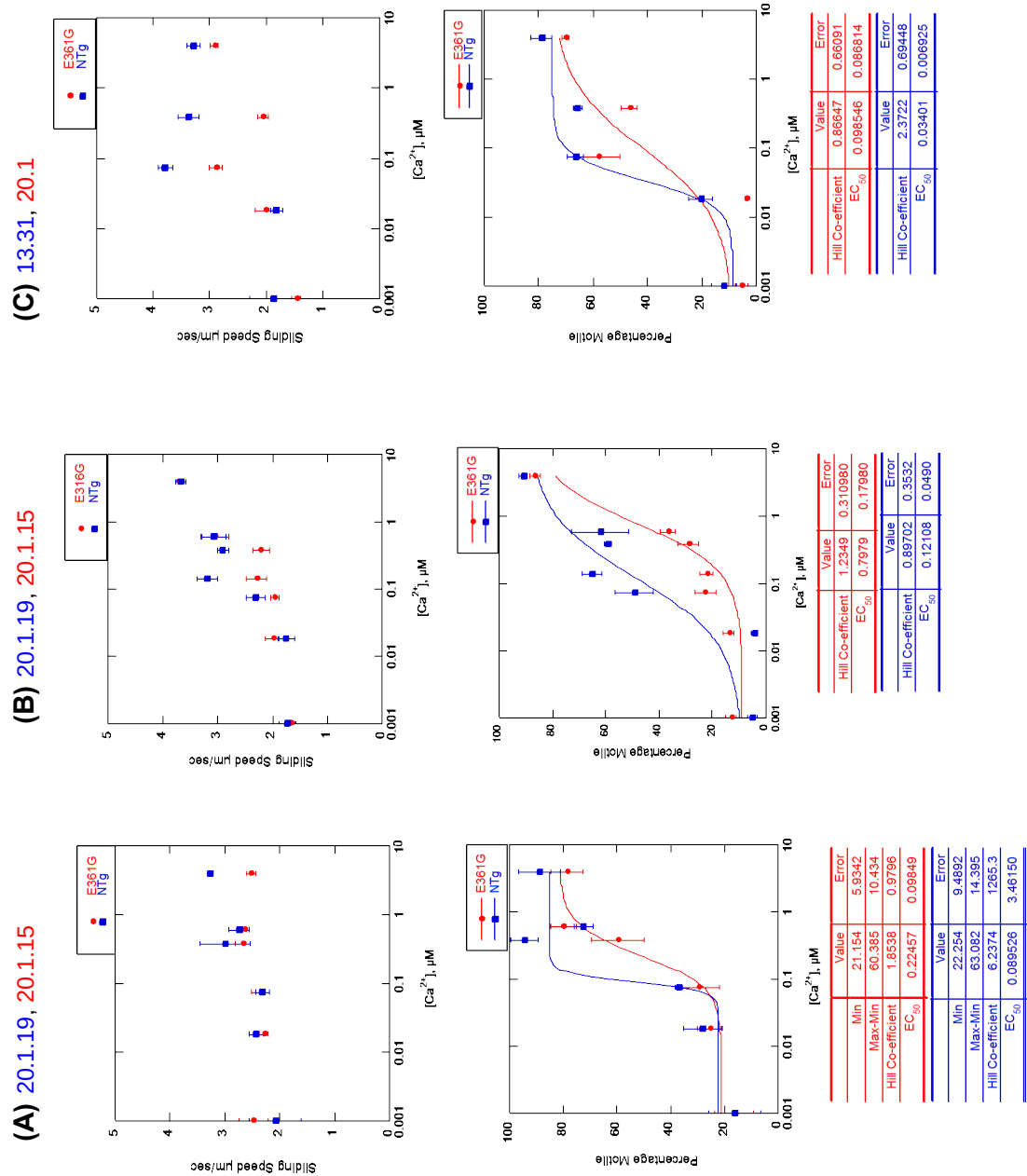
Table 4.4 Effect of Rabbit Skeletal Troponin on Filament Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)

Mouse Line		% Motility Actin.Tm		% Motility Actin.Tm.Tn (3.9μM Ca ²⁺)		Relative % Motility of Actin.Tm.Tn: Actin.Tm	
NTg	E361G	NTg	E361G	NTg	E361G	NTg	E361G
20.1.19	20.1.15	83 ± 1.41	80 ± 8.26	85 ± 3.30	74 ± 3.81	1.02	0.93
		78 ± 6.51	75 ± 5.32	77 ± 8.33	87 ± 11.5	0.99	1.16
		65 ± 2.65	63 ± 6.23	67 ± 5.68	71 ± 3.83	1.03	1.13
		87 ± 4.26	86 ± 1.56	89 ± 7.5	79 ± 6.13	1.02	0.92
13.31	20.1	89 ± 2.16	85 ± 1.32	91 ± 1.58	87 ± 2.02	1.02	1.02
		75 ± 3.16	77 ± 2.63	79 ± 3.93	70 ± 1.31	1.05	0.91
		75 ± 2.36	61 ± 2.31	68 ± 1.00	57 ± 8.0	0.91	0.93
		85 ± 5.12	81 ± 5.63	88 ± 2.74	83 ± 1.32	1.04	1.02
Mean ± SE n = 8		79.63 ± 2.81	76.00 ± 3.32	80.50 ± 3.31	76.00 ± 3.60	1.01 ± 0.02	1.00± 0.03
T-test P Value		Paired = 0.06 Unpaired = 0.42		Paired = 0.14 Unpaired = 0.37		Paired = 0.85 Unpaired = 0.85	
T-test of Actin.Tm: Actin.Tm.Tn				Paired = 0.50 Unpaired = 0.84		Paired = 1.0 Unpaired = 1.0	

4.1.1 Comparing Ca^{2+} Concentration Curves of Non-Transgenic and E361G Actin with Rabbit Skeletal Troponin

Thin filaments were reconstituted with 10nM E361G or non-transgenic actin, 60nM human cardiac tropomyosin, and 20nM rabbit skeletal troponin. The Ca^{2+} sensitivity of sliding and the percentage motile of actin filaments were measured 3 times from 2 separate paired non-transgenic and E361G mouse hearts (Figure 4.2 A-C). No accurate fit was possible with the sliding speeds, and the points have simply been plotted.

Figure 4.2 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Thin Filament Actin with Skeletal Troponin



The percentage motility fits have had the following parameters fixed to obtain an accurate sigmoidal curve: Graph B (Min and Max values fixed at 9% and 80%), Graph C (Min and Max values fixed at 8.75% and 66.25%).

Table 4.5 Ca^{2+} Regulation of Thin Filaments containing Non-Transgenic and E361G Actin with Skeletal Troponin

Mouse Line		EC ₅₀ Percentage Motility, μM		EC ₅₀ NTg EC ₅₀ E361G
Non-Transgenic	E361G	Non-Transgenic	E361G	
20.1.19	20.1.15	0.090 ± 3.462	0.225 ± 0.099	0.40
20.1.19	20.1.15	0.121 ± 0.049	0.798 ± 0.180	0.15
13.31	20.1	0.034 ± 0.007	0.099 ± 0.087	0.34
Mean: $\pm$ SE		0.082 ± 0.025	0.374 ± 0.215	0.297 ± 0.075

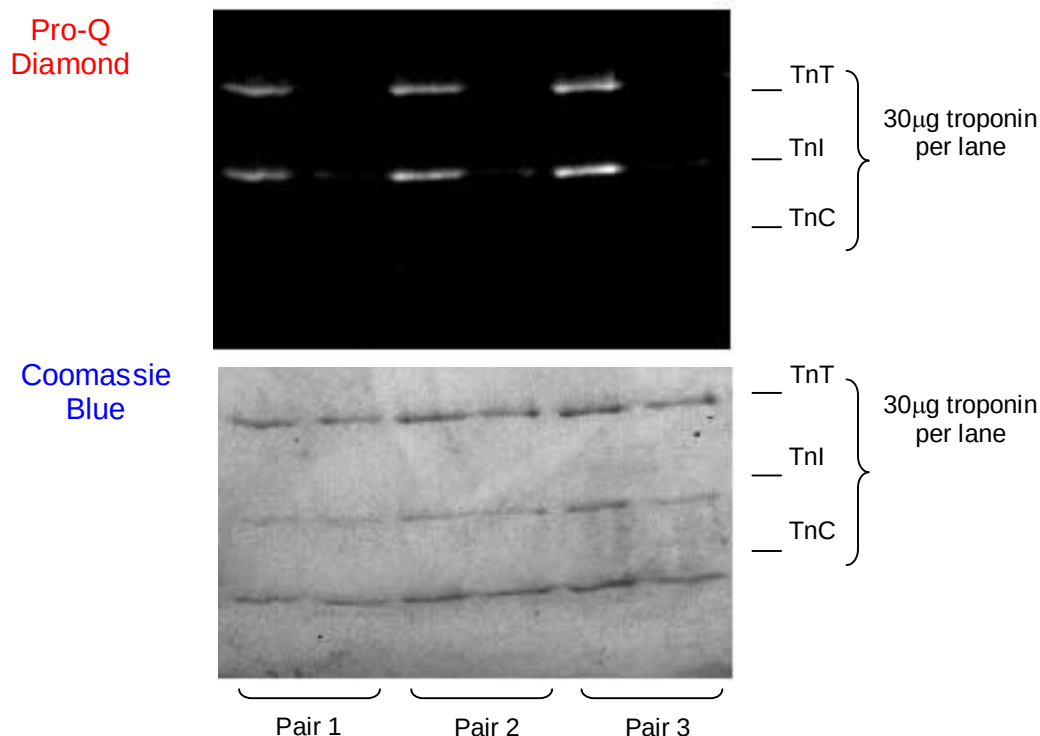
When skeletal muscle troponin was incorporated into the thin filament in place of the cardiac troponin, E361G actin had a lower Ca^{2+} sensitivity compared to non-transgenic actin. The E361G actin EC₅₀ was approximately 4.6-fold higher (Table 4.5). The use of skeletal rabbit troponin therefore unmasked a functional difference between non-transgenic and E361G actin which was not detectable in the system that used human cardiac regulatory proteins. A similar functional effect was produced to previous studies that reconstituted unphosphorylated recombinant troponin into the thin filament (Robinson *et al.*, 2002, Mirza *et al.*, 2005).

One obvious difference between skeletal and cardiac troponin is the N-terminal extension of cardiac troponin I which is phosphorylated by PKA. The phosphorylation status of the troponin used in the E361G thin filament therefore appeared to be potentially important. The effect of human cardiac troponin dephosphorylation on the Ca^{2+} regulation of motility was investigated to determine whether the E361G thin filaments were responsive to altered phosphorylation levels of cardiac troponin.

4.2 Effect of Cardiac Troponin Dephosphorylation on the Ca^{2+} dependence of E361G and Non-transgenic Actin Motility

Dephosphorylation was achieved by using 1.4 units/ml acid phosphatase. Samples were analysed by SDS-PAGE and stained with Pro-Q Diamond.

Figure 4.3 SDS-PAGE of Non-Failing Human Cardiac Troponin



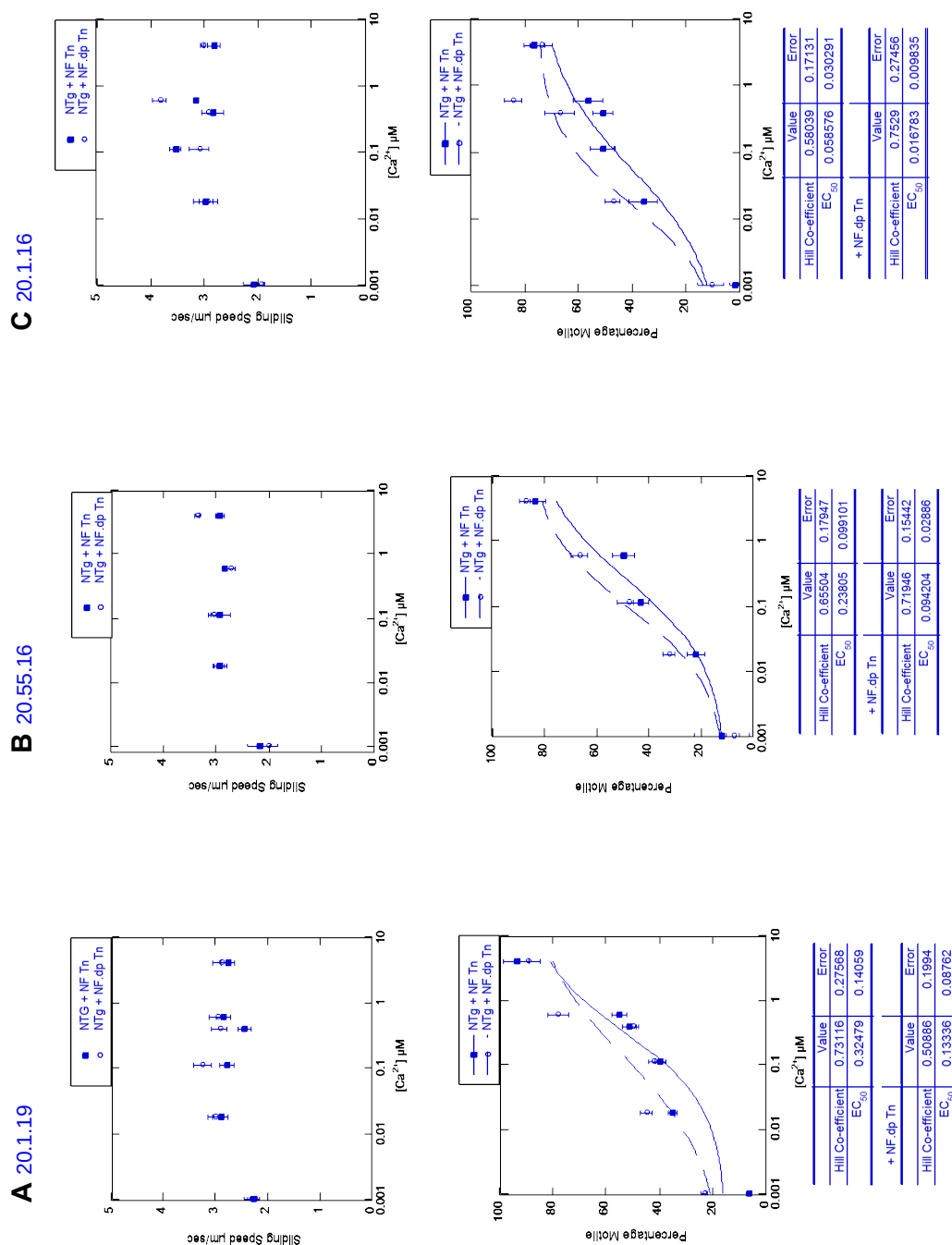
SDS-PAGE gel of 3 non-failing troponin pairs showing the effect of acid phosphatase on troponin T and I phosphorylation. Stained with (A) Pro-Q Diamond (for phosphoproteins) and (B) Coomassie Blue for total protein levels

There were three types of experiments that reconstituted dephosphorylated non-failing troponin into the thin filament. (1) Control experiments used thin filaments reconstituted with non-transgenic actin in combination with either native or dephosphorylated non-failing human cardiac troponin (Figure 4.4). (2) Mutant E361G actin was reconstituted into thin filaments in combination with either native or dephosphorylated non-failing human cardiac troponin (Figure 4.5), and finally (3) non-transgenic and E361G actin was reconstituted with non-failing dephosphorylated troponin (Figure 4.6).

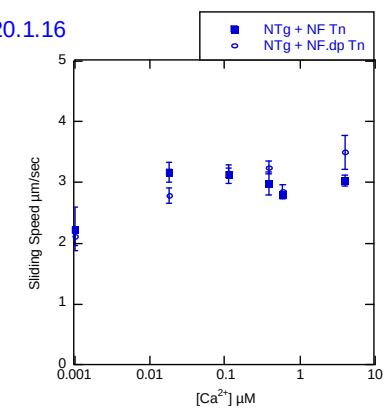
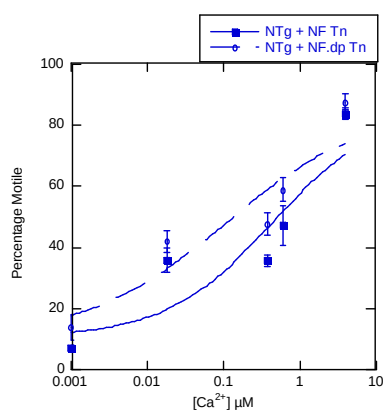
The control experiments will first be described (Figure 4.4). The Ca^{2+} sensitivity of the sliding speed and the percentage motile of actin filaments were measured for 5 non-transgenic mouse hearts. The actin sliding speeds produced variable measurements across all Ca^{2+} concentrations. Only the percentage motility could be accurately fitted to the Hill Equation.

Dephosphorylation of the troponin shifted the percentage motility curve to the left and increased the Ca^{2+} sensitivity of non-transgenic actin filaments. The dephosphorylated troponin produced a 2.98-fold increase in Ca^{2+} sensitivity, and this difference was significant ($p = 0.003$) (Table 4.6).

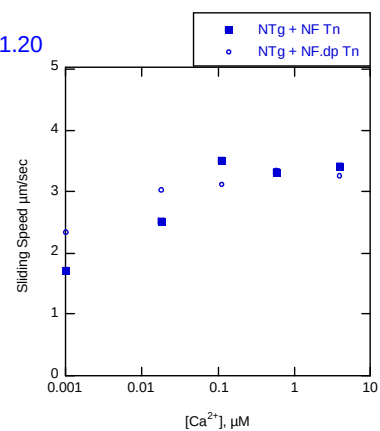
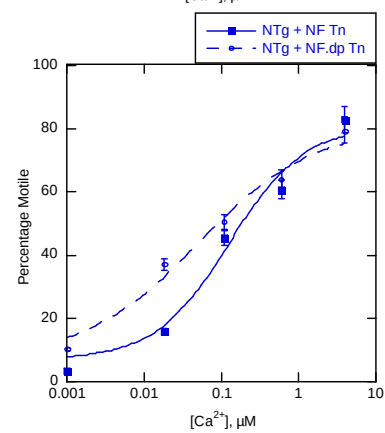
Figure 4.4 Effect of Non-Transgenic Actin in Combination with Native or Dephosphorylated Troponin



The Ca²⁺ curves have had the following maximum and minimum parameters fixed to accurately represent the fit: Graph A (min fixed at 15%, max fixed at 77%), Graph B (min fixed at 10%, max fixed at 76%).

D 20.1.16

	Value	Error
Hill Co-efficient	0.62033	0.36679
EC ₅₀	0.43474	0.33053
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	0.47565	0.18884
EC ₅₀	0.10855	0.09092

E 20.1.20

	Value	Error
Hill Co-efficient	0.90311	0.19849
EC ₅₀	0.12827	0.033112
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	0.58107	0.087598
EC ₅₀	0.050939	0.013296

Graph D (min fixed at 10.5%, max fixed at 75.25%), Graph E (min fixed at 7%, max fixed at 74%)

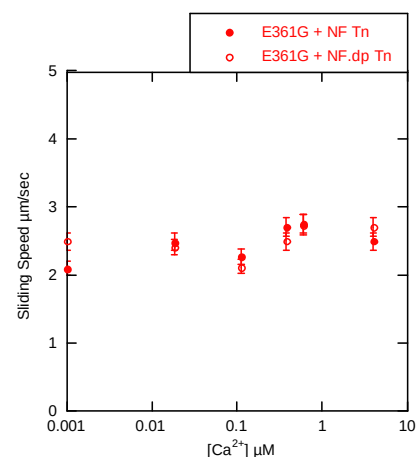
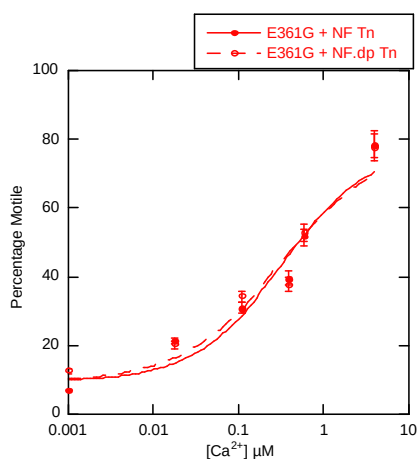
Table 4.6 Ca^{2+} Sensitivity Data for Non-Transgenic Actin Incorporated with Native or Dephosphorylated Troponin

Non-Transgenic Mouse Line	EC ₅₀ Percentage Motility, μM		$\frac{\text{EC}_{50} \text{ NF}}{\text{EC}_{50} \text{ NF.dp}}$
	Non-Failing	Non-Failing.dp	
20.1.19	0.325 ± 0.141	0.133 ± 0.088	2.44
20.55.16	0.238 ± 0.099	0.094 ± 0.029	2.53
20.1.16	0.059 ± 0.030	0.017 ± 0.010	3.47
20.1.16	0.433 ± 0.331	0.109 ± 0.091	3.97
20.1.20	0.128 ± 0.033	0.051 ± 0.013	2.51
Mean: ± SE	0.237 ± 0.067	0.081 ± 0.021	2.984 ± 0.3146
T test (paired)	p = 0.03		single group, p = 0.003 (compared with 1)
T test (Unpaired)	p = 0.06		

The effect on the Ca^{2+} sensitivity of E361G actin filaments when reconstituted with dephosphorylated troponin was then investigated (Figure 4.5). Incorporating E361G actin with either native or dephosphorylated troponin resulted in no Ca^{2+} sensitivity difference between the filaments ($p = 0.61$). The ratio of percentage motility EC₅₀ values was not significantly different from 1 ($p = 0.74$) (Table 4.7).

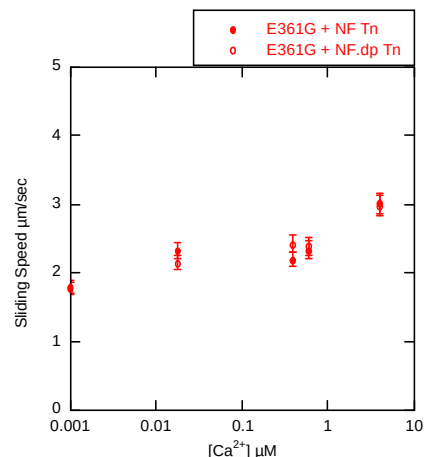
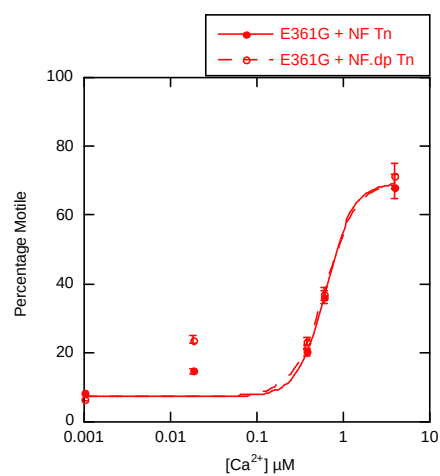
Figure 4.5 Effect of Troponin Dephosphorylation on E361G Actin Motility

A 20.55.19



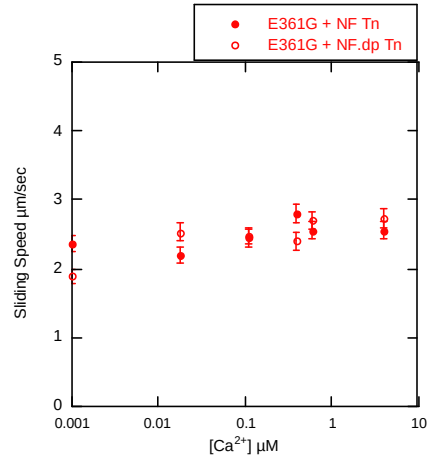
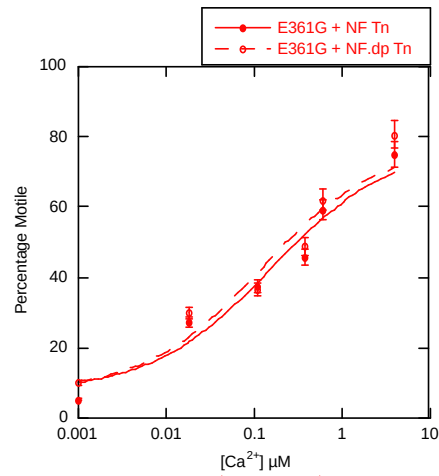
	Value	Error
Hill Co-efficient	0.77114	0.20582
EC ₅₀	0.30941	0.090761
+ Nf.dp Tn		
Hill Co-efficient	0.84425	0.22111
EC ₅₀	0.33076	0.086995

B 20.1.20.1

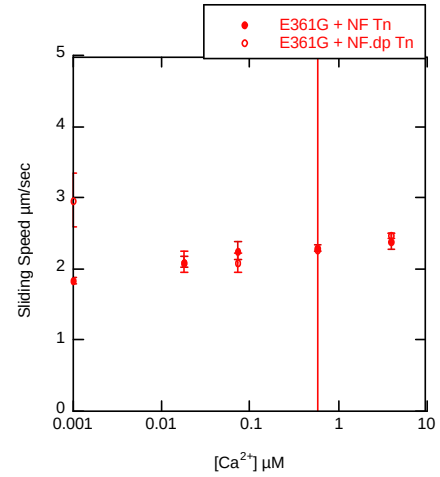
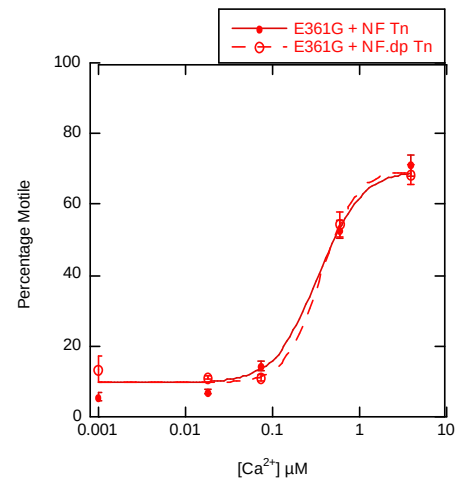


	Value	Error
Hill Co-efficient	2.3893	2.2134
EC ₅₀	0.60428	0.16027
+ NF.dp Tn		
Hill Co-efficient	2.6805	1.1086
EC ₅₀	0.62323	0.072605

The following Ca^{2+} curves had the maximum and minimum parameters fixed to accurately represent the fit: Graph A (min fixed at 9.5%, max fixed at 68.5%), Graph B (min fixed at 7.4%, max fixed at 62%).

C 20.55.26

	Value	Error
Hill Co-efficient	0.64289	0.13725
EC ₅₀	0.15313	0.045936
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	0.66003	0.17332
EC ₅₀	0.11992	0.045325

D 20.55.26

	Value	Error
Hill Co-efficient	1.7556	0.42143
EC ₅₀	0.33529	0.058115
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	2.1741	0.63574
EC ₅₀	0.35813	0.058378

Graph C (min fixed at 7.5%, max fixed at 70%), Graph D (min fixed at 9.6%, max fixed at 60%).

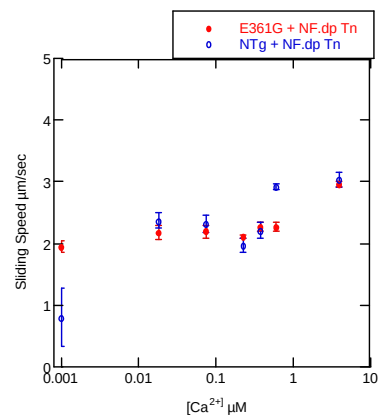
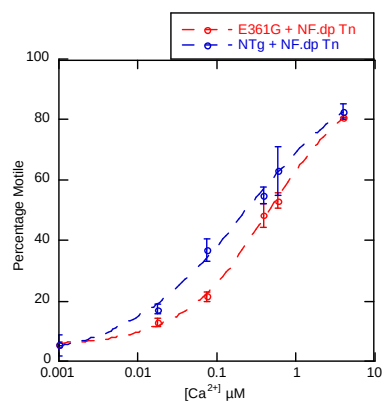
Table 4.7 Ca^{2+} Sensitivity Data for E361G Actin Incorporated with Native or Dephosphorylated Troponin

Mouse Line	EC ₅₀ Percentage Motility, μM		$\frac{\text{EC}_{50} \text{ NF}}{\text{EC}_{50} \text{ NF.dp}}$
	Non-Failing	Non-Failing.dp	
20.55.19	0.309 ± 0.091	0.331 ± 0.087	0.93
20.1.20.1	0.604 ± 0.160	0.623 ± 0.073	0.97
20.55.26	0.153 ± 0.046	0.120 ± 0.045	1.28
20.55.26	0.335 ± 0.058	0.358 ± 0.058	0.94
Mean: $\pm$ SE	0.350 ± 0.094	0.358 ± 0.103	1.03 ± 0.08
T test (paired)	p = 0.61		Single group, p = 0.74 (compare with 1)

The final set of experiments used E361G and non-transgenic actin in combination with non-failing dephosphorylated troponin (Figure 4.6). Dephosphorylating the troponin shifted the non-transgenic actin percentage motility curve to the left. E361G actin had a 2-fold lower Ca^{2+} sensitivity than non-transgenic actin, and this difference was significant ($p = 0.01$).

Figure 4.6 Effect of Troponin Dephosphorylation on Non-Transgenic and E361G Actin Motility

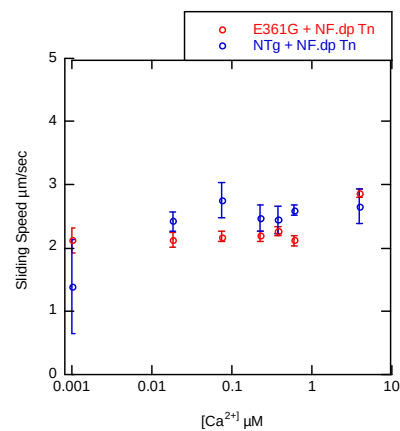
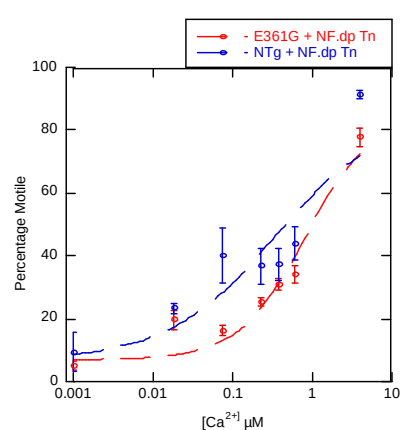
A 20.1.14, 20.1.22



	Value	Error
Min	5.3605	1.9937
Max-Min	89.169	7.5635
Hill Co-efficient	0.78088	0.10782
EC ₅₀	0.45761	0.094151

	Value	Error
Min	0.70687	4.9411
Max-Min	98.275	15.005
Hill Co-efficient	0.56855	0.12871
EC ₅₀	0.23206	0.095655

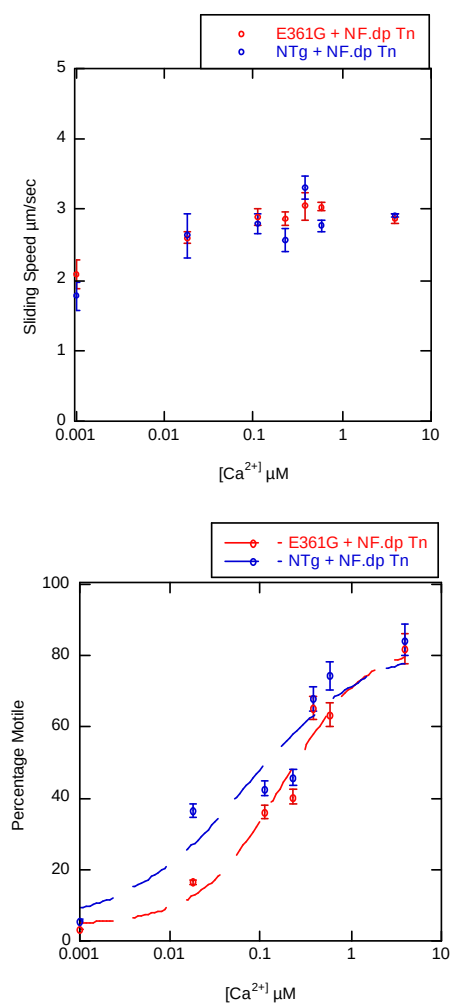
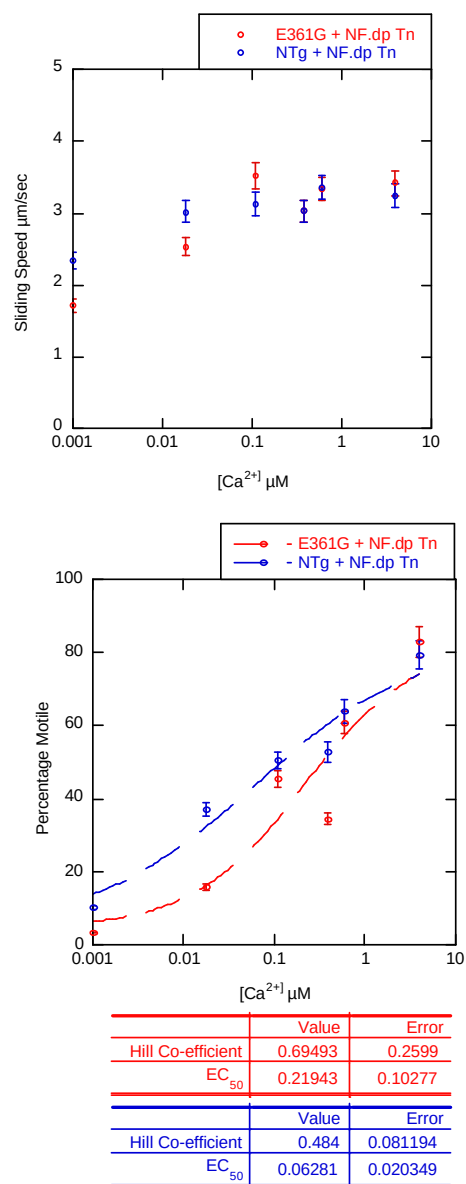
B 20.1.14, 20.1.22



	Value	Error
Hill Co-efficient	1.0378	0.23329
EC ₅₀	0.80656	0.18067

	Value	Error
Hill Co-efficient	0.64506	0.26323
EC ₅₀	0.33485	0.16421

Ca²⁺ curve B had the maximum and minimum parameters fitted to accurately represent the fit: (min fixed at 7%, max fixed at 78%)

C 20.1.16, 20.55.19**D** 20.1.16, 20.55.19

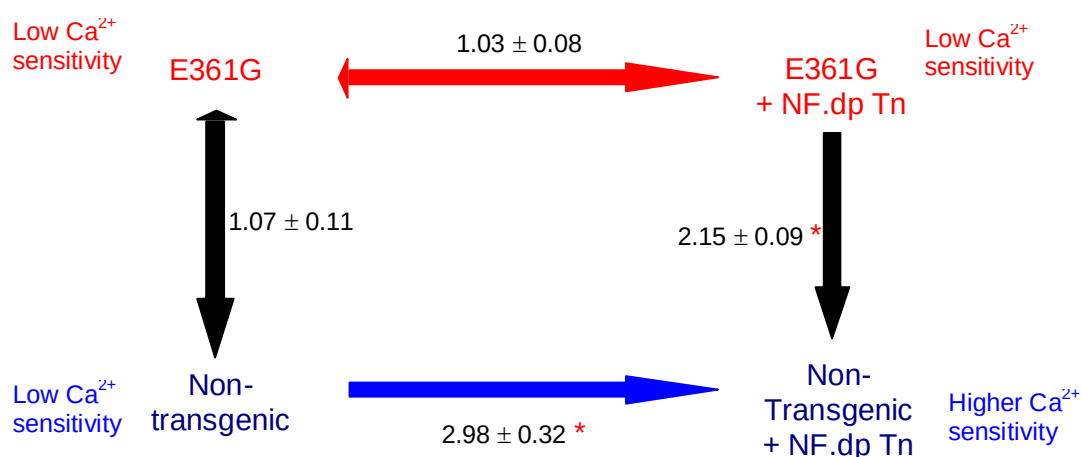
Graph C (min fixed at 4.5%, max fixed at 79%), Graph D (min fixed at 4.5%, max fixed at 79%).

Table 4.8 Ca^{2+} Sensitivity Data for Non-Transgenic and E361G Actin Incorporated with Dephosphorylated Troponin

Mouse Line		EC ₅₀ Percentage Motility, μM		$\frac{\text{EC}_{50} \text{ NTg}}{\text{EC}_{50} \text{ E361G}}$
		Non-Transgenic	E361G	
20.1.14	20.1.22	0.569 ± 0.129	0.781 ± 0.108	0.73
20.1.14	20.1.22	0.335 ± 0.164	0.807 ± 0.181	0.42
20.1.16	20.55.19	0.073 ± 0.031	0.175 ± 0.030	0.42
20.1.16	20.55.19	0.063 ± 0.020	0.219 ± 0.103	0.29
Mean: $\pm$ SE		0.260 ± 0.121	0.496 ± 0.173	0.465 ± 0.09
T test (paired)		p = 0.06		Single group, p = 0.01 (compared to 1)
T test (Unpaired)		p = 0.31		

To summarise, E361G-transgenic and non-transgenic actins were compared using dephosphorylated and native non-failing human cardiac troponin in all possible combinations. The figure below shows the difference in EC₅₀ values for the percentage motile of filaments. Thin filaments containing E361G actin did not appear to respond to changes in troponin I phosphorylation.

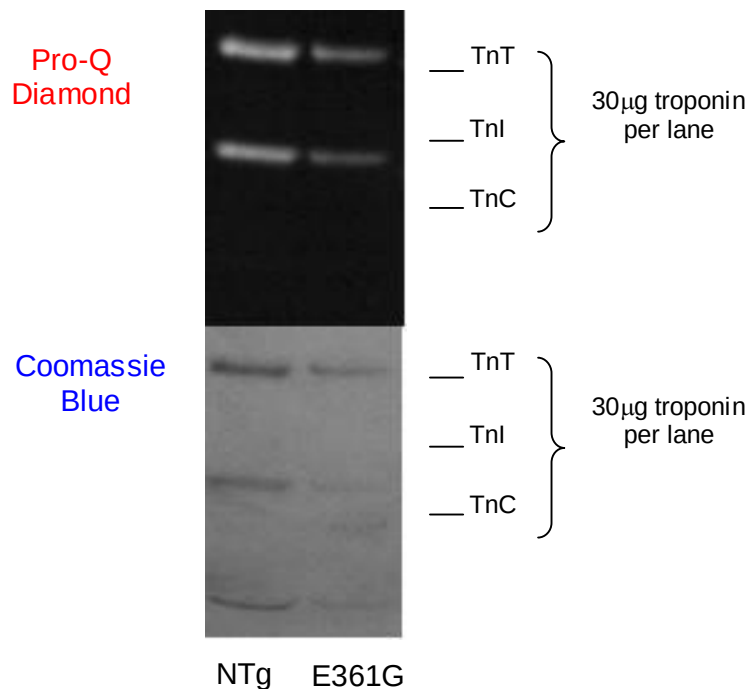
Figure 4.7 E361G and Non-transgenic Actin in combination with Phosphorylated and Dephosphorylated Troponin



The average EC₅₀ value differences (percentage motility) of E361G-transgenic and non-transgenic actin. The larger of the two sided arrows denotes the direction of higher EC₅₀ value. Starred values* denote a statistically significant difference.

