

**β-ADRENERGIC SIGNALLING IN
THE NEONATAL VENTRICULAR
CARDIOMYOCYTES OF THE *POPDC2*^{W188X}
MUTANT AND CHARACTERIZATION OF
THE *POPDC2*^{W188X} MUTANT MOUSE**

**THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY**

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ABSTRACT

The β -adrenergic receptor - cyclic adenosine 3',5'-monophosphate (β AR-cAMP) signalling transduces the extracellular sympathetic stimulus to intracellular effector proteins to adapt cardiac output during stress or exercise. The transmembrane Popeye domain containing (POPDC) protein family represent a novel class of cAMP effectors of which POPDC1 and POPDC2 are abundantly expressed in the working myocardium and in the cardiac conduction system. Dysregulation of POPDC expression has been associated with a defective heart rate response to stress. A nonsense variant of *POPDC2* (*POPDC2*^{W188X}) discovered in patients was selected to generate a knock-in mouse model to study the role of POPDC proteins in the β AR-cAMP mediated stress response of cardiac pacemaking and conduction.

POPDC2^{W188X} mutant tissue and cultured neonatal mouse cardiomyocytes (NMCM) displayed a loss of *Popdc2* transcripts and a loss and mislocalisation of POPDC1 proteins. To understand how this loss affected sinoatrial pacemaking, isolated *POPDC2*^{W188X} mutant hearts were studied by Langendorff perfusion. The *POPDC2*^{W188X} mutant demonstrated an increase in heart rate at baseline, and a tachy-bradycardia in response to β AR activation. A dysregulation of β_2 AR signalling was identified in cultured *POPDC2*^{W188X} mutant NMCM, resulting in low cAMP nanodomain signalling as revealed by different Förster resonance energy transfer sensors following sympathetic activation but not at baseline. POPDC proteins appear to be specifically involved in β AR signalling as cAMP nanodomain signalling was not impaired in mutant NMCM when the prostaglandin receptor was stimulated. Likewise, direct stimulation of adenylyl cyclases by forskolin was not impacted by the *POPDC2*^{W188X} mutation. The phenotype was rescued by pertussis toxin suggestive of abnormal G_i (inhibitory G-protein)-coupling of the β_2 AR in mutant NMCM. Interfering with the interaction of phosphodiesterase 4A and POPDC1 using a disruptor peptide in wild type NMCM was shown to mimic the *POPDC2*^{W188X} phenotype. These results start unravelling the intricate role of POPDC proteins within the β AR signalling complexes.

STATEMENT OF ORIGINALITY

All the work presented in this thesis was conducted by me, Lena Gruscheski, unless stated otherwise. Where applicable contributions from other people are acknowledged. I conducted the experiments, analysed the data, produced the graphs and figures, and wrote the text with the guidance of my supervisor Professor Thomas Brand.

Lena Gruscheski

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AKAP	A Kinase anchoring protein
ANO5	Anoctamin 5
AP	Action potential
aPKC	atypical protein kinase C
ATP	Adenosine triphosphat
AV	Atrioventricular
β AR	β -adrenergic receptor
cAMP	Cyclic 3',5'-adenosine monophosphate
CaMKII	Calmodulin-dependent protein kinase II
CAP	Catabolite activator protein
Cav	Caveolin
CCS	Cardiac conduction system
cGMP	Cyclic 3',5'-guanosine monophosphate
CICR	Calcium-induced-calcium-release
CM	Cardiomyocyte
CNBD	Cyclic nucleotide-binding domain
CNG	Cyclic nucleotide-gated ion channels
CRP	cAMP receptor protein
CTD	Carboxyterminal domain
DAG	Diaglycerol
ECC	Excitation-contraction-coupling
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EPAC	Exchange protein activated by cyclic AMP
ERK1/2	Extracellular signal-regulated kinases
FRET	Förster resonance energy transfer
FSK	Forskolin
GDP	Guanosine diphosphat
GEF	Guanine nucleotide exchange factors
GEFT	Guanine nucleotide exchange factor for Rho family of small GTPases
GPCR	G-protein coupled receptor
GRK	GPCR kinase
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
HCN4	Hyperpolarization-activated cyclic-nucleotide gated ion channel 4
HPS	His-Purkinje system
HR	Heart rate
IBMX	3-isobutyl-1-methylxanthine