A reported feature of acquired human heart failure is the substantial dephosphorylation of troponin I, which may contribute towards a blunted inotropic and lusitropic response (Messer *et al.*, 2006). It was therefore important to evaluate the phosphorylation status of non-transgenic and E361G mouse troponin (Figure 4.8).

Figure 4.8 Phosphorylation of Non-Transgenic and E361G-Transgenic Mouse Troponin



	Non- Transgenic Troponin		E361G Troponin	
	ProQ/Coomassie	Mols Pi/mol—TnI	ProQ/Coomassie	Mols Pi/mol TnI
TnT	1.49	2.54	1.54	2.62
TnI	2.12	3.60	2.07	3.52

No difference was found in the phosphorylation of troponin I and troponin T of non-transgenic and E361G mouse troponin. Compared with human cardiac troponin, the mouse cardiac troponin T was less highly phosphorylated (2.5 compared to 3.1 mols Pi/mol TnI), and the troponin I was more phosphorylated (3.6 compared to 2.1 mols Pi/mol TnT). However, the E361G mutation did not

affect this ratio. In addition to measuring the phosphorylation level of pure troponin, myofibrils from non-transgenic and E361G hearts were analysed (Figure 4.9). Myofibrils were separated by SDS-PAGE and the bands detected by the Pro-Q Diamond phosphoprotein gel stain were normalised to the total protein levels in each band (stained by SYPRO Ruby). E361G transgenic myofibrils showed a high level of phosphorylation for myosin binding protein-C (MyBP-C), troponin T (TnT), troponin I (TnI) and myosin light chain 2 (MLC-2) that was indistinguishable from non-transgenic myofibrils.

Figure 4.9 SDS-PAGE and Ratios of E361G and Non-Transgenic Myofibrils

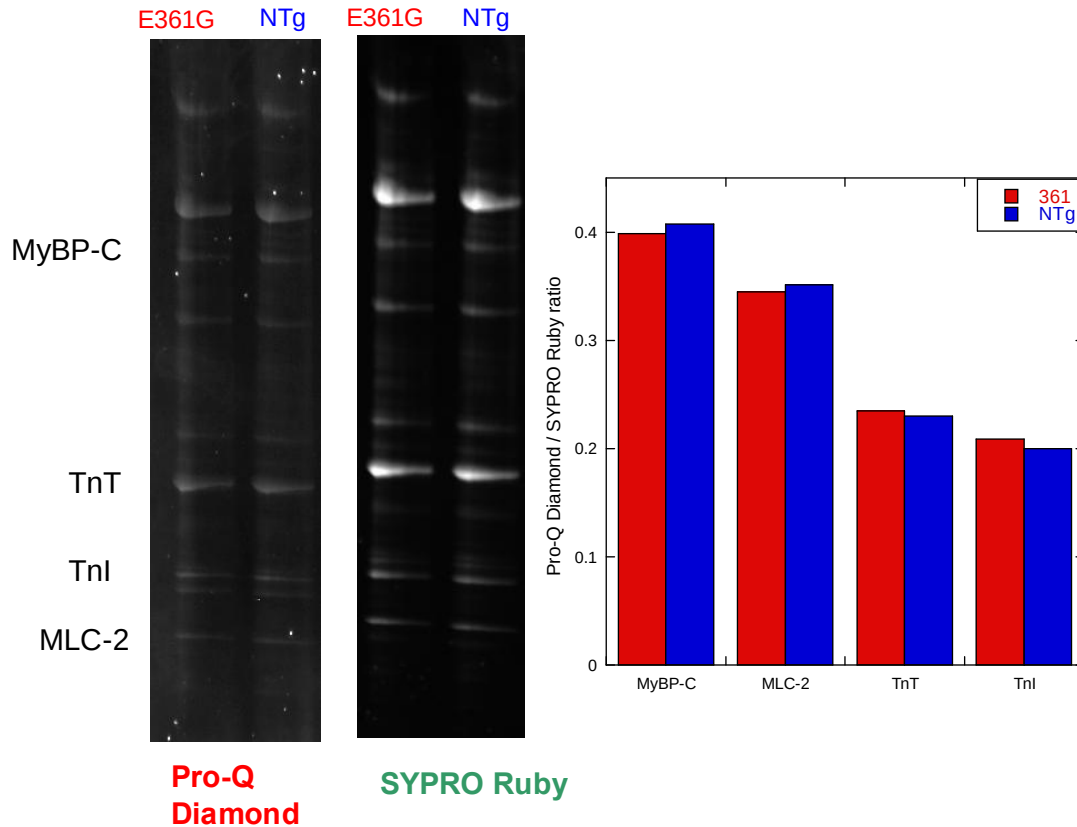


Table 4.9 Pro-Q/SYPRO ratios from 5 Myofibril Experiments

Expt. No.	MyBP-C		MLC-2		TnT		TnI	
	361	NTg	361	NTg	361	NTg	361	NTg
1	0.411	0.400	0.331	0.355	0.190	0.178	0.209	0.199
2	0.395	0.422	0.276	0.295	0.164	0.187	0.179	0.189
3	0.468	0.472	0.299	0.276	0.219	0.230	0.177	0.160
4	0.388	0.412	0.404	0.396	0.295	0.268	0.269	0.258
5	0.332	0.340	0.416	0.431	0.306	0.289	0.209	0.192
Mean $\pm$ SE	0.399 $\pm$ 0.022	0.409 $\pm$ 0.021	0.345 $\pm$ 0.028	0.351 $\pm$ 0.029	0.235 $\pm$ 0.028	0.230 $\pm$ 0.022	0.209 $\pm$ 0.017	0.200 $\pm$ 0.016
T-test (paired/ unpaired)	p = 0.209 / 0.741, n=5		p = 0.580 / 0.897, n=5		p = 0.660 / 0.905, n=5		p = 0.144 / 0.707, n=5	

4.3 Discussion

Our strategy for investigating the E361G actin mutation expressed in transgenic mouse hearts, was to reconstitute thin filaments that were as similar as possible to the human cardiac system. Using human cardiac tropomyosin and troponin, the mutant thin filaments had a Ca^{2+} sensitivity indistinguishable from non-transgenic thin filaments. Bing *et al.* reported that altering the troponin that was incorporated into the thin filament allowed a functional difference to be exposed between mutant and wild type proteins that was not detectable in a fully native system (Bing *et al.*, 2000). Indeed, experiments that used rabbit skeletal troponin unmasked a functional difference between non-transgenic and E361G actin that was not present when native human cardiac troponin was incorporated into the thin filament. The E361G actin had 4.5-fold lower Ca^{2+} sensitivity and 9% slower sliding speed at high Ca^{2+} . The reduced maximum sliding speed was significant over 8 measurements ($p = 0.03$). This was the first evidence of a functional effect caused by the mutation and indicated that the phosphorylation state of the troponin incorporated into the E361G thin filament was a crucial part of the E361G phenotype.

The E361G phenotype that was exposed when using rabbit skeletal troponin appeared similar to previously studied DCM mutations (Robinson *et al.*, 2002, Mirza *et al.*, 2005). Mirza *et al.* studied the five TnT mutants (R131W, R141W, R205L, Δ Lys-210, D270N), two β -tropomyosin mutants (E40K and E54K), and the single TnC mutation (G159D). There was a single decrease in the maximum sliding speed at high Ca^{2+} by approximately 10% ($p < 0.001$ for

TnT R131W; $p < 0.01$ for TnT R141W, TnT R205L, TnT Δ Lys-210, and TnC G159D; and $p < 0.05$ for β -tropomyosin E40K), and a decrease in the Ca^{2+} sensitivity by 0.18 (TnT D270N) and 0.4 (TnT R205L) pCa units in 50:50 wild type: mutant mixtures (1.3-2 fold increase in EC_{50}). This was accompanied by a decrease in the Hill co-efficient, which reflected a loss of cooperativity. The decrease in sliding speed and Ca^{2+} sensitivity was also observed in ATPase assays using the same 50: 50 mixtures of recombinant proteins.

Rabbit skeletal troponin therefore appeared to produce the same phenotype as recombinant human cardiac troponin, but native human cardiac troponin did not. It is important to note that skeletal troponin does not have a phosphorylation site and that the recombinant human cardiac troponin was not phosphorylated, whereas native human cardiac troponin has 3.1 mols Pi /mol TnT and 2.1 mols Pi /mol TnI (Messer *et al.*, 2006). When non-transgenic and E361G actin were tested with native human cardiac troponin dephosphorylated by acid phosphatase, the DCM phenotype previously observed by Mirza *et al.* and Robinson *et al.* was replicated. The E361G thin filaments had a lower Ca^{2+} sensitivity than non-transgenic thin filaments (1.9 increase in EC_{50}). The sliding speed at $3.9\mu\text{M}$ Ca^{2+} was not different, however in this system the sliding speed appeared not to depend upon the Ca^{2+} concentration, unlike with skeletal troponin (E361G actin 9% slower, $p = 0.03$).

Non-transgenic mouse actin in the presence of dephosphorylated troponin produced a high Ca^{2+} sensitivity compared to reconstitution with natively phosphorylated troponin (EC_{50} decreased by 2.9, $p = 0.03$). Similarly, a 2.5 times higher Ca^{2+} sensitivity of dephosphorylated human cardiac troponin with rabbit skeletal actin was previously found by A.E Messer (PhD Thesis, 2007). In order to examine whether the mutant affected the contractility in a similar way when the troponin was dephosphorylated, E361G actin was reconstituted into a thin filament containing dephosphorylated non-failing human cardiac troponin. The increased Ca^{2+} sensitivity of non-transgenic actin in the presence of dephosphorylated troponin was significantly depressed (0.35 ± 0.09 vs 0.36 ± 0.10 , $p = 0.61$). We can therefore conclude that this actin DCM mutant significantly blunts the contractile response to troponin phosphorylation levels. These observations were made by direct comparisons of Ca^{2+} curves from

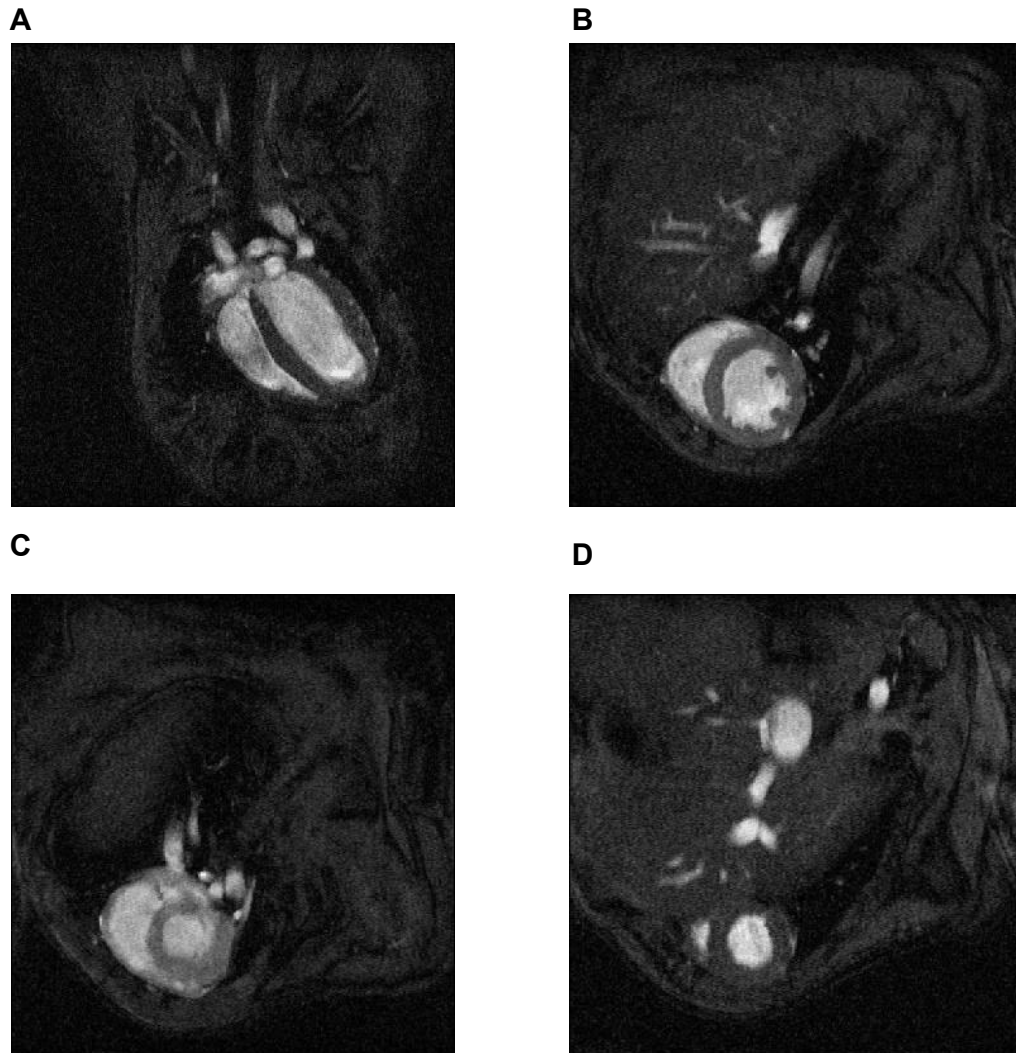
E361G or non-transgenic actin measured with phosphorylated or dephosphorylated troponin in the same cell.

Changing the phosphorylation of troponin that is incorporated into the E361G thin filament has therefore elucidated a potential mechanism for familial DCM. E361G thin filaments are not responsive to altered phosphorylation levels and the cardiac contractile reserve could be reduced. It was shown that the actual levels of troponin I and troponin T phosphorylation in non-transgenic and E361G hearts are the same. Troponin T is phosphorylated at 2.54 mols Pi/mol TnT in non-transgenic hearts compared to 2.62 mols Pi/mol TnT in E361G hearts. Troponin I is phosphorylated at 3.6 mols Pi/mol TnI in non-transgenic hearts compared to 3.52 mols Pi/mol TnI in E361G hearts. Myosin light chain and myosin binding protein C are also unchanged. The levels of troponin phosphorylation thus appear normal in the E361G transgenic mouse heart but the myofilaments cannot respond to changes in phosphorylation. It is also interesting to make comparisons with the phosphorylation levels of human cardiac troponin. Messer *et al.* showed non-failing human cardiac troponin contained 2.1 mol Pi/mol TnI. This is 42% less than in mouse troponin, so it could be that PKA or PKC sites are more phosphorylated in mouse than in human troponin. Non-failing human cardiac troponin contained 3.1 mol Pi/mol TnT (22% more than in mouse troponin) (Messer *et al.*, 2006).

Mice carrying the ACTC E361G mutation have a normal lifespan and *in vivo* investigations using MRI have shown no overt failing heart phenotype exists. The cardiac morphology of six transgenic and six non-transgenic mice was investigated by Caroline Carr and Daniel Stuckey (Department of Physiology, University of Oxford). Dr. Weihua Song provided the data (Figure 4.10). No differences were found between the E361G-transgenic and non-transgenic mice in the left ventricular mass (E361G = 95.92mg $\pm$ 7.53, NTg = 94.52mg $\pm$ 9.04, p=0.38), stroke volume (E361G = 39.32ul $\pm$ 4.81, NTg = 38.02ul $\pm$ 5.0, p=0.32), or ejection fraction (E361G = 61.06% $\pm$ 8.46, NTg = 58.25% $\pm$ 7.46, p=0.27). Cardiac output was slightly different in left ventricular function (E361G = 16.8ul/min $\pm$ 2.39, NTg = 14.35ul/min $\pm$ 2.66, p=0.06), although this was because of the slightly higher heart rates of the transgenic mice (E361G = 427.78 bpm $\pm$ 37.72, NTg = 376.65 bpm $\pm$ 38.07, p=0.02). The

higher heart rates were considered to be due to experimental variation rather than a systematic difference.

Figure 4.10 MRI Scanning of E361G Transgenic Mouse Hearts



(A) 4 chamber long axis, (B) Mid-papillary short axis, (C) Basal short axis, (D) apical short axis

Subsequent investigations of unloaded cardiac myocyte contractility have shown that the E361G mutation does also not induce any difference in the basal contraction, response to Ca^{2+} changes, response to isoprenaline, or the response to frequency changes (Figure 4.11 - Figure 4.14). Data has been provided by Dr. Weihua Song.

Figure 4.11 Non-Transgenic and E361G Basal Contraction Data

Basal Contraction: Non-transgenic 41 (9) VS Transgenic 66(11)

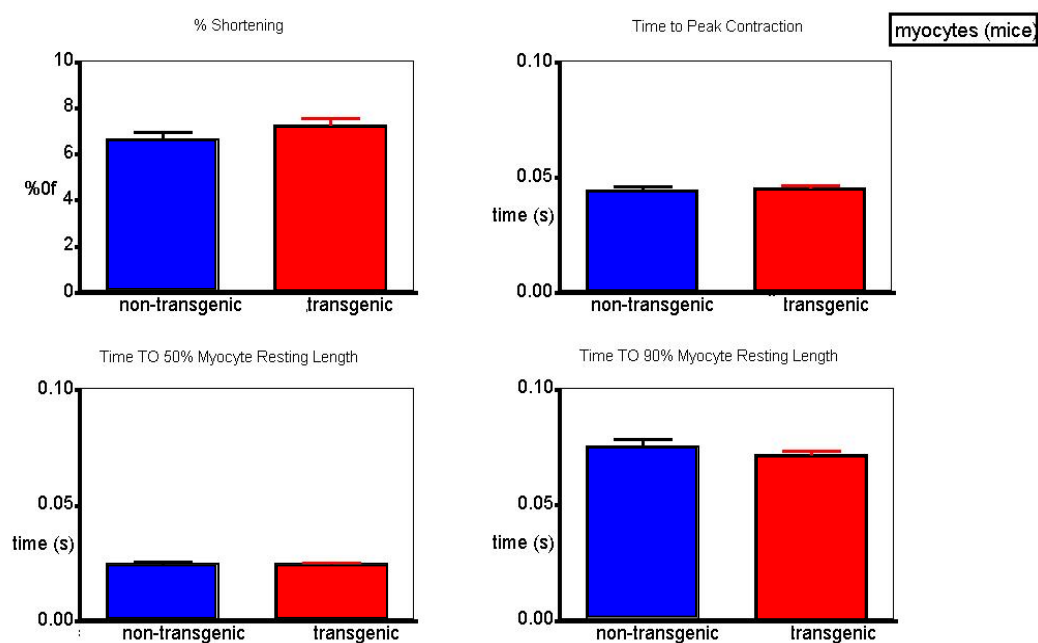


Figure 4.12 Non-Transgenic and E361G Isoprenaline Response Data

Isoprenaline Response: Non-transgenic 7 (4) VS Transgenic 9 (5)

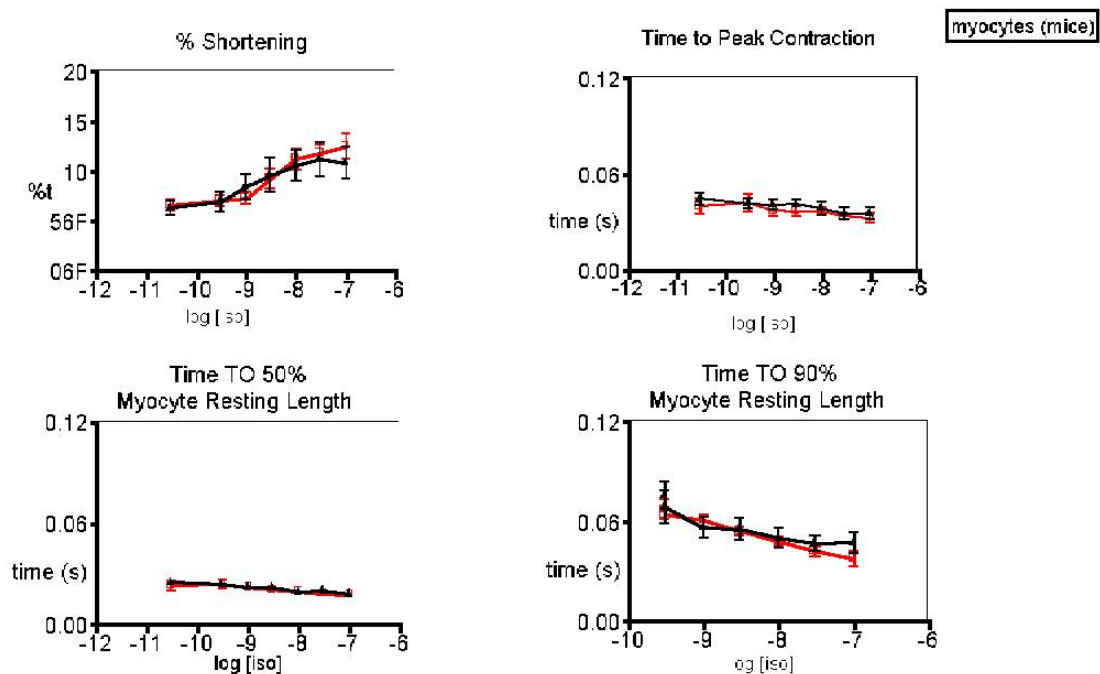
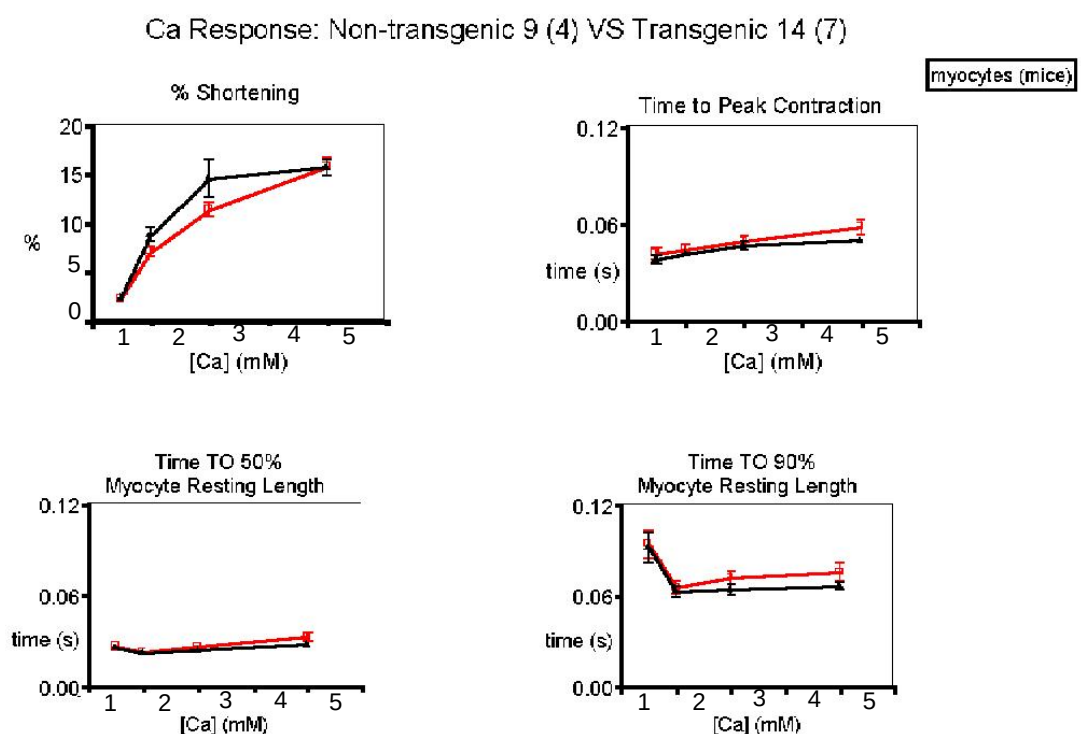
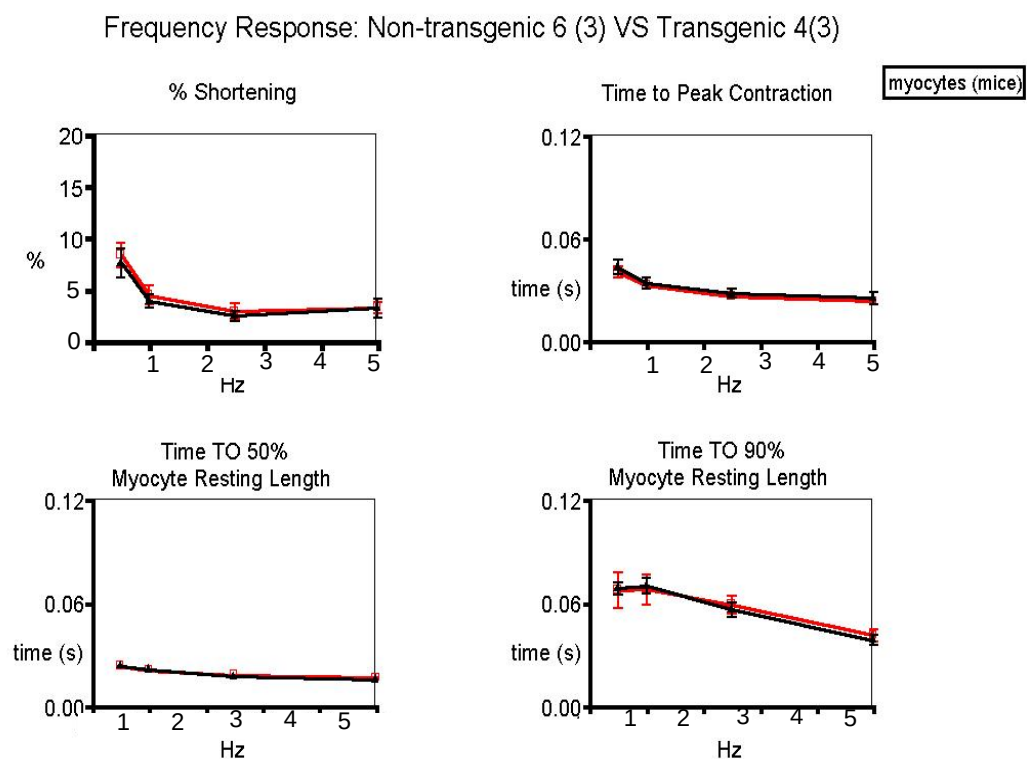


Figure 4.13 Non-Transgenic and E361G Ca^{2+} Response Data**Figure 4.14 Non-Transgenic and E361G Frequency Response Data**

The nature of the experimental system has proven to have a strong influence on the results obtained with the E361G thin filament. Although E361G is the only actin DCM associated mutant to be tested in combination with troponin phosphorylation, similar studies that have used a recombinant troponin C DCM mutant G159D have also revealed insensitivity to troponin phosphorylation (Biesiadecki *et al.*, 2007, Preston *et al.*, 2007a). There is therefore convincing evidence that this novel mechanism may contribute to the pathogenic effect of DCM. We have been fortunate enough to obtain a sample of human heart muscle containing the troponin C DCM-causing mutation G159D. Our results with this tissue are reported in the next chapter.

5. FUNCTIONAL INVESTIGATION OF A DCM TROPONIN C MUTANT GLY159ASP

The missense mutation Gly159Asp (G159D) in cardiac troponin C is associated with DCM. It was first identified by Mogensen *et al.* through a genetic case study of cardiac troponin C and troponin T in 235 consecutive patients with idiopathic DCM (Mogensen *et al.*, 2004b). We have obtained a cardiac muscle sample from a patient belonging to the original family with the DCM troponin C G159D mutation. This chapter will describe the functional investigation of this mutant troponin using the *in vitro* motility assay. Our strategy has been to reconstitute human cardiac partner proteins into the thin filament to accurately replicate a human system. The human cardiac troponin isolated from the biopsy should have the physiological post-translational modifications and mutant to wild type ratios characteristic of this DCM phenotype.

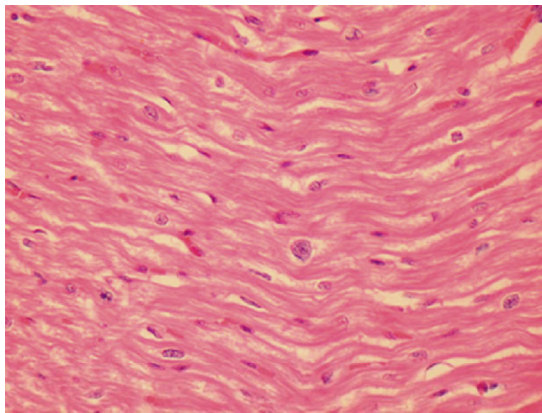
5.1 Patient History and Diagnosis

The three-year old male patient was admitted to hospital with a 3-day history of vomiting and reduced urine output (Kaski *et al.*, 2007). He had been unwell for the previous three months with lethargy, dyspnoea, and a cough. A presumptive diagnosis was made of cardiomyopathy, given his chest X-ray findings (increased cardiothoracic ratio, pulmonary oedema) and the strong family history of DCM (refer to (Mogensen *et al.*, 2004b)). On the maternal side his mother has dilated cardiomyopathy, uncle has had a heart transplant aged 21, aunt is awaiting a transplant and grandmother died at the age of 45. During admission he had a short lasting tachyarrhythmia (rate 210 bpm) detected on the monitor, but not captured on the 12-lead echocardiogram. The ECG demonstrated a normally connected heart, with 4-chamber dilatation, left ventricular end-diastolic dimensions of 54mm, with end-systolic dimension of 50mm, poor biventricular systolic function, and the left ventricular ejection fraction being at 20%. Despite inotropic support, he developed respiratory distress and increasing agitation, which progressed to bradycardia and respiratory arrest. He was therefore intubated for extracorporeal membrane oxygenation support. An orthotopic heart transplantation followed and at the 7-

month review after transplantation he remained well and active, with no episodes of rejection detected clinically or on endomyocardial biopsy.

Histological analysis of the explanted heart showed non-specific patchy fibrosis, particularly in the subendocardium (Figure 5.1). There was no myocyte disarray or hypertrophy. Mutation analysis on the patient's genomic DNA extracted from a peripheral blood sample confirmed the presence of the G159D mutation.

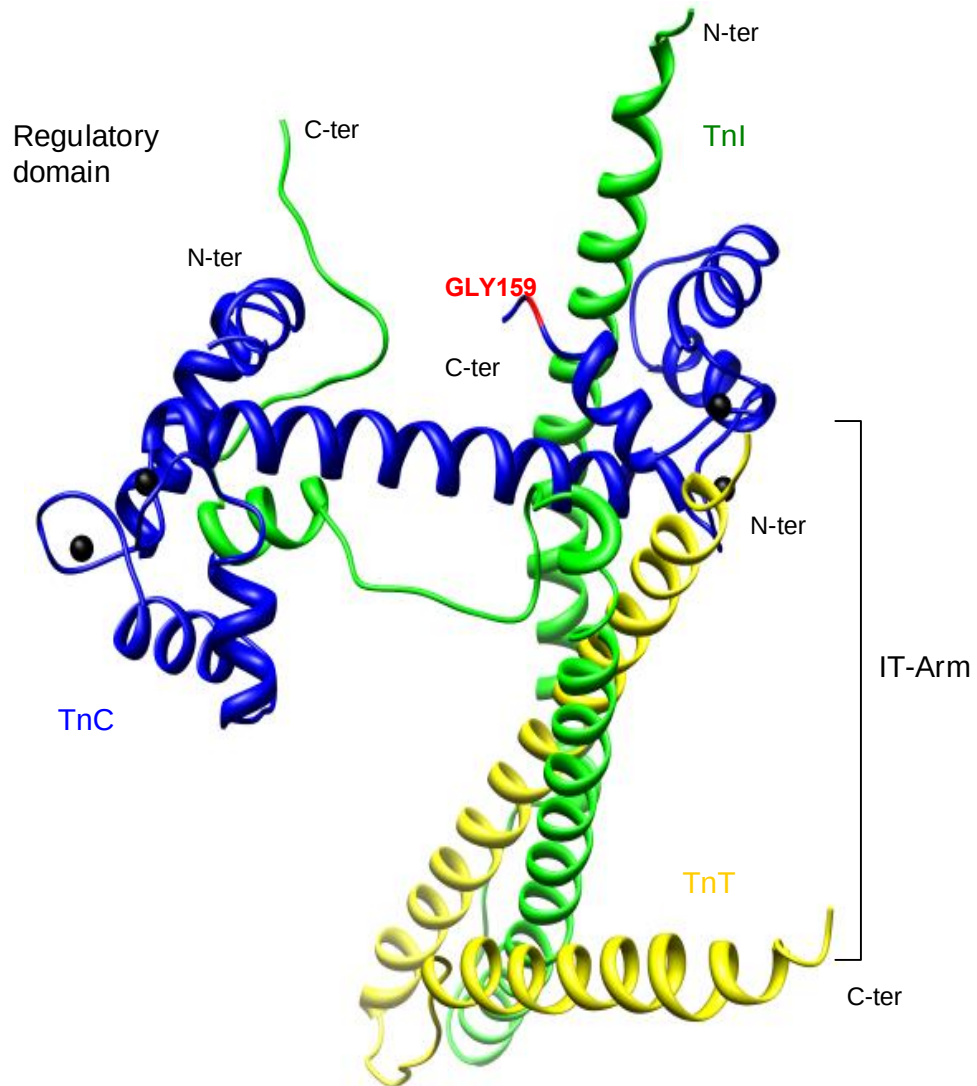
Figure 5.1 Histological Analysis of Left Ventricular Myocardium



A section of left ventricular myocardium from the explanted heart. A few elongated myofibers with enlarged nuclei are visible. There is no fibrosis, inflammation or necrosis. Obtained from (Kaski *et al.*, 2007)

5.2 Location of the TNNC1 G159D Mutation

The representation in Figure 5.2 shows that the structure of human cardiac troponin is composed of two domains, (1) the regulatory head, and (2) the troponin I-troponin T arm. The G159D mutation is located within the C-terminal lobe of cardiac troponin C (Biesiadecki *et al.*, 2007). This is considered a structural rather than a regulatory site (reviewed in (Kobayashi and Solaro, 2005)). NMR analysis of troponin C-troponin I complexes have shown that the N-terminal domain of Troponin I makes contact with the C-terminal domain of troponin C, at a site close to the Troponin C residue G159D (Finley *et al.*, 1999).

Figure 5.2 Troponin C Gly159 in the Whole Troponin Complex

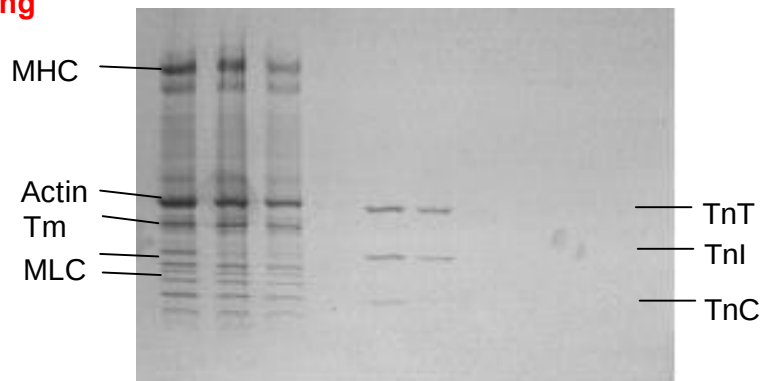
Ribbon representation of the troponin complex, highlighting the position of Troponin C Gly159 mutation in red. Troponin C and T are shown in blue and yellow respectively. Troponin I is shown in green. Black spheres show the Ca^{2+} ions. Molecular graphics were prepared with Chimera.

5.3 Isolation of Non-Failing and G159D Troponin

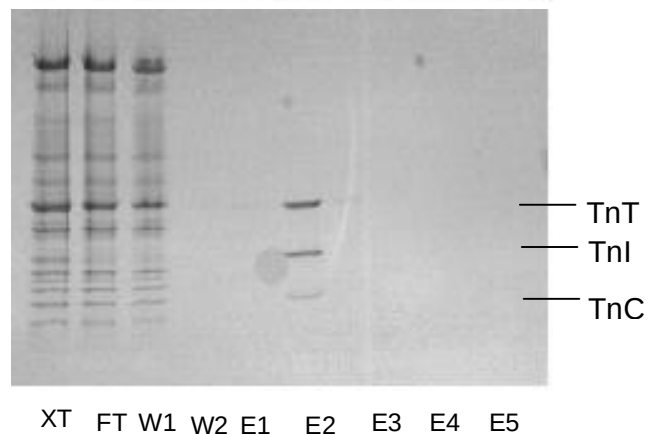
Troponin was isolated from 150-200mg of non-failing and G159D heart tissue by affinity chromatography with the anti-troponin I monoclonal antibody 16A11 (section 2.8). Figure 5.3 shows SDS-PAGE analysis of the affinity purification. Pure non-failing troponin has eluted in fractions E1 and E2. Pure G159D troponin has eluted in E2. The yield was normally 300-700 μ l of 5-7 μ M from 150mg of tissue.

Figure 5.3 SDS-PAGE Showing Extraction of Troponin from Non-Failing and G159D Heart Tissue

Non-Failing



G159D

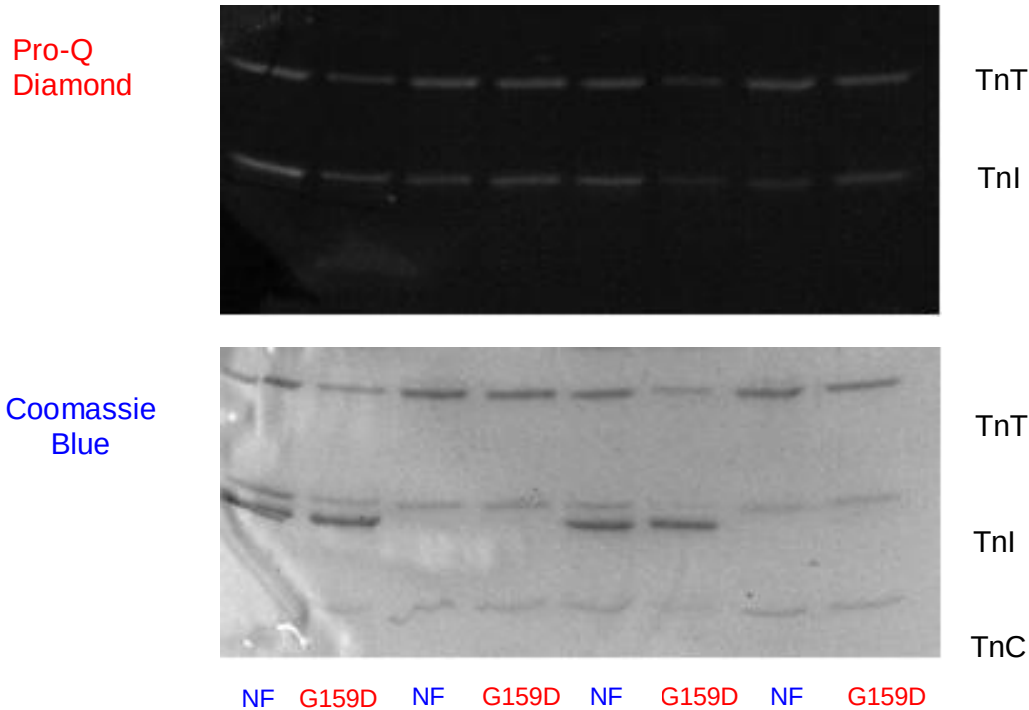


XT ÷ Extract, FT ÷ Flow through, W ÷ Wash, E ÷ Elute fraction, MHC ÷ Myosin heavy chain, MLC ÷ Myosin light chain, Tm ÷ Tropomyosin, Tn ÷ Troponin.

5.4 Phosphorylation Measurements

Levels of phosphorylation from native non-failing and G159D troponin were measured directly using SDS-PAGE with the Pro-Q Diamond phosphoprotein gel stain (Figure 5.4).

Figure 5.4 SDS-PAGE of Non-Failing and G159D Troponin

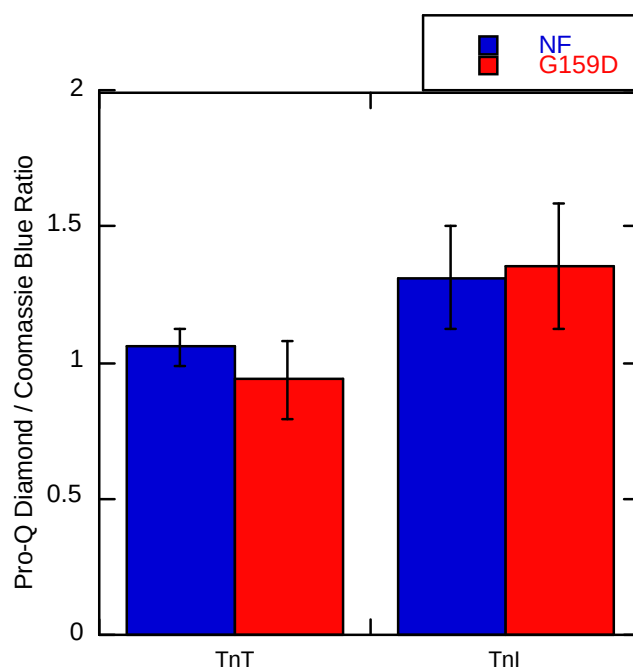


Phosphorylation levels were measured from 4 paired troponin isolations. G159D troponin had an 11% reduction in troponin T phosphorylation compared to non-failing troponin, and a 3% increase in troponin I phosphorylation. However, these differences were not significantly different from non-failing troponin, $p = 0.45$ and 0.92 respectively (Table 5.1, Figure 5.5).

Table 5.1 Phosphorylation Differences Between Non-Failing and G159D Troponin

	Pro-Q Diamond/Coomassie Ratio		% Difference
	Non-Failing	G159D	
TnT	1.06 ± 0.07	0.94 ± 0.014	- 11.3%
t-test (n = 4)	Paired = 0.45 Unpaired = 0.47		
TnI	1.31 ± 0.19	1.36 ± 0.23	+ 3.8%
t-test (n = 4)	Paired = 0.92 Unpaired = 0.89		

Figure 5.5 Average Phosphorylation Levels of Troponin T and Troponin I

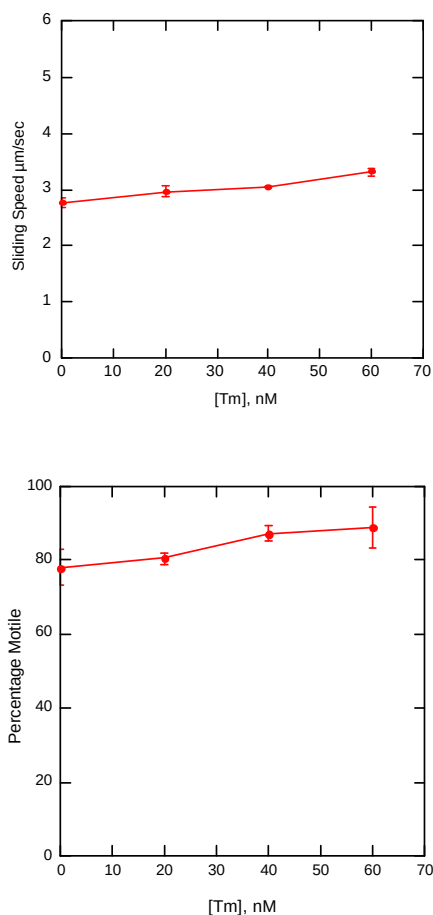


5.5 *In Vitro* Motility Analysis

5.5.1 Effects of Human Cardiac Tropomyosin on Actin Filament Motility

Over 19 separate measurements, skeletal rabbit actin moved an average of $2.9 \pm 0.15 \mu\text{m}/\text{sec}$ with $78 \pm 3.12\%$ motility. 20-60nM human cardiac tropomyosin was titrated against 10nM skeletal rabbit actin. This resulted in the actin sliding speed increasing by 12% to $3.12 \pm 0.15 \mu\text{m}/\text{sec}$, and the percentage of filaments motile to increase by 9% to $84 \pm 2.01\%$. A typical titration is shown in Figure 5.6.

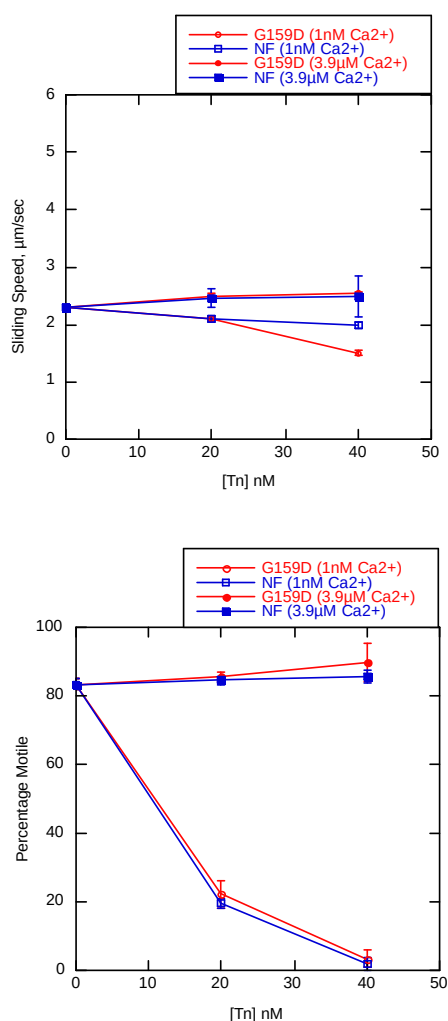
Figure 5.6 Effect of Human Cardiac Tropomyosin on Rabbit Actin Motility (from Expt. 1; Table 5.2 and Table 5.5)



5.5.2 Effects of Human Cardiac Troponin on Actin Filament Motility

Thin filaments were assembled using rabbit skeletal muscle actin, human cardiac tropomyosin, and non-failing or G159D troponin. Troponin at increasing concentrations (20-40nM) was titrated with 10nM actin and 60nM human cardiac tropomyosin. Initial titrations at 1nM Ca^{2+} showed at which concentration troponin produced a maximum switch off. This was usually obtained with 40nM Troponin. With increasing troponin concentrations the filament sliding speed reached a maximum value and then levelled off.

Figure 5.7 Effects of Non-failing and G159D Human Cardiac Troponin (40nM) on Actin Motility at 3.9 μM Ca^{2+} and 1nM Ca^{2+}



At 1nM Ca^{2+} , the actin filament motility for both non-failing and G159D troponin was switched off by 40nM troponin with the percentage motility falling to approximately 5% and the sliding speed reduced by 38% for non-failing troponin and 35% for G159D troponin (Table 5.2 and Table 5.3). There was no significant difference between non-failing and G159D troponin in terms of the percentage motility ($p = 0.53$) or the sliding speed ($p = 0.52$). Both inhibited actin filament motility to a similar degree. The G159D mutation has previously been studied in our laboratory using the *in vitro* motility assay, and a similar result was obtained (Mirza *et al.*, 2005). Troponin complexes were reconstituted from recombinant human cardiac troponin I, C, and T with wild type troponin C and G159D mutant troponin C. Under relaxing conditions of 1nM Ca^{2+} , filament motility of thin filaments containing wild type and G159D mutant troponin C were indistinguishable. The percentage motility was switched off by troponin such that <10% of filaments were motile and the sliding speed was reduced by approximately 40%.

At 3.9 μM Ca^{2+} , 40nM of both non-failing and G159D troponin increased actin sliding speeds by 7% over actin-tropomyosin (Table 5.4). The increase in sliding speed with non-failing troponin can be contrasted with the larger increase of 26% reported by Messer *et al.* who similarly used human cardiac troponin and tropomyosin (Messer *et al.*, 2006), and the 34% increase reported by Mirza *et al.* when using recombinant wild type troponin. Using recombinant G159D troponin with human cardiac tropomyosin, Mirza *et al.* found that G159D troponin conferred a significantly lower maximum sliding speed compared to non-failing troponin. The recombinant G159D troponin caused a 14% increase in sliding speed compared to the 34% increase with non-failing troponin.

Our studies found the percentage motility at high Ca^{2+} was also not significantly different ($p = 0.63$) (Table 5.5). Non-failing troponin caused an increase of just 2% and G159D human cardiac troponin increased by 1%. Between 81-82% of actin filaments were motile at saturating concentrations of tropomyosin and non-failing and G159D troponin. Similarly, Mirza *et al.* found no significant difference between the percentage motility of non-failing and G159D troponin at high Ca^{2+} conditions ($p = 0.9$) (Mirza *et al.*, 2005).

Table 5.2 Effect of Human Cardiac Troponin on Filament Sliding Speed at Low Ca^{2+} (1nM Ca^{2+})

Exp. No.	Sliding Speed, $\mu\text{m}/\text{sec}$			Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
	Actin-Tm	NF	G159D	NF	G159D
1	3.3 ± 0.07	1.4 ± 0.03	2.0 ± 0.22	0.4	0.6
2	3.0 ± 0.04	1.4 ± 0.11	1.4 ± 0.08	0.5	0.5
3	2.3 ± 0.03	1.4 ± 0.01	2.0 ± 0.23	0.6	0.9
4	2.3 ± 0.04	2.0 ± 0.06	1.5 ± 0.04	0.9	0.7
5	2.0 ± 0.04	1.6 ± 0.07	1.6 ± 0.03	0.8	0.8
6	2.5 ± 0.09	1.9 ± 0.09	2.0 ± 0.10	0.8	0.8
7	2.5 ± 0.12	1.7 ± 0.03	1.6 ± 0.10	0.7	0.6
Mean $\pm$ SE n = 7	2.56 ± 0.17	1.63 ± 0.09	1.73 ± 0.10	0.67 ± 0.07	0.70 ± 0.05
T-test P Value		Paired = 0.52 Unpaired = 0.48		Paired = 0.67 Unpaired = 0.75	

Table 5.3 Effect of Human Cardiac Troponin on Filament Percentage Motility at Low Ca^{2+} (1nM Ca^{2+})

Exp. No.	Percentage Motile, %			Relative % Motility of Actin.Tm.Tn: Actin.Tm	
	Actin-Tm	NF	G159D	NF	G159D
1	89 ± 5.57	3 ± 2.63	3 ± 5.62	0.03	0.03
2	90 ± 2.61	7 ± 1.43	5 ± 3.28	0.08	0.06
3	61 ± 1.63	3 ± 1.22	1 ± 1.00	0.05	0.02
4	83 ± 1.64	2 ± 1.84	4 ± 2.63	0.02	0.05
5	79 ± 3.92	6 ± 0.75	12 ± 2.89	0.08	0.15
6	87 ± 1.31	6 ± 2.96	8 ± 1.26	0.07	0.09
7	75 ± 1.03	3 ± 1.64	2 ± 4.01	0.04	0.03
Mean $\pm$ SE n = 7	80.57 ± 3.85	4.28 ± 0.75	5.00 ± 1.45	0.05 ± 0.01	0.06 ± 0.02
T-test P Value		Paired = 0.53 Unpaired = 0.67		Paired = 0.53 Unpaired = 0.67	

Table 5.4 Effect of Human Cardiac Troponin on Filament Sliding Speed at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)

Exp. No.	Sliding Speed, $\mu\text{m/sec}$			Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
	Actin-Tm	NF	G159D	NF	G159D
1	3.3 ± 0.07	2.8 ± 0.05	2.8 ± 0.02	0.9	0.9
2	3.0 ± 0.04	3.1 ± 0.09	3.2 ± 0.03	1.0	1.1
3	2.3 ± 0.03	2.8 ± 0.03	2.8 ± 0.01	1.2	1.2
4	2.3 ± 0.04	2.5 ± 0.36	2.5 ± 0.02	1.1	1.1
5	2.0 ± 0.04	2.9 ± 0.25	2.8 ± 0.24	1.5	1.4
6	2.5 ± 0.09	2.5 ± 0.15	2.8 ± 0.11	1.0	1.1
7	2.5 ± 0.12	2.5 ± 0.02	2.2 ± 0.05	1.0	0.9
Mean $\pm$ SE n = 7	2.56 ± 0.17	2.73 ± 0.09	2.73 ± 0.12	1.08 ± 0.07	1.08 ± 0.08
T-test P Value		Paired = 1.0 Unpaired = 1.0		Paired = 0.92 Unpaired = 0.98	

Table 5.5 Effect of Human Cardiac Troponin on Filament Percentage Motility at High Ca^{2+} ($3.9\mu\text{M Ca}^{2+}$)

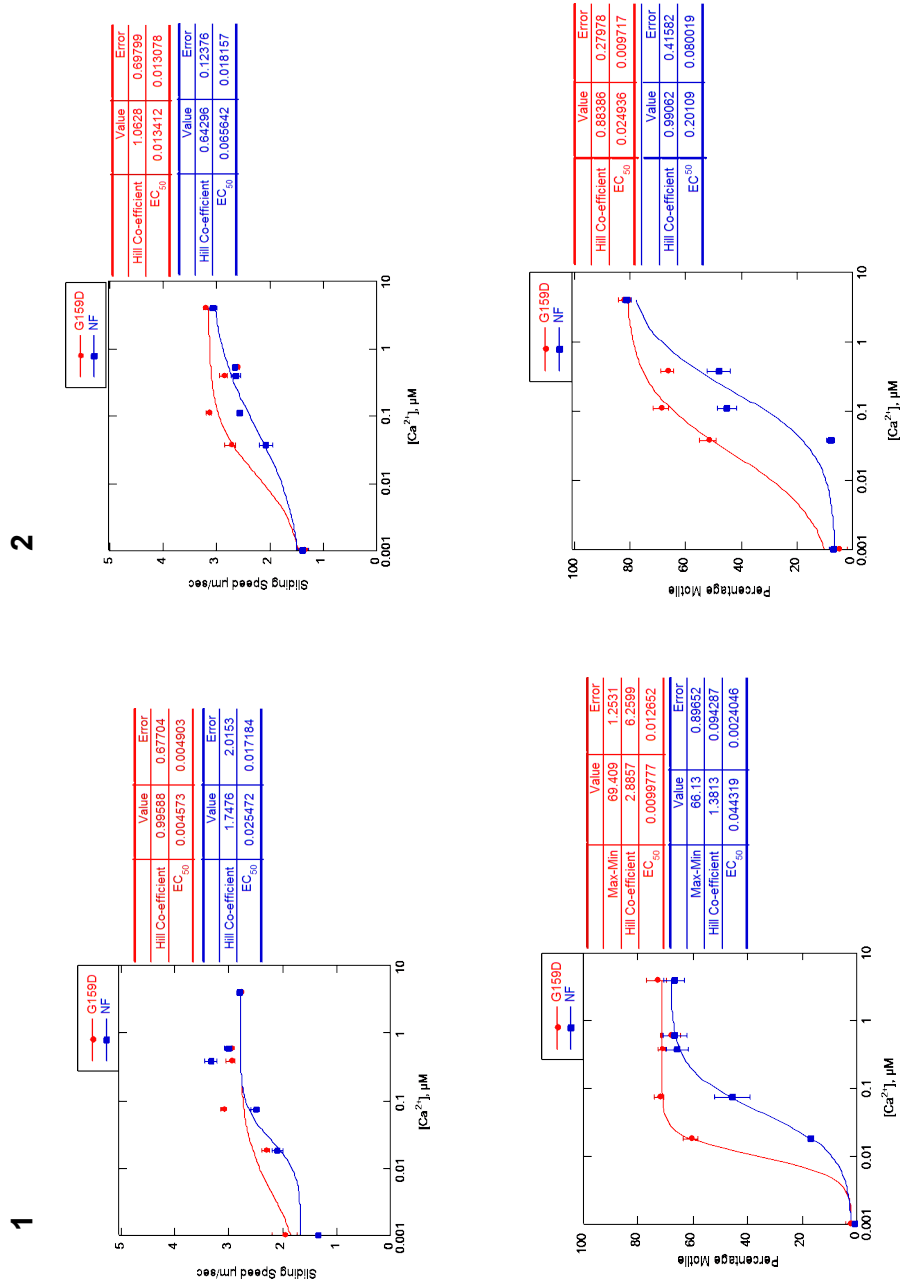
Exp. No.	Percentage Motile, %			Relative % Motility of Actin.Tm.Tn: Actin.Tm	
	Actin-Tm	NF	G159D	NF	G159D
1	89 ± 5.57	92 ± 2.65	89 ± 3.62	1.0	1.0
2	90 ± 2.61	81 ± 1.65	82 ± 2.05	0.9	0.9
3	61 ± 1.63	73 ± 3.57	67 ± 3.76	1.2	1.1
4	83 ± 1.64	86 ± 1.89	90 ± 5.62	1.0	1.1
5	79 ± 3.92	65 ± 1.44	59 ± 4.48	0.8	0.8
6	87 ± 1.31	94 ± 1.84	99 ± 4.51	1.1	1.1
7	75 ± 1.03	87 ± 1.33	86 ± 2.36	1.2	1.1
Mean $\pm$ SE n = 7	80.57 ± 3.85	82.57 ± 3.95	81.71 ± 5.28	1.03 ± 0.05	1.01 ± 0.05
T-test P Value		Paired = 0.63 Unpaired = 0.90		Paired = 0.60 Unpaired = 0.85	

5.5.3 Comparing Ca^{2+} Sensitivity Curves of Non-Failing and G159D Cardiac Troponin

Concentrations of troponin that gave maximal inhibition of filament activity at 1nM Ca^{2+} were used to measure the Ca^{2+} dependence of thin filament *in vitro* motility. The Ca^{2+} sensitivity of the sliding speed and the percentage motile of actin filaments were investigated for 3 paired non-failing and G159D heart muscle samples. Reconstituted thin filaments consisted of 10nM skeletal rabbit actin, 60nM human cardiac tropomyosin, and 40nM non-failing or G159D troponin. In these experiments, the human cardiac troponin was in its native phosphorylation state.

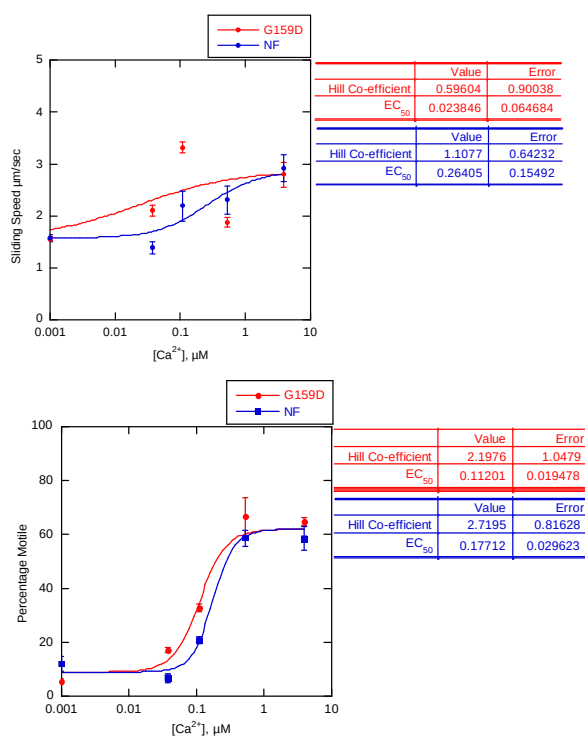
The Ca^{2+} sensitivity curves for the sliding speed proved inaccurate when fitted to the Hill Equation since the overall increase in sliding speeds from 1nM Ca^{2+} to 3.9 μM Ca^{2+} was small. The resulting fits are shown in Figure 5.8, however the sliding speeds did not change enough for the calculated EC_{50} value to be accurate. The Ca^{2+} sensitivity curves for the percentage motility of thin filaments containing G159D mutant troponin C could be accurately fitted. The curves were shifted to the left and the calculated Ca^{2+} sensitivity was increased. The G159D mutation caused a 4.7 ± 1.87 -fold decrease in percentage motility EC_{50} compared to non-failing troponin (Table 5.6).

Figure 5.8 Ca^{2+} Regulation of Thin Filaments containing Non-failing or G159D Troponin.



The following data had the parameters set to obtain an accurate sigmoidal curve:
 Graph 1 (Min values set at 1.6µm/sec and 2%, max-min at 1.1µm/sec)
 Graph 2 (Min values set at 1.4µm/sec and 6%, max-min at 1.8µm/sec and 76%).

3



Graph 3 (Min values set at 1.3μm/sec and 9%, max-min at 1.3μm/sec and 53%).

Table 5.6 Ca²⁺ Sensitivity Data for Non-Failing and G159D Troponin incorporated into the Native Thin Filament

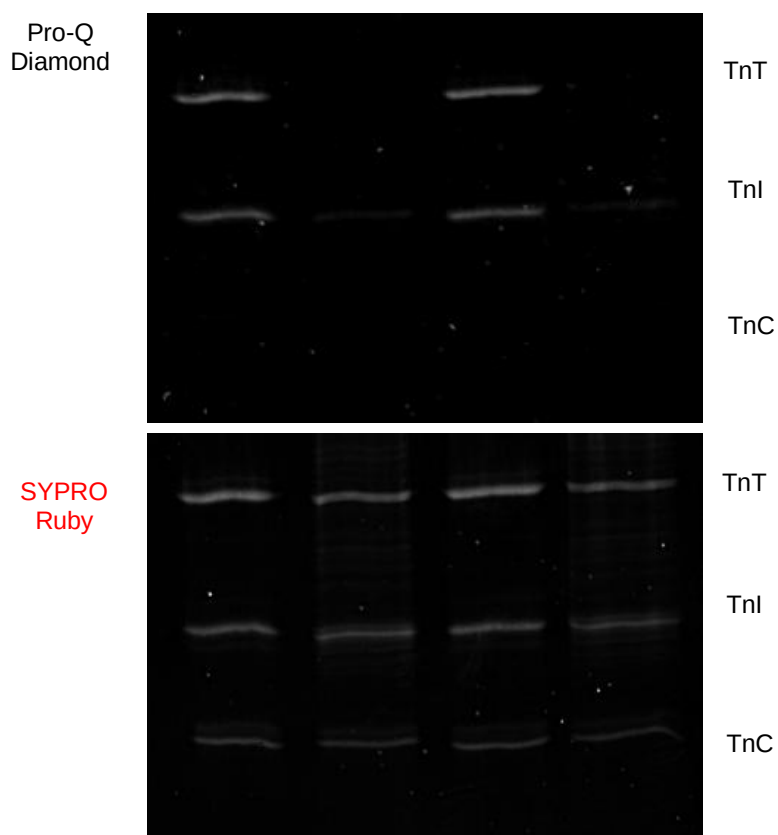
Expt. No.	EC ₅₀ Sliding Speed, μM		EC ₅₀ NF EC ₅₀ G159D	EC ₅₀ Percentage Motility, μM		EC ₅₀ NF EC ₅₀ G159D
	Non-Failing	G159D		Non-Failing	G159D	
1	0.025 ± 0.018	0.005 ± 0.005	5.00	0.044 ± 0.002	0.010 ± 0.013	4.40
2	0.066 ± 0.018	0.013 ± 0.013	5.08	0.201 ± 0.080	0.025 ± 0.009	8.04
3	0.264 ± 0.155	0.024 ± 0.065	11.00	0.177 ± 0.029	0.112 ± 0.019	1.58
Mean ± SD	0.118 ± 0.07	0.014 ± 0.01	7.03 ± 1.99	0.141 ± 0.049	0.049 ± 0.032	4.67 ± 1.87

These Ca^{2+} sensitivity results that used native troponin from a human G159D heart sample can be compared with those produced when wild type and G159D troponin C was expressed in *Eschericia.coli* and then substituted into recombinant troponin (Mirza *et al.*, 2005). The recombinant G159D troponin produced an opposite phenotype in the *in vitro* motility assay. The Ca^{2+} sensitivity was decreased (increase in EC_{50} by 1.6-fold $\pm$ 0.01 and 1.5-fold $\pm$ 0.03 for sliding speed and percentage motility respectively), and the Hill coefficient also decreased for both parameters. However, the recombinant troponin incorporated was not phosphorylated and our native human cardiac troponin was phosphorylated at 3.1 mol Pi/molTnT and 2.1mol Pi/mol TnI (Messer *et al.*, 2006). The Ca^{2+} sensitivity of phosphorylated recombinant G159D troponin was never tested. The *in vitro* motility results from the DCM actin E361G mutation implicate insensitivity to troponin phosphorylation levels may be a critical causative factor in familial DCM. We therefore tested the troponin from the G159D biopsy in its native and dephosphorylated state.

5.5.4 Effect of Troponin Dephosphorylation on the Ca^{2+} -Dependence of Motility

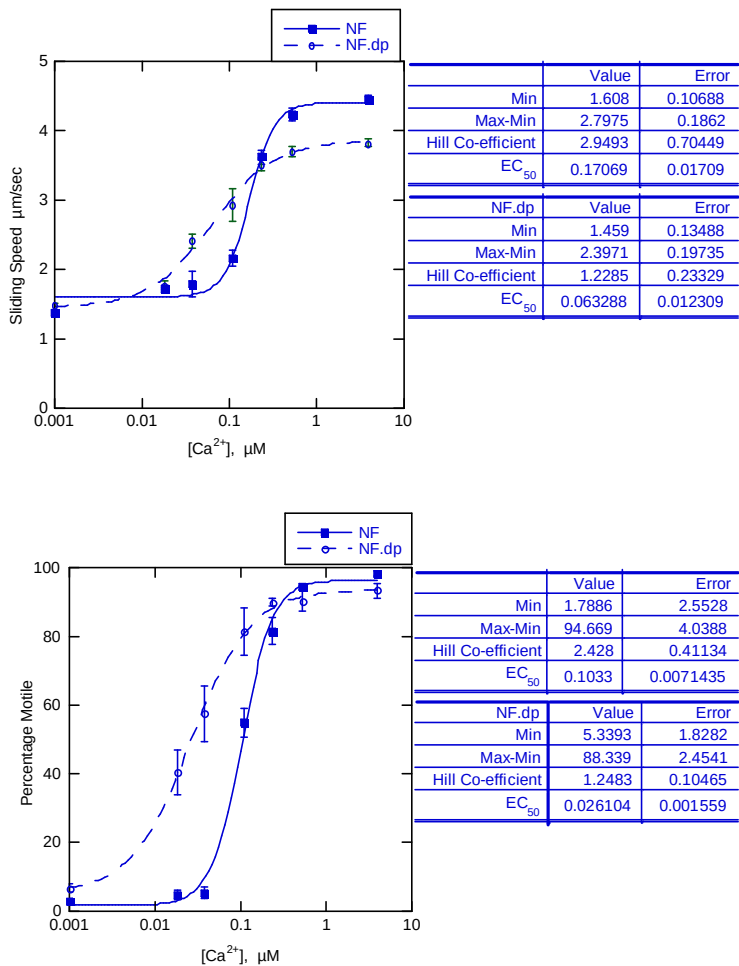
Dephosphorylation was achieved by incubation of the troponin with 1.4 units/ml acid phosphatase (Figure 5.9). Thin filaments were assembled from combinations of native and dephosphorylated non-failing and G159D troponin Figure 5.10 demonstrates the well-known impact of dephosphorylation on non-failing troponin. There is a significant leftward shift of the sliding speed/percentage motility- Ca^{2+} relationship and a lower sliding speed at $3.9\mu\text{M}$ Ca^{2+} . The Ca^{2+} sensitivity of the myofilaments increased 2.8-fold $\pm$ 0.66 for the sliding speed and 4.8-fold $\pm$ 2.05 for the percentage motility (Messer *et al.*, 2006).

Figure 5.9 SDS-PAGE of Non-Failing and G159D Human Cardiac Troponin



SDS-PAGE gel of two non-failing and G159D troponin pairs, showing the effect of acid phosphatase on troponin T and I phosphorylation. Stained with (A) Pro-Q Diamond (for phosphoproteins) and (B) Sypro Ruby for total protein levels.

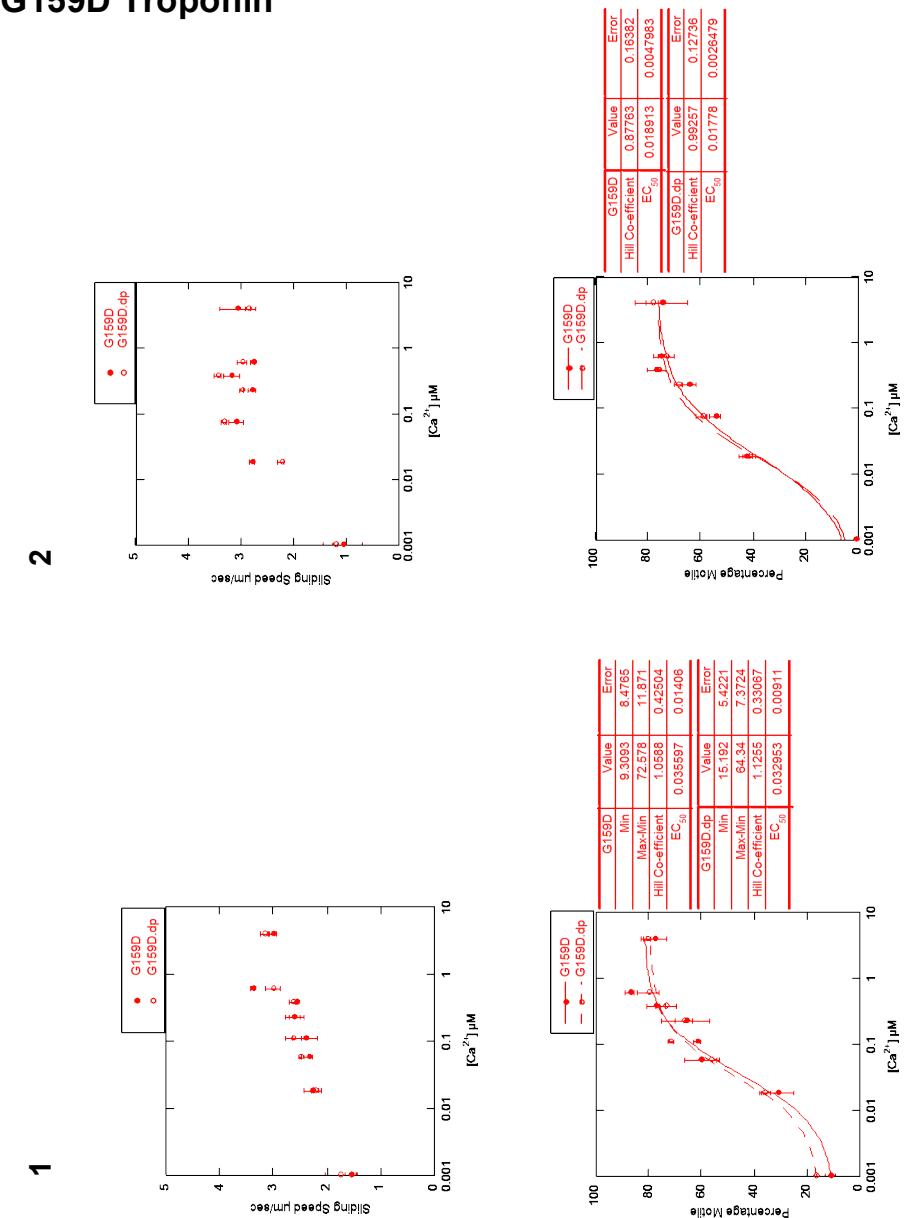
Figure 5.10 Effect of Dephosphorylation on Non-Failing Troponin



Figures obtained from A.E Messer PhD Thesis, 2007.

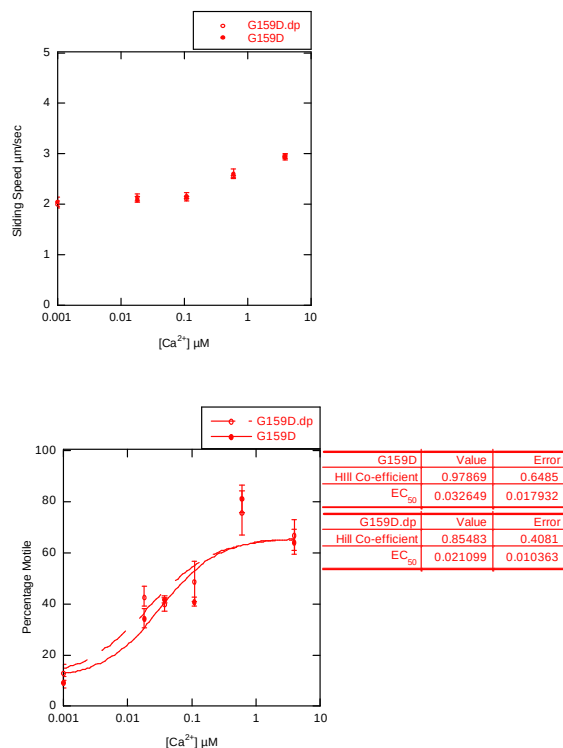
The impact of dephosphorylation on G159D filament Ca²⁺-sensitivity was investigated (Figure 5.11, **Error! Reference source not found.**). The characteristic increase in Ca²⁺ sensitivity that normally accompanies troponin I dephosphorylation was abolished. Native and dephosphorylated G159D troponins produced similar Ca²⁺ sensitivities (EC₅₀ 159/159.dp = 1.24 ± 0.17). The dephosphorylated G159D thin filaments were not responsive to the phosphorylation change shown by the non-failing troponin in Figure 5.10 and behaved as if phosphorylated at all times.

Figure 5.11 Effect of Dephosphorylation on the Regulation by G159D Troponin



The sliding speeds did not change enough for accurate EC₅₀ values to be obtained. The points have simply been plotted. Graph B had the minimum percentage motility parameter values set at 1%, and maximum-minimum at 75.5%.

3



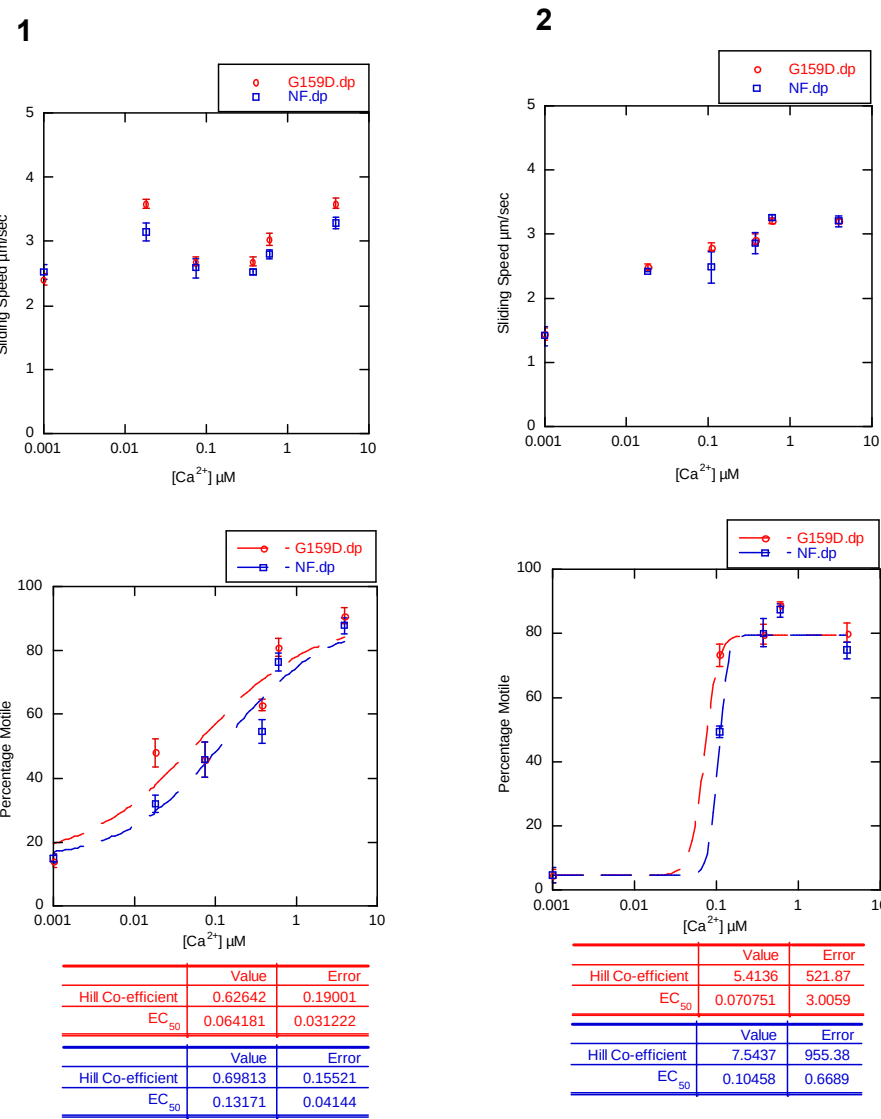
Graph C had the minimum values set at 11%, and maximum-minimum set at 55% to obtain accurate sigmoidal curves.

Table 5.7 Ca²⁺ Sensitivity Data for Native and Dephosphorylated G159D Troponin

Expt. No.	EC ₅₀ Percentage Motility, µM		$\frac{EC_{50} \text{ G159D}}{EC_{50} \text{ G159D.dp}}$
	G159D	G159D.dp	
1	0.036 ± 0.014	0.033 ± 0.009	1.09
2	0.019 ± 0.005	0.018 ± 0.003	1.06
3	0.033 ± 0.018	0.021 ± 0.010	1.57
Mean ± SD	0.029 ± 0.005	0.024 ± 0.006	1.24 ± 0.17

Dephosphorylating both non-failing and G159D troponin and reconstituting them into the thin filament produced a slight leftward shift of G159D troponin (Figure 5.12). Over 2 separate measurements, G159D showed a 1.77 ± 0.29 -fold higher Ca²⁺ sensitivity of the percentage motility (Table 5.8).

Figure 5.12 Effect of Dephosphorylation of Non-Failing and G159D Troponin



Graph 1 (Min values set at 14%, max-min at 75%), Graph 2 (Min values set at 4.6%, max-min at 75%).