ICI	ICI 118,551
iCNS	Intrinsic cardiac nervous system
ID	Intercalated disk
I_f	<i>Funny current</i>
I_{K1}	Potassium channels
I_{Kr}	Potassium channels rapidly
I_{Ks}	Potassium channels slowly
I_{Kur}	Potassium channels ultra-rapidly
I_{Na}	Sodium current
I_{NaL}	Late sodium current
I_{to}	Transient K^+ current
IP3	Inositol 1,4,5-triphosphate
iPSC	Induced pluripotent stem cells
KO	Knock-out
KCNQ1	Potassium voltage-gated channel subfamily Q member 1
LQTS	Long-QT syndrome
LTCC	L-type calcium channel
M2	Muscarinic receptor 2
MAPK	Mitogen-activated protein kinase
M β CD	Methyl- β -Cyclodextrin
Meis1	Meis homeobox protein 1
MI	Myocardial infarction
Nav	Voltage-gated sodium channels
NCX	Sodium calcium exchanger
NDGR4	N-myc downstream regulated gene 4
NE	Norepinephrine
NMCM	Neonatal mouse cardiomyocyte
NMD	Nonsense mediated decay
PBC	Phosphate binding cassette
PCNA	Proliferating cell nuclear antigen
PIP2	Phosphatidylinositol bisphosphate
PKA	Protein kinase A
PLB	Phospholamban
POPDC	Popeye Domain Containing
PP1	Protein phosphatase 1
PPI	Protein-protein interaction
PSN	Parasympathetic nervous system
PTC	Premature termination codon
RyRs	Ryanodine receptors
SAN	Sinoatrial node
SCN5A	Sodium voltage-gated channel alpha subunit 5

SDD	Slow diastolic depolarisation
SERCA2	Sarcoplasmic/endoplasmic reticulum Ca ²⁺ ATPase 2a pump
SND	Sinus node dysfunction
SNS	Sympathetic nervous system
Sodium/potassium ATPase (Na ⁺ /K ⁺ ATPase)	
SR	Sarcoplasmic reticulum
TREK-1	Two-pore potassium channel TWIK-related K ⁺ channel
T-tubules or TATs	Transverse tubules
UCR	Upstream conserved domains
ZO-1	Zona occludens 1
ZONAB/DbpA	ZO-1 associated nucleic acid-binding protein/DNA binding protein A
WT	Wild type

CHAPTER 1 General Introduction

1.1 The heart

The heart functions as a mechanical pump that starts beating during early embryonic development. Throughout lifetime it has the vital role to maintain adequate blood and therefore oxygen and nutrient supply to the various organ systems of the body. The heartbeat is driven by the cardiac conduction system (CCS) composed of different cardiac myocyte types that set an intrinsic heart rate by the means of an electrical impulse also called action potential (AP). Physiological heart rate adaptation is achieved under the regulation of the autonomic nervous system. The delicate interplay between the electrical and mechanical components driving a synchronized contraction of the heart chambers is mediated by cycling of calcium ions.

1.1.1 The heart chambers

The heart begins to form as early as the third week of human embryonic development from the splanchnic mesoderm. Several additional cell lineages such as the neural crest or the proepicardium also contribute cells to the heart during later development¹. The heart is surrounded and held in place by a supportive and protective fibrous sac called pericardium which can be divided into the fibrous (outer layer) and serous (inner layer) pericardia². In mammals and birds, the heart is composed of four chambers: the upper left and right atria, which are the receiving chambers that are separated by the interatrial septum, and the lower left and right ventricles, which are the discharging chambers that are separated by a thick interventricular septum. For an efficient pumping of blood through the body, the four chambers work in concert i.e., when the ventricles contract (systole) the atria are relaxed and when the ventricles are relaxed (diastole) the atria contract.

1.1.2 The cardiac cycle

The right atrium receives deoxygenated circulatory blood through the superior and inferior vena cava. A small amount of blood from the coronary circulation also drains into the right atrium from the coronary sinus which is located near the inferior vena cava. From the right atrium, during early diastole, deoxygenated blood flows into the right ventricle through the

open tricuspid valve. Simultaneously, the left atrium receives reoxygenated blood from the pulmonary system through the pulmonary veins, which flows into the left ventricle via the mitral valve. The valves are embedded into the atrioventricular septum which separates the atria from the ventricles. It is part of a fibrous cardiac skeleton of a high-density connective tissue that forms and anchors the valves and provides electrical isolation between the upper and lower heart chambers. During late diastole, the atrial AP spreads initiating atrial contraction (atrial systole) to eject any remaining blood into the ventricles under pressure. As the electrical signal propagates towards the lower chambers, the ventricles begin to enter systole which generates back pressure on the tricuspid and mitral valves forcing them to close. Further contraction of the ventricles increases the pressure in the trunks of the aorta and pulmonary arteries, forcing the aortic valve in the left ventricle and the pulmonary valve in the right ventricle to open so that blood can be ejected from both ventricles into the pulmonary and circulatory systems, respectively. A fraction of the blood is also ejected into coronary arteries through the aortic sinuses found above the aortic valve. The ejection of blood results in a drop of the ventricular pressure below its peak which allows the aortic and pulmonary valves to close. As the ventricles relax further, the atria start refilling with blood and the mitral and tricuspid valves open so that the cardiac cycle returns to its starting point, the ventricular diastole³.

1.1.3 The cardiac muscle

The cardiac wall is composed of three distinct layers, the epicardium, the myocardium and the endocardium⁴. The epicardium is formed by the aforementioned proepicardium, which establishes the epicardium after proepicardial cells are getting in contact with the forming ventricular and atrial chambers. The endocardium lines the inner walls of the four cardiac chambers and also gives rise to the valvular apparatus of the heart with some material contributions from the proepicardial and neural crest lineages². The endocardium forms a barrier between the cardiac muscle (myocardium) and the blood stream, the lymphatic vessels and nerves⁴. Additionally, adjacent to the endocardium in the so-called subendocardial layer, is located the ventricular CCS^{5,6}.