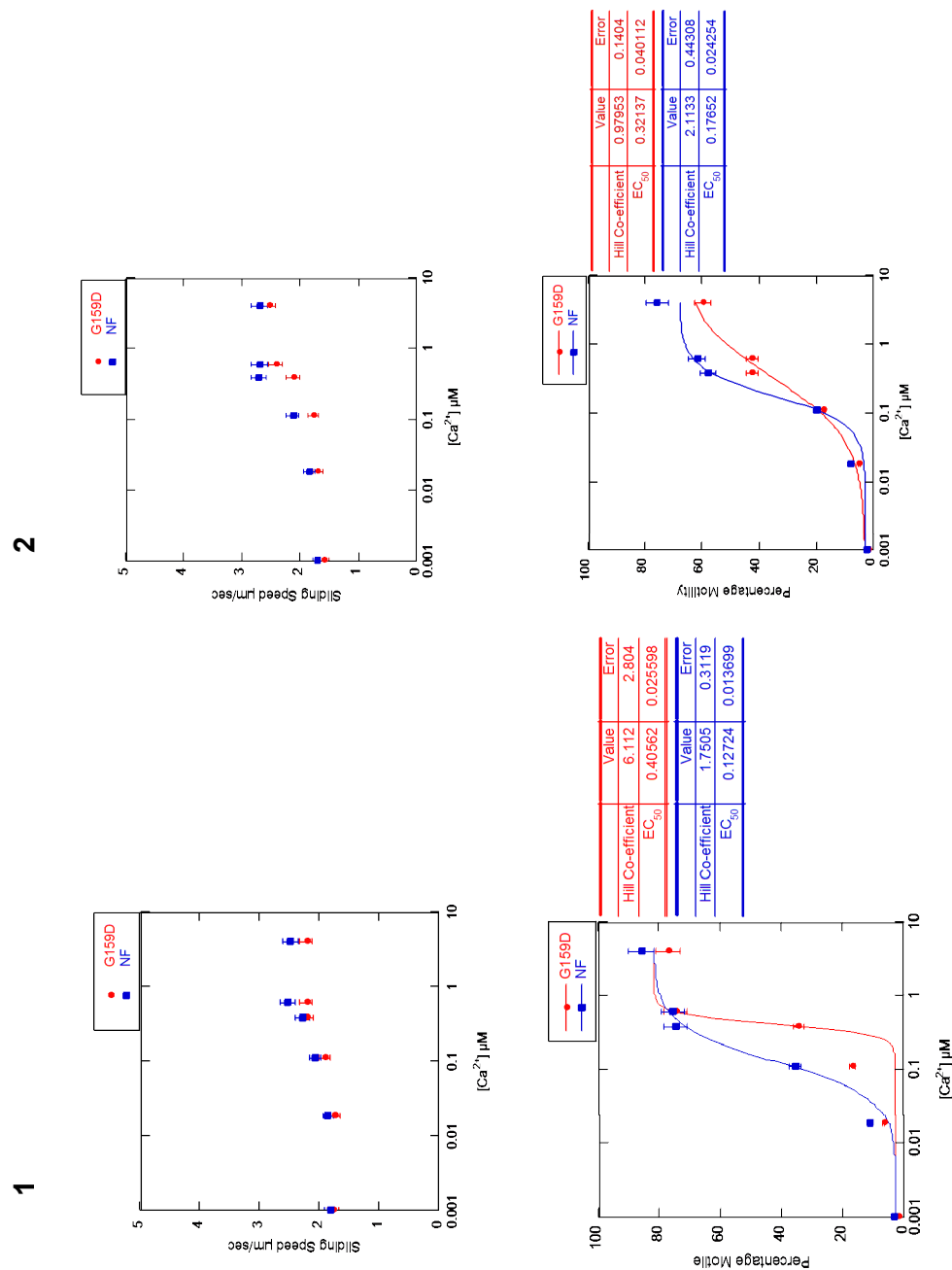
Table 5.8 Ca^{2+} sensitivity Data for Dephosphorylated Non-Failing and G159D Troponin

Expt. No.	EC ₅₀ Percentage Motility, μM		EC ₅₀ NF.dp EC ₅₀ G159D.dp
	NF.dp	G159D.dp	
1	0.132 $\pm$ 0.041	0.064 $\pm$ 0.031	2.06
2	0.105 $\pm$ 0.669	0.071 $\pm$ 3.006	1.48
Mean $\pm$ SD	0.119 $\pm$ 0.014	0.068 $\pm$ 0.004	1.77 $\pm$ 0.29

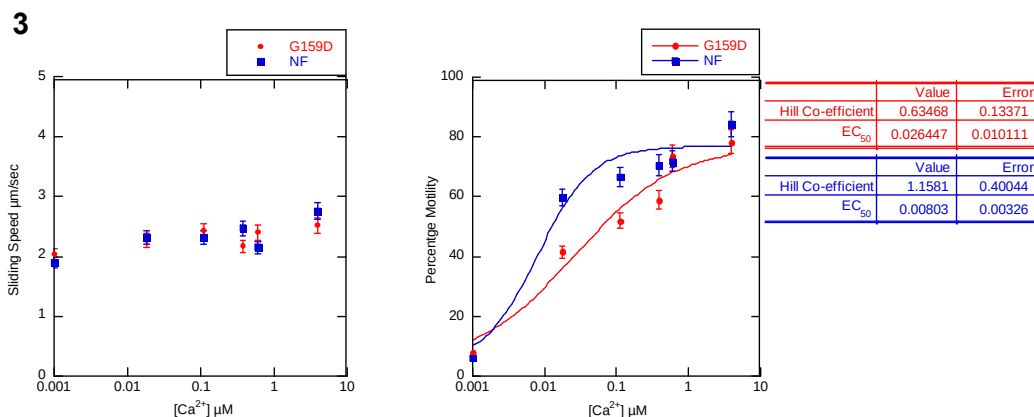
Our strategy has been to reconstitute a system that is as close as possible to the human myofilament. The regulatory properties of thin filaments depend upon all the components. This was illustrated by Bing *et al.*, who reported that different effects of HCM mutations in tropomyosin occur when using either rabbit skeletal muscle troponin or human cardiac troponin (Bing *et al.*, 2000). Two examples concerning the G159D mutation also confirm the importance of the nature of the experimental system. The previous *in vitro* motility results with wild type and G159D troponin expressed in *Escherichia.coli* and substituted into native troponin used human cardiac tropomyosin in the thin filament (Mirza *et al.*, 2005). However, since this troponin was not phosphorylated, an unnatural thin filament was experimented upon. The opposite results found to ours of decreased Ca^{2+} sensitivity and a lower maximum sliding speed reflected this. The second example comes from previous *in vitro* motility studies in our laboratory that used troponin from the same G159D human heart muscle sample (Jacques, unpublished data 2007). In contrast to our findings however, a lower Ca^{2+} sensitivity and a lower maximum sliding speed was found. However, these experiments had AS- $\ddot{\text{U}}$ -tropomyosin reconstituted into the thin filament, producing another unnatural thin filament system. In order to clarify the situation, we have repeated these *in vitro* motility experiments on thin filaments assembled from AS- $\ddot{\text{U}}$ -tropomyosin and native non-failing and G159D human cardiac troponin. Results of a similar direction and magnitude to those reported by Jacques were found in this system. The G159D troponin had a 2.6-fold lower Ca^{2+} sensitivity of percentage

motility (EC_{50} NF/G159D = 0.39) and an 8% lower maximum sliding speed (Figure 5.13, Table 5.9, Table 5.10).

Figure 5.13 Effect of Recombinant Tropomyosin incorporated into the Non-Failing and G159D Thin Filament



Graph 1 (Min values set at 2.6%, max-min at 79%), Graph 2 (Min values set at 2.8%, max-min at 65%).



Graph 3 (Min values set at 4%, max-min at 73%)

Table 5.9 Ca²⁺ Sensitivity Data for Non-failing and G159D Thin Filaments Incorporated with AS- α -Tropomyosin

Expt. No.	EC ₅₀ Percentage Motility, μ M		$\frac{EC_{50} \text{ NF}}{EC_{50} \text{ G159D}}$
	NF	G159D	
1	0.127 $\pm$ 0.014	0.406 $\pm$ 0.026	0.31
2	0.177 $\pm$ 0.024	0.321 $\pm$ 0.040	0.55
3	0.008 $\pm$ 0.003	0.026 $\pm$ 0.010	0.31
Mean $\pm$ SD (n = 3)	0.104 $\pm$ 0.050	0.251 $\pm$ 0.115	0.39 $\pm$ 0.08

Table 5.10 Non-Failing and G159D Thin Filament Sliding Speeds at 3.9 μ M Ca²⁺

	3.9 μ M Sliding Speeds μ m/sec	
Expt No.	Non-Failing	G159D
1	2.75 $\pm$ 0.11	2.52 $\pm$ 0.15
2	2.70 $\pm$ 0.06	2.54 $\pm$ 0.06
3	2.48 $\pm$ 0.02	2.22 $\pm$ 0.05
Mean: $\pm$ SE (n = 3)	2.64 $\pm$ 0.08	2.43 $\pm$ 0.10

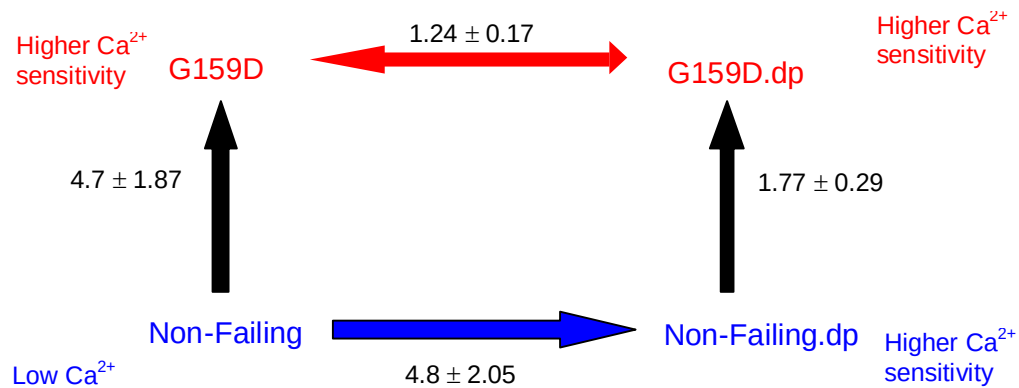
5.6 Discussion

The TNNC1 G159D mutation was first identified by Mogensen *et al.* in a genetic screen study of DCM families (Mogensen *et al.*, 2004b). One family was affected, and we were fortunate enough to obtain a muscle sample from a three-year old child belonging to the original family that underwent an orthotopic heart transplant. To date this is the only known DCM tissue sample that has been genotyped. The aim was to isolate pure troponin (fully phosphorylated) from the patient with the G159D troponin C mutation and investigate the functional effects of the mutation in combination with troponin phosphorylation using the *in vitro* motility assay. Previous *in vitro* motility studies have been done on thin filaments assembled from combinations of recombinant and native troponin and tropomyosin subunits (Mirza *et al.*, 2005). However, since we have had access to a muscle sample we have been able work with proteins that were all isolated from human cardiac muscle. Troponin was isolated from the human muscle sample via an affinity separation method that used an anti-cardiac troponin I monoclonal antibody conjugated to CNBr Sepharose CL-4B (section 2.8.1). The yield was normally 600µl of 7µl from 100mg of tissue. Troponin was dephosphorylated by incubation with 1.4 U/ml acid phosphatase (section 2.8.1.1). Staining of this troponin with a Pro-Q Diamond Phosphoprotein Gel Stain confirmed its full dephosphorylation (Figure 5.9). Thin filaments were reconstituted with skeletal rabbit actin (same amino acid sequence as human ACTA1), human cardiac troponin (from non-failing heart or the G159D heart muscle sample) and human cardiac β -tropomyosin.

When G159D troponin and non-failing troponin were incorporated into the thin filaments in their native phosphorylation states (with both troponin I and troponin T phosphorylated), the Ca^{2+} sensitivity of G159D troponin was 4.67 ± 1.87 -fold higher than NF troponin in the percentage motility parameter. The sliding speed did not change enough for an accurate EC_{50} value to be determined. When dephosphorylated, G159D troponin had a Ca^{2+} sensitivity that was similar to G159D troponin in its native phosphorylated state, $\text{EC}_{50} 159/159.\text{dp} = 1.24 \pm 0.17$. This can be directly compared with the 4.8 -fold ± 2.05 increase in Ca^{2+} sensitivity of dephosphorylated non-failing troponin compared to native non-failing troponin. This lack of response to troponin I

phosphorylation levels was also found with the actin E361G DCM mutation. Comparisons of the G159D with non-failing troponin when both dephosphorylated showed the G159D troponin to have a slightly raised Ca^{2+} sensitivity compared to dephosphorylated non-failing troponin (percentage motility $\text{EC}_{50} \text{ NF.dp/159.dp} = 1.77 \pm 0.29$), although the difference is less than with the native troponin (percentage motility $\text{EC}_{50} \text{ NF/159} = 4.67 \pm 1.87$). These findings are summarised in Figure 5.14.

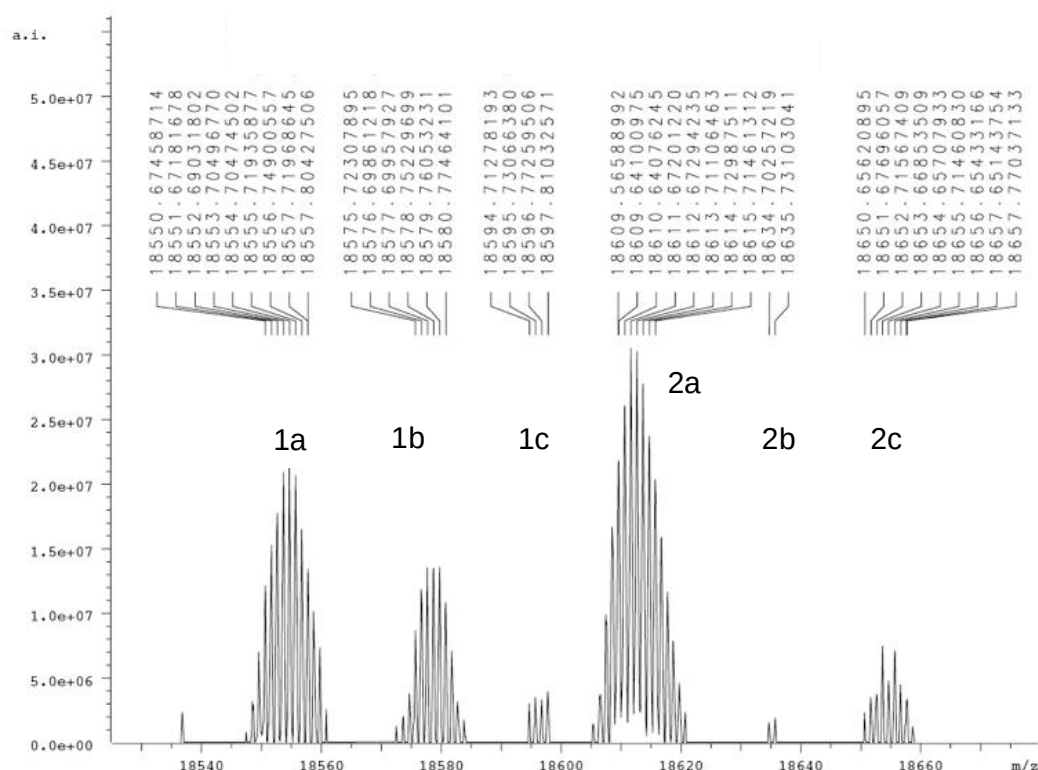
Figure 5.14 Comparison of Phosphorylated and Dephosphorylated Non-failing and G159D Troponin



The average EC_{50} value differences (percentage motility) of non-failing and G159D troponin. The larger of the two sided arrows denotes the direction of higher EC_{50} value.

The troponin isolated from the human muscle sample should have the mutant to wild type ratios characteristic of the DCM phenotype. Preliminary mass spectrometry data has been conducted to try and determine what this ratio is. Initial ESI-FTICR studies of a trial mixture of 1:1 wild type: G159D recombinant troponin C mixture conducted in Peter Derrick's laboratory has shown the FTICR technique can clearly distinguish the mutant troponin C and quantify the amount present (Figure 5.15).

Figure 5.15 ESI-FTICR of 1:1 Recombinant Troponin C and G159D Troponin C Mixture

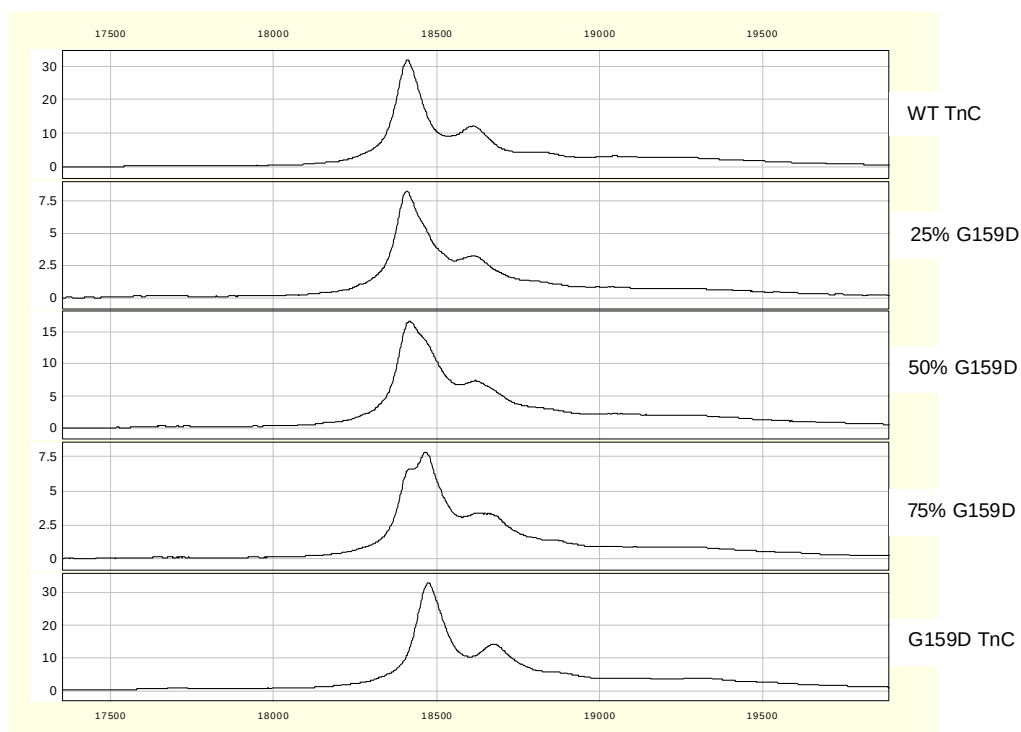
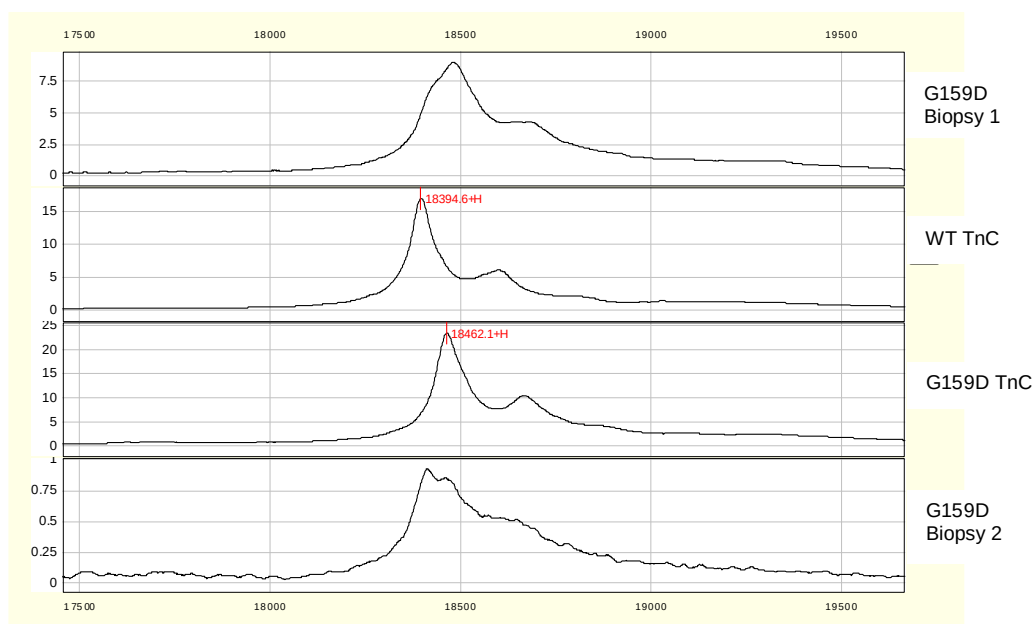


Two species with major bands are clearly resolved. Peak 1a corresponds to troponin C (18,403Da from amino acid sequence), and peak 2a corresponds to troponin C G159D (+150Da). The satellite peaks 1b and 2b are +Mg, and 1c and 2c are Ca^{2+} . The proportion of each of the 3 bands is different for troponin C and G159D troponin C. Adding up the 3 bands on peak height gives 10.7 units for troponin C and 11.1 units for G159D troponin C. This equals the 1:1 ratio of species actually present, thus G159D is 51% of cardiac troponin C.

Evidence for the presence of the G159D mutation in whole troponin isolated from our G159D human muscle biopsy has been confirmed by SELDI-Mass Spectrometry carried out by Dr. Douglas Ward (CRC Cancer Studies at the University of Birmingham), and Dr. Clare Gallon (NHLI, Imperial College London). The whole troponin was purified using an NP20 SELDI Chip. Figure 5.16 shows 100% recombinant wild type and G159D troponin C traces and mixtures of 25%, 50%, and 75% G159D troponin C. The wild type mass obtained of 18395Da was close to the theoretical value of 18403Da. The G159D mass obtained of 18462Da was close to the theoretical value of

18461Da. The identity of the peak to the right of the wild type troponin C is unknown.

Figure 5.17 shows two mass spectra obtained from the human muscle sample of cardiac G159D troponin. Sample 2 has a poor resolution, although both show 2 peaks corresponding to cardiac troponin C and G159D cardiac troponin C. The quantity of mutant to wild type troponin C in our human muscle biopsy seems to be between 50-75%. SELDI-MS of troponin is able to detect cardiac troponin C at a much lower resolution than FTICR. Further mass spectroscopy studies are being conducted on the G159D human muscle biopsy in Prof. Jeffery Walker's laboratory at the University of Wisconsin.

Figure 5.16 SELDI-MS of Recombinant Troponin C Mixtures**Figure 5.17 SELDI-MS of G159D Whole Troponin**

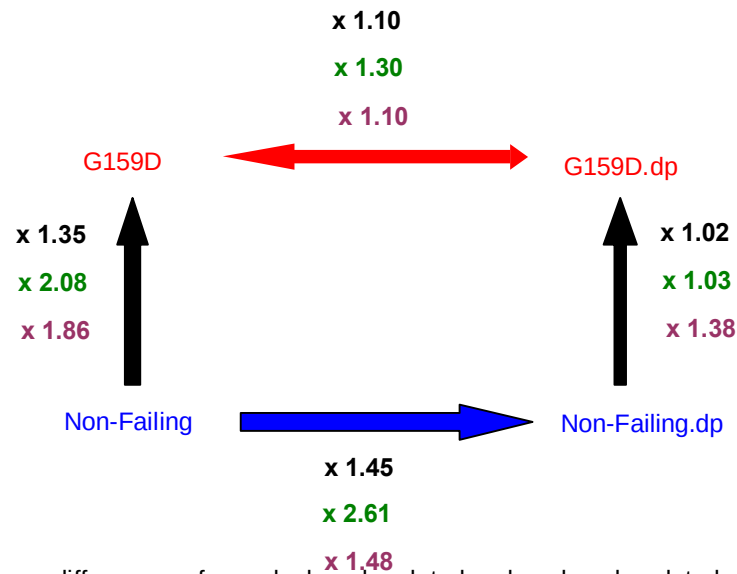
The findings from this G159D human muscle study can be directly compared with recently published studies that used pseudophosphorylated and unphosphorylated recombinant G159D and wild type troponin (Biesiadecki *et al.*, 2007, Preston *et al.*, 2007a). When Biesiadecki *et al.* incorporated recombinant wild type and G159D troponin (with no phosphorylation) into chemically skinned rat trabeculae, no differences were found between wild type and G159D troponin (WT-Tn $EC_{50} = 2.34 \pm 0.11$, G159D-Tn $EC_{50} = 2.29 \pm 0.14$). However, when pseudophosphorylated troponin I was incorporated into the skinned rat trabeculae, the Ca^{2+} sensitivity of G159D troponin was increased 1.35-fold compared with exchange with wild type-pseudophosphorylated troponin (G159D-Tn-DD $EC_{50} = 2.51 \pm 0.17$, WT-Tn-DD $EC_{50} = 3.39 \pm 0.18$). ATPase measurements also demonstrated ATPase hydrolysis was significantly enhanced 1.32-fold after exchange with the pseudophosphorylated G159D troponin compared with wild type pseudophosphorylated troponin (G159D-Tn-DD $EC_{50} = 2.14 \pm 0.15$, WT-Tn-DD $EC_{50} = 2.82 \pm 0.2$). These experiments are equivalent to the native G159D and non-failing troponin comparison described in this thesis, and a similar result was obtained. In comparison, Preston *et al.* reported that G159D troponin C in the presence of a PKA phosphomimic of troponin I (Ser23Asp, Ser24Asp) had an equal Ca^{2+} sensitivity to wild type troponin I PKA pseudophosphorylated troponin (exchanged into skinned rabbit psoas fibres) (Preston *et al.*, 2007a).

Both Biesiadecki *et al.* and Preston *et al.* reported that the Ca^{2+} sensitivity of G159D troponin was insensitive to troponin pseudophosphorylation. Biesiadecki *et al.* reported no differences in the ATPase and tension of G159D containing a PKA pseudophosphorylated Troponin I exchanged into trabeculae compared to those containing unphosphorylated Troponin I (G159D-Troponin-DD $EC_{50} = 2.51 \pm 0.17$, G159D-Troponin $EC_{50} = 2.29 \pm 0.14$). Preston *et al.* showed that the G159D mutation abolished the significant increase in maximal force (but not the decrease in Ca^{2+} sensitivity) that resulted from the incorporation of the troponin I phosphomimic in the presence of wild type Troponin C. The mutation blunted the reduction in Ca^{2+} sensitivity caused by a mimic of PKC troponin T phosphorylation (Thr203Glu). These findings showing the blunted response of G159D troponin

to phosphorylation correlate with the *in vitro* motility assay results reported in this study.

It is a widely established finding that a lower Ca^{2+} sensitivity is obtained when troponin is phosphorylated and this is associated with a decrease in Ca^{2+} binding affinity to the troponin complex. Measurements of Ca^{2+} binding by troponin I-C complexes *in vitro* have shown that the dependence of Ca^{2+} binding affinity on troponin I phosphorylation was lost due to the G159D mutation (Biesiadecki *et al.*, 2007, Dong *et al.*, 2007). Biesiadecki *et al.* has shown that the presence of the G159D mutation did not alter Ca^{2+} binding affinity when unphosphorylated (EC_{50} WT-Troponin/G159D-Troponin = 1.03). However when phosphorylated, the decrease in Ca^{2+} binding shown with wild type troponin was prevented with G159D troponin. Dong *et al.* reported using Förster resonance energy transfer (FRET) combined with equilibrium Ca^{2+} titration and stopped-flow kinetic measurements, that the kinetics of the Ca^{2+} induced opening or closing of the N-domain was altered by G159D and when troponin I was phosphorylated by PKA, the G159D mutation completely abolished the effect of phosphorylation on the kinetics of troponin C N-domain opening and closing. The Ca^{2+} binding affinity results of Biesiadecki *et al.* and Dong *et al.* are in direct correlation with our *in vitro* motility studies on G159D, and are summarised in Figure 5.18. In contrast to Biesiadecki *et al.* and Dong *et al.*, Robinson *et al.* have found no difference in Ca^{2+} binding when troponin I was unphosphorylated. They showed using an IAANS fluorescent probe attached to Cys34 of troponin C followed by reconstitution into regulated thin filaments, that a 50: 50 mixture of unphosphorylated G159D troponin had a 2.6-fold decrease in the Ca^{2+} binding affinity (EC_{50} WT-troponin = 0.61 ± 0.02 , EC_{50} G159D-troponin = 1.58 ± 0.02) (Robinson *et al.*, 2007). However, the Ca^{2+} binding affinity of G159D troponin C in combination with troponin I phosphorylation was not investigated.

Figure 5.18 Comparison of Pseudophosphorylated and Unphosphorylated Non-failing and G159D Recombinant Troponin



The average differences of pseudophosphorylated and unphosphorylated non-failing and G159D troponin from Biesiadecki *et al.* and Dong *et al.* experiments are shown above. Biesiadecki (Isometric Force measurements), Biesiadecki (Ca^{2+} binding measurements), Dong (Ca^{2+} binding measurements). The larger of the two sided arrows denotes the direction of higher EC_{50} value.

A reduced response to troponin I phosphorylation levels and a higher Ca^{2+} sensitivity were two of the main effects produced by the G159D mutation described in this thesis. The results of Biesiadecki *et al.*, Preston *et al.* and Dong *et al.* are in agreement with our *in vitro* motility data (Biesiadecki *et al.*, 2007, Preston *et al.*, 2007a, Dong *et al.*, 2007). The reduced response of mutant thin filaments to troponin phosphorylation levels also corresponds with the E361G ACTC mutation, and could on its own cause a DCM phenotype. However, the higher Ca^{2+} sensitivity does not correspond with either the E361G mutation or with previous studies on G159D (Jacques, unpublished data 2007) (Mirza *et al.*, 2005).

Previous *in vitro* motility studies have been done on troponin extracted from this same G159D human biopsy sample in our laboratory (Jacques, unpublished data 2007). In contrast to our results however, the native G159D troponin showed a lower Ca^{2+} sensitivity (EC_{50} increased by 1.63) and a 17% lower maximum sliding speed than native non-failing troponin. It is important to note however, that these results were obtained when Ala-Ser $\ddot{U}$ -tropomyosin

(AS- $\checkmark$ Tm) was reconstituted into the non-failing and G159D thin filaments. Results reported in this chapter of an increased Ca^{2+} sensitivity of native G159D troponin and insensitivity to troponin I phosphorylation, were produced when human cardiac tropomyosin was incorporated into the thin filament. We have been able to reproduce this AS- $\checkmark$ Tm result and a similar decrease in Ca^{2+} sensitivity was found (EC_{50} increased by 2.4). The G159D mutation has therefore been shown to cause a lower or higher Ca^{2+} sensitivity compared to non-failing troponin depending on the incorporation of either native human cardiac tropomyosin or AS- $\checkmark$ Tm into the thin filament. Recombinant AS- $\checkmark$ Tm differs from native human cardiac tropomyosin in its N-terminal Ala-Ser extension, which mimics acetylation. Studies on the cooperative allosteric regulation of tropomyosin isoforms have shown AS- $\checkmark$ Tm to have a significantly lower cooperative unit size of 6.5 compared to a cooperative unit size of 10 produced with native N-acetylated $\checkmark$ -tropomyosin (Mirza *et al.*, 2007). Cooperative control of the thin filament is dependent upon the end-to-end interactions that occur between tropomyosin dimers at the N-terminus, and the N-terminal Ala-Ser extension of AS- $\checkmark$ Tm could explain the change in G159D troponin function that was produced in this study.

The higher Ca^{2+} sensitivity of the native G159D troponin found in our study is also in contrast to results produced from a 50:50 mixture of wild type: recombinant G159D troponin, which showed that G159D troponin had a 14% lower sliding speed at $3.9\mu\text{M}$ Ca^{2+} and a lower Ca^{2+} sensitivity in the *in vitro* motility assay (EC_{50} increased by 1.54) (Mirza *et al.*, 2005). This phenotype was found by all 8 DCM mutations studied in cardiac troponin T, troponin C, and $\checkmark$ -tropomyosin using the *in vitro* motility and ATPase assays by Mirza *et al.*, and was the same direction and magnitude to results produced by A. Jacques using the human G159D biopsy. However, both these studies used a mixture of recombinant and human cardiac proteins in the thin filaments and did not therefore produce an entirely physiological system. In contrast, the results described in this chapter used troponin from a human G159D biopsy which should have the post-translational modifications and mutant: wild type ratios characteristic of the DCM phenotype. Incorporation of this troponin with human cardiac tropomyosin should represent the conditions of an actual human system.

In conclusion, our results demonstrate that cardiac thin filaments regulated by G159D troponin have a higher Ca^{2+} sensitivity and lack the Ca^{2+} desensitisation that normally results when Troponin I is phosphorylated by PKA. It is important to determine what may be physiological and what may cause the DCM phenotype. The blunting of response to troponin I phosphorylation has been found in all native systems for G159D, as well as for the actin E361G mutation and could occur for other DCM tropomyosin and troponin T mutations. This may indeed be enough to cause the DCM phenotype. In comparison, the higher Ca^{2+} sensitivity of G159D troponin is different from all other DCM mutations that have been previously studied, although these previous studies were in non-physiological systems so it could just be an artefact of this system.

To date, these are the first set of measurements from human DCM tissue tested in combination with troponin phosphorylation, and the natively modified partner proteins used in this study are more likely to mimic the real *in vivo* situation than previous systems.

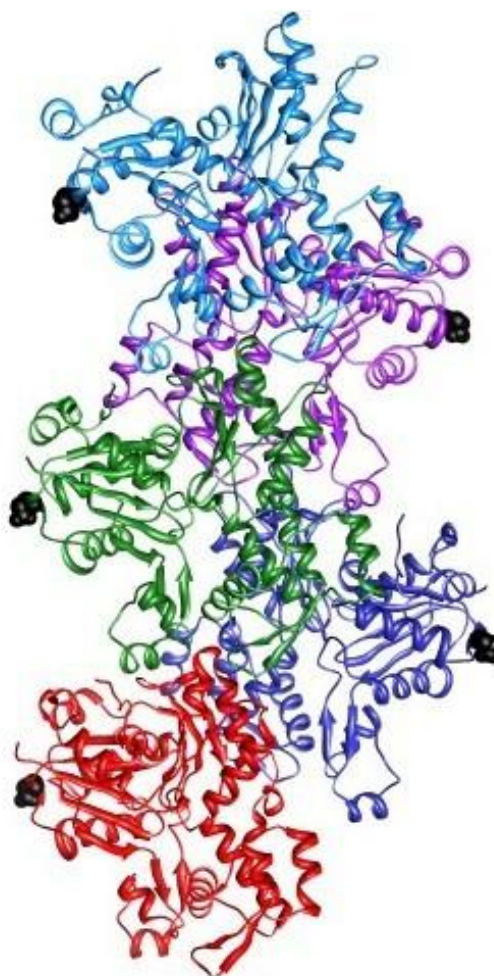
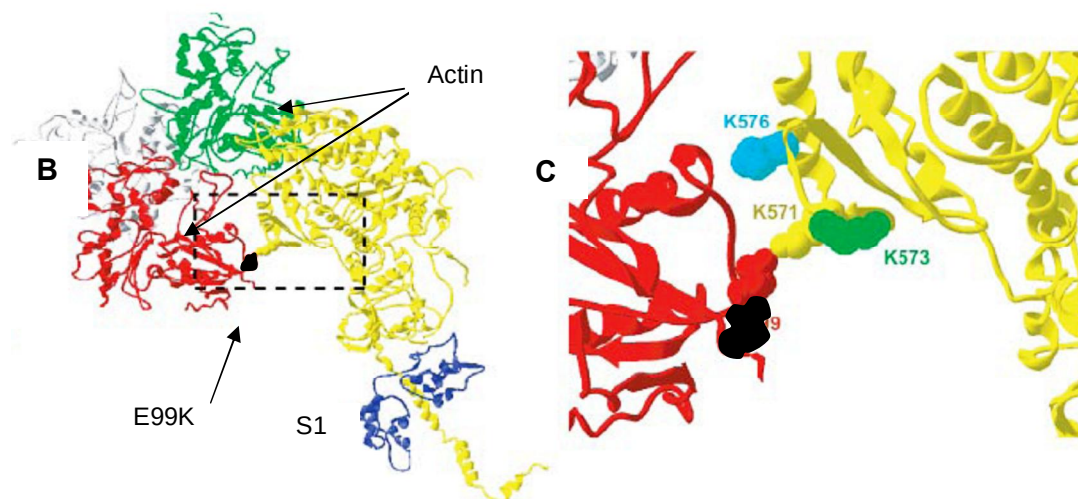
6. FUNCTIONAL INVESTIGATION OF HCM ACTIN MUTANT GLU99LYS

6.1 Introduction

To date, there are 7 mutations in the human alpha-cardiac actin gene (ACTC) known to cause HCM. In this study, a transgenic mouse model has been developed that over expresses the HCM actin mutation Glu99Lys (E99K) in their hearts. The E99K mutation has been identified in numerous genetic screening studies (Arad *et al.*, 2005a, Olson *et al.*, 2000, Monserrat *et al.*, 2007). Many affected families show an uncommon apical form of HCM. The mutation is also associated with atrial septal defects. Using the *in vitro* motility assay, this chapter will describe the functional investigation of E99K actin when reconstituted into a regulated thin filament.

6.2 Location of E99K in the Actin Molecule

The E99K mutation is located in a surface loop in subdomain 1 of the actin molecule (Figure 6.1A). This is one of the secondary binding sites for the myosin head on each actin monomer. The E99K region is thought to interact with the positively charged cluster located in the lower 50-kDa domain of the myosin motor domain. The E99K mutant, caused by a single charge reversion, is likely to have an impact on actomyosin interactions (Bookwalter and Trybus, 2006).

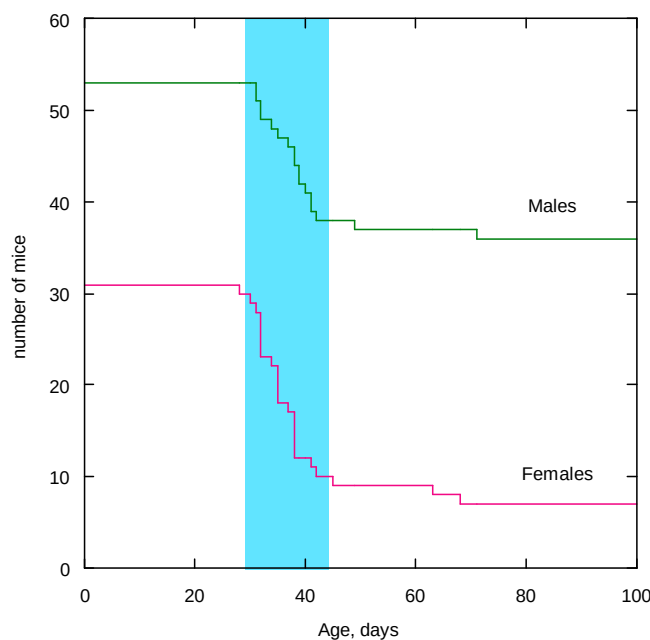
Figure 6.1 Location of the E99K Mutation in Actin**A**

[A] A ribbon representation of actin with E99K shown in black (produced using Chimera). [B] S1 docking onto actin. S1 interacts with 2 actins (shown in green and red). E99K is on the red actin subunit and is part of the secondary binding site that interacts with residue Lys-571 of S1. A close up is shown in [C]. Two other nearby Lys residues (Lys-573 (green) and Lys-576(cyan) are also indicated. Obtained from (Bookwalter and Trybus, 2006).

6.3 E99K Transgenic Mouse Phenotype

The E99K transgenic mice have not shown any cardiac hypertrophy and no septal defects have been observed. The mice appear healthy and breed normally. However, the mice show a high rate of sudden death. Prior to death, there is no weight loss and post-mortem mice have the same weight as their non-transgenic littermates. Assuming all sudden deaths are in transgenic individuals, the females exhibiting early death die in the range of 28-45 days (mean 35 days). The males are very similar, with sudden death ranging from 31-49 days (mean 40 days) (Figure 6.2). As a percentage of the total of genotyped transgenics, 71% of the females die at this time, and 34% of the males. After this period, there is low sporadic mortality that is more prominent in females than males and is higher than normal for this genetic background. However, there was no obvious pre-mortem clinical signs or post-mortem abnormalities.

Figure 6.2 Age of Death in Male and Female E99K Transgenic Mice

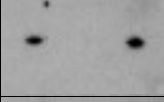
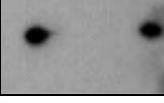


There was no difference between males and females in terms of breeding. The higher numbers of male mice was due to fewer genotyped females.

6.4 Screening of E99K Transgenic Lines

The charge change of glutamic acid to lysine (+2) allowed the mutant to be identified and quantified by 2-D Electrophoresis. Four E99K transgenic lines were generated (Table 6.1). Just one line (N11) successfully incorporated the transgene and expressed E99K mutant cardiac actin in their hearts. This expression amounted to approximately 55% of the total actin.

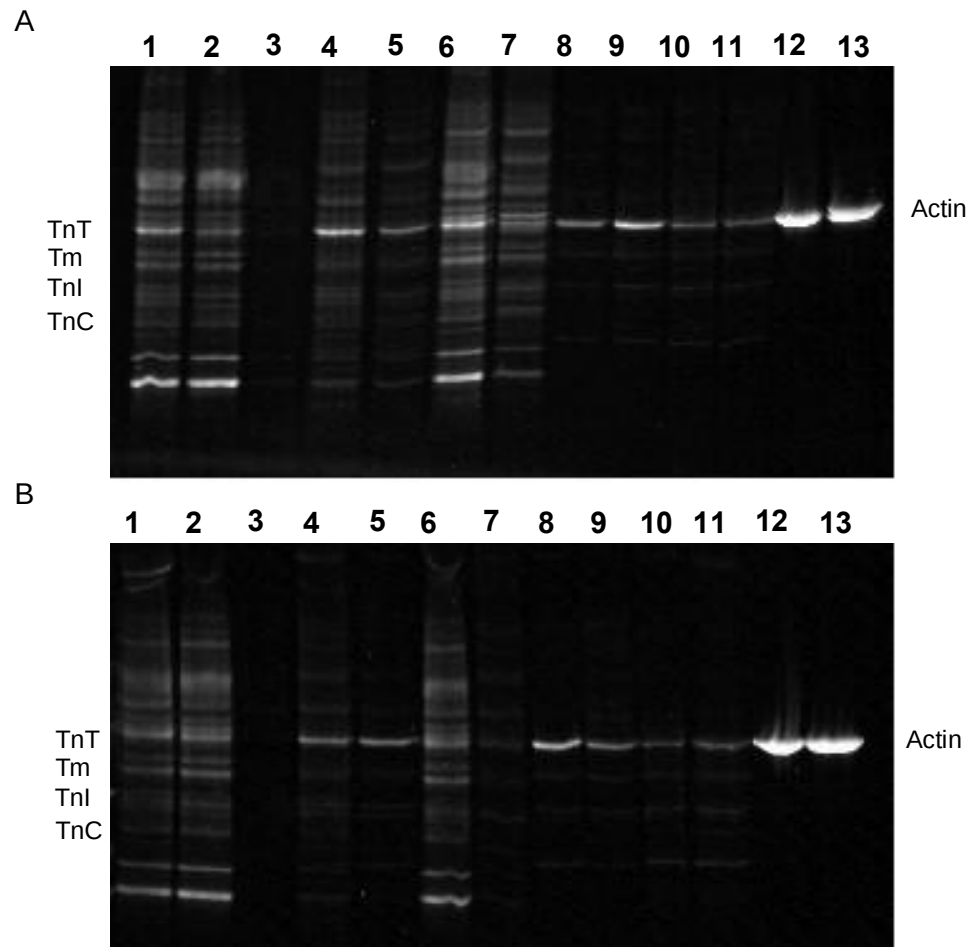
Table 6.1 Screening of E99K Transgenic Lines

	Sample	Heart Weight (g)	PCR Result	2-DE	% E99K actin
Line 16	16.5	0.10	+		0 %
	16.6	0.11	-		0 %
	16.7	0.12	+		0 %
Line N2	3 +		+		0 %
Line N5	9 +		+		0 %
Line N11	17 +		+		56 %
	16 +		+		45%

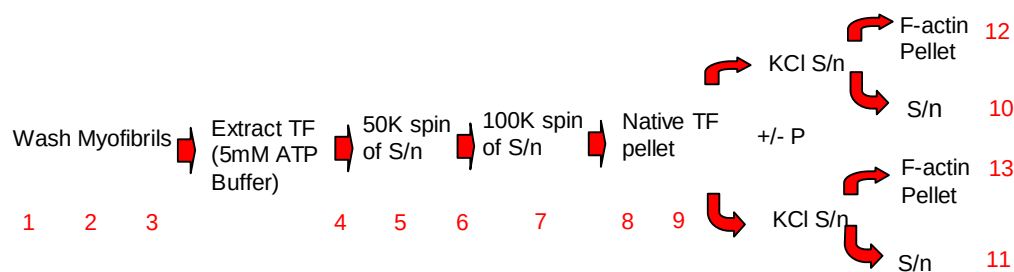
6.5 Isolation of F-Actin from E99K Transgenic Mouse Hearts

Actin was isolated directly from native thin filaments using the method described in section 2.5. This successfully preserved the 50% expression of the E99K mutation in the F-actin.

Figure 6.3 SDS-PAGE of (A) E99K and (B) Non-Transgenic Actin Isolation Fractions

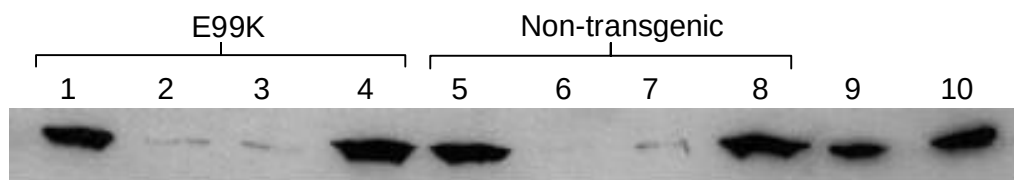


1) Whole muscle homogenate, (2) first wash s/n, (3) last wash s/n, (4) S/n after 2 extractions in thin filament extraction buffer, (5) S/n after 50K spin, (6) Pellet after 50K spin, (7) S/n after 100K spin, (8) Resuspended pellet of 100K spin before dialysis, (9) S/n after dialysis, (10) KCl s/n + 150nM phalloidin, (11) KCl s/n - 150nM phalloidin, (12) f-actin (+ phalloidin), (13) f-actin (- phalloidin).



The whole thin filaments were pelleted after the 100K spin of the thin filament extract supernatants (fraction 8). The pellet was resuspended in an ATP buffer and dialysed for 3 hours. After dialysis (fraction 9), the thin filaments were mixed with 0.8M KCl, with and without phalloidin (fraction 10 and 11), and the F-actin was pelleted (fraction 12 and 13). No differences were detected during the isolation of non-transgenic and E99K actin. The final F-actin pellet was pure for both non-transgenic and E99K actin.

Figure 6.4 Western Blot of Actin Isolation Fractions

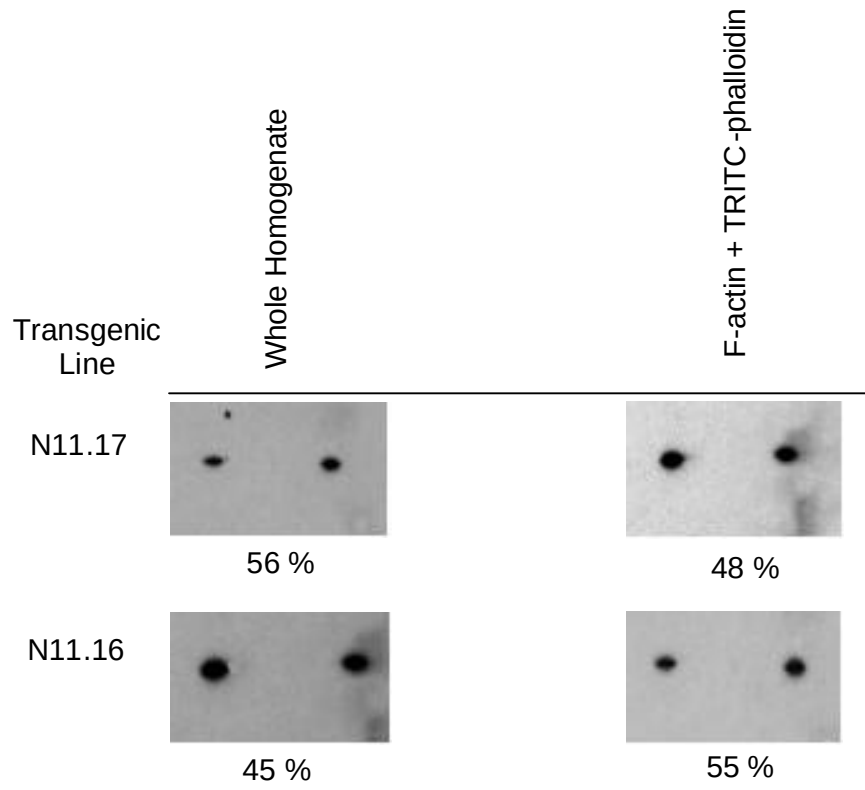


(1) E99K whole homogenate, (2) E99K supernatant after 100K spin, (3) E99K KCl supernatant + 150nM TRITC-phalloidin, (4) E99K F-actin pellet + TRITC-phalloidin, (5) NTg whole homogenate, (6) NTg supernatant after 100K spin, (7) NTg KCl supernatant + 150nM TRITC-phalloidin, (8) NTg F-actin pellet + TRITC-phalloidin, (9) 0.02µg actin marker, (10) 0.05µg marker.

Western blotting analysis was used to determine the final concentration and overall yield of E99K F-actin, which was found to be the same as the non-transgenic F-actin. The concentration of both E99K and non-transgenic actin was approximately 0.4 mg/ml and the yield of actin approximately 1µg per 1mg of tissue.

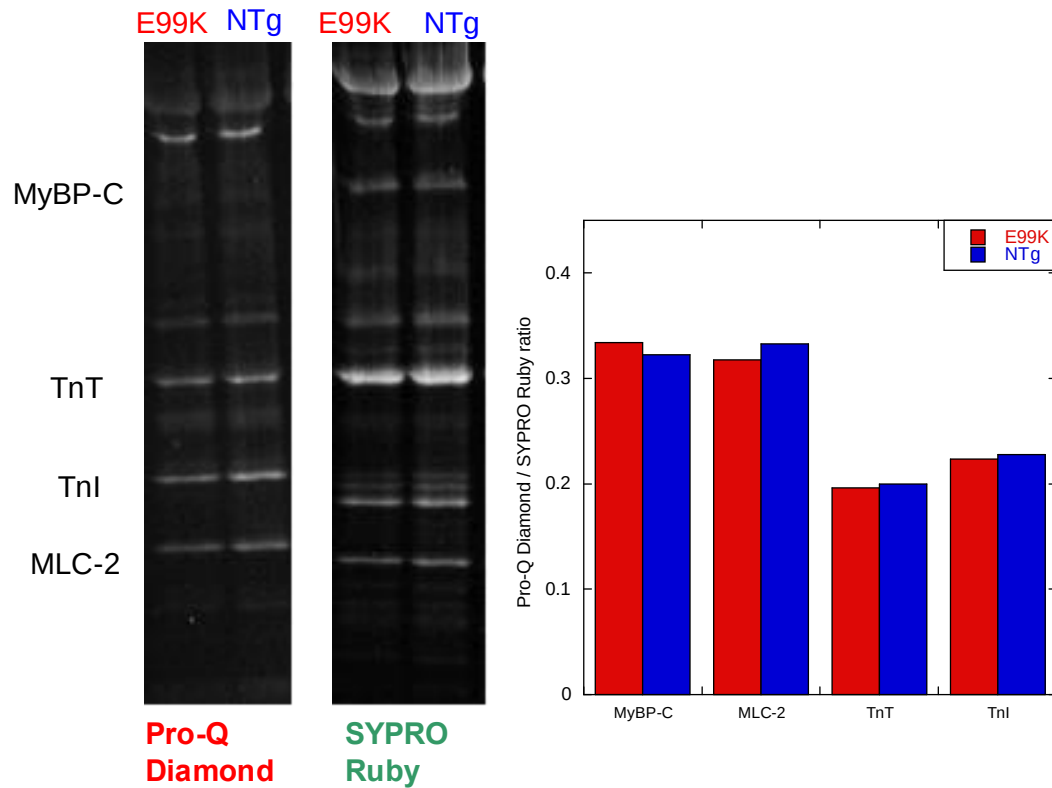
2-D Electrophoresis was carried out on the whole muscle homogenate and f-actin from 2 separate actin preparations. Extracting actin from whole thin filaments preserved the expression at approximately 50% in the final f-actin (Figure 6.5).

Figure 6.5 Preservation of E99K Expression



6.6 Measurement of Phosphorylation Levels

Myofibrils from non-transgenic and E99K hearts were analysed to determine the phosphorylation level of all proteins. Myofibrils were separated by SDS-PAGE, and bands detected by the Pro-Q Diamond phosphoprotein gel stain were normalised to the total protein levels in each band (stained by SYPRO Ruby) (Figure 6.6). No difference was found between the phosphorylation of myosin binding protein C, troponin T, troponin I, and myosin light chain 2 in non-transgenic and E99K heart myofibrils.

Figure 6.6 SDS-PAGE of E361G and Non-Transgenic Myofibrils**Table 6.2 Pro-Q/SYPRO ratios from 5 Myofibril Experiments**

Expt. No.	MyBP-C		MLC-2		TnT		TnI	
	E99K	NTg	E99K	NTg	E99K	NTg	E99K	NTg
1	0.334	0.361	0.321	0.342	0.175	0.192	0.284	0.275
2	0.422	0.393	0.243	0.275	0.188	0.204	0.261	0.232
3	0.278	0.263	0.290	0.298	0.181	0.169	0.185	0.207
4	0.256	0.244	0.339	0.375	0.275	0.266	0.176	0.207
5	0.376	0.350	0.393	0.372	0.164	0.170	0.210	0.220
Mean ± SE	0.333 ± 0.031	0.322 ± 0.029	0.317 ± 0.025	0.333 ± 0.020	0.197 ± 0.020	0.200 ± 0.018	0.223 ± 0.021	0.228 ± 0.013
T-test (paired/unpaired)	p = 0.327 / 0.799, n=5		p = 0.210 / 0.648, n=5		p = 0.611 / 0.901, n=5		p = 0.693 / 0.854, n=5	

6.7 Length Distribution of E99K and Non-Transgenic Actin

Non-transgenic and E99K actin filaments were of similar appearance when labelled with TRITC-phalloidin (Figure 6.7). Filaments were short and bright and present in high yield relative to the concentration at a 1/1000 dilution. Measurements using the DIAS 3.2 programme showed non-transgenic and E99K actin had similar filament length distributions (Figure 6.8). The mean length of non-transgenic actin was $7.4\mu\text{m} \pm 0.46$ ($n = 6$) and E99K actin was $7.8\mu\text{m} \pm 0.4$ ($n = 6$).

Figure 6.7 Non-Transgenic and E99K Actin Filaments Labelled with TRITC-phalloidin

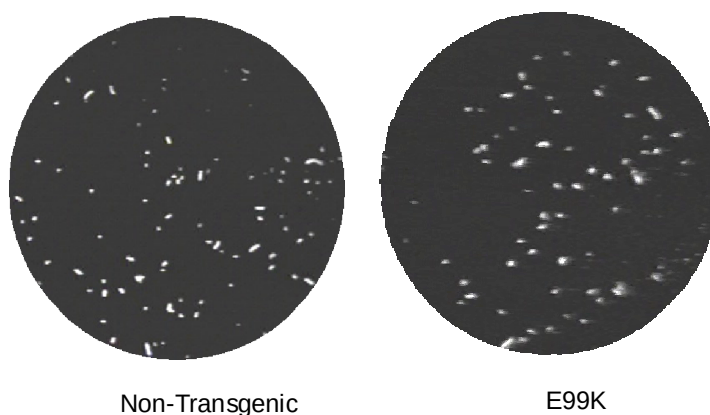
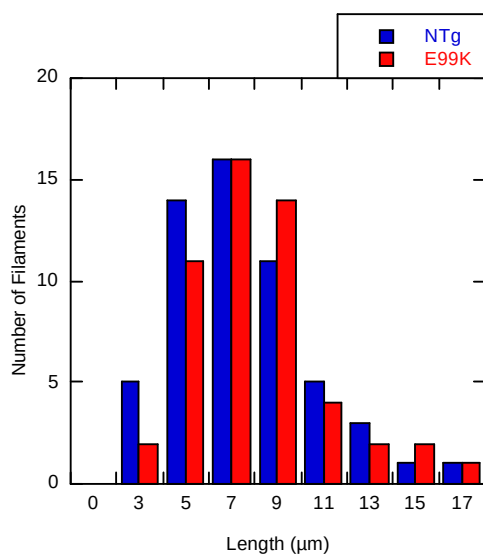


Figure 6.8 Length Distribution of Non-Transgenic and E99K Actin



6.8 *In Vitro* Motility Assay

6.8.1 Non-Transgenic and E99K Actin Filament Motility

14 separate measurements were taken using 6 pairs of hearts and the averages are shown in Table 6.3. The sliding speed of E99K actin was consistently lower than non-transgenic actin. This difference was 8%, which was significant in the paired t-test ($p = 0.004$). E99K actin also had a 4% lower percentage motile of filaments than non-transgenic actin, although this was not significant (paired t-test, $p = 0.22$).

Table 6.3 Comparison of Non-Transgenic and E99K Actin

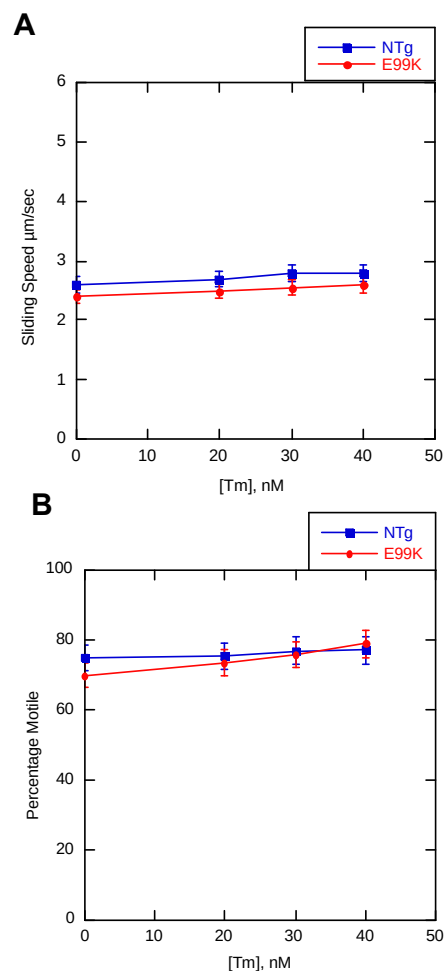
Transgenic Line		Actin Sliding Speed ($\mu\text{m}/\text{sec}$)		Actin Percentage Motile	
NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	3.3 ± 0.03 2.7 ± 0.06 3.0 ± 0.05 3.1 ± 0.11	2.9 ± 0.04 2.7 ± 0.08 2.5 ± 0.14 3.0 ± 0.11	89 ± 1.80 75 ± 2.93 80 ± 2.86 85 ± 4.42	58 ± 1.80 82 ± 1.73 74 ± 3.62 88 ± 3.40
N2.3	N11.17	3.4 ± 0.09 3.3 ± 0.05 3.0 ± 0.12 3.0 ± 0.18	3.3 ± 0.23 3.3 ± 0.09 2.2 ± 0.04 2.6 ± 0.17	97 ± 0.71 92 ± 3.19 80 ± 0.41 71 ± 6.77	90 ± 4.94 96 ± 1.78 71 ± 4.01 74 ± 2.40
N11.30.9	N11.16	2.6 ± 0.06 3.0 ± 0.07	2.4 ± 0.08 2.5 ± 0.10	66 ± 0.50 76 ± 5.37	73 ± 4.10 71 ± 1.93
N.16.30	N11.30.16	2.3 ± 0.05	2.3 ± 0.12	72 ± 3.23	67 ± 1.78
N5.3	N11.30.12	2.1 ± 0.07	2.1 ± 0.07	57 ± 3.50	51 ± 2.33
N5.9	N11.30.10	2.3 ± 0.11 2.7 ± 0.08	2.3 ± 0.10 2.4 ± 0.03	76 ± 4.15 75 ± 4.27	79 ± 4.57 70 ± 5.02
Mean $\pm$ SE n = 14		2.84 ± 0.11	2.61 ± 0.10	78 ± 2.80	75 ± 3.22
T Test P Value		Paired = 0.004 Unpaired = 0.13		Paired = 0.22 Unpaired = 0.44	

The significantly slower sliding speed of E99K actin compared to non-transgenic actin correlates with findings by Bookwalter *et al.* on E99K actin expressed and purified from the baculovirus/insect cell expression (Bookwalter and Trybus, 2006). They found E99K actin had a reduced velocity of between 13-21% relative to wild type actin.

6.8.2 Effects of Human Cardiac Tropomyosin on Actin Filament Motility

20-60nM tropomyosin was titrated with 10nM non-transgenic and E99K actin. 10 measurements were taken from 6 paired non-transgenic and E99K hearts, and the averages are shown in Table 6.4 and Table 6.5.

Figure 6.9 Effect of Tropomyosin on Sliding Speed (A) and Percentage Motility (B)



An optimal increase in non-transgenic and E99K actin sliding speeds and percentage motility was seen upon titration with 40nM human cardiac tropomyosin (Table 6.4). The sliding speed of non-transgenic actin increased by 6.5%, which was significant in the paired t-test ($p = 0.007$). E99K actin increased by 10% ($p = 0.06$). Overall, E99K actin.tropomyosin had a 4% slower

sliding speed compared to non-transgenic actin.tropomyosin, although the difference was not significant in the paired t-test ($p = 0.30$).

Table 6.4 Effect of 40nM Human Cardiac Tropomyosin on Non-Transgenic and E99K Actin Sliding Speeds

Filament Sliding Speed(μm/sec)					
Transgenic Line		Actin		Actin.Tm	
NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	3.3 ± 0.03 2.7 ± 0.06	2.9 ± 0.04 2.7 ± 0.08	3.2 ± 0.15 2.9 ± 0.11	2.6 ± 0.08 2.8 ± 0.15
N2.3	N11.17	3.4 ± 0.09 3.3 ± 0.05 3.0 ± 0.12	3.3 ± 0.23 3.3 ± 0.09 2.2 ± 0.04	3.5 ± 0.05 3.7 ± 0.14 3.3 ± 0.28	3.6 ± 0.07 3.8 ± 0.07 3.0 ± 0.27
N11.30.9	N11.16	2.6 ± 0.06	2.4 ± 0.08	2.8 ± 0.07	2.6 ± 0.11
N.16.30	N11.30.1 6	2.3 ± 0.05	2.3 ± 0.12	2.4 ± 0.02	2.4 ± 0.03
N5.3	N11.30.1 2	2.1 ± 0.07	2.1 ± 0.07	2.5 ± 0.07	2.0 ± 0.16
N5.9	N11.30.1 0	2.3 ± 0.11 2.7 ± 0.08	2.3 ± 0.10 2.4 ± 0.03	2.5 ± 0.11 2.7 ± 0.03	2.4 ± 0.12 3.2 ± 0.10
Mean ± SE n = 10		2.77 ± 0.15	2.59 ± 0.14	2.95 ± 0.14	2.84 ± 0.18
T Test P Value		Paired = 0.06 Unpaired = 0.39		Paired = 0.30 Unpaired = 0.64	
T-test of Actin: Actin.Tm				Paired = 0.007 Unpaired = 0.39	Paired = 0.06 Unpaired = 0.28

The percentage motility of non-transgenic actin actually decreased by 3% upon titration with human cardiac tropomyosin (Table 6.5). E99K actin increased by 10%, which was significant in the paired t-test ($p = 0.03$). At 40nM tropomyosin, E99K actin had a 5.5% higher percentage motility, however this was not significantly different from the non-transgenic actin ($p = 0.16$).

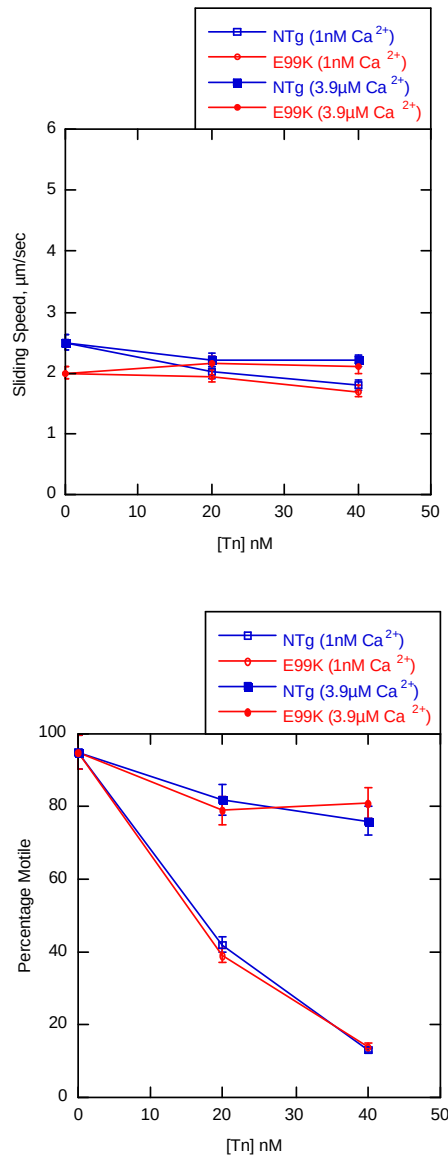
Table 6.5 Effect of 40nM Human Cardiac Tropomyosin on Non-Transgenic and E99K Actin Percentage Motility

Filaments Percentage Motile					
Transgenic Line		Actin		Actin.Tm	
NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	89 ± 1.80	58 ± 1.80	80 ± 5.75	85 ± 4.04
		75 ± 2.93	82 ± 1.73	81 ± 1.11	87 ± 1.41
N2.3	N11.17	97 ± 0.71	90 ± 4.94	95 ± 1.70	95 ± 3.44
		92 ± 3.19	96 ± 1.78	94 ± 2.69	95 ± 2.40
		80 ± 0.41	71 ± 4.01	58 ± 8.00	92 ± 1.00
N11.30.9	N11.16	75 ± 4.27	70 ± 5.02	77 ± 2.40	78 ± 4.40
N.16.30	N11.30.16	72 ± 3.23	67 ± 1.78	55 ± 1.20	65 ± 1.55
N5.3	N11.30.12	57 ± 3.50	51 ± 2.33	73 ± 4.61	62 ± 8.37
N5.9	N11.30.10	76 ± 4.15	79 ± 4.57	75 ± 6.70	80 ± 3.50
		66 ± 0.50	73 ± 4.10	70 ± 0.63	74 ± 3.17
Mean ± SE		77.9 ± 3.84	73.7 ± 4.33	75.8 ± 4.13	81.3 ± 3.71
T Test P Value		Paired = 0.27 Unpaired = 0.48		Paired = 0.16 Unpaired = 0.34	
T-test of Actin: Actin.Tm				Paired = 0.57 Unpaired = 0.71	Paired = 0.03 Unpaired = 0.20

6.8.3 Effects of Non-Failing Human Cardiac Troponin on Non-Transgenic and E99K Actin at High and Low Ca²⁺

20-60nM human cardiac troponin was titrated with 10nM actin and 40nM human cardiac tropomyosin at 3.9μM and 1nM Ca²⁺. An optimal effect was usually seen upon titration with 40nM troponin. 7 measurements were taken from 6 separate paired non-transgenic and E99K mouse hearts.

Figure 6.10 Effects of Non-Failing Human Cardiac Troponin on Actin Motility at $3.9\mu\text{M Ca}^{2+}$ and 1nM Ca^{2+}



Under relaxing conditions of 1nM Ca^{2+} , non-transgenic and E99K actin motility was indistinguishable. The percentage motility was switched off by 40nM non-failing troponin, such that approximately 10% of both non-transgenic and E99K actin filaments were moving ($p = 0.53$) (Table 6.7). This was a reduction of approximately 67% from the actin.tropomyosin percentage motility. No significant differences were also found in the speed of non-transgenic and

E99K actin ($p = 0.65$) (Table 6.6). Sliding speeds were reduced from actin.tropomyosin speeds by 35% for non-transgenic actin and 34% for E99K actin, such that speeds at 1nM Ca^{2+} were similar.

At $3.9\mu\text{M Ca}^{2+}$, non-transgenic and E99K actin actually decreased in both their sliding speed and percentage motility. Reductions were of a similar magnitude for both actins (Table 6.8 and Table 6.9). Sliding speeds were reduced by 3% and the percentage motile of filaments reduced by 7%. This lack of a switch on in high Ca^{2+} conditions could be due to the quality of the human cardiac troponin or tropomyosin incorporated into the thin filaments, or was a characteristic of the mouse actin isolated via the whole thin filament method.

Table 6.6 Effect of 40nM Human Cardiac Troponin on Actin**Sliding Speed at 1nM Ca²⁺**

Transgenic Line		Sliding Speed Actin.Tm		Sliding Speed Actin.Tm.Tn (1 nM Ca ²⁺)		Relative Sliding Speed of Actin.Tm.Tn:Actin.Tm	
NTg	E99K	NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	2.9 ± 0.11 3.2 ± 0.15	2.8 ± 0.15 2.6 ± 0.08	2.3 ± 0.07 2.1 ± 0.19	2.1 ± 0.16 2.0 ± 0.07	0.79 0.66	0.75 0.77
N2.3	N11.17	3.5 ± 0.05	3.6 ± 0.07	1.8 ± 0.21	1.8 ± 0.14	0.51	0.50
N11.3 0.9	N11.16	2.8 ± 0.07	2.6 ± 0.11	1.6 ± 0.28	1.2 ± 0.05	0.57	0.46
N.16.3 0	N11.30. 16	2.5 ± 0.07	2.0 ± 0.16	1.8 ± 0.07	1.7 ± 0.41	0.72	0.85
N5.3	N11.30. 12	2.5 ± 0.11	2.4 ± 0.12	1.6 ± 0.03	1.8 ± 0.21	0.64	0.75
N5.9	N11.30. 10	2.7 ± 0.03	3.2 ± 0.10	1.8 ± 0.08	2.1 ± 0.06	0.67	0.66
Mean ± SE n = 7		2.87 ± 0.14	2.74 ± 0.20	1.86 ± 0.10	1.81 ± 0.12	0.65 ± 0.03	0.68 ± 0.06
T Test P Value		Paired = 0.39 Unpaired = 0.61		Paired = 0.65 Unpaired = 0.78		Paired = 0.50 Unpaired = 0.71	
T-test of Actin.Tm: Actin.Tm.Tn				Paired = 0.0003 Unpaired = <.0001	Paired = 0.004 Unpaired = 0.002		

Table 6.7 Effect of Human Cardiac Troponin on Actin**Percentage Motility at 1nM Ca²⁺**

Transgenic Line		% Motile Actin.Tm		% Motile at 1 nM Ca ²⁺		Relative % Motility of Actin.Tm.Tn:Actin.Tm	
NTg	E99K	NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	80 ± 5.75 81 ± 1.11	85 ± 4.04 87 ± 1.41	30 ± 2.35 2 ± 1.73	26 ± 5.09 11 ± 4.7	0.37 0.025	0.31 0.13
N2.3	N11.17	73 ± 4.61	62 ± 8.37	8 ± 0.41	5 ± 1.71	0.11	0.081
N11.3 0.9	N11.16	70 ± 0.63	74 ± 3.17	1 ± 0.25	1 ± 0	0.014	0.014
N.16. 30	N11.30. 16	95 ± 1.70	95 ± 3.44	13 ± 0.5	14 ± 2.72	0.14	0.15
N5.3	N11.30. 12	75 ± 6.70	80 ± 3.50	12 ± 0.67	10 ± 3.06	0.16	0.13
N5.9	N11.30. 10	77 ± 2.40	78 ± 4.40	25 ± 1.00	13 ± 4.95	0.32	0.17
Mean ± SE n = 7		78.7 ± 3.13	80.1 ± 3.97	13.0 ± 4.15	11.43 ± 3.04	0.16	0.14
T Test P Value		Paired = 0.55 Unpaired = 0.78		Paired = 0.53 Unpaired = 0.76		Paired = 0.42 Unpaired = 0.69	
T-test of Actin.Tm: Actin.Tm.Tn				Paired = <.0001 Unpaired = <.0001	Paired = <.0001 Unpaired = <.0001		

Table 6.8 Effect of 40nM Human Cardiac Troponin on Actin Sliding Speed at 3.9 μ M Ca²⁺

Transgenic Line		Sliding Speed Actin.Tm		Sliding Speed at 3.9 μ M Ca^{2+}		Relative Sliding Speed of Actin.Tm.Tn: Actin.Tm	
NTg	E99 K	NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	2.9 $\pm$ 0.11 3.2 $\pm$ 0.15	2.8 $\pm$ 0.15 2.6 $\pm$ 0.08	3.0 $\pm$ 0.07 3.5 $\pm$ 0.14	2.8 $\pm$ 0.09 3.1 $\pm$ 0.18	1 1.1	1 1.2
N2.3	N11.17	3.5 $\pm$ 0.05	3.6 $\pm$ 0.07	3.2 $\pm$ 0.09	3.4 $\pm$ 0.09	0.91	0.94
N11.30.9	N11.16	2.8 $\pm$ 0.07	2.6 $\pm$ 0.11	2.5 $\pm$ 0.07	2.4 $\pm$ 0.22	0.89	0.92
N.16.30	N11.30.16	2.5 $\pm$ 0.07	2.0 $\pm$ 0.16	2.2 $\pm$ 0.05	2.1 $\pm$ 0.04	0.88	1
N5.3	N11.30.12	2.5 $\pm$ 0.11	2.4 $\pm$ 0.12	2.4 $\pm$ 0.10	2.7 $\pm$ 0.08	0.96	1.1
N5.9	N11.30.10	2.7 $\pm$ 0.03	3.2 $\pm$ 0.10	2.2 $\pm$ 0.04	2.4 $\pm$ 0.04	0.81	0.75
Mean $\pm$ SE n = 7		2.87 $\pm$ 0.14	2.74 $\pm$ 0.20	2.71 $\pm$ 0.20	2.70 $\pm$ 0.17	0.94 $\pm$ 0.04	0.99 $\pm$ 0.05
T Test P Value		Paired = 0.39 Unpaired = 0.61		Paired = 0.89 Unpaired = 0.96		Paired = 0.16 Unpaired = 0.41	
T-test of Actin.Tm: Actin.Tm.Tn				Paired = 0.18 Unpaired = 0.53	Paired = 0.80 Unpaired = 0.87		

Table 6.9 Effect of Human Cardiac Troponin on Actin Percentage Motility at 3.9 μ M Ca²⁺

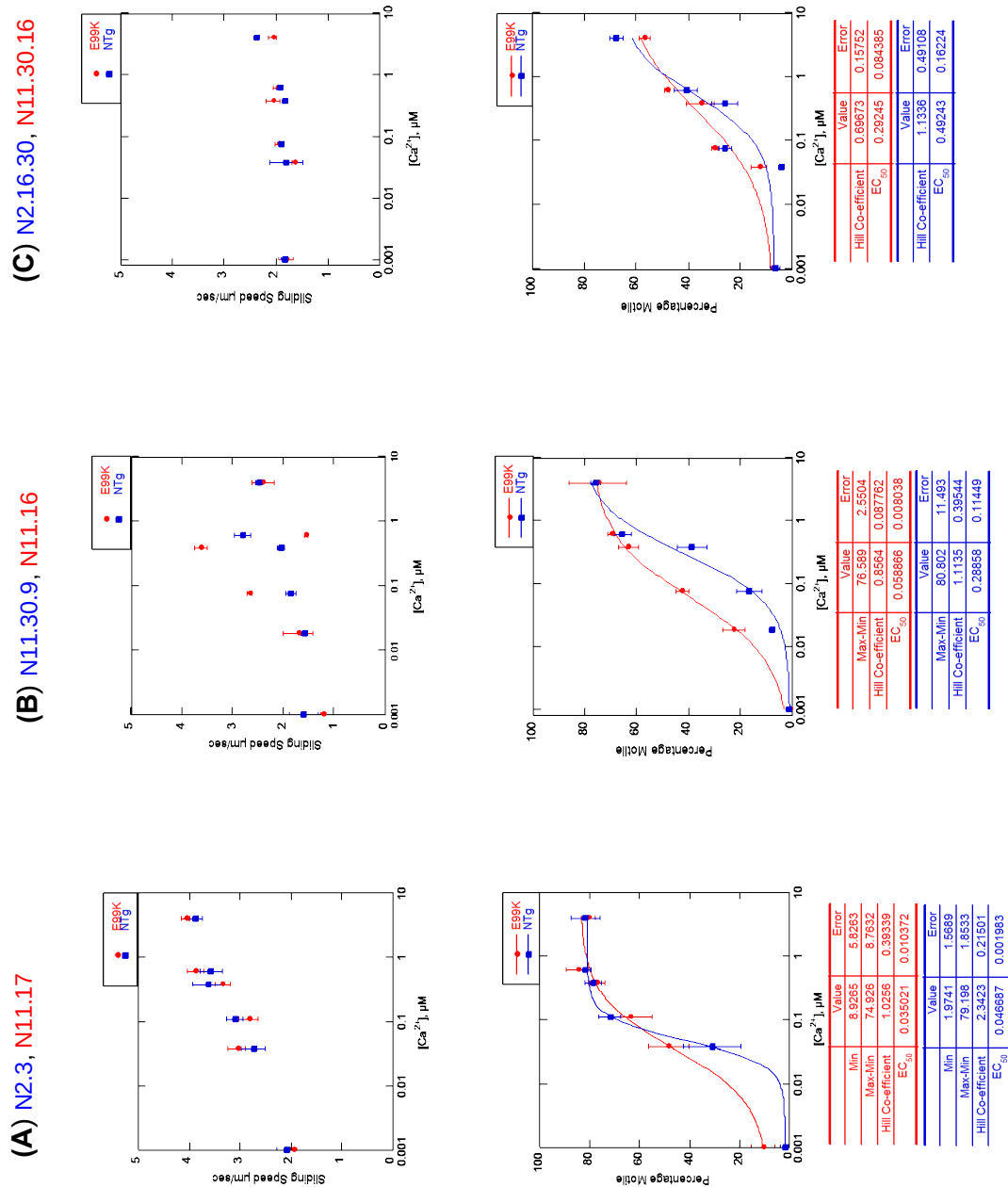
Transgenic Line		% Motile Actin.Tm		% Motile at 3.9 μ M Ca ²⁺		Relative % Motility of Actin.Tm.Tn:Actin.Tm	
NTg	E99 K	NTg	E99K	NTg	E99K	NTg	E99K
N2.4	N11.17	80 $\pm$ 5.75 81 $\pm$ 1.11	85 $\pm$ 4.04 87 $\pm$ 1.41	90 $\pm$ 0.87 81 $\pm$ 3.84	76 $\pm$ 4.02 67 $\pm$ 4.73	1.1 1	0.89 0.77
N2.3	N11.17	73 $\pm$ 4.61	62 $\pm$ 8.37	66 $\pm$ 3.12	69 $\pm$ 3.40	0.9	1.1
N11.30.9	N11.16	70 $\pm$ 0.63	74 $\pm$ 3.17	76 $\pm$ 1.73	75 $\pm$ 11.1	1.1	1
N.16.30	N11.30.16	95 $\pm$ 1.70	95 $\pm$ 3.44	76 $\pm$ 3.39	81 $\pm$ 4.10	0.8	0.85
N5.3	N11.30.12	75 $\pm$ 6.70	80 $\pm$ 3.50	61 $\pm$ 2.52	72 $\pm$ 3.47	0.81	0.9
N5.9	N11.30.10	77 $\pm$ 2.40	78 $\pm$ 4.40	60 $\pm$ 3.69	70 $\pm$ 0.91	0.78	0.9
Mean $\pm$ SE n = 7		78.7 $\pm$ 3.13	80.1 $\pm$ 3.97	72.9 $\pm$ 4.18	72.9 $\pm$ 1.82	0.93	0.92
T Test P Value		Paired = 0.55 Unpaired = 0.78		Paired = 1.0 Unpaired = 1.0		Paired =0.89 Unpaired = 0.89	
T-test of Actin.Tm: Actin.Tm.Tn				Paired = 0.23 Unpaired = 0.28	Paired = 0.07 Unpaired = 0.12		

6.8.4 Comparing Ca^{2+} Concentration Curves of Non-Failing and E99K Actin

Initial Ca^{2+} sensitivity measurements were done with thin filaments that were reconstituted with 10nM E99K or non-transgenic actin, 40nM human cardiac tropomyosin, and 40nM cardiac troponin that was purified from non-failing human heart muscle (troponin I phosphorylation 2.1 mols/mol). The Ca^{2+} sensitivity of sliding and the percentage motile of thin filament actin were measured with actin from 5 paired non-transgenic and E99K mouse hearts Figure 6.11 A-E). The individual mouse heart sample numbers for non-transgenic and mutant actin are highlighted in blue and red respectively.