The myocardium forms the thickest layer of the heart wall, although the thickness of the myocardium varies between chambers in line with the contractile force that the different chambers generate to propel the blood. This muscular layer of the heart contains cardiomyocytes, which are branched, striated and predominantly mononucleated cells (in the adult human heart, while being binucleated in the adult mouse heart) with a high density of mitochondria and a basal membrane⁷. The basal membrane is an extracellular matrix composed of the glycoprotein's laminin and fibronectin, type IV collagen and proteoglycans. It surrounds each individual cardiac myocyte and provides a scaffold to which the myocyte can adhere. To promote seamless propagation of an AP, cardiac myocytes are branched and electrically coupled through gap junctions at the intercalated disks (ID)⁸. Gap junctions, made of 6 protein subunits called connexins, promote a quick AP propagation between individual myocytes by reducing the internal resistance and facilitating cell-cell communication by permitting the passage of sodium, potassium and calcium ions as well as various compounds such as second messenger molecules, metabolites and water⁹. Notably, sodium, potassium and calcium ions are essential for cardiac pacemaking, the cardiac action potential as well as regulating cardiac contraction and their intricate interplay, which is discussed in the section '1.1.6 The cardiac pacemaker potential'. Intercalated disks also contain desmosomes and adherens junctions which function as mechanical anchors between adjacent cardiomyocytes to enable controlled transmission of contractile force between cardiomyocytes during contraction¹⁰. Another important feature of cardiac myocytes are the transverse tubules (T-tubules or TATs)¹¹. This extensive network of transversal and longitudinal invaginations of the plasma membrane (sarcolemma) allows for an increased surface area and rapid transmission of the AP deep into the cell. Furthermore, T-tubules form cardiac microdomains that house key receptors, ion channels and exchangers important for the regulation of cellular calcium concentrations as part of the excitation-contraction coupling (ECC). ECC is a process which converts the AP into contraction by the action of the contractile apparatus, which gets activated via a mechanism explained by the sliding filament theory^{12,13}. Myocyte striations are a visual product of the crystalline-like alignment of contractile proteins into cardiac myofibrils forming distinct, repeating units called sarcomeres. Parallel arranged thick (consisting of myosin with a globular head which possess ATPase activity) and thin myofilaments (consisting mainly of the actin filament comprised of a linear polymer of globular actin subunits and troponin subunits involved in calcium sensing) representing the

basic contractile elements of a muscle cell that by binding calcium can generate contraction¹⁴. T-tubules make it possible to simultaneously deliver calcium to sarcomeres across and at the core of a cardiac myocyte, for an efficient and simultaneous activation of the entire contractile apparatus within the cell¹⁵. In line with its function, the T-tubule network is most extensively developed in ventricular myocytes (the working myocardium) and much less developed in respect to density, or even absent in atrial myocytes and in pacemaker myocytes found in the sinoatrial and atrioventricular nodes^{16,17}. Additionally, it has been shown that T-tubules undergo extensive remodelling during heart disease^{15,17}.

Alongside T-tubules, caveolae have been recognised as an important feature of cardiac myocytes¹⁸. These small, flask-shaped membrane pockets are rich in cholesterol and glycosphingolipids and are structurally stabilized by scaffolding proteins called caveolins (Cav1–Cav3) and cavinins (cavin1-4), with Cav3 and cavin4 being the striated muscle-specific isoforms^{19,20}. Interestingly, it has been shown that cavin4 is re-localised during caveolae-associated muscle disease²¹. Initially, caveolae were thought to serve as membrane lipid reservoirs, that allowed for an instantaneous increase in the surface area upon osmotic pressure²². However, now they are also understood to be at the centre of various processes such as vesicular trafficking, mechanosensation and transduction, as well as signalling processes such as β -adrenergic receptor (β AR) signalling¹⁸. Several different ion channels and transporters as well as all components of β AR signalling, have been localized to caveolae in cardiac myocytes including the L-type calcium channel (LTCC), cardiac voltage-gated sodium channel, hyperpolarization-activated cyclic-nucleotide gated ion channel 4 (HCN4) and sodium calcium exchanger (NCX) and as such they provide specialised microdomains for signalling and crosstalk to take place.

Mammalian cardiomyocytes are generally understood to exhibit minimal regenerative capacity due to their relative inability, in the adult heart, to re-enter the cell cycle and complete mitosis after undergoing DNA synthesis and exiting the cell cycle shortly after birth. Moreover, no cardiac stem cell population has been identified, that is able to repopulate lost cardiac myocytes (CM) following injury such as myocardial infarction (MI)^{23,24,25}. In recent studies, however, evidence has been obtained that CM turnover rate in humans is approximately 1% at the age of 25, declining to less than 0.45 % past the age of 75, resulting

in nearly half of the CM population to be substituted during postnatal life^{26,27}. Recent studies revealed that during embryogenesis myocytes can form from non-myocyte populations, however in the adult heart, new myocytes only arise from pre-existing cardiac myocytes and not from any non-myocyte population present in the heart²⁸. As evidenced by the progressive deterioration of heart function and continual fibrotic deposition, the natural capacity of cardiac myocyte regeneration in adult mammals is insufficient to compensate for myocyte loss in different cardiac diseases. However, CM of amphibians and teleost were found to have a powerful regenerative capacity through their ability to re-enter cell cycle and undergo mitosis²⁹. Further study of signalling mechanisms that induce CM proliferation in, for example, zebrafish may elucidate novel strategies to induce CM proliferation in human heart after MI. Other approaches such as therapeutic implantation of iPSC-derived cardiomyocytes and direct reprogramming of cardiac fibroblasts into cardiomyocyte-like cells are also being investigated^{30,31}.

Generally, growth of the postnatal heart is achieved by hypertrophy of cardiac myocytes, a process, which is described as an increase of the size of individual myocytes without substantial increase in cell numbers³². Hypertrophy can be physiological as observed in athletes, but is mostly pathological, with a prevalence of 1:500 persons. It can arise as the result of volume overload or high blood pressure, but long-term hypertrophic growth generally degenerates into heart failure^{33,34}. On a wider scope it is part of many pathological conditions affecting the heart muscle such as cardiomyopathies³⁵.

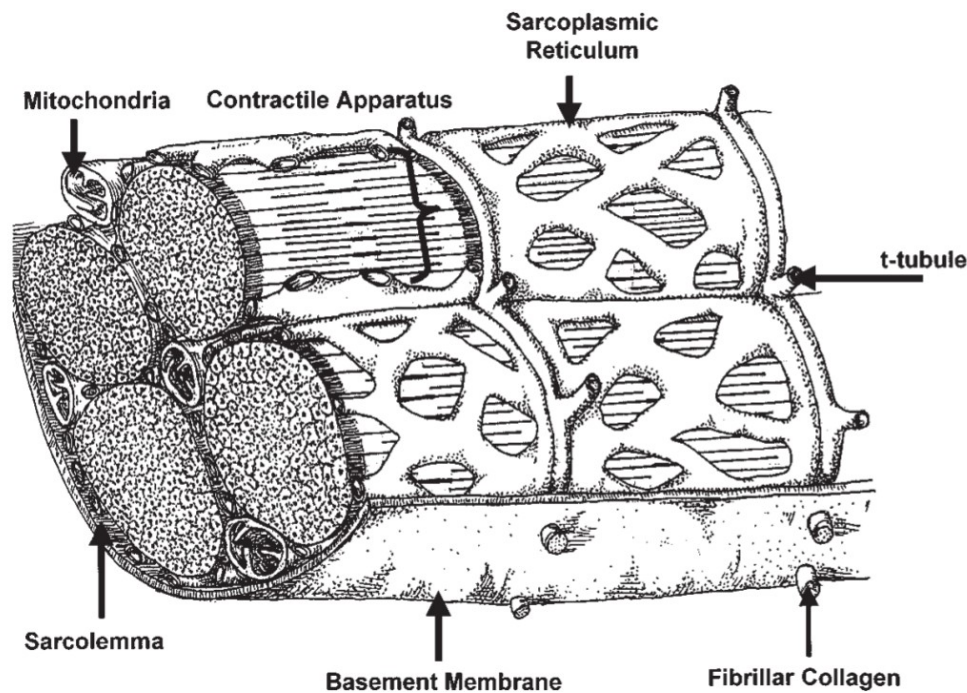


Figure 1.1. Individual cardiac myocyte depicted in a longitudinal cross section. From the outside-in shown are: the basement membrane – provides continuity with extracellular matrix, the sarcolemma – separates the intra and extra cellular spaces, t-tubules – transverse and longitudinal sarcolemma folds, the sarcoplasmic reticulum - an internal store and source of cytosolic calcium for the ECC, the contractile apparatus – predominantly actin and myosin myofilaments, mitochondria – situated close to the myofilament apparatus they generate high volumes of ATP to prevent cardiac fatigue (adapted from³⁶).

1.1.4 The Cardiac Conduction System

The CCS is a network of specialized cells that is responsible for the initiation and co-ordination of the heartbeat by means of cardiac electrical signalling. Cells of the CCS have the unique ability to generate an AP without an external stimulus; a property that was termed automaticity. There are three main components of the CCS, namely the sinoatrial node (SAN), the atrioventricular (AV) node and the His-Purkinje system (HPS).