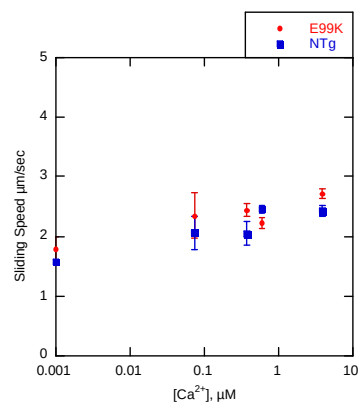
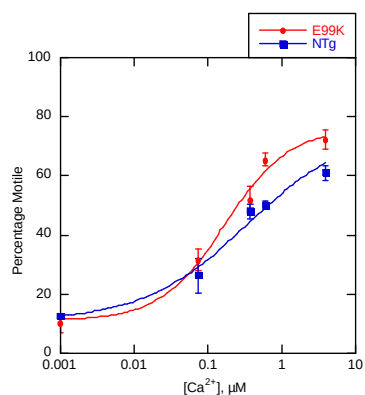
Mouse actin produced a large variability in the sliding speed across all Ca^{2+} concentrations. No accurate fits have been possible and the points have simply been plotted. There is no significant difference in the sliding speeds of non-transgenic and E99K actin at $3.9\mu\text{M}$ Ca^{2+} (Table 6.10). For the percentage motility parameter, thin filaments reconstituted with mutant E99K actin had a higher Ca^{2+} sensitivity than thin filaments reconstituted with non-transgenic actin. The shift in Ca^{2+} sensitivity corresponds to a 2.5-fold decrease in EC_{50} of the percentage motility parameter ($p = 0.07$). Table 6.11 shows all the percentage motile EC_{50} values for 5 pairs of non-transgenic and E99K hearts.

Figure 6.11 Ca^{2+} Regulation of Thin Filaments Containing Non-Transgenic and E99K Actin



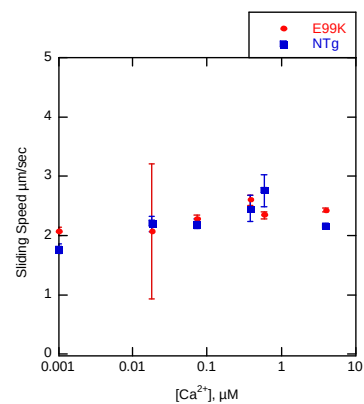
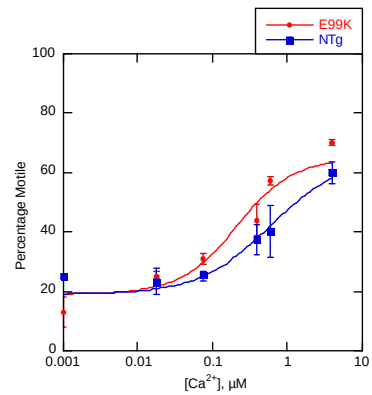
The following percentage motility curves have had parameters fixed to obtain an accurate sigmoidal curve: Graph B (Min value fixed at 1%), Graph C (Min and Max values fixed at 7% and 60%).

(D) N5.3, N11.30.12



	Value	Error
Hill Co-efficient	0.99729	0.15908
EC ₅₀	0.17019	0.027354
Hill Co-efficient	0.62076	0.098178
EC ₅₀	0.33995	0.06446

(E) N5.9, N11.30.10



	Value	Error
Hill Co-efficient	1.1298	0.37524
EC ₅₀	0.211700	0.069183
Hill Co-efficient	0.90901	0.19912
EC ₅₀	0.5934	0.1274

Graph D (Min and Max values fixed at 11% and 55%), Graph E (Min and Max values fixed at 19% and 46%).

Table 6.10 Non-Transgenic and E99K Actin Sliding Speeds at 3.9 μ M Ca²⁺

Mouse Line	3.9 μ M Sliding Speeds μ m/sec	
	Non-Transgenic	E99K
N2.3	3.9 $\pm$ 0.13	4.1 $\pm$ 0.08
N11.30.9	2.5 $\pm$ 0.07	2.4 $\pm$ 0.22
N2.16.30	2.4 $\pm$ 0.02	2.1 $\pm$ 0.08
N5.3	2.5 $\pm$ 0.43	2.7 $\pm$ 0.08
N5.9	2.2 $\pm$ 0.04	2.4 $\pm$ 0.04
Mean: $\pm$ SE (n = 5)	2.70 $\pm$ 0.30	2.74 $\pm$ 0.35
Student's t-test (paired)	p = 0.72	
(Unpaired)	p = 0.93	

Table 6.10 shows the actual EC₅₀ values and the difference in EC₅₀ values expressed as a ratio of Non-Transgenic to E99K actin for the percentage motility parameter.

Table 6.11 Ca²⁺-Sensitivity data for all Non-Transgenic and E99K Actin in a Non-Failing Thin Filament

Mouse Line		EC ₅₀ Percentage Motility, μ M		$\frac{\text{EC}_{50} \text{ NTg}}{\text{EC}_{50} \text{ E99K}}$
Non-Transgenic	E99K	Non-Transgenic	E99K	
N2.3	N11.17	0.047 $\pm$ 0.002	0.035 $\pm$ 0.010	1.34
N11.30.9	N11.16	0.289 $\pm$ 0.114	0.059 $\pm$ 0.008	4.90
N2.16.30	N11.30.16	0.492 $\pm$ 0.162	0.292 $\pm$ 0.084	1.68
N5.3	N11.30.12	0.340 $\pm$ 0.064	0.170 $\pm$ 0.027	2.00
N5.9	N11.30.10	0.593 $\pm$ 0.127	0.212 $\pm$ 0.069	2.80
Mean: $\pm$ SE (n = 5)		0.352 $\pm$ 0.09	0.154 $\pm$ 0.05	2.54 $\pm$ 0.64
Student's t-test (paired)		p = 0.03		single group, p = 0.07 (compared with 1)
(Unpaired)		p = 0.10		

6.8.5 Effect of Troponin Dephosphorylation on the Ca²⁺

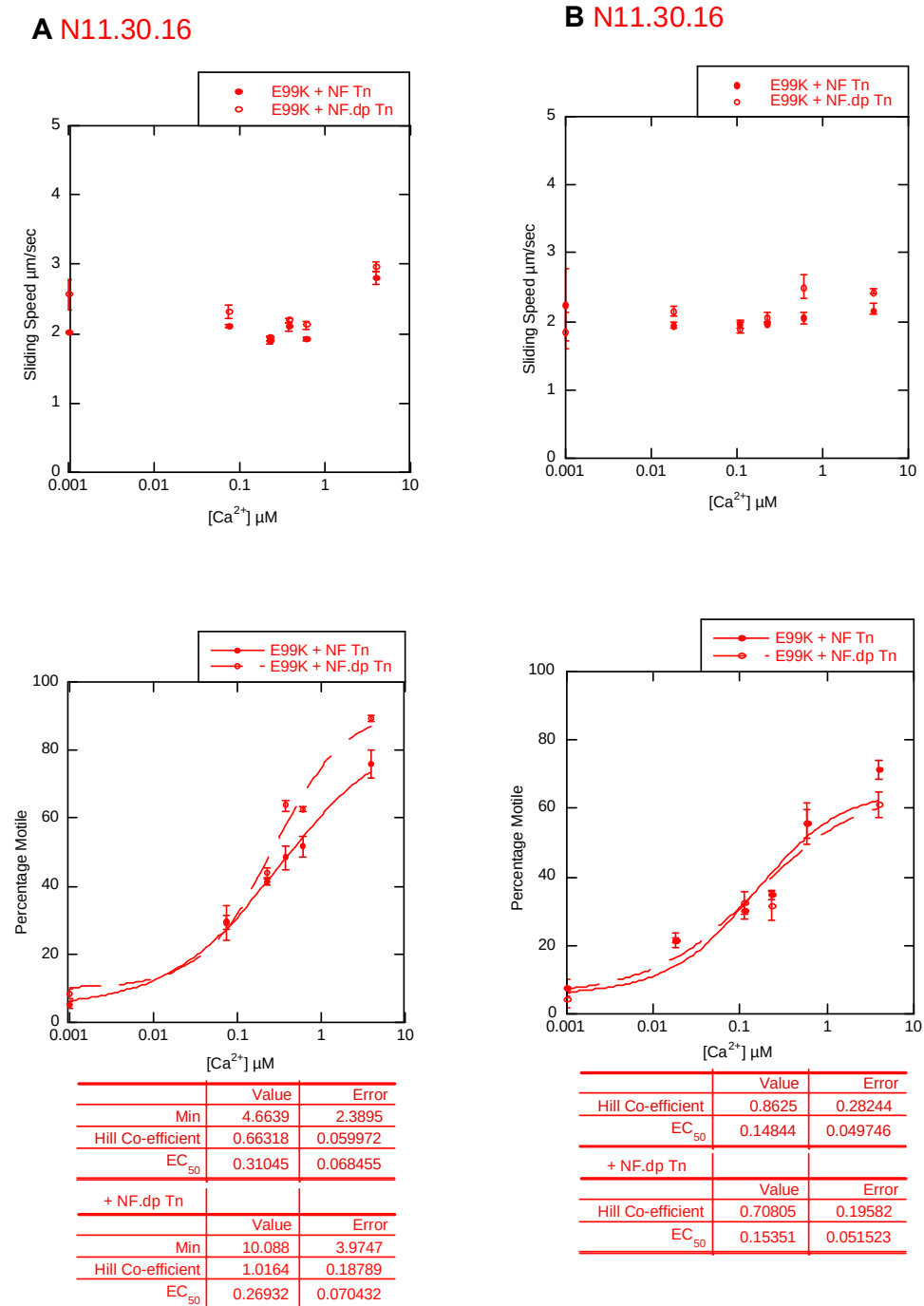
Dependence of Motility

E99K and non-transgenic thin filaments were assembled from a combination of native (2.1 mol Pi/mol TnI) or dephosphorylated troponin and human cardiac tropomyosin. Troponin was dephosphorylated with 1.4 units/ml acid phosphatase (Figure 4.3). Using non-transgenic mouse actin, Figure 4.4 illustrated the well-known impact of dephosphorylation on non-failing troponin.

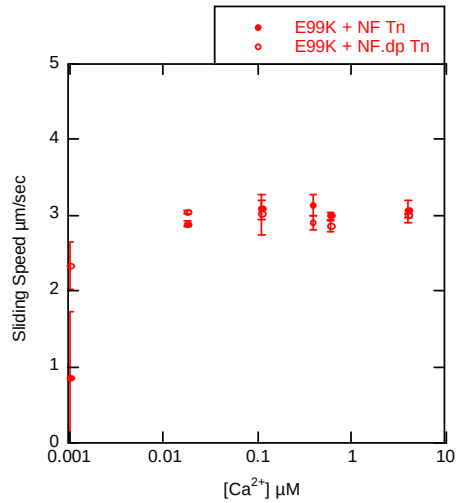
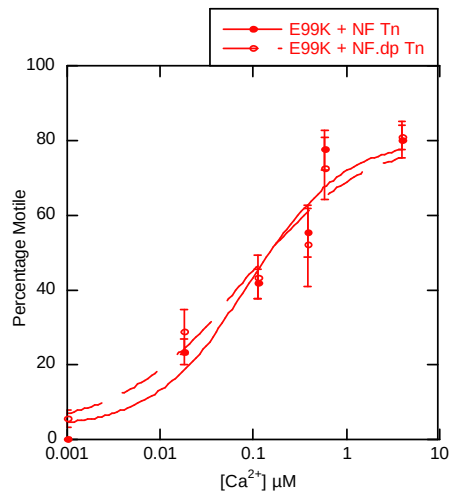
Dephosphorylation of the troponin produced a leftward shift in the percentage motility- Ca^{2+} relationship and an increase in Ca^{2+} sensitivity, which corresponded to a 2.98 ± 0.31 -fold decrease in the EC_{50} value.

Figure 6.12 and Table 6.12 illustrate the Ca^{2+} response of E99K actin when incorporated into the thin filament with either native or dephosphorylated troponin. The higher Ca^{2+} sensitivity produced with non-transgenic actin incorporated with dephosphorylated troponin was not seen with the E99K actin and there was no difference in the Ca^{2+} sensitivity of the filaments ($p = 0.16$). The ratio of percentage motility EC_{50} values was not significantly different from 1 ($p = 0.12$). E99K actin therefore shows insensitivity to troponin I phosphorylation levels.

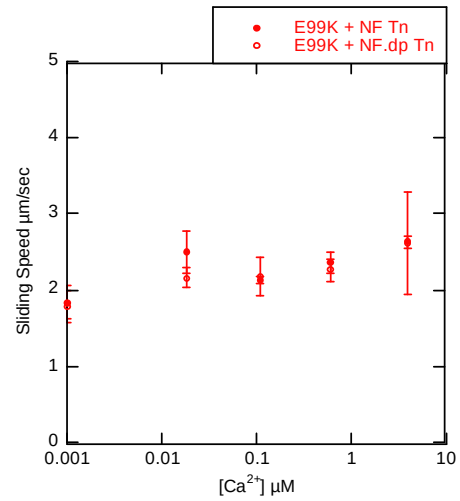
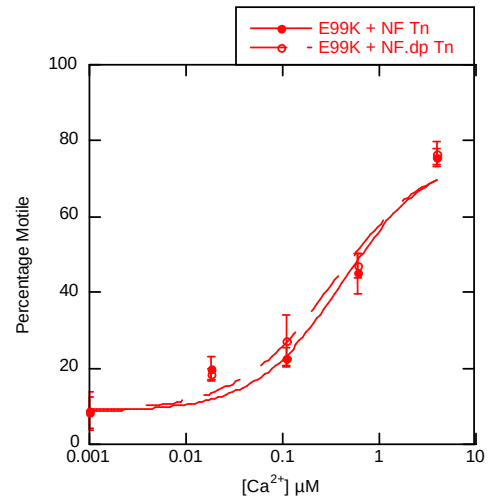
Figure 6.12 Effect of Troponin Dephosphorylation on E99K Actin Motility



The percentage motility curves have had the following parameters fixed: Graph A (Min and Max values fixed at 6.6% and 76%), Graph B (Min and Max values fixed at 6% and 60%).

C N11.30.12

	Value	Error
Hill Co-efficient	0.85225	0.2055
EC ₅₀	0.08999	0.027899
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	0.66642	0.15199
EC ₅₀	0.075811	0.026691

D N11.30.12

	Value	Error
Hill Co-efficient	0.96519	0.26576
EC ₅₀	0.41374	0.12613
+ NF.dp Tn		
	Value	Error
Hill Co-efficient	0.88777	0.20245
EC ₅₀	0.33223	0.090734

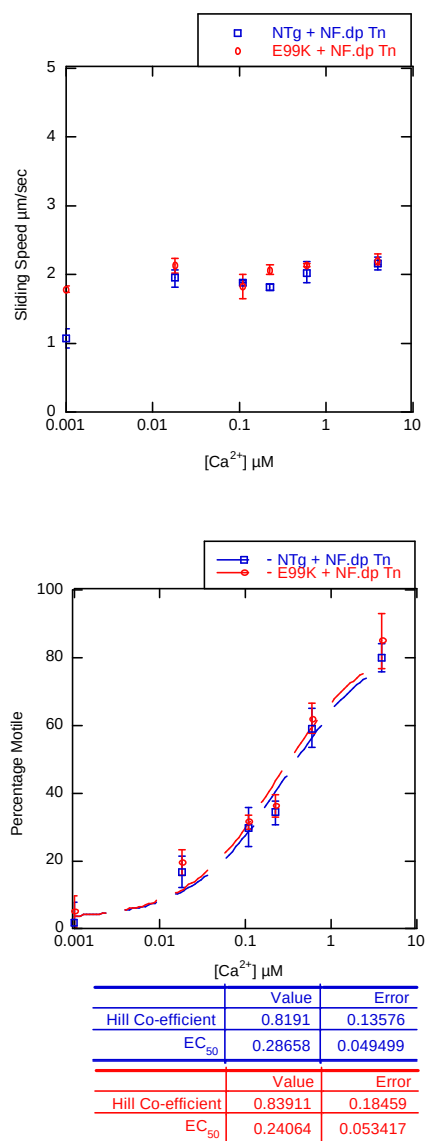
Graph C (Min and Max values fixed at 3% and 78%), Graph D (Min and Max values fixed at 9% and 68%).

Table 6.12 Ca^{2+} Sensitivity Data for E99K Actin Incorporated with Native or Dephosphorylated Troponin

Mouse Line	EC ₅₀ Percentage Motility, μM		$\frac{\text{EC}_{50} \text{ NF}}{\text{EC}_{50} \text{ NF.dp}}$
	Non-Failing Troponin	Non-Failing Troponin. dp	
N11.30.16	0.310 ± 0.068	0.269 ± 0.07	1.15
N11.30.16	0.148 ± 0.050	0.154 ± 0.052	0.96
N11.30.12	0.090 ± 0.028	0.076 ± 0.027	1.18
N11.30.12	0.414 ± 0.126	0.332 ± 0.091	1.25
Mean ± SE (n = 4)	0.234 ± 0.072	0.187 ± 0.054	1.14 ± 0.06
Student's t-test (paired)	p = 0.16		Single group, p = 0.12 (compared with 1)
(Unpaired)	p = 0.62		

The final Ca^{2+} sensitivity measurements used E99K and non-transgenic actin incorporated with non-failing dephosphorylated troponin. A single experiment was performed. The percentage motility curve in Figure 6.13 had the minimum parameter fixed at 3% and the maximum at 83%. When the troponin was dephosphorylated, the E99K thin filaments had a 1.2-fold higher Ca^{2+} sensitivity of motility compared to non-transgenic thin filaments. However, this was not statistically significant.

Figure 6.13 Effect of Troponin Dephosphorylation on Non-Transgenic and E99K Actin Motility

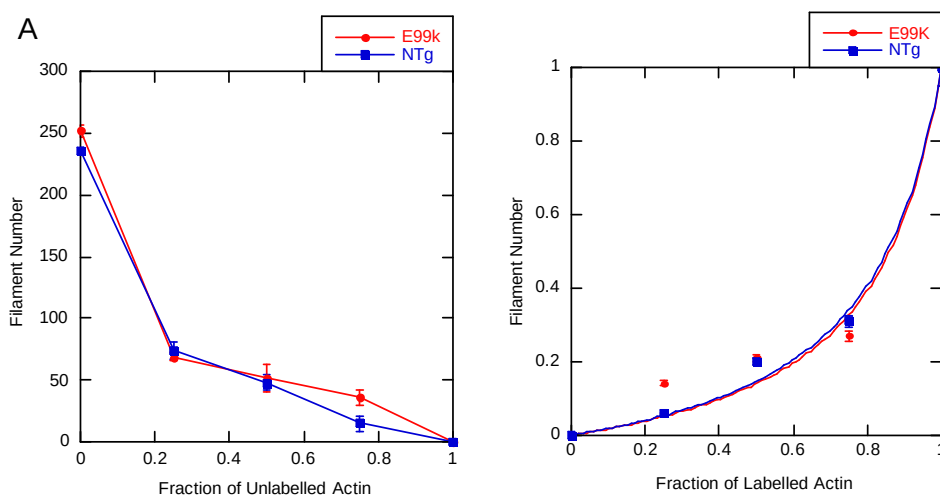


6.8.6 Competitive Binding Assay of Fluorescent and Non-Fluorescent Actin to α -Actinin

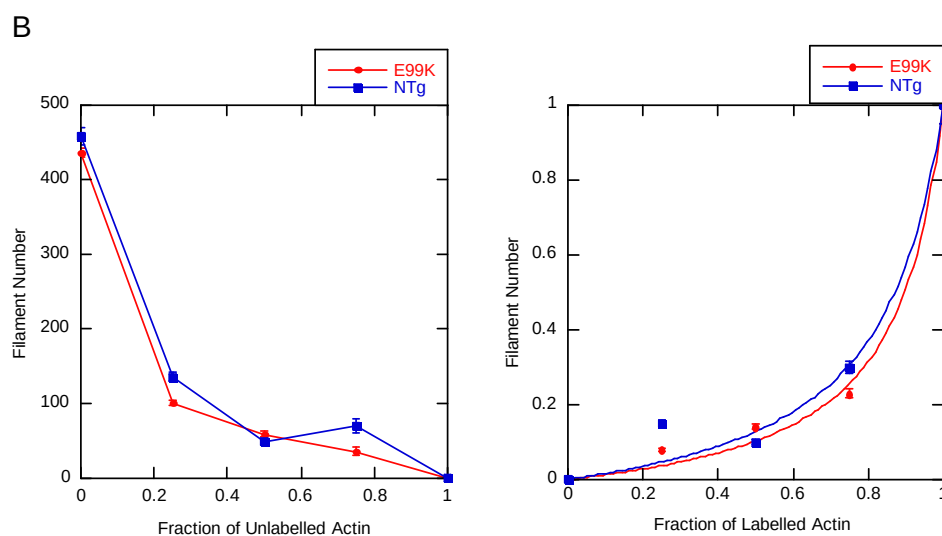
The $\bar{U}$ -actinin binding assay described in section 2.12 was used to establish that E99K actin has the same affinity for $\bar{U}$ -actinin as non-transgenic actin. This assay was repeated twice and the results are shown in Figure 6.14 A and B. Fitting the number of filaments to the $\bar{U}$ -actinin binding equation

produces the graph on the right. The average K value for E99K actin was 0.14 ± 0.02 and for non-transgenic actin was 0.16 ± 0.01 . Since the K values were less than 1, both E99K and non-transgenic mouse actins had an affinity for $\bar{U}$ -actinin that was less than rabbit actin.

Figure 6.14 α -Actinin Competitive Binding Assay of E99K Actin and Non-Transgenic Actin Filaments



E99K actin has a k value of 0.16 ± 0.04 , and non-transgenic actin has a k value of 0.17 ± 0.02 (k E99K/NTg = 0.94).



E99K actin has a k value of 0.12 ± 0.02 , and non-transgenic actin has a k value of 0.15 ± 0.03 (k E99K/NTg = 0.8).

These results were similar to those reported by Bookwalter *et al.* using E99K and wild type actin expressed and purified from the baculovirus/insect cell expression system (Bookwalter and Trybus, 2006). A control pelleting assay showed that E99K actin had the same affinity for $\bar{\text{U}}$ -actinin as wild type actin. However, using $\bar{\text{U}}$ -actinin as a load in the *in vitro* motility assay to measure relative isometric force, they showed that E99K actin supported a lower average force than wild type actin. Approximately 30% less $\bar{\text{U}}$ -actinin was needed to stop the movement of E99K actin compared to wild type.

6.9 Discussion

This study has focused on the apical HCM-causing actin mutation E99K, which is located in a surface loop in subdomain 1 of actin. Apical HCM is an uncommon hypertrophic morphology in which non-obstructive hypertrophy is localised to the cardiac apex. Individuals present with a later onset of the disease and display a variable degree of hypertrophy (Olson *et al.*, 2000). Several clinical studies have reported the prevalence of the E99K mutation in patients with apical hypertrophy (Arad *et al.*, 2005b, Olson *et al.*, 2000, Monserrat *et al.*, 2007). Arad *et al.* reported that the E99K mutation was unique in producing apical HCM in all clinically affected members from two distantly related families. This genotype has also been reported in another small family with apical HCM (Olson *et al.*, 2000). Haplotype data has indicated that this family was genetically unrelated to the two families reported by Arad *et al.*, which consolidates a primary relationship between this genotype and apical hypertrophy. A further study by Monserrat *et al.* screened specifically for the E99K mutation in 247 individuals with phenotypes of HCM, DCM, or left ventricular non-compaction (LVNC). It was reported that the E99K mutation was found in 42 individuals. In addition to being associated with apical HCM, it could also cause LVNC and produce a high incidence of septal defects. The mutation was found in one family with LVNC and four with HCM. The penetrance of the mutation was also high, as the mutant carriers all had pathological manifestations.

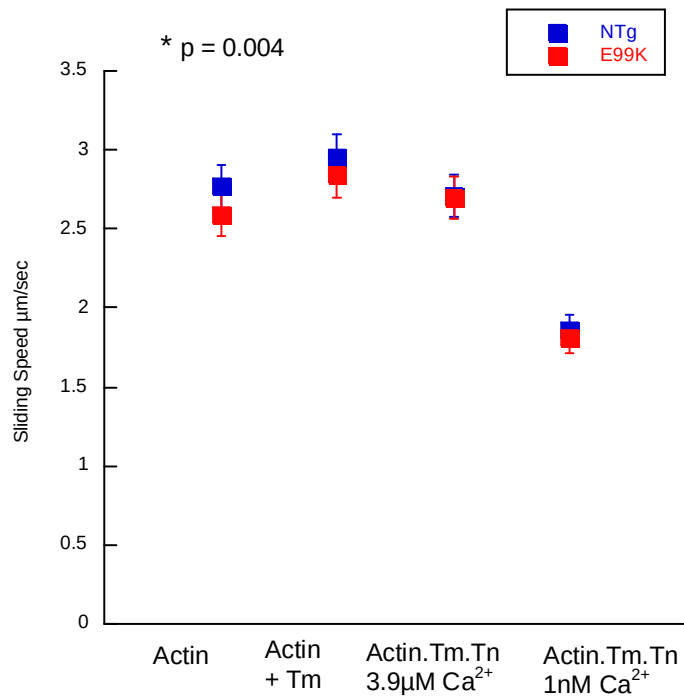
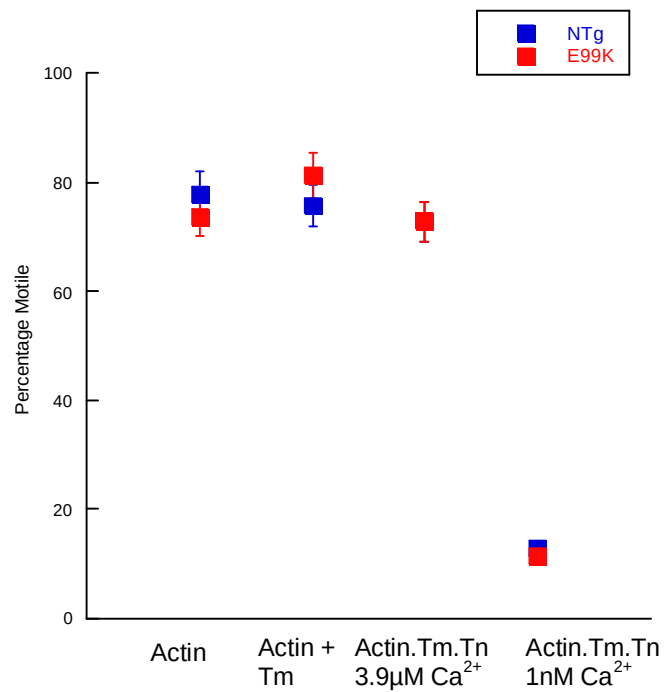
To understand the functional effects produced by the E99K actin mutation in the development of these cardiac diseases, transgenic mice were

generated to express the E99K mutant actin in their hearts. Transgenic mice that carried the E99K transgene showed a high rate of sudden death. Approximately 70% of females and 34% of males died between 35-40 days, although no abnormal clinical features were present upon post-mortem. No inotropic stimulation was applied. Although several of the PCR positive lines did not contain the transgene, one line (N11) transmitted successfully at 55% in whole tissue and F-actin. *In vitro* motility studies were carried out on this line.

6.9.1 Defects of Transgenic E99K Actin

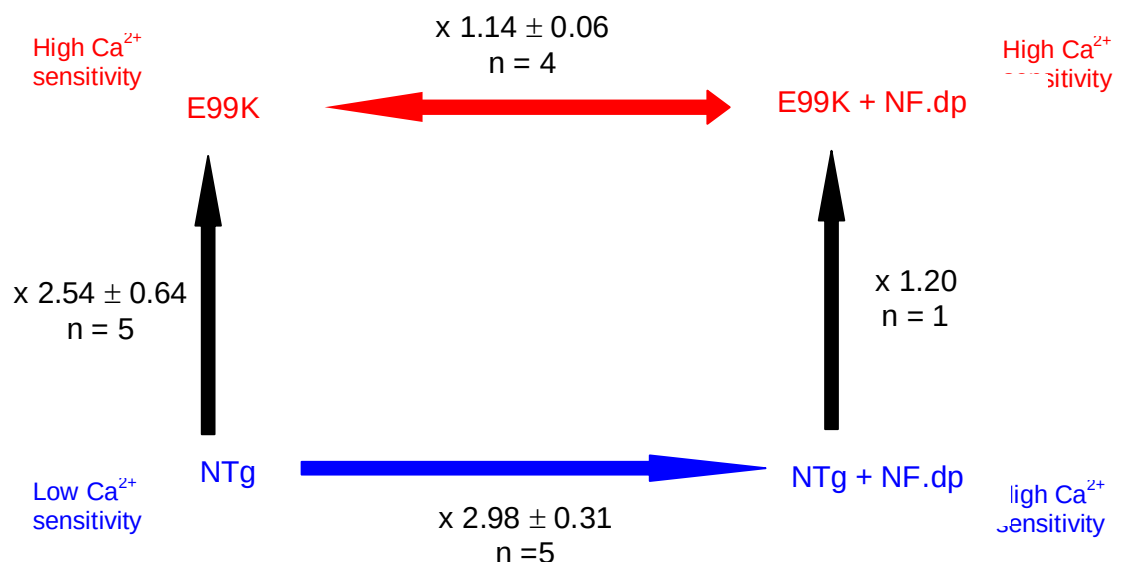
F-actin was extracted from E99K mutant hearts via the thin filament method to produce the same yield as non-transgenic hearts. The *in vitro* motility results suggested that one of the primary defects the E99K actin produced was a slower motility over HMM (8% lower sliding speed, $p = 0.004$, 4% lower percentage motility, $p = 0.22$). In the presence of 40nM human cardiac tropomyosin, E99K actin exhibited a greater increase in sliding speed (10%, $p = 0.06$) compared to non-transgenic actin (6.5%, $p = 0.007$), and reduced the difference in sliding speed to 4%. The average sliding speeds at optimum tropomyosin concentrations were not significantly different from each other ($p = 0.30$), however E99K actin had a 7% higher percentage motility ($p = 0.16$) at optimum tropomyosin concentrations.

Reconstitution with 40nM human cardiac tropomyosin and 40nM human cardiac troponin removed the slower sliding speed exhibited by E99K actin so that both were the same at $3.9\mu\text{M Ca}^{2+}$ ($p = 0.89$). Both E99K and non-transgenic actin had a percentage motility that was reduced at $3.9\mu\text{M Ca}^{2+}$ by 3-7%, which was possibly due to the other contractile proteins in the thin filament. At 1nM Ca^{2+} , both were fully switched off. These results are summarised in Figure 6.15 and Figure 6.16.

Figure 6.15 *In vitro* Motility Assay Summary of Sliding Speed**Figure 6.16 *In vitro* Motility Assay Summary of Percentage Motile**

Across a range of Ca^{2+} concentrations, the mutant thin filaments had a higher Ca^{2+} sensitivity of percentage motility ($\text{EC}_{50} \text{ NTg/E99K} = 2.54 \pm 0.64$, $p = 0.07$) compared to non-transgenic actin. The sliding speeds could not be fitted to the Hill Equation as the net increase in actin filament sliding speed from 1nM Ca^{2+} to 3.9 μM Ca^{2+} was small. E99K actin had a 1.5% faster sliding speed at 3.9 μM Ca^{2+} than non-transgenic actin, although this was not a significant difference ($p = 0.72$). When reconstituted with dephosphorylated non-failing human cardiac troponin, non-transgenic mouse actin exhibited a 2.98 ± 0.31 -fold increase in Ca^{2+} -sensitivity (Figure 4.4). This characteristic response with non-transgenic actin can be compared with the much blunted Ca^{2+} sensitivity response produced when E99K actin was incorporated with dephosphorylated troponin ($\text{EC}_{50} \text{ E99K/E99K+Tn.dp} = 1.14 \pm 0.06$, $p = 0.12$). In a single experiment, E99K actin still exhibited a slightly higher Ca^{2+} sensitivity than non-transgenic actin when both were incorporated with dephosphorylated troponin ($\text{EC}_{50} \text{ NTg/E99K} = 1.2$), although this was not a statistically significant difference. The presence of the E99K mutation therefore appeared to blunt the Ca^{2+} sensitivity decrease caused by troponin I PKA phosphorylation (Figure 6.17).

Figure 6.17 Comparison of E99K and Non-Transgenic Actin Incorporated into Native or Dephosphorylated Thin Filaments



6.9.2 Comparison with Another E99K Mutation Study

Comparisons of our E99K actin *in vitro* motility results can be made with similar functional studies reported by Bookwalter and Trybus, who were successful in expressing high yields of E99K and wild type actin in the baculovirus/insect cell expression system (Bookwalter and Trybus, 2006). Consistent with the *in vitro* motility results reported in our study, Bookwalter and Trybus showed that E99K actin had a reduced velocity, although the effect was greater at between 13-21% relative to wild type actin over a range of KCl concentrations ($p = <0.01$). It is important to note though that they had 100% mutant expression compared to the 55% expression in our study which would account for the greater effect reported. Also consistent with our data was the similar affinity of E99K actin for $\bar{U}$ -actinin compared to the affinity showed by wild type actin. In addition though, they reported that E99K actin supported a 30% lower force than wild type actin when the $\bar{U}$ -actinin was used as a load in the *in vitro* motility assay. The relative isometric force of E99K actin was not measured in our study.

Bookwalter and Trybus proposed that the slower motility, reduced average force, and a weakened interaction with myosin in the presence of ATP might be sufficient to trigger the HCM phenotype by the E99K mutation. However, this phenotype is opposite to most HCM molecular phenotypes, and in our study the effects of the mutation disappeared when tropomyosin and troponin were added. It is important to note that unlike our transgenic mouse study, the baculovirus/insect cell expression system that was used did not closely mimic the *in vivo* situation since no other thin filament proteins were reconstituted into the system with the mutant actin. It is also important to note that the expression of human cardiac wild type and E99K actin in the Sf9 cells was not entirely pure. Although western blotting with antibodies specific for cardiac muscle actins recognised the recombinant cardiac actin but not endogenous Sf9 cell actin, probing with a monoclonal antibody specific to β -cytoplasmic actin showed a small carryover of endogenous Sf9 cell actin.

6.9.3 Comparisons with Other HCM-Associated Mutations

Most HCM-associated mutations have been reported to produce a common hypercontractile response. One of the main goals of this study was to determine if the actin E99K mutant produces the same or different properties as other HCM mutations in the regulation of muscle contraction. The troponin T HCM mutation R92Q has previously been studied by *in vitro* motility assay and by ATPase measurements (Robinson *et al.*, 2002). In contrast with our study, Robinson *et al.* used mutant and wild type recombinant troponin that was unphosphorylated at troponin I and troponin T. Our experimental system used the native phosphorylation state of the non-failing troponin. Therefore, comparisons can only be made with the system in our study that used dephosphorylated troponin. Similarly to E99K actin however, the R92Q troponin increased thin filament sliding speed with a higher Ca^{2+} sensitivity. R92Q also produced less inhibition of filament motility than wild type at 1nM Ca^{2+} . These results were similar to the actomyosin ATPase assay, in which R92Q had a 1.7-fold enhanced activation (EC_{50} WT = 0.33 ± 0.02 , EC_{50} R92Q = 0.19 ± 0.03). In addition, at 1nM Ca^{2+} thin filaments containing R92Q troponin T gave less inhibition of ATPase (79% compared with 90% with wild type troponin).

The results of Robinson *et al.* are in agreement with other R92Q studies in other systems. Morimoto *et al.* investigated the mechanical properties of R92Q expressed in *Escherichia coli* and exchanged into skinned rabbit trabeculae that had native phosphorylation levels (Morimoto *et al.*, 1998). R92Q had a 1.5-fold higher Ca^{2+} sensitivity compared to wild type (EC_{50} WT = 1.78 ± 0.03 , EC_{50} R92Q = 1.20 ± 0.02). Similarly, using porcine fibres Szczesna *et al.* reported a 1.5-fold increased Ca^{2+} sensitivity of force production (EC_{50} WT = 3.31 ± 0.01 , EC_{50} R92Q = 2.19 ± 0.02) (Szczesna *et al.*, 2000), and Yanaga *et al.* showed a 1.4-fold increase in Ca^{2+} sensitivity when measuring ATPase in reconstituted rabbit cardiac myofibrils (EC_{50} WT = 0.98 ± 0.04 , EC_{50} R92Q = 0.69 ± 0.08) (Yanaga *et al.*, 1999).