The SAN has the highest intrinsic rate of automaticity and thus is responsible for the primary initiation of the cardiac AP³⁷. It is located at the border between the vena cava and the right atrium where loosely arranged nodal cells are electrically isolated from each other and the atrial myocardium by an extensive extracellular matrix to prevent hyperpolarization from the much larger atrial myocardium³⁸. Moreover, the SAN is composed of three specialized cell types: pale cells (P), transitional cells (T) and fibroblast-like cells³⁹⁻⁴⁰. P cells are small and relatively devoid of microfilament skeleton and sarcomeres compared with those of the

surrounding cardiac tissue. P cells were shown to be the origin of automaticity, however, they do not form any junctions with atrial myocytes, instead T cells, located in the transitional tissue at the periphery of the SAN, gradually transmit the AP to the myocardium and internodal conduction pathways (mosaic effect)^{41,42}, although it is to be said that the internodal pathways and the mosaic model are still subject of scientific debate as not all research agrees with this model. Interestingly, the SAN is heavily innervated by parasympathetic and sympathetic neurons, yet some reports did not identify any direct synaptic contact with P or T cells⁴³ (Cardiac innervation is discussed in more detail under section 1.6 *The cardiac innervation*). Recent studies have shown the existence of superior and inferior SAN clusters, located near the superior and inferior vena cava, respectively. Utilising optical mapping it was shown, that during physiological heart rate changes the superior SAN cluster generally controlled higher heart rates and the inferior SAN cluster controlled lower heart rates⁴⁴. This mechanism may also contribute to bradycardia in sinus node dysfunction (SND) through a caudal shift in the leading pacemaker⁴⁵. A synchronized network rhythm of a heterogeneous population of SAN cells is achieved by the principle of entrainment, whereby faster discharging cells are slowed by neighbouring cells that are firing more slowly and vice versa⁴⁶.

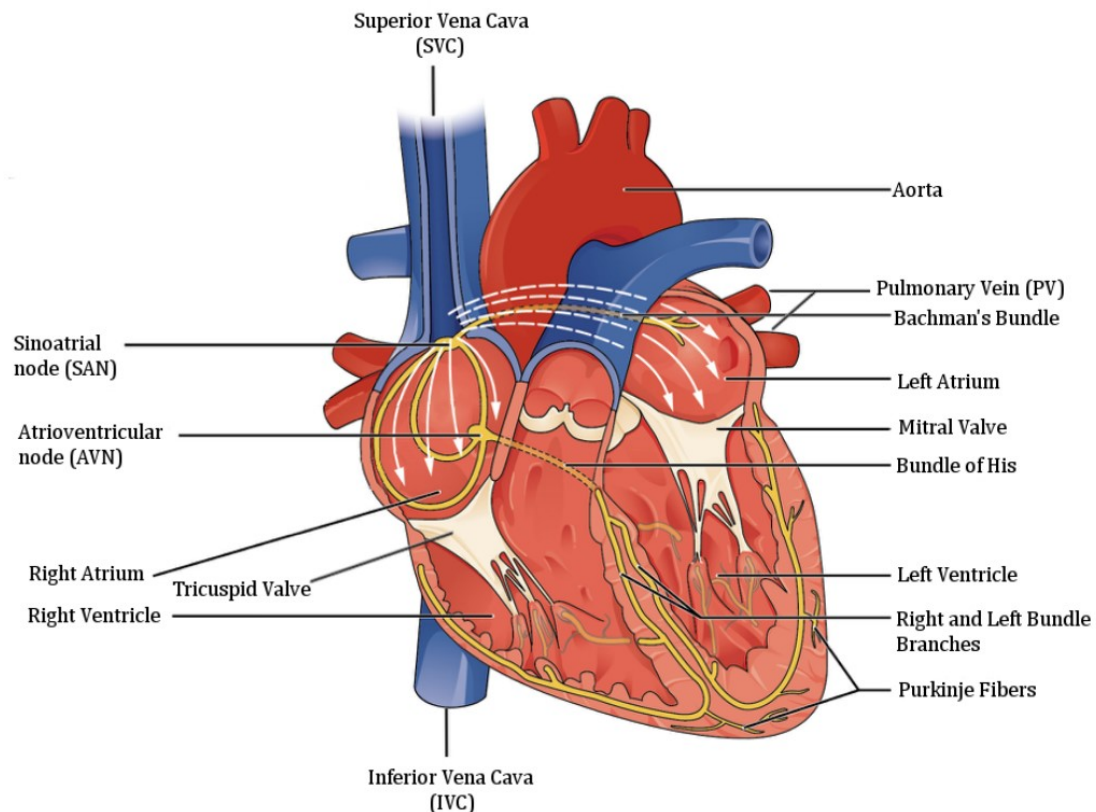


Figure 1.2. A schematic of the heart and CCS. Shown is a visualization of the cardiac anatomy, with the various elements of the CCS (sinoatrial and atrioventricular nodes, bundle of His, right and left bundle branches and Purkinje fibres) being highlighted in yellow. Furthermore, white arrows indicate the movement of the electrical stimulus beginning at the SAN and progressing through the left and right atria (adapted from⁴⁷).

The initiated AP travels from the SAN to the AV node in the atrioventricular septum via a fast-conducting pathway. Consistent with its role as functional delay by virtue of its unique property of decremental conduction, the AV node delays the impulse arriving from the atria to permit ventricular filling before atrial systole^{48,49}. The particular importance of this feature becomes evident in the event of atrial tachyarrhythmias such as atrial flutter or atrial fibrillation, when the AV node plays an important role in limiting the number of APs conducted to the ventricles. Moreover, the AV node is considered to have two zones: compact zone consists of true AV nodal cells and transitional zone which is made up of cells that are intermediate in morphology and function between the compact nodal cells and atrial cells. The transitional zone envelops the compact AV node producing a buffer layer between the compact node and the atrial myocardium⁵⁰. The nodal zone gives rise to a dual physiology consisting of a slow pathway (SP): slow conduction velocity but shorter refractory period, and a fast pathway (FP): fast conduction velocity but longer refractory period, that connect to

each other before entering the bundle of His ⁵¹. Typically, during sinus rhythm the AP is conducted through the fast pathway due its faster conduction rate. This feature of slow and fast pathways has been shown to induce atrioventricular nodal re-entrant tachycardia in some patients^{52,53}. As with the SAN the AV node receives sympathetic and parasympathetic input from the autonomic nervous system, and it might have important physiological and pathophysiological effects on AV node function. That said, the leading role of the AV node is to transmit an AP from the SAN to the ventricles through the His-Purkinje system to initiate ventricular contraction. Additionally, in case of SAN dysfunction (e.g., reduced intrinsic SAN automaticity, sinus arrest or SAN exit block), AV node myocytes, due to their automaticity, can function as a latent pacemaker, albeit at a slower rate as they have an intrinsically slower diastolic depolarization. Clinically, such pathological change of pacemaker location would present itself as bradycardia.

From the AV node the AP moves through HPS which splits into the left and right bundle branches that reach into the ventricles from the apex of the heart via a network of terminal Purkinje fibres. The terminal Purkinje fibres form multicellular bundles in longitudinal strands on the endocardial surface to transmit the atrial AP to the ventricular myocytes and stimulate their simultaneous electrical activation and contraction.