Previously in Prof. Marston's laboratory, several other mutations that cause HCM have been investigated by the *in vitro* motility assay. Of these mutations, two were in β -tropomyosin (Asp175Asn and Glu180Gly) (Bing *et al.*, 2000), one was in troponin I (Arg145Gly) (Burton *et al.*, 2002) and one was a

truncated mutation in troponin T (Redwood *et al.*, 2000). Collectively, these previous functional studies of HCM mutants all show one or more of the following changes: (1) an increase in Ca^{2+} sensitivity, (2) a faster sliding speed/ATPase activity at $3.9\mu\text{M Ca}^{2+}$, and (3) failure of inhibition at 1nM Ca^{2+} . When E99K actin was reconstituted with dephosphorylated non-failing troponin, there was a 1.2-fold increase in Ca^{2+} sensitivity, but no difference was seen in motility at $3.9\mu\text{M Ca}^{2+}$ or 1nM Ca^{2+} compared to non-transgenic actin. The change in Ca^{2+} sensitivity of E99K thin filament motility was of a similar direction to all previously studied HCM mutations. The magnitude of the change was however less than previous studies, including measurements from the R92Q troponin T mutation, which reported a 1.7-fold increase in Ca^{2+} sensitivity of R92Q troponin. These experiments were done with recombinant troponin that was not phosphorylated before reconstitution into the thin filament, so it may be possible that if these experiments were repeated using pseudophosphorylated R92Q and wild type troponin, the changes in Ca^{2+} sensitivity may be larger, like our E99K studies found with native non-failing troponin. This suggests that the effect of HCM mutations can be increased or decreased depending on the type of partner protein reconstituted into the thin filament (either native or recombinant). Our studies have shown that it is important to closely mimic the *in vivo* situation by using native human contractile proteins.

Several transgenic animal studies have enabled the functional consequences of HCM mutations to be studied in real *in vivo* conditions (Knollmann *et al.*, 2001, Miller *et al.*, 2001). Miller *et al.* investigated skinned fibres dissected from transgenic mice with the HCM troponin T mutation 179N and found, similarly to E99K actin, an increased the Ca^{2+} sensitivity of myofilaments. Subsequent studies by Knollman *et al.* on the cardiac function of 179N isolated hearts showed at baseline conditions the increased Ca^{2+} sensitivity is followed by an increase in contractility. The systolic function was higher than non-transgenic mice, with little effect on diastolic function. Echocardiography and Doppler measurements confirmed that baseline systolic function was higher in 179N mice. Interestingly, during inotropic stimulation with high extracellular Ca^{2+} or with isoproterenol, the diastolic function was impaired with increased end-diastolic pressure and time to 90% relaxation. Although chronic swimming exercise had no effect on mortality, the isoproterenol caused

significant mortality in the 179N mice. This may be due to the lower maximal force with high Ca^{2+} decreasing the contractile reserve available to increase cardiac output in response to isoproterenol. This HCM troponin T 179N study is therefore evidence that the effect of a mutation can be largely dependent upon the inotropic state of the heart, an effect that has also been reported by Deng *et al.* using ATPase and *in vitro* motility studies on the HCM cardiac troponin I mutation K206Q (Deng *et al.*, 2003). Upon phosphorylation of the troponin I, this mutation had a 1.6-fold increase in the Ca^{2+} sensitivity of acto S1-ATPase activity (EC_{50} WT = 0.93 ± 0.02 , EC_{50} K206Q = 0.59 ± 0.03) and also showed the same increase in maximum unloaded sliding speed in the *in vitro* motility assay compared to wild type cardiac troponin I (EC_{50} WT = 0.4 ± 0.02 , EC_{50} K206Q = 0.25 ± 0.04). However, the characteristic increase in Ca^{2+} sensitivity with unphosphorylated wild type cardiac troponin I (EC_{50} WT.bisphosphorylated/WT = 1.6) was abolished by the K206Q mutation (EC_{50} K206Q.bisphosphorylated/K206Q = 1).

Similar to these previous HCM studies, a dependency on the inotropic state of the heart has been shown with the E99K mutation in the *in vitro* motility assay. The E99K HCM phenotype of an enhanced Ca^{2+} sensitivity therefore appears to correlate with all previous HCM phenotypes, but interestingly both the enhanced Ca^{2+} sensitivity and the blunted response to troponin I phosphorylation in the E99K native thin filament is similar to the G159D DCM phenotype.

7. GENERAL DISCUSSION

7.1 Rationale for this Study

The overall aim of this study was to investigate the role that contractile protein mutations have in determining the phenotype of HCM and DCM. It has been the first study that has used cardiac muscle with defined disease-causing mutations in actin and troponin C. Results with these samples can be compared with previous experiments that have used recombinant proteins in synthetic systems (Tobacman *et al.*, 1999, Redwood *et al.*, 2000, Knollmann and Potter, 2001, Robinson *et al.*, 2002, Mirza *et al.*, 2005, Chang *et al.*, 2005).

I have chosen to study mutations in actin because it is the only contractile protein that is identical in man and mouse. Additionally, by expressing actin mutations in transgenic mice, the protein will have native post-translational modifications. It was therefore possible to accurately replicate and experiment upon a virtually perfect native human thin filament, when the mouse actin was reconstituted with human cardiac troponin (natively phosphorylated) and tropomyosin. This assay also made it possible to express actin without any adducts. Since the ACTC mutations (E361G and E99K) induce a charge change, it was possible to determine by 2-D Electrophoresis that the tissue content of mutant actin was approximately 50% of the total actin. This is a level similar to that predicted for a DCM/HCM patient with a dominant mutation. In previous experiments, levels of mutant expression were 50% or less (Cuda *et al.*, 1997, Bottinelli *et al.*, 1998, Nier *et al.*, 1999).

I have also studied a unique human heart sample from a patient with DCM requiring heart transplantation. The G159D troponin C mutation was identified in this family member with familial DCM (Mogensen *et al.*, 2003b) and the heart muscle sample was available for functional analysis. The troponin extracted from this sample therefore had the post-translational modifications and native molecular ratios of mutant: wild type characteristic of this DCM phenotype. Moreover, this ratio was measurable by mass spectroscopy at approximately 50% of troponin C. This is the only study in which both the structural properties (expression level, phosphorylation levels) and functional properties of the G159D mutation has been investigated. This sample has yielded unexpected

results that emphasise the importance of being able to study native contractile proteins rather than synthetic systems.

Both the transgenic mouse models and the human muscle sample have allowed the conditions in muscle of HCM and DCM patients to be closely replicated.

7.2 Limitations of the Study

There was a major difference between the way I prepared actin from mouse hearts compared to all previous experimentation. The inability of the mouse actin to polymerise lead to the mutant actin being lost when isolated via the usual acetone powder method (Straub, 1942). This presumably was because the mutation destabilised the G-actin. Actin polymerisation was stabilised upon incubation with TRITC-phalloidin but this lead to only 25-40% of the mutant actin being incorporated. Full mutant expression was retained in the F-actin upon extraction from native thin filaments, however this actin exhibited different behaviour in the *in vitro* motility assay compared with the actin extracted from an acetone powder. The lack of an increase in the thin filament sliding speed with mouse actin across the entire range of Ca^{2+} concentrations from $3.9\mu\text{M}$ to 1nM limited the information that could be obtained from the *in vitro* motility assay. Thin filaments reconstituted with acetone powder actin show a 2-3 fold Ca^{2+} dependent increase (Bing *et al.*, 1998, Purcell *et al.*, 1999, Bing *et al.*, 2000). Results were therefore based entirely on the percentage motility parameter. However, it has been repeatedly shown that changes in the velocity and percentage motility parameters of Ca^{2+} sensitivity correlate with each other (Mirza *et al.*, 2005, Messer *et al.*, 2006).

7.3 The *in vitro* Motility Assay

As a functional technique, the *in vitro* motility assay was essential for investigating the effects of the mutations associated with HCM and DCM. Comparative experiments were run in split motility cells where the only variable was the mutated protein. The technique enabled small differences to be detected that were highly reproducible. It also allowed the proteins of the thin filaments to be manipulated, in particular the levels of phosphorylation. This has

been the first study to test the effect of cardiac muscle with defined disease-causing mutations in combination with altered phosphorylation.

This thesis has investigated transgenic mice at the molecular level primarily using the *in vitro* motility assay, but the mouse models will also be suitable for investigating the whole heart and whole animal physiological consequences of these mutations.

7.4 Contractile Abnormalities caused by DCM Mutations

Previous work in our laboratory has shown there are features common to all DCM mutations investigated by the *in vitro* motility assay. These features are independent of both the location of the mutation and the contractile protein bearing the mutation (Mirza *et al.*, 2005). They include a decrease in the Ca^{2+} sensitivity of 50: 50 mixtures, a significant decrease in the maximum sliding speed at high Ca^{2+} (except E54K), and a decrease in the Hill coefficient reflecting a loss of cooperativity in several cases. The decrease in sliding speed and Ca^{2+} sensitivity correlates with ATPase measurements (Robinson *et al.*, 2002, Mirza *et al.*, 2005). It is also in agreement with the reduction in the Ca^{2+} sensitivity caused by the troponin T mutations ΔLys210 , Arg141Trp and Arg205Leu in partially substituted rabbit skeletal muscle fibres (Morimoto *et al.*, 2002, Mirza *et al.*, 2005) and in fully substituted pig heart fibres using recombinant troponin subunits (Venkatraman *et al.*, 2003).

These *in vitro* studies suggest that DCM mutations will produce myofilaments that are less responsive to Ca^{2+} and will cause a decrease in force across a range of Ca^{2+} concentrations. This was considered sufficient to be a cause of DCM. My results with native-sequence thin filaments containing the E361G mutation were completely different from the previous results, since E361G transgenic and non-transgenic thin filaments were indistinguishable. However, dephosphorylation of both troponin I and troponin T (or substitution with skeletal muscle troponin) uncovered a difference between E361G transgenic and non-transgenic, where the E361G thin filaments reduced the Ca^{2+} sensitivity in common with previous experiments.

7.4.1 E361G: Blunted Response to Troponin I Phosphorylation

When E361G actin was reconstituted into the thin filament in combination with natively phosphorylated human cardiac troponin, it had a low Ca^{2+} sensitivity and was indistinguishable from non-transgenic actin. But when E361G troponin was dephosphorylated the Ca^{2+} sensitivity remained low, whereas it increased 3-fold in the non-transgenic. Thus, the mutant thin filaments were not responsive to a change in the troponin phosphorylation. In our E361G DCM mouse, the level of phosphorylation of all contractile proteins, including troponin I and troponin T, were the same as non-transgenic. This was also the case for the TNNC1 G159D muscle sample. Therefore the abnormality is in the Ca^{2+} sensitivity of the dephosphorylated troponin. It is unlikely that the reduced Ca^{2+} sensitivity with unphosphorylated troponin is relevant for the development of DCM. It is more likely that the critical factor is the associated lack of response to changes in the level of phosphorylation. This provides a novel mechanism for DCM.

It is well established that troponin I phosphorylation plays a major role in the normal response to adrenergic stimulation. For instance, transgenic mouse studies have shown that phosphorylation by PKA at troponin I Ser23/24, along with the phosphorylation of the membrane proteins involved in Ca^{2+} homeostasis, are essential to increase cardiac relaxation which is needed to maintain adequate diastolic ventricular filling with increased heart rate (Wolska *et al.*, 2002). The E361G mutation may therefore impair myocardial relaxation and trigger maladaptive cardiac remodelling leading to the DCM phenotype. In the normal heart, phosphorylation by PKA at troponin I Ser23/24 causes a decreased Ca^{2+} sensitivity and an increase cross-bridge cycling rate (Kentish *et al.*, 2001, Hunlich *et al.*, 2005). Such modifications result in an increase in power output in both the myocyte and the whole heart. Our results indicate that the E361G mutation suppresses the β -adrenergic stimulation of systolic function. It could be predicted that this would lead to a reduced cardiac output and the development of heart failure under stress. This mechanism is quite compatible with all the previously published work on DCM mutations since the effect of changing the phosphorylation levels were not tested.

7.4.2 Reduced α -actinin Binding

In addition to the reduced response to troponin I phosphorylation, the E361G mutation also significantly reduces the binding of actin affinity to $\tilde{U}$ -actinin. This could lead to impaired cardiac muscle function through the defective transmission of force and could be enough alone to cause the DCM phenotype. It is worth noting however that it is unlikely that this happens with DCM mutations in troponin subunits since they do not interact with $\tilde{U}$ -actinin. Data from our laboratory (Mirza 2005, unpublished) found no differences in $\tilde{U}$ -actinin force measurements with skeletal troponin T and two tropomyosin mutants.

7.4.3 The DCM Transgenic Mouse Phenotype

The mice exhibited no overt phenotype at rest and have a normal lifespan. Single cell contractility measurements and MRI analysis detected no functional abnormalities. This does not necessarily indicate that the mutation has no effect *in vivo*, since transgenic models are often compensated. Indeed, the TNNT Δ Lys210 DCM transgenic mouse showed a similar lack of phenotype at the whole cell level whilst the contractile apparatus was clearly abnormal (Morimoto *et al.*, 2006). This suggests the systolic function was fully compensated. Du *et al.* showed a lower Ca^{2+} sensitivity of force generation but no difference in the maximum isometric force produced by intact Δ Lys210 cardiac muscle fibres, indicating an increase in the intracellular Ca^{2+} transient. It seems likely that it is necessary to subject the mice to stress in order to reveal the phenotype (Geisterfer-Lowrance *et al.*, 1996). These physiological experiments are planned.

7.4.4 The G159D Phenotype

The G159D mutation has been found in a single family with a history of heart failure and sudden death over 5 generations and with a 100% association of the mutation with heart disease (penetrance). The individual whose heart we studied was on life-support at the age of 3 years, indicating that this is a particularly severe mutation (Kaski *et al.*, 2007).

Previous studies have investigated recombinant troponin with several troponin T and tropomyosin mutations, as well as the single TNNC1 mutation G159D (Mirza *et al.*, 2005). Mirza *et al.* showed the G159D mutation produced a lower Ca^{2+} sensitivity and cross-bridge turnover rate in common with the other DCM mutations. Despite this, our investigation of the troponin extracted from the human muscle sample with this mutation produced a strikingly different result, with the mutation producing a higher Ca^{2+} sensitivity both with natural phosphorylation levels and with dephosphorylated troponin. A similar result was obtained in the experiments of Biesiadecki *et al.* (Biesiadecki *et al.*, 2007). What the G159D troponin did share with the ACTC E361G DCM mutation was a lack of response to changes in troponin phosphorylation. Interestingly, the report from Preston *et al.* indicates that troponin T phosphorylation is critical for this effect to be observed (Preston *et al.*, 2007b).

An initial conclusion from our observations is that the common feature of all DCM mutations may be insensitivity to changes in troponin phosphorylation, with accompanying blunting of the response to adrenergic stimulation. The DCM phenotype does not seem to correlate with the altered Ca^{2+} sensitivity since both increased and decreased sensitivity has been observed in DCM phenotypes. Nor does the phenotype correlate with the reduced cross-bridge turnover rate, since this was not observed with the G159D mutation. The increased Ca^{2+} sensitivity in G159D is characteristic of HCM, but in this case it would seem not to result in any HCM-like phenotype. It may however contribute to the unusually high penetrance and severity of this mutation (Mogensen *et al.*, 2004b, Kaski *et al.*, 2007), perhaps by a mechanism similar to restrictive cardiomyopathy. Restrictive cardiomyopathy has been shown to have a molecular phenotype similar to, but more extreme than HCM (Mogensen *et al.*, 2003a).

This is the first study of a DCM mutation using the troponin extracted from the muscle of a patient. It illustrates in an extreme way the principle that we were already aware of, namely that the behaviour of any mutation is dependent upon the nature of the partner proteins. This has been shown previously for the truncated troponin T HCM mutation (Redwood *et al.*, 2000), and the D175N and E180G TPM1 HCM mutations (Bing *et al.*, 2000). Here, we find that G159D reverts to the classical *in vitro* phenotype when either the

troponin T and troponin I are not native (Mirza *et al.*, 2005), or the Û-tropomyosin is not native (reported in this thesis). The tropomyosin result is remarkable, since the only known difference is the substitution of the N terminal acetyl group for Ala-Ser, which does not alter any function except cooperativity (Monteiro *et al.*, 1994, Bing *et al.*, 2000, Mirza *et al.*, 2007). The implication is that there may be additional post-translational modifications in native tropomyosin that we are not aware of.

7.5 Contractile Abnormalities caused by HCM Mutations

The E99K HCM mice produce a strikingly different pattern of results to DCM mutations of an increase in the Ca^{2+} sensitivity, irrespective of the level of troponin phosphorylation. This HCM phenotype correlates well with the common mechanism when a dephosphorylated system is used. Previous *in vitro* motility studies and ATPase assays of the HCM mutation R92Q showed an increase in maximum filament sliding speed and an increase in the Ca^{2+} sensitivity (Mirza *et al.*, 2005). This also correlates with *in vitro* motility data of the tropomyosin mutations (Asp175Asn, Glu180Gly), and a truncated troponin T mutation (Bing *et al.*, 2000, Redwood *et al.*, 2000). In addition, a transgenic mouse model of the troponin I mutation R145E showed an increase in the Ca^{2+} sensitivity of skinned muscle fibres and at the whole organ level there was hypercontractility with diastolic dysfunction (James *et al.*, 2000).

The hypothesis has previously been put forward of HCM mutations causing a hypercontractile response, and they do so by affecting the Ca^{2+} sensitivity and cross-bridge cycling rate. However, this has been the first study to test the effect of a HCM mutation in combination with altering the phosphorylation of the troponin in the thin filament, and the E99K mutation also seems to produce a reduced but not completely suppressed response to troponin I phosphorylation. This seems to be at odds with the conclusion that the lack of response to troponin phosphorylation is a key feature of the DCM phenotype. In reality it may be a matter of degree, such that the same response is enough to avoid the DCM phenotype.

A feature of these transgenic mice is the substantial HCM-like sudden death (30% in males, 75% in females), which occurs between 30-45 days of

age. At the protein level, E99K transgenic mouse muscle had high levels of phosphorylation similar to non-transgenic muscle. This can be compared with the low level of phosphorylation seen in hypertrophic obstructive cardiomyopathy (HOCM) muscle obtained from septal myectomy operations. Myectomy muscle shows substantial reductions in the phosphorylation of myosin binding protein C (45%), troponin T (12%), troponin I (90%), and myosin light chain-2 (45%) (Gallon *et al.*, 2007). However, the phosphorylation changes in myectomy muscle seem to be secondary to the hypercontractile response caused by HCM mutations and the hypertrophy that is caused. In the mouse muscle, no secondary changes of post translational modifications were reported. This may have been due to the size of the mouse heart, the higher heart rates of the mice, or the short lifespan. Whatever the reason, it appears that the mouse model may reproduce the initial stages of HCM but not the later stages observed in patients undergoing myectomy.

The HCM phenotype caused by the E99K mutation is in great contrast to those caused by the DCM mutations that have been studied in this thesis. DCM is a chronic progressive disorder of the heart muscle characterised by a poor functioning and dilated ventricle. In the patient genotyped with the G159D mutation the clinical diagnosis of DCM was made, but at the molecular level G159D shows characteristics of being both HCM and DCM-like. The paradox of the HCM-like higher Ca^{2+} sensitivity of G159D may just reflect the fact that there are multiple pathways for a mutation to cause DCM. In contrast to DCM, HCM is characterised by the thickening of the ventricles which causes obstruction of the outflow tract and heart failure in some cases. A hypercontractile response has been commonly reported for HCM mutations and was indeed shown for the E99K mutation. This study of defined disease-causing mutations using the *in vitro* motility assay has revealed the importance of the nature of the experimental system in establishing the real *in vivo* effect of HCM and DCM and has opened up many questions.

7.6 Future Work

The altered response of myofilaments to changes in troponin I phosphorylation provides a novel mechanism for DCM, and there is indirect

evidence that this mechanism may also occur with several other DCM mutations studied in the past but in synthetic thin filament systems. All known DCM mutations should therefore be re-investigated in experimental systems that are close as possible to native human cardiac thin filaments. These should contain native post-translational modifications and human cardiac sequences. In the case of troponin I and troponin T, this can be done by exchange. It will be interesting to determine whether the blocking of the Ca^{2+} -desensitisation effect of troponin I phosphorylation is common to all DCM mutations, or whether there are multiple pathways for mutations to cause the disease.

The phenotype caused by the ACTC E361G mutation was exposed by altering the phosphorylation and by changing the partner proteins within the thin filament, and the G159D phenotype was completely different in native and synthetic systems. To determine whether the tropomyosin or troponin T is responsible for the two different phenotypes reported for this one mutation, it will be necessary to exchange the mutant troponin C or troponin T into human cardiac troponin with native phosphorylation levels.

HCM investigated in human heart muscle appears to be caused by a different and more complex mechanism than DCM. It is evident that the E99K mutation induces a clear phenotype *in vitro* under basal conditions but further functional studies are required. Mouse cardiac myofibrillar force measurements could be investigated, allowing the kinetics of Ca^{2+} activation and relaxation to be studied. These measurements should involve manipulation of protein phosphorylation levels within the myofibril.

This study has successfully combined human and mouse studies to investigate the molecular phenotype of genetic HCM and DCM. To further our understanding it will be necessary to study the disease mechanisms at the level of the intact heart and whole animal through physiological measurements (PV loop, MRI, 3D echo), allowing the development of HCM and DCM to be tracked. It will also be necessary to induce stress upon the E361G mice to perhaps uncover a phenotype. This could be done using drugs (dobutamine), exercise, or through surgery (aortic constriction).

8. REFERENCES

- Akbar, M. T., Lundberg, A. M., Liu, K., Vidyadaran, S., Wells, K. E., Dolatshad, H., Wynn, S., Wells, D. J., Latchman, D. S. & De Bellerocche, J. (2003) The Neuroprotective Effects Of Heat Shock Protein 27 Overexpression In Transgenic Animals Against Kainate-Induced Seizures And Hippocampal Cell Death. *J Biol Chem*, 278, 19956-65.
- Alahyan, M. & El-Mezgueldi, M. (2005) *Characterisation Of The Molecular Mechanism Of Inhibition Of The Actomyosin Atpase By Caldesmon-Tropomyosin. *Biophysical Journal*, 88, 133a.
- Alahyan, M., Webb, M. R., Marston, S. B. & El-Mezgueldi, M. (2006) The Mechanism Of Smooth Muscle Caldesmon-Tropomyosin Inhibition Of The Elementary Steps Of The Actomyosin Atpase. *J Biol Chem*, 281, 19433-48.
- Alpert, N. R., Mulieri, L. A. & Warshaw, D. (2002) The Failing Human Heart. *Cardiovasc Res*, 54, 1-10.
- Antoniv, T. T., Tanaka, S., Sudan, B., De Val, S., Liu, K., Wang, L., Wells, D. J., Bou-Gharios, G. & Ramirez, F. (2005) Identification Of A Repressor In The First Intron Of The Human Alpha2(I) Collagen Gene (Col1a2). *J Biol Chem*, 280, 35417-23.
- Arad, M., Lahat, H. & Freimark, D. (2005a) Genetic Ideology Of Dilated Cardiomyopathy. *Isr Med Assoc J*, 7, 392-6.
- Arad, M., Penas-Lado, M., Monserrat, L., Maron, B. J., Sherrid, M., Ho, C. Y., Barr, S., Karim, A., Olson, T. M., Kamisago, M., Seidman, J. G. & Seidman, C. E. (2005b) Gene Mutations In Apical Hypertrophic Cardiomyopathy. *Circulation*, 112, 2805-11.
- Bergen, H. R., 3rd, Ajtai, K., Burghardt, T. P., Nepomuceno, A. I. & Muddiman, D. C. (2003) Mass Spectral Determination Of Skeletal/Cardiac Actin Isoform Ratios In Cardiac Muscle. *Rapid Commun Mass Spectrom*, 17, 1467-71.
- Bers, D. M., Patton, C. W., Nuccitelli, R (1994). A practical guide to the preparation of calcium buffers. *Methods in Cell Biology*, 40, 3i 29
- 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., Fraser, I. D. C. & 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.

-
- Bing, W., Knott, A., Redwood, C. S., Esposito, G., Purcell, I., Watkins, S. & Marston, S. B. (2000) Effect Of Hypertrophic Cardiomyopathy Mutations In Human Cardiac A-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., Razzaq, A., Sparrow, J. & 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.
- Bione, S., Maestrini, E., Rivella, S., Mancini, M., Regis, S., Romeo, G. & Toniolo, D. (1994) Identification Of A Novel X-Linked Gene Responsible For Emery-Dreifuss Muscular Dystrophy. *Nat Genet*, 8, 323-7.
- Bonne, G., Carrier, L., Bercovici, J., Cruaud, C., Richard, P., Hainque, B., Gautel, M., Labeit, S., James, M., Beckmann, J., Weissenbach, J., Vosberg, H. P., Fiszman, M., Komajda, M. & Schwartz, K. (1995) Cardiac Myosin Binding Protein-C Gene Splice Acceptor Site Mutation Is Associated With Familial Hypertrophic Cardiomyopathy. *Nat Genet*, 11, 438-40.
- Bookwalter, C. S. & Trybus, K. M. (2006) Functional Consequences Of A Mutation In An Expressed Human Alpha-Cardiac Actin At A Site Implicated In Familial Hypertrophic Cardiomyopathy. *J Biol Chem*, 281, 16777-84.
- Borrego-Diaz, E., Kerff, F., Lee, S. H., Ferron, F., Li, Y. & Dominguez, R. (2006) Crystal Structure Of The Actin-Binding Domain Of Alpha-Actinin 1: Evaluating Two Competing Actin-Binding Models. *J Struct Biol*, 155, 230-8.
- Bottinelli, R., Coviello, D. A., Redwood, C. S., Pellegrino, M. A., Maron, B. J., Spirito, P., Watkins, H. & Reggiani, C. (1998) A Mutant Tropomyosin That Causes Hypertrophic Cardiomyopathy Is Expressed In Vivo And Associated With An Increased Calcium Sensitivity. *Circ Res*, 82, 106-115.
- Broderick, M. J. & Winder, S. J. (2005) Spectrin, Alpha-Actinin, And Dystrophin. *Adv Protein Chem*, 70, 203-46.
- Burton, D., Abdulrazzak, H., Knott, A., Elliott, K., Redwood, C. S., Watkins, H., Marston, S. & Ashley, C. (2002) Two Mutations In Troponin I That Cause Hypertrophic Cardiomyopathy Have Contrasting Effects On Cardiac Muscle Contractility. *Biochem J*, 362, 443-451.
- Chandra, M., Rundell, V. L., Tardiff, J. C., Leinwand, L. A., De Tombe, P. P. & Solaro, R. J. (2001) Ca(2+) Activation Of Myofilaments From Transgenic
-

-
- Mouse Hearts Expressing R92q Mutant Cardiac Troponin T. *Am J Physiol Heart Circ Physiol*, 280, H705-13.
- Chang, A. N., Harada, K., Ackerman, M. J. & Potter, J. D. (2005) Functional Consequences Of Hypertrophic And Dilated Cardiomyopathy-Causing Mutations In Alpha-Tropomyosin. *J Biol Chem*, 280, 34343-9.
- Chang, A. N. & Potter, J. D. (2005) Sarcomeric Protein Mutations In Dilated Cardiomyopathy. *Heart Fail Rev*, 10, 225-35.
- Codd, M. B., Sugrue, D. D., Gersh, B. J. & Melton, L. J., 3rd (1989) Epidemiology Of Idiopathic Dilated And Hypertrophic Cardiomyopathy. A Population-Based Study In Olmsted County, Minnesota, 1975-1984. *Circulation*, 80, 564-72.
- Cohn, J. N., Bristow, M. R., Chien, K. R., Colucci, W. S., Frazier, O. H., Leinwand, L. A., Lorell, B. H., Moss, A. J., Sonnenblick, E. H., Walsh, R. A., Mockrin, S. C. & Reinlib, L. (1997) Report Of The National Heart, Lung, And Blood Institute Special Emphasis Panel On Heart Failure Research. *Circulation*, 95, 766-70.
- Costa, C. F., Rommelaere, H., Waterschoot, D., Sethi, K. K., Nowak, K. J., Laing, N. G., Ampe, C. & Machesky, L. M. (2004) Myopathy Mutations In Alpha-Skeletal-Muscle Actin Cause A Range Of Molecular Defects. *J Cell Sci*, 117, 3367-77.
- Craig, S. W., Lancashire, C. L. & Cooper, J. A. (1982) Preparation Of Smooth Muscle A-Actinin. *Methods In Enzymology*, 85, 516-521.
- Cuda, G., Fananapazir, L., Epstein, N. D. & Sellers, J. R. (1997) The In Vitro Motility Activity Of Beta-Cardiac Myosin Depends On The Nature Of The Beta-Myosin Heavy Chain Gene Mutation In Hypertrophic Cardiomyopathy. *J Muscle Res Cell Motil*, 18, 275-83.
- D'amico, A., Graziano, C., Pacileo, G., Petrini, S., Nowak, K. J., Boldrini, R., Jacques, A., Feng, J. J., Porfirio, B., Sewry, C. A., Santorelli, F. M., Limongelli, G., Bertini, E., Laing, N. & Marston, S. B. (2006) Fatal Hypertrophic Cardiomyopathy And Nemaline Myopathy Associated With Acta1 K336e Mutation. *Neuromuscul Disord*, 16, 548-552.
- Daehmlow, S., Erdmann, J., Knueppel, T., Gille, C., Froemmel, C., Hummel, M., Hetzer, R. & Regitz-Zagrosek, V. (2002) Novel Mutations In Sarcomeric Protein Genes In Dilated Cardiomyopathy. *Biochem Biophys Res Commun*, 298, 116-20.
- Dalloz, F., Osinska, H. & Robbins, J. (2001) Manipulating The Contractile Apparatus: Genetically Defined Animal Models Of Cardiovascular Disease. *J Mol Cell Cardiol*, 33, 9-25.
- Davies, J. K., Wells, D. J., Liu, K., Whitrow, H. R., Daniel, T. D., Grignani, R., Lygate, C. A., Schneider, J. E., Noel, G., Watkins, H. & Carling, D. (2006)
-

Characterization Of The Role Of Gamma2 R531g Mutation In Amp-Activated Protein Kinase In Cardiac Hypertrophy And Wolff-Parkinson-White Syndrome. *Am J Physiol Heart Circ Physiol*, 290, H1942-51.

Davis, J. S. & Harrington, W. F. (1993) Kinetic And Physical Characterization Of Force Generation In Muscle: A Laser Temperature-Jump And Length-Jump Study On Activated And Contracting Rigor Fibers. *Adv Exp Med Biol*, 332, 513-24; Discussion 525-6.

Davis, J. S. & Rodgers, M. E. (1995) Indirect Coupling Of Phosphate Release To De Novo Tension Generation During Muscle Contraction. *Proc Natl Acad Sci U S A*, 92, 10482-6.

Debold, E. P., Schmitt, J. P., Patlak, J. B., Beck, S. E., Moore, J. R., Seidman, J. G., Seidman, C. & Warshaw, D. M. (2007) Hypertrophic And Dilated Cardiomyopathy Mutations Differentially Affect The Molecular Force Generation Of Mouse Alpha-Cardiac Myosin In The Laser Trap Assay. *Am J Physiol Heart Circ Physiol*, 293, H284-91.

Deng, Y., Schmidtman, A., Kruse, S., Filatov, V., Heilmeyer, L. M., Jr., Jaquet, K. & Thieleczek, R. (2003) Phosphorylation Of Human Cardiac Troponin I G203s And K206q Linked To Familial Hypertrophic Cardiomyopathy Affects Actomyosin Interaction In Different Ways. *J Mol Cell Cardiol*, 35, 1365-74.

Dong, W., An, J., Xing, J. & Cheung, H. (2007) Structural Kinetics Of Cardiac Troponin C Mutants Linked To Familial Hypertrophic Cardiomyopathy And Dilated Cardiomyopathy In Troponin Complexes. *Biophysical Journal*, 91, 628a.

Doolan, A., Nguyen, L. & Semsarian, C. (2004) Hypertrophic Cardiomyopathy: From "Heart Tumour" To A Complex Molecular Genetic Disorder. *Heart Lung Circ*, 13, 15-25.

Dos-Remedios, C. G. & Moens, P. D. (1995) Actin And The Actomyosin Interface: A Review. *Biochimica Et Biophysica Acta*, 1228, 99-124.

Du, C. K., Morimoto, S., Nishii, K., Minakami, R., Ohta, M., Tadano, N., Lu, Q. W., Wang, Y. Y., Zhan, D. Y., Mochizuki, M., Kita, S., Miwa, Y., Takahashi-Yanaga, F., Iwamoto, T., Ohtsuki, I. & Sasaguri, T. (2007) Knock-In Mouse Model Of Dilated Cardiomyopathy Caused By Troponin Mutation. *Circ Res*, 101, 185-94.

Ebashi, S. & Endo, M. (1968) Calcium Ion And Muscle Contraction. *Prog. Biophys. Molec. Biol.*, 18, 125-183.

Ebashi, S., Kodama, A. & Ebashi, F. (1968) Troponin. I. Preparation And Physiological Function. *J Biochem (Tokyo)*, 64, 465-77.

-
- Ebashi, S. & Ohtsuki, I. (2007) Regulatory Mechanisms Of Striated Muscle Contraction. *Advances In Experimental Medicine And Biology*, 592.
- Eisenberg, E. & Moos, C. (1968) The Adenosine Triphosphatase Activity Of Acto-Heavy Meromyosin. A Kinetic Analysis Of Actin Activation. *Biochemistry*, 7, 1486-9.
- El-Armouche, A., Pohlmann, L., Schlossarek, S., Starbatty, J., Yeh, Y. H., Nattel, S., Dobrev, D., Eschenhagen, T. & Carrier, L. (2007) Decreased Phosphorylation Levels Of Cardiac Myosin-Binding Protein-C In Human And Experimental Heart Failure. *J Mol Cell Cardiol*, 43, 223-9.
- Elliott, P. M., Poloniecki, J., Dickie, S., Sharma, S., Monserrat, L., Varnava, A., Mahon, N. G. & McKenna, W. J. (2000) Sudden Death In Hypertrophic Cardiomyopathy: Identification Of High Risk Patients. *J Am Coll Cardiol*, 36, 2212-8.
- Elliott, P. M., Sharma, S., Varnava, A., Poloniecki, J., Rowland, E. & McKenna, W. J. (1999) Survival After Cardiac Arrest Or Sustained Ventricular Tachycardia In Patients With Hypertrophic Cardiomyopathy. *J Am Coll Cardiol*, 33, 1596-601.
- Fananapazir, L., Dalakas, M. C., Cyran, F., Cohn, G. & Epstein, N. D. (1993) Missense Mutations In The Beta-Myosin Heavy-Chain Gene Cause Central Core Disease In Hypertrophic Cardiomyopathy. *Proc Natl Acad Sci U S A*, 90, 3993-7.
- Fatkin, D., Christe, M. E., Aristizabal, O., McConnell, B. K., Srinivasan, S., Schoen, F. J., Seidman, C. E., Turnbull, D. H. & Seidman, J. G. (1999) Neonatal Cardiomyopathy In Mice Homozygous For The Arg403Gln Mutation In The Alpha Cardiac Myosin Heavy Chain Gene. *J Clin Invest*, 103, 147-53.
- Feng, J-J. (2007) A Functional Investigation of Actin Mutations in Disease. NHLI, London. Imperial College London.
- Fentzke, R. C., Buck, S. H., Patel, J. R., Lin, H., Wolska, B. M., Stojanovic, M. O., Martin, A. F., Solaro, R. J., Moss, R. L. & Leiden, J. M. (1999) Impaired Cardiomyocyte Relaxation And Diastolic Function In Transgenic Mice Expressing Slow Skeletal Troponin I In The Heart. *J Physiol*, 517, 143-157.
- Filatov, V. L., Katrukha, A. G., Bereznikova, A. V., Esakova, T. V., Bulargina, T. V., Kolosova, O. V., Severin, E. S. & Gusev, N. B. (1998) Epitope Mapping Of Anti-Troponin I Monoclonal Antibodies. *Biochem. Mol. Biol. Int.*, 45, 1179-87.
- Finley, N., Abbott, M. B., Abusamhadneh, E., Gaponenko, V., Dong, W., Gasmi-Seabrook, G., Howarth, J. W., Rance, M., Solaro, R. J., Cheung, H. C. & Rosevear, P. R. (1999) NMR Analysis Of Cardiac Troponin C-Troponin I Complexes: Effects Of Phosphorylation. *Febs Lett*, 453, 107-12.

- Fraser, I. D. C. & Marston, S. B. (1995a) *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.
- Fraser, I. D. C. & Marston, S. B. (1995b) *Troponin Regulation Of Thin Filament Motility Studied With An *In Vitro* Motility Assay. *J.Muscle Res.Cell Motil.*, 16, 148.
- Fuchs, F. & Briggs, F. N. (1968) The Site Of Calcium Binding In Relation To The Activation Of Myofibrillar Contraction. *J Gen Physiol*, 51, 655-76.
- Gallon, C. E., Jacques, A., Messer, A. E., Tsang, V., Mckenna, W.J. & Marston, S. B. (2007) Altered Functions And Post-Translational Modification Of Contractile Proteins In Myectomy Samples From Hocm Patients. *J Mol Cell Cardiol.*, 42:S167.
- Geeves, M. A. & Halsall, D. J. (1987) Two Step Ligand Binding And Cooperativity. A Model To Describe The Cooperative Binding Of Myosin Subfragment 1 To Regulated Actin. *Biophysical Journal*, 52, 215-220.
- Geeves, M. A. & Holmes, K. C. (1999) Structural Mechanism Of Muscle Contraction. *Annu. Rev. Biochem.*, 68, 687-728.
- Geeves, M. A. & Holmes, K. C. (2005) The Molecular Mechanism Of Muscle Contraction. *Adv Protein Chem*, 71, 161-93.
- Geisterfer-Lowrance, A. A., Christe, M., Conner, D. A., Ingwall, J. S., Schoen, F. J., Seidman, C. E. & Seidman, J. G. (1996) A Mouse Model Of Familial Hypertrophic Cardiomyopathy. *Science*, 272, 731-4.
- Geisterfer-Lowrance, 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.
- Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitas, K., Sasse-Klaassen, S., Seidman, J. G., Seidman, C., Granzier, H., Labeit, S., Frenneaux, M. & Thierfelder, L. (2002) Mutations Of Ttn, Encoding The Giant Muscle Filament Titin, Cause Familial Dilated Cardiomyopathy. *Nat Genet*, 30, 201-4.
- Gordon, A. M., Huxley, A. F. & Julian, F. J. (1966) The Variation In Isometric Tension With Sarcomere Length In Vertebrate Muscle Fibres. *J Physiol*, 184, 170-92.
- Greaser, M. L. & Gergely, J. (1971) Reconstitution Of Troponin Activity From Three Protein Components. *J.Biol.Chem.*, 246, 4226-4233.