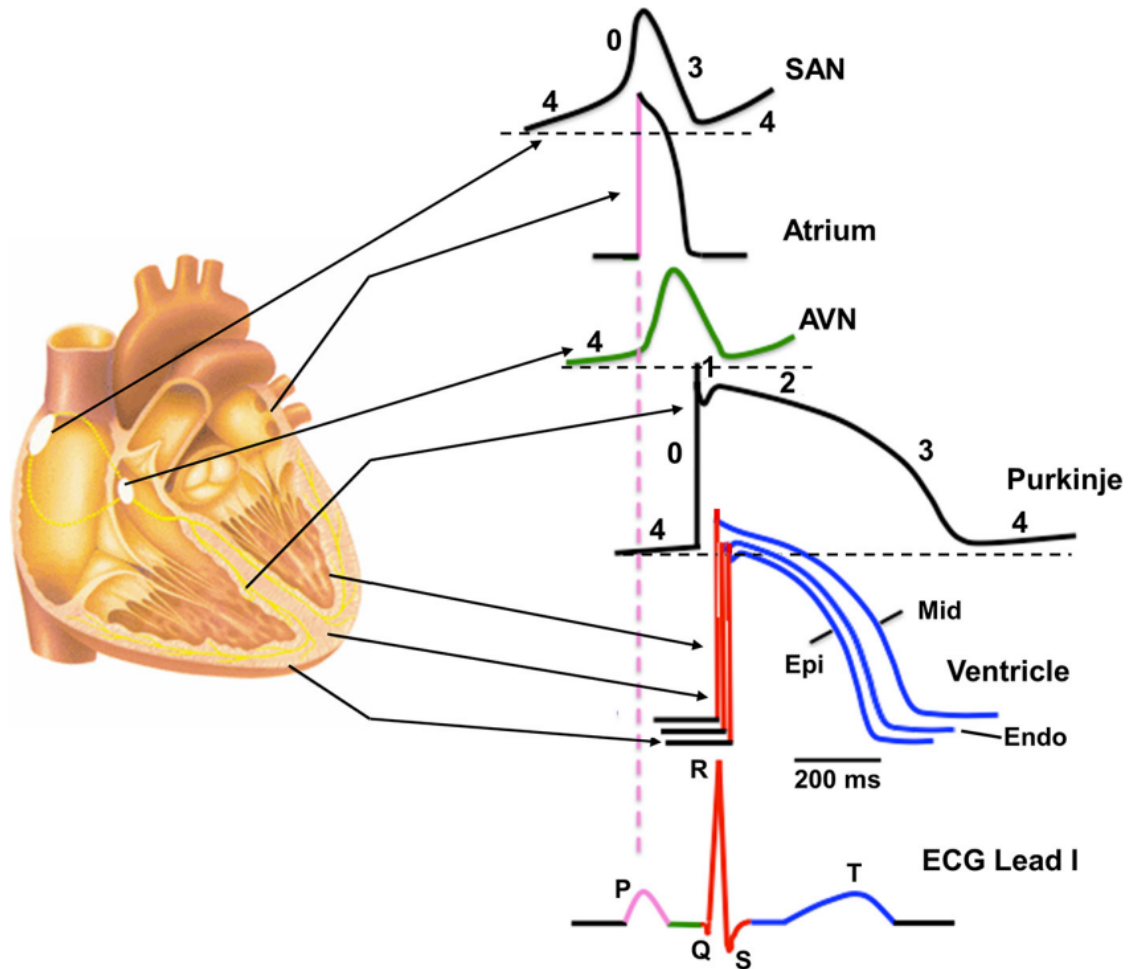


Figure 1.3. Cardiac AP waveform in different cardiac myocyte lineages. The arrows point to an AP waveform generated by a specific region of the heart as indicated in the cross-section through the heart. The ECG lead and AP waveforms are colour-coded for the corresponding sections. Abbreviations: SAN – Sinoatrial node; AV node – Atrioventricular node; Epi – epicardial; Endo – Endocardial; Mid – Middle layer; ECG – electrocardiogram. (Diagram was copied from⁵⁴).

1.1.5 The Cardiac Action Potential

All cells including cardiac myocytes are enclosed in a semipermeable plasma membrane (sarcolemma), which harbours the essential machinery such as active ion pumps and transporters to establish an unequal ion distribution along the membrane resulting in an electrolyte gradient between the inside of the cell and the extracellular fluid^{7,55}. The differential electrolyte concentration on either side of the cell membrane, produce an intracellular potential called resting membrane potential. The flow of ions down the concentration gradient, when ion channels are opening produces a localized electrical change across the cell membrane which is the underlying principle of the cardiac AP.

The AP is determined by the opening and closing of various, spatially defined, complex transmembrane proteins, which consist of ion-selective channels such as sodium or potassium ion channels, transporters such as sodium/potassium ATPase (Na^+/K^+ ATPase) and exchangers such as NCX. These proteins may be formed by several subunits and can have accessory proteins. Particularly the latter influence a range of channel properties including trafficking of the pore forming subunit(s) to the membrane as in case of sodium channels. Additionally, the permeation of specific ions through conducting ion channels can be influenced by ligand binding, second messengers such as cAMP and post-translational modifications such as phosphorylation^{56,57}.

An AP can be structured into five subsequent phases reflecting the major ionic movement across the membrane: the resting membrane potential of about -85mV, which is maintained by a slow outward current of potassium ions through potassium channels I_{K1} and I_{KACh} , represents phase 4. It is followed by phase 0, which comprises a rapid depolarization (rapid upstroke) of the membrane, mainly caused by an inward depolarizing sodium current, I_{Na} , through the activation of fast voltage-gated sodium channels (Nav). During this, Nav channels open in response to an electrical current from an adjacent myocyte through gap junctions, resulting in a rapid, positive change in voltage across the cell membrane. Activation of fast transient voltage-gated outward potassium channels, I_{to} , I_f , and inactivation of Nav channels produce an early repolarization phase or phase 1. During phase 2 a current plateau is reached as the membrane potential remains largely constant due to the activation of LTCC generating a depolarizing inward calcium current, I_{CaI} , and the voltage-gated potassium channels causing repolarizing outward current (ultra-rapidly (I_{Kur}), rapidly (I_{Kr}), and slowly (I_{Ks})).

As the membrane potential declines due to inactivation of voltage gated calcium channels, phase 3 is initiated and inward rectifying potassium currents are activated, resulting in a complete repolarization^{58,59,60}.

The depolarizing AP that travels across the membrane ultimately causes voltage-gated calcium channels to open and calcium ions to enter the cell during the plateau phase. The initial influx of calcium induces the opening of ryanodine receptors (RyRs) located on the surface of the sarcoplasmic reticulum (SR)^{61,62}. The SR functions as an intracellular calcium

store, and the opening of RyRs greatly increases cytosolic calcium concentration, a process termed calcium-induced-calcium-release (CICR). Free calcium, as part of the ECC, can activate sarcomeres to produce contractile force and the amount of calcium released is directly proportional to the contractile force that the heart muscle generates⁶³. Physiologically, this corresponds with parameters such as stroke volume, ejection fraction, and cardiac output. The efficient delivery of calcium to sarcomeres deep inside the cell is enabled by the aforementioned T-tubules. These are arranged in proximity with the terminal cisternae of the SR, together forming the so-called dyads. At the end of each cycle, during diastole, calcium is removed from the cytosol back into the SR by the action of SERCA2 (sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase 2a) pump and to a minor extent via the sarcolemmal Ca^{2+} -ATPase and mitochondrial Ca^{2+} -uniporter⁶². The sodium-potassium ATPase and NCX located in the sarcoplasm outside of T-tubules, restore the cardiomyocyte membrane potential so the cycle can repeat with the next incoming depolarization⁶².