-
- Hamid, M. S., Norman, M., Quraishi, A., Firoozi, S., Thaman, R., Gimeno, J. R., Sachdev, B., Rowland, E., Elliott, P. M. & McKenna, W. J. (2002) Prospective Evaluation Of Relatives For Familial Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia Reveals A Need To Broaden Diagnostic Criteria. *J Am Coll Cardiol*, 40, 1445-50.
- Hennessey, E. S., Drummond, D. R. & Sparrow, J. C. (1993) Molecular Genetics Of Actin Function. *Biochem.J.*, 282, 657-671.
- Herron, T. J., Korte, F. S. & McDonald, K. S. (2001) Power Output Is Increased After Phosphorylation Of Myofibrillar Proteins In Rat Skinned Cardiac Myocytes. *Circ Res*, 89, 1184-90.
- Hershberger, R. E. (2005) Familial Dilated Cardiomyopathy. *Progress In Pediatric Cardiology*, 20, 161-168.
- Hoffmann, B., Schmidt-Traub, H., Perrot, A., Osterziel, K. J. & Gessner, R. (2001) First Mutation In Cardiac Troponin C, L29q, In A Patient With Hypertrophic Cardiomyopathy. *Hum Mutat*, 17, 524.
- Hoh, J. F., Rossmanith, G. H., Kwan, L. J. & Hamilton, A. M. (1988) Adrenaline Increases The Rate Of Cycling Of Crossbridges In Rat Cardiac Muscle As Measured By Pseudo-Random Binary Noise-Modulated Perturbation Analysis. *Circ Res*, 62, 452-61.
- Holmes, K. C., Popp, D., Gebhard, W. & Kabsch, W. (1990) Atomic Model Of The Actin Filament. *Nature*, 347, 44-49.
- Houser, S. R. & Margulies, K. B. (2003) Is Depressed Myocyte Contractility Centrally Involved In Heart Failure? *Circ Res*, 92, 350-8.
- Hunlich, M., Begin, K. J., Gorga, J. A., Fishbaugher, D. E., Lewinter, M. M. & Vanburen, P. (2005) Protein Kinase A Mediated Modulation Of Acto-Myosin Kinetics. *J Mol Cell Cardiol*, 38, 119-25.
- Huxley, A. F. (1957) Muscle Structure And Theories Of Contraction. *Prog. Biophys. Biophys. Chem.*, 7, 255-318.
- Huxley, H. E. & Hanson, J. (1954) Changes In Cross-Striations Of Muscle During Contraction And Stretch And Their Structural Interpretation. *Nature*, 173, 973-976.
- Ilkovski, B., Nowak, K. J., Domazetovska, A., Maxwell, A. L., Clement, S., Davies, K. E., Laing, N. G., North, K. N. & Cooper, S. T. (2004) Evidence For A Dominant-Negative Effect In Acta1 Nemaline Myopathy Caused By Abnormal Folding, Aggregation And Altered Polymerization Of Mutant Actin Isoforms. *Hum Mol Genet*, 13, 1727-43.
- James, J., Zhang, Y., Osonska, H., Sanbe, A., Klevitsky, R., Hewett, T. E. & Robbins, J. (2000) Transgenic Modelling Of A Cardiac Troponin I

- Mutation Linked To Familial Hypertrophic Cardiomyopathy. *Circ Res*, 87, 805-811.
- Johara, M., Toyoshima, Y. Y., Ishijima, A., Kojima, H., Yanagida, T. & Sutoh, K. (1993) Charge-Reversion Mutagenesis Of *Dictyostelium* Actin To Map The Surface Recognised By Myosin During Atp-Driven Sliding Motion. *Proc.Natl.Acad.Sci.U.S.A.*, 90, 2127-2131.
- Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. & Holmes, K. C. (1990) Atomic Structure Of Actin:Dnase I Complex. *Nature*, 347, 37-44.
- Kamisago, M., Sharma, S. D., Depalma, S. R., Solomon, S., Sharma, P., Mcdonough, B., Smoot, L., Mullen, M. P., Woolf, P. K., Wigle, E. D., Seidman, J. G. & Seidman, C. E. (2000) Mutations In Sarcomere Protein Genes As A Cause Of Dilated Cardiomyopathy. *N Engl J Med*, 343, 1688-96.
- Kaski, J. P., Burch, M. & Elliott, P. (2007) Mutations In The Dilated Cardiac Troponin C Gene Are A Cause Of Idiopathic Dilated Cardiomyopathy In Childhood. *Cardiol Young*, 17, 675-677.
- Katrakha, 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.
- Kentish, J. C., Mccloskey, D. T., Layland, J., Palmer, S., Leiden, J. M., Martin, A. F. & Solaro, R. J. (2001) Phosphorylation Of Troponin I By Protein Kinase A Accelerates Relaxation And Crossbridge Cycle Kinetics In Mouse Ventricular Muscle. *Circ Res*, 88, 1059-65.
- Kimura, A., Harada, H., Park, J. E., Nishi, H., Satoh, M., Takahashi, M., Hiroi, S., Sasaoka, T., Ohbuchi, N., Nakamura, T., Koyanagi, T., Hwang, T. H., Choo, J. A., Chung, K. S., Hasegawa, A., Nagai, R., Okazaki, O., Nakamura, H., Matsuzaki, M., Sakamoto, T., Toshima, H., Koga, Y., Imaizumi, T. & Sasazuki, T. (1997) Mutations In The Cardiac Troponin I Gene Associated With Hypertrophic Cardiomyopathy. *J Biochem (Tokyo)*, 321, 873-878.
- Knoll, R., Hoshijima, M., Hoffman, H. M., Person, V., Lorenzen-Schmidt, I., Bang, M. L., Hayashi, T., Shiga, N., Yasukawa, H., Schaper, W., Mckenna, W., Yokoyama, M., Schork, N. J., Omens, J. H., Mcculloch, A. D., Kimura, A., Gregorio, C. C., Poller, W., Schaper, J., Schultheiss, H. P. & Chien, K. R. (2002) The Cardiac Mechanical Stretch Sensor Machinery Involves A Z Disc Complex That Is Defective In A Subset Of Human Dilated Cardiomyopathy. *Cell*, 111, 943-55.
- Knollmann, B. C., Blatt, S. A., Horton, K., De Freitas, F., Miller, T., Bell, M., Housmans, P. R., Weissman, N. J., Morad, M. & Potter, J. D. (2001) Inotropic Stimulation Induces Cardiac Dysfunction In Transgenic Mice

-
- Expressing A Troponin T (I79n) Mutation Linked To Familial Hypertrophic Cardiomyopathy. *J Biol Chem*, 276, 10039-48.
- Knollmann, B. C. & Potter, J. D. (2001) Altered Regulation Of Cardiac Muscle Contraction By Troponin T Mutations That Cause Familial Hypertrophic Cardiomyopathy. *Trends In Cardiovascular Medicine*, 11, 206-212.
- Knott, A., Purcell, I. F. & Marston, S. (2002) In Vitro Motility Analysis Of Thin Filaments From Failing And Non-Failing Human Hearts Induces Slower Filament Sliding And Higher Ca²⁺-Sensitivity. *J Mol Cell Cardiol*, 34, 469-482.
- Kobayashi, T. & Solaro, R. J. (2005) Calcium, Thin Filaments, And The Integrative Biology Of Cardiac Contractility. *Annu Rev Physiol*, 67, 39-67.
- Kron, S. J. & Spudich, J. A. (1986) Fluorescent Actin Filaments Move On Myosin Fixed To A Glass Surface. *Proc.Natl.Acad.Sci.U.S.A.*, 83, 6272-6276.
- Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q. P. & Spudich, J. A. (1991) Assays For Actin Sliding Movement Over Myosin Coated Surfaces. *Methods In Enzymology.*, 196, 399-416.
- Layland, J., Solaro, R. J. & Shah, A. M. (2005) Regulation Of Cardiac Contractile Function By Troponin I Phosphorylation. *Cardiovasc Res*, 66, 12-21.
- Lebart, M. C., Mejean, C., Roustan, C. & Benyamin, Y. (1993) Further Characterization Of The Alpha-Actinin-Actin Interface And Comparison With Filamin-Binding Sites On Actin. *J Biol Chem*, 268, 5642-8.
- Lehrer, S. S. & Geeves, M. A. (1998) The Muscle Thin Filament As A Classical Cooperative/Allosteric Regulatory System. *J Mol Biol*, 277, 1081-1089.
- Li, D., Tapscoft, T., Gonzalez, O., Burch, P. E., Quinones, M. A., Zoghbi, W. A., Hill, R., Bachinski, L. L., Mann, D. L. & Roberts, R. (1999) Desmin Mutation Responsible For Idiopathic Dilated Cardiomyopathy. *Circulation*, 100, 461-4.
- Lionel, H. O. (2005) Mechanisms Of Cardiac Contraction And Relaxation. In Zipes, P. D., Libby, P., Bonow, O. R. & Braunwald, E. (Eds.) *Heart Disease: A Textbook Of Cardiovascular Medicine*. 7th Ed., Elsevier Saunders.
- Lorenz, M., Poole, K. J. V., Popp, D., Rosenbaum, G. & Holmes, K. C. (1995) An Atomic Model Of The Unregulated Thin Filament Obtained By X-Ray Diffraction On Orientated Actin-Tropomyosin Gels. *J Mol Biol.*, 246, 108-119.
-

-
- Lorenz, M., Popp, D. & Holmes, K. C. (1993) Refinement Of The F-Actin Model Against X-Ray Fiber Diffraction Data By The Use Of A Directed Mutation Algorithm. *J Mol Biol.*, 234, 826-836.
- 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. (1971a) Mechanism Of Adenosine Triphosphate Hydrolysis By Actomyosin. *Biochemistry*, 10, 4617-24.
- Lymn, R. W. & Taylor, E. W. (1971b) Meshanism Of Adenosine Triphosphate Hydrolysis By Actomyosin. *Biochemistry*, 10, 4617-4623.
- Malmqvist, U. P., Aronshtam, A. & Lowey, S. (2004) Cardiac Myosin Isoforms From Different Species Have Unique Enzymatic And Mechanical Properties. *Biochemistry*, 43, 15058-65.
- 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.
- Margossian, S. S. & Lowey, S. (1982) Preparation Of Myosin And Its Subfragments From Rabbit Skeletal Muscle. *Methods In Enzymology*, 85, 55-71.
- 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., Shirani, J., Poliac, L. C., Mathenge, R., Roberts, W. C. & Mueller, F. O. (1996) Sudden Death In Young Competitive Athletes. Clinical, Demographic, And Pathological Profiles. *Jama*, 276, 199-204.
- Maron, B. J., Spirito, P., Wesley, Y. & Arce, J. (1986) Development And Progression Of Left Ventricular Hypertrophy In Children With Hypertrophic Cardiomyopathy. *N Engl J Med*, 315, 610-4.
- Marston, S. (2003) Random Walks With Thin Filaments: Application Of In Vitro Motility Assay To The Study Of Actomyosin Regulation. *J Muscle Res Cell Motility*, 24, 149-156.
- Marston, S. & Hodgkinson, J. (2001) News And Views: Cardiac And Skeletal Myopathies: Can Genotype Explain Phenotype? *J Muscle Res Cell Motility*, 22, 1-4.
- Marston, S., Mirza, M., Abdulrazzak, H. & Sewry, C. (2004) Functional Characterisation Of A Mutant Actin (Met132val) From A Patient With Nemaline Myopathy. *Neuromuscul Disord*, 14, 167-74.

-
- 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. & Smith, C. W. J. (1984) Purification And Properties Of Ca^{2+} -Regulated Thin Filaments And F-Actin From Sheep Aorta Smooth Muscle. *J Muscle Res Cell Motility*, 5, 559-575.
- Marston, S.B., Redwood, C. S., Purcell, I. F., Esposito, G., Watkins, H. & Wu, B. (1998) Effects Of Fhc Mutations In Alpha Tropomyosin, Asp175asn And Glu180gly, Upon The Functional Properties Of Reconstituted Thin Filaments Studied By In Vitro Motility Assay. *Circulation*, 98 (Supp. 1), 466.
- Mayosi, B. M., Khogali, S., Zhang, B. & Watkins, H. (1999) Cardiac And Skeletal Actin Gene Mutations Are Not A Common Cause Of Dilated Cardiomyopathy. *J Med Genet*, 36, 796-7.
- Maytum, R., Lehrer, S. & Geeves, M. (1999) Cooperativity And Switching Within The Three-State Model Of Muscle Regulation. *Biochemistry*, 38, 1102-1110.
- Mcgough, A., Way, M. & Derosier, D. (1994) Determination Of The Alpha-Actinin-Binding Site On Actin Filaments By Cryoelectron Microscopy And Image Analysis. *J Cell Biol*, 126, 433-43.
- McGuigan, J. A., Luthi, D. & Burri, A. (1991) Calcium buffer solutions and how to make them: a do it yourself guide. *Can. J. Physiol. Pharmacol.*, 69, 1733-1749.
- Mckenna, W. (2003) The Cardiomyopathies: Hypertrophic, Dilated, Restrictive, And Right Ventricular. *Oxford Textbook Of Medicine*, 2, 1021-1035.
- 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. *Biophysical Journal*, 65, 693-701.
- Mclaughlin, P. J., Gooch, J. T., Mannherz, H. G. & Weeds, A. G. (1993) Structure Of Gelsolin Segment 1-Actin Complex And The Mechanism Of Filament Severing. *Nature*, 364, 685-92.
- Messer, A. E., Jacques, A. M. & Marston, S. B. (2006) 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. (2007) Structural And Functional Polymorphisms Of Troponin In Failing Heart. NHLI. London, Imperial College London.

-
- Mestroni, L., Maisch, B., McKenna, W. J., Schwartz, K., Charron, P., Rocco, C., Tesson, F., Richter, A., Wilke, A. & Komajda, M. (1999a) Guidelines For The Study Of Familial Dilated Cardiomyopathies. Collaborative Research Group Of The European Human And Capital Mobility Project On Familial Dilated Cardiomyopathy. *Eur Heart J*, 20, 93-102.
- Mestroni, L., Rocco, C., Gregori, D., Sinagra, G., Di Lenarda, A., Miocic, S., Vatta, M., Pinamonti, B., Muntoni, F., Caforio, A. L., McKenna, W. J., Falaschi, A., Giacca, M. & Camerini (1999b) Familial Dilated Cardiomyopathy: Evidence For Genetic And Phenotypic Heterogeneity. Heart Muscle Disease Study Group. *J Am Coll Cardiol*, 34, 181-90.
- Metzger, J. M. & Westfall, M. V. (2004) Covalent And Noncovalent Modification Of Thin Filament Action. The Essential Role Of Troponin In Cardiac Muscle Regulation. *Circ Res*, 94, 146-158.
- Miller, T., Szczesna, D., Housmans, P. R., Zhao, J., De Freitas, F., Gomes, A. V., Culbreath, L., Mccue, J., Wang, Y., Xu, Y., Kerrick, W. G. & Potter, J. D. (2001) Abnormal Contractile Function In Transgenic Mice Expressing A Familial Hypertrophic Cardiomyopathy-Linked Troponin T (I79n) Mutation. *J Biol Chem*, 276, 3743-55.
- Mirza, M. (2005) Pathological Mutations In Thin Filament Proteins Investigated By In Vitro Motility Assay. NHLI. London, 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.
- Mogensen, J., Klausen, I. C., Pedersen, A. K., Egeblad, H., Bross, P., Kruse, T. A., Gregersen, N., Hansen, P. S., Baandrup, U. & Borghlum, A. D. (1999) Alpha-Cardiac Actin Is A Novel Disease Gene In Familial Hypertrophic Cardiomyopathy. *J Clin Invest*, 103, R39-43.
- Mogensen, J., Kubo, T., Duque, M., Uribe, W., Shaw, A., Murphy, R., Gimeno, J. R., Elliott, P. & McKenna, W. J. (2003a) Idiopathic Restrictive Cardiomyopathy Is Part Of The Clinical Expression Of Cardiac Troponin I Mutations. *J Clin Invest*, 111, 209-16.
- 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, A., Bahl, A., Elliott, P. M. & McKenna, W. J. (2003b) *Cardiac Troponin C And T Mutations In 288 Patients With Idiopathic Dilatedcardiomyopathy: Prevalence, Clinical Features And Impact On The Troponin Complex. *Circulation*, 108, Supp Iv P50.
- 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.
- Mogensen, J., Perrot, A., Andersen, P. S., Havndrup, O., Klausen, I. C., Christiansen, M., Bross, P., Egeblad, H., Bundgaard, H., Osterziel, K. J., Haltern, G., Lapp, H., Reinecke, P., Gregersen, N. & Borglum, A. D. (2004c) Clinical And Genetic Characteristics Of Alpha Cardiac Actin Gene Mutations In Hypertrophic Cardiomyopathy. *J Med Genet*, 41, E10.
- Mohapatra, B., Jimenez, S., Lin, J. H., Bowles, K. R., Coveler, K. J., Marx, J. G., Chrisco, M. A., Murphy, R. T., Lurie, P. R., Schwartz, R. J., Elliott, P. M., Vatta, M., McKenna, W., Towbin, J. A. & Bowles, N. E. (2003) Mutations In The Muscle Lim Protein And Alpha-Actinin-2 Genes In Dilated Cardiomyopathy And Endocardial Fibroelastosis. *Mol Genet Metab*, 80, 207-15.
- Mollica, L. R., Crawley, J. T., Liu, K., Rance, J. B., Cockerill, P. N., Follows, G. A., Landry, J. R., Wells, D. J. & Lane, D. A. (2006) Role Of A 5'-Enhancer In The Transcriptional Regulation Of The Human Endothelial Cell Protein C Receptor Gene. *Blood*, 108, 1251-9.
- Monseerrat, 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.
- Monteiro, P. B., Lataro, R. C., Ferro, J. A. & Reinach, F. (1994) Functional Alpha-Tropomyosin Produced In Escherichia Coli. A Dipeptide Extension Can Substitute The Amino-Terminal Acetyl Group. *J Biol Chem*, 269, 10461-10466.
- Morimoto, S., Du, C., Lu, Q., Nishii, K., Minakami, R., Ohta, M., Oka, N., Tadano, N., Miyazaki, J., Yamamura, K. & Ohtsuki, I. (2006) A Knock-In Mouse Model For Dilated Cardiomyopathy Caused By Dk210 Mutation In Cardiac Troponin T And Its Molecular Pathogenic Mechanism. *Biophysical Journal*, 90.

-
- Morimoto, S., Lu, Q. W., Harada, K., Takahashi-Yanaga, F., Minakami, R., Ohta, M., Sasaguri, T. & Ohtsuki, I. (2002) Ca²⁺-Desensitizing Effect Of A Deletion Mutation Delta K210 In Cardiac Troponin T That Causes Familial Dilated Cardiomyopathy. *Proc Natl Acad Sci Usa*, 99 (2): 913-18
- Morimoto, S., Yanaga, F., Minakami, R. & Ohtsuki, I. (1998) Ca²⁺-Sensitizing Effects Of The Mutations At Ile-79 And Arg-92 Of Troponin T In Hypertrophic Cardiomyopathy. *Am J Physiol*, 275, C200-7.
- Mornet, D., Bertrand, R. U., Pantel, P., Audemard, E. & Kassab, R. (1981) Proteolytic Approach To Structure And Function Of Actin Recognition Site In Myosin Heads. *Biochemistry*, 20, 2110-20.
- Murphy, R. T., Mogensen, J., Shaw, A., Kubo, T., Hughes, S. & McKenna, W. J. (2004) Novel Mutation In Cardiac Troponin I In Recessive Idiopathic Dilated Cardiomyopathy. *Lancet*, 363, 371-2.
- Nier, V., Schultz, I., Brenner, B., Forssmann, W. & Raida, M. (1999) Variability In The Ratio Of Mutant To Wildtype Myosin Heavy Chain Present In The Soleus Muscle Of Patients With Familial Hypertrophic Cardiomyopathy. A New Approach For The Quantification Of Mutant To Wildtype Protein. *Febs Lett*, 461, 246-52.
- Niimura, H., Bachinski, L. L., Sangwatanaroj, S., Watkins, H., Chudley, A. E., McKenna, W., Kristinsson, A., Roberts, R., Sole, M., Maron, B. J., Seidman, J. G. & Seidman, C. E. (1998) Mutations In The Gene For Cardiac Myosin-Binding Protein C And Late-Onset Familial Hypertrophic Cardiomyopathy. *N Engl J Med*, 338, 1248-57.
- Noguchi, T., Camp, P., Jr., Alix, S. L., Gorga, J. A., Begin, K. J., Leavitt, B. J., Ittleman, F. P., Alpert, N. R., Lewinter, M. M. & Vanburen, P. (2003) Myosin From Failing And Non-Failing Human Ventricles Exhibit Similar Contractile Properties. *J Mol Cell Cardiol*, 35, 91-7.
- Oberst, L., Zhao, G., Park, J. T., Brugada, R., Michael, L. H., Entman, M. L., Roberts, R. & Marian, A. J. (1998) Dominant-Negative Effect Of A Mutant Cardiac Troponin T On Cardiac Structure And Function In Transgenic Mice. *J Clin Invest*, 102, 1498-505.
- Okamoto, Y. & Sekine, T. (1985) A Streamlined Method For Subfragment-1 Preparation From Myosin. *J.Biochem.(Tokyo)*, 98, 1143-1145.
- 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.
- Otterbein, L. R., Graceffa, P. & Dominguez, R. (2001) The Crystal Structure Of Uncomplexed Actin In The Adp State. *Science*, 293, 708-11.
- Pagani, E., Alousi, A., Grant, A., Older, T., Dziuban, S. & Allen, P. (1988) Changes In Myofibrillar Content And Mg-ATPase Activity In Ventricular Tissues From Patients With Heart Failure Caused By Coronary Artery Disease, Cardiomyopathy, Or Mitral Valve Insufficiency. *Circ Res*, 63, 380-385.
- Palmer, B. M. (2005) Thick Filament Proteins And Performance In Human Heart Failure. *Heart Fail Rev*, 10, 187-97.
- 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.
- Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. & Ferrin, T. E. (2004) Ucsf Chimera--A Visualization System For Exploratory Research And Analysis. *J Comput Chem*, 25, 1605-12.
- Pirani, A., Vinogradova, M. V., Curmi, P. M., King, W. A., Fletterick, R. J., Craig, R., Tobacman, L. S., Xu, C., Hatch, V. & Lehman, W. (2006) An Atomic Model Of The Thin Filament In The Relaxed And Ca(2+)-Activated States. *J Mol Biol*, 357, 707-717.
- Pirani, A., Xu, C., Hatch, V., Craig, R., Tobacman, L. S. & Lehman, W. (2005) Single Particle Analysis Of Relaxed And Activated Muscle Thin Filaments. *J Mol Biol*, 346, 761-72.
- Poetter, K., Jiang, H., Hassanzadeh, S., Master, S. R., Chang, A., Dalakas, M. C., Rayment, I., Sellers, J. R., Fananapazir, L. & Epstein, N. D. (1996) Mutations In Either The Essential Or Regulatory Light Chains Of Myosin Are Associated With A Rare Myopathy In Human Heart And Skeletal Muscle. *Nat Genet*, 13, 63-9.
- Pollard, T. D. & Cooper, J. A. (1986) Actin And Actin-Binding Proteins. A Critical Evaluation Of Mechanisms And Functions. *Annu Rev Biochem*, 55, 987-1035.
- Potter, J. D. (1982) Preparation Of Troponin And Its Subunits. *Methods In Enzymology*, 85, 241-263.

-
- Potter, J. D., Sheng, Z., Pan, B. & Zhao, J. (1995) A Direct Regulatory Role For Troponin T And A Dual Role For Troponin C In The Ca^{2+} Regulation Of Muscle Contraction. *J.Biol.Chem.*, 270, 2557-2562.
- Preston, L. C., Ashley, C. C. & Redwood, C. S. (2007a) Dcm Troponin C Mutation Gly159asp Blunts The Response To Troponin Phosphorylation. *Biochem Biophys Res Commun*, In Press.
- Preston, L. C., Lipscomb, S., Robinson, P., Mogensen, J., McKenna, W. J., Watkins, H., Ashley, C. C. & Redwood, C. S. (2007b) Functional Effects Of The Dcm Mutant Gly159asp Troponin C In Skinned Muscle Fibres. *Pflugers Arch*, 453, 771-6.
- Purcell, I. F., Bing, W. & Marston, S. B. (1999) Functional Analysis Of Human Cardiac Troponin By The In Vitro Motility Assay: Comparison Of Adult, Foetal And Failing Hearts. *Cardiovasc Res*, 43, 884-891.
- Rajan, S., Ahmed, R. P. H., 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, 205-214.
- 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, 11-6.
- Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C. & Milligan, R. A. (1993a) Structure Of The Actin-Myosin Complex And Its Implications For Muscle Contraction. *Science*, 261, 58-65.
- Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelman, D. A., Wesenberg, G. & Holden, H. (1993b) Three Dimensional Structure Of Myosin Subfragment-1: A Molecular Motor. *Science*, 261, 50-58.
- Razzaq, A., Schmitz, S., Veigel, C., Molloy, J. E., Geeves, M. A. & Sparrow, J. C. (1999) Actin Residue Glu(93) Is Identified As An Amino Acid Affecting Myosin Binding. *J Biol Chem*, 274, 28321-8.
- Redwood, C., Lohmann, K., Bing, W., Esposito, G., Elliott, K., Abdulrazzak, H., Knott, A., Purcell, I., Marston, S. & Watkins, H. (2000) Investigation Of A Truncated Troponin T That Causes Familial Hypertrophic Cardiomyopathy: Ca^{2+} Regulatory Properties Of Reconstituted Thin Filaments Depend On The Ratio Of Mutant To Wild-Type Peptide. *Circ Res*, 86, 1146-1152.
- Richard, P., Charron, P., Carrier, L., Ledeuil, C., Cheav, T., Pichereau, C., Benaiche, A., Isnard, R., Dubourg, O., Burban, M., Gueffet, J. P., Millaire,

-
- A., Desnos, M., Schwartz, K., Hainque, B. & Komajda, M. (2003) Hypertrophic Cardiomyopathy: Distribution Of Disease Genes, Spectrum Of Mutations, And Implications For A Molecular Diagnosis Strategy. *Circulation*, 107, 2227-32.
- Richardson, P., McKenna, W., Bristow, M., Maisch, B., Mautner, B., O'Connell, J., Olsen, E., Thiene, G., Goodwin, J., Gyarsas, I., Martin, I. & Nordet, P. (1996) Report Of The 1995 World Health Organization/International Society And Federation Of Cardiology Task Force On The Definition And Classification Of Cardiomyopathies. *Circulation*, 93, 841-2.
- Robinson, P., Watkins, H. & Redwood, C. (2007) *Using Fluorescence Emission Spectroscopy To Assess The Ca^{2+} Affinity Of Reconstituted Troponin And Thin Filaments Containing Cardiomyopathy Causing Mutations Of Troponin And γ -Tropomyosin. *Biophysical Journal*, 91, 362a.
- Robinson, P. J. R., Mirza, M., Knott, A., Abdulrazzak, H., Marston, S., Watkins, H. & Redwood, C. S. (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.
- Saeki, Y., Shiozawa, K., Yanagisawa, K. & Shibata, T. (1990) Adrenaline Increases The Rate Of Cross-Bridge Cycling In Rat Cardiac Muscle. *J Mol Cell Cardiol*, 22, 453-60.
- 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.
- Satoh, M., Takahashi, M., Sakamoto, T., Hiroe, M., Marumo, F. & Kimura, A. (1999) Structural Analysis Of The Titin Gene In Hypertrophic Cardiomyopathy: Identification Of A Novel Disease Gene. *Biochem Biophys Res Commun*, 262, 411-7.
- Schmitt, J. P., Kamisago, M., Asahi, M., Li, G. H., Ahmad, F., Mende, U., Kranias, E. G., MacLennan, D. H., Seidman, J. G. & Seidman, C. E. (2003) Dilated Cardiomyopathy And Heart Failure Caused By A Mutation In Phospholamban. *Science*, 299, 1410-3.
- Schulenberg, B., Aggeler, R., Beechem, J. M., Capaldi, R. A. & Patton, W. F. (2003) Analysis Of Steady-State Protein Phosphorylation In Mitochondria Using A Novel Fluorescent Phosphosensor Dye. *J Biol Chem*, 278, 27251-5.
- Schutt, C. E., Myslik, J. C., Rozycki, M. D., Goonsekere, N. C. & Lindberg, U. (1993) The Structure Of Crystalline Profilin-Beta-Actin. *Nature*, 365, 810-816.
-

-
- Seidman, J. G. & Seidman, C. (2001) The Genetic Basis For Cardiomyopathy: From Mutation Identification To Mechanistic Paradigms. *Cell*, 104, 557-67.
- Sevdali, M. & Sparrow, J. (2007) A Human Skeletal Actin (Actc1) Myopathy Mutation (R372h): Studies Using *Drosophila* Suggest The Primary Defect Is In Alpha-Actinin-Actin Binding. *Amc Abstract* 2007.
- Severs, N. J. (2000) The Cardiac Muscle Cell. *Bioessays*, 22, 188-199.
- Sheetz, M. P. & Spudich, J. A. (1983) Movement Of Myosin-Coated Fluorescent Beads On Actin Cables In Vitro. *Nature*, 303, 31-5.
- Sheterline, P., Clayton, P. & Sparrow, J. C. (1998) *Actin*, Oxford, Oxford University Press.
- Sleep, J., Lewalle, A. & Smith, D. (2006) Reconciling The Working Strokes Of A Single Head Of Skeletal Muscle Myosin Estimated From Laser-Trap Experiments And Crystal Structures. *Proc Natl Acad Sci U S A*, 103, 1278-82.
- 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. & Rarick, H. M. (1998) Troponin And Tropomyosin: Proteins That Switch On And Tune In The Activity Of Cardiac Myofilaments. *Circ Res*, 83, 471-80.
- Spudich, J. A. (2001) The Myosin Swinging Cross-Bridge Model. *Nat Rev Mol Cell Biol*, 2, 387-92.
- Spudich, J. A. & Watt, S. (1971) The Regulation Of Rabbit Skeletal Muscle Contraction. *J Biol Chem*, 246, 4866-4871.
- Steinberg, T. H., Agnew, B. J., Gee, K. R., Leung, W. Y., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J. M., Haugland, R. P. & Patton, W. F. (2003) Global Quantitative Phosphoprotein Analysis Using Multiplexed Proteomics Technology. *Proteomics*, 3, 1128-44.
- Straub, F. B. (1942) Actin. *Studies From The Institute Of Medical Chemistry, University Of Szeged*, 2, 3-16.
- Subramaniam, A., Jones, W. K., Gulick, J., Wert, S., Neumann, J. & Robbins, J. (1991) Tissue-Specific Regulation Of The Alpha-Myosin Heavy Chain Gene Promoter In Transgenic Mice. *J Biol Chem*, 266, 24613-20.
- Sugden, P. H., Severs, N. J., Macleod, K. T. & Poole-Wilson, P. A. (2003) Biochemistry And Cellular Physiology Of Heart Muscle. *Oxford Textbook Of Medicine*, Fourth Edition (2), 809-820.
-

- Sundberg, M., Rosengren, J. P., Bunk, R., Lindahl, J., Nicholls, I. A., Tagerud, S., Omling, P., Montelius, L. & Mansson, A. (2003) Silanized Surfaces For In Vitro Studies Of Actomyosin Function And Nanotechnology Applications. *Anal Biochem*, 323, 127-38.
- Sutoh, K. (1982) An Actin-Binding Site On The 20k Fragment Of Myosin Subfragment 1. *Biochemistry*, 21, 4800-4.
- Szczesna, D., Zhang, R., Zhao, J., Jones, M., Guzman, G. & Potter, J. D. (2000) Altered Regulation Of Cardiac Muscle Contraction By Troponin T Mutations That Cause Familial Hypertrophic Cardiomyopathy. *J Biol Chem*, 275, 624-630.
- Szilagyi, L., Balint, M., Sreter, F. A. & Gergely, J. (1979) Photoaffinity Labelling With An Atp Analog Of The N-Terminal Peptide Of Myosin. *Biochem Biophys Res Commun*, 87, 936-45.
- Takai, E., Akita, H., Shiga, N., Kanazawa, K., Yamada, S., Terashima, M., Matsuda, Y., Iwai, C., Kawai, K., Yokota, Y. & Yokoyama, M. (1999) Mutational Analysis Of The Cardiac Actin Gene In Familial And Sporadic Dilated Cardiomyopathy. *Am J Med Genet*, 86, 325-7.
- Takeda, S., 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.
- Tardiff, J. C., Hewett, T. E., Palmer, B. M., Olsson, C., Factor, S. M., Moore, R. L., Robbins, J. & Leinwand, L. A. (1999) Cardiac Troponin T Mutations Result In Allele-Specific Phenotypes In A Mouse Model For Hypertrophic Cardiomyopathy. *J Clin Invest*, 104, 469-81.
- Tesson, F., Sylvius, N., Pilotto, A., Dubosq-Bidot, L., Peuchmaurd, M., Bouchier, C., Benaiche, A., Mangin, L., Charron, P., Gavazzi, A., Tavazzi, L., Arbustini, E. & Komajda, M. (2000) Epidemiology Of Desmin And Cardiac Actin Gene Mutations In A European Population Of Dilated Cardiomyopathy. *Eur Heart J*, 21, 1872-6.
- Thierfelder, L., Watkins, H., Macrae, C., Lamas, R., McKenna, W., Vosberg, H.-P., Seidman, J. G. & Seidman, C. E. (1994) A-Tropomyosin And Cardiac Troponin T Mutations Cause Familial Hypertrophic Cardiomyopathy: A Disease Of The Sarcomere. *Cell*, 77, 701-712.
- Tobacman, L. & Sawyer, D. (1990) Calcium Binds Cooperatively To The Regulatory Sites Of The Cardiac Thin Filament. *J Biol Chem*, 265, 931-939.
- Tobacman, L. S., Lin, D., Butters, C., Landis, C., Back, N., Pavlov, D. & Homsher, E. (1999) Functional Consequences Of Troponin T Mutations Found In Hypertrophic Cardiomyopathy. *J Biol Chem*, 274, 28363-70.

-
- Towbin, J. A. & Bowles, N. E. (2002) The Failing Heart. *Nature*, 415, 227-33.
- Van Der Velden, J., Klein, L. J., Van Der Bijl, M., Huybregts, M. A., Stooker, W., Witkop, J., Eijssman, L., Visser, C. A., Visser, F. C. & Stienen, G. J. (1998) Force Production In Mechanically Isolated Cardiac Myocytes From Human Ventricular Muscle Tissue. *Cardiovasc Res*, 38, 414-23.
- Van Der Velden, J., Papp, Z., Zaremba, R., Boontje, N. M., De Jong, J. W., Owen, V. J., Burton, P. B., Goldmann, P., Jaquet, K. & Stienen, G. J. (2003) Increased Ca^{2+} -Sensitivity Of The Contractile Apparatus In End-Stage Human Heart Failure Results From Altered Phosphorylation Of Contractile Proteins. *Cardiovasc Res*, 57, 37-47.
- Vandekerckhove, J., Bugaisky, G. & Buckingham, M. (1986) Simultaneous Expression Of Skeletal Muscle And Heart Actin Proteins In Various Striated Muscle Tissues And Cells. A Quantitative Determination Of The Two Actin Isoforms. *J Biol Chem*, 261, 1838-43.
- Venkatraman, G., Harada, K., Gomes, A. V., Kerrick, G. W. & Potter, J. D. (2003) Different Functional Properties Of Troponin T Mutants That Cause Dilated Cardiomyopathy. *J Biol Chem*.
- Vilfan, A. (2001) The Binding Dynamics Of Tropomyosin On Actin. *Biophysical Journal*, 81, 3146-3155.
- Vinogradova, M. V., Stone, D. B., Malanina, G. G., Karatzaferi, C., Cooke, R., Mendelson, R. A. & Fletterick, R. J. (2005) Ca^{2+} -Regulated Structural Changes In Troponin. *Proc Natl Acad Sci U S A*, 102, 5038-5045.
- Voet, D. & Voet, J. G. (1995) Biochemistry, John Wiley & Sons.
- Watkins, H., Conner, D., Thierfelder, L., Jarcho, J. A., Macrae, C., McKenna, W. J., Maron, B. J., Seidman, J. G. & Seidman, C. E. (1995) Mutations In The Cardiac Myosin Binding Protein-C Gene On Chromosome 11 Cause Familial Hypertrophic Cardiomyopathy. *Nat Genet*, 11, 434-7.
- Weeds, A. G. & Lowey, S. (1971) Substructure Of The Myosin Molecule. II. The Light Chains Of Myosin. *J Mol Biol*, 61, 701-25.
- Wells, D. J., Wells, K. E., Asante, E. A., Turner, G., Sunada, Y., Campbell, K. P., Walsh, F. S. & Dickson, G. (1995) Expression Of Human Full-Length And Minidystrophin In Transgenic Mdx Mice: Implications For Gene Therapy Of Duchenne Muscular Dystrophy. *Hum Mol Genet*, 4, 1245-50.
- Wilcken, D. E. L. (2003) Clinical Physiology Of The Normal Heart. *Oxford Textbook Of Medicine*, Fourth Edition (2), 820-828.
- Wolff, M., Buck, S., Stoker, S., Greaser, M. & Mentzer, R. (1996) Myofibrillar Calcium Sensitivity Of Isometric Tension Is Increased In Human Dilated Cardiomyopathies. *J Clin Invest*, 98, 167-176.
-

- Wolska, B. M., Arteaga, G. M., Pena, J. R., Nowak, G., Phillips, R. M., Sahai, S., De Tombe, P. P., Martin, A. F., Kranias, E. G. & Solaro, R. J. (2002) Expression Of Slow Skeletal Troponin I In Hearts Of Phospholamban Knockout Mice Alters The Relaxant Effect Of Beta-Adrenergic Stimulation. *Circ Res*, 90, 882-8.
- Wong, W. W., Doyle, T. C., Cheung, P., Olson, T. M. & Reisler, E. (2002) Functional Studies Of Yeast Actin Mutants Corresponding To Human Cardiomyopathy Mutations. *J Muscle Res Cell Motil*, 22, 665-674.
- Yanaga, F., Morimoto, S. & Ohtsuki, I. (1999) Ca^{2+} Sensitization And Potentiation Of The Maximum Level Of Myofibrillar Atpase Activity Caused By Mutations Of Troponin T Found In Familial Hypertrophic Cardiomyopathy. *J Biol Chem*, 274, 8806-12.
- Yang, Q., Sanbe, A., Osinska, H., Hewett, T. E., Klevitsky, R. & Robbins, J. (1998) A Mouse Model Of Myosin Binding Protein C Human Familial Hypertrophic Cardiomyopathy. *J Clin Invest*, 102, 1292-300.
- Yasui, B., Fuchs, F. & Briggs, F. N. (1968) The Role Of The Sulfhydryl Groups Of Tropomyosin And Troponin In The Calcium Control Of Actomyosin Contractility. *J Biol Chem*, 243, 735-42.