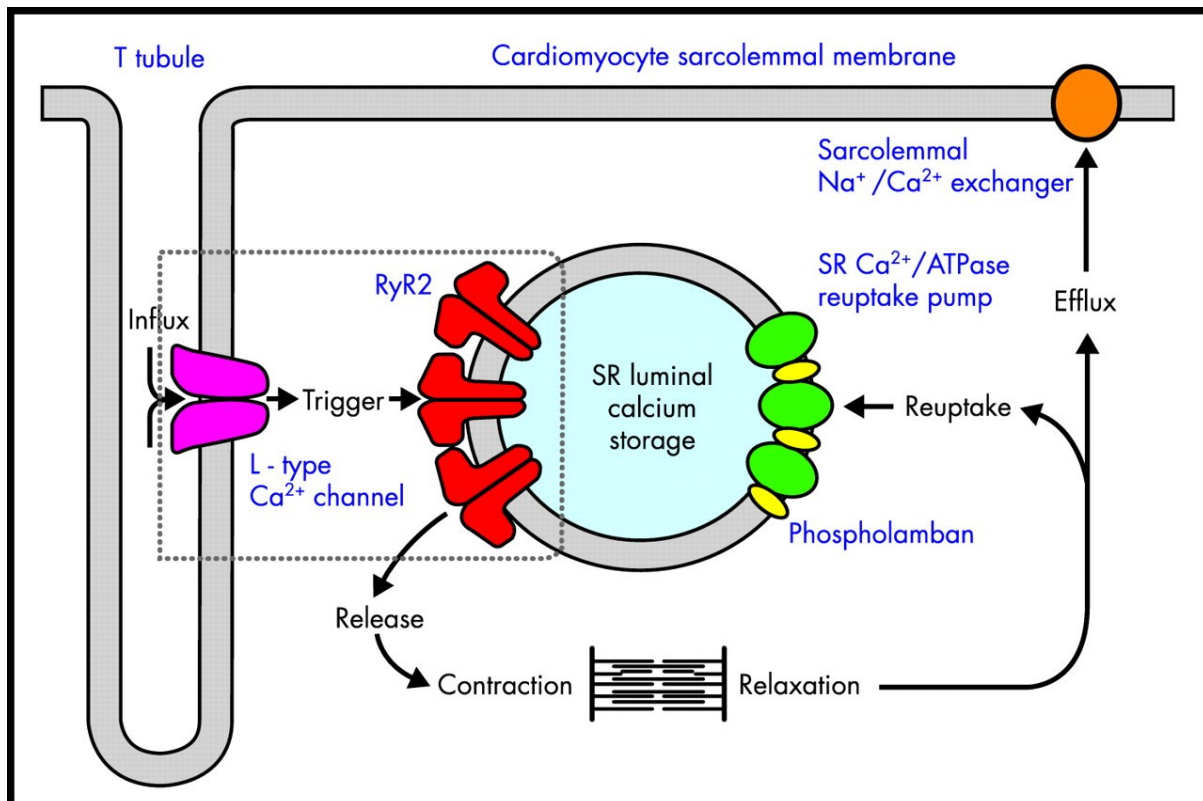


Figure 1.4. The excitation-contraction coupling. Ca^{2+} enters the cell via the activated L-type calcium channel (LTCC). This Ca^{2+} induces a conformational change in the ryanodine receptor (RyR) resulting in the release of Ca^{2+} from the sarcoplasmic reticulum (SR). Release Ca^{2+} interacts with contractile proteins to mediate cellular contraction. Relaxation of the cell occurs through the removal of Ca^{2+} from the cell via sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) or sodium–calcium exchanger (NCX) (Diagram was taken from⁶⁴).

1.1.6 The Cardiac Pacemaker Potential

Within the heart different regions of the myocardium such as ventricular and atrial cardiomyocytes and pacemaker cells of the SAN and AV node, produce different AP waveforms (Figure 1.3). For example, the plateau phase in the atrial myocardium is less prominent than in ventricular cardiomyocytes, where it is strongest. In pacemaker cells phase 0 is slower than in the working myocardium, and the transient repolarisation (phase 1) and the plateau phase are completely missing. However, despite the differences in the AP morphology between the atrial and ventricular myocytes, they are more like each other than to the AP morphology and the underlying ionic movement of pacemaker cells. In fact, cells of the SAN and AV node don't have a resting membrane potential, but a pacemaker [membrane] potential which is a slow depolarisation of the membrane potential by virtue of the pacemaker current or funny (I_f) current⁶⁵. I_f is generated by an inward flow of sodium and potassium ions through the hyperpolarization-activated cyclic-nucleotide gated (HCN) ion

channels^{65,66}. These channels are exclusive to pacemaker cells and absent in the cells of the working myocardium. HCN channels are also regulated by cAMP signalling and so they are an important pathway for the heart rate regulation by the autonomic nervous system⁶⁷ (a detailed discussion of HCN channels as cAMP effectors can be found in section 1.2.4.3).

HCN channels are activated early in phase 4 (diastole) when the cell is hyperpolarised, to drive the membrane potential of the pacemaker cell to a more positive diastolic potential. This, in turn, leads to an influx of calcium through the, now activated, transient voltage-gated calcium channels, continuing diastolic depolarisation. Additionally, by the mechanism of CICR a larger volume of calcium is released from the SR that produces local, disorganised calcium sparks. The relatively higher intracellular calcium concentration activates further action of the NCX, which exchanges one calcium for three sodium ions, producing additional net positive charge influx⁶².

Once the diastolic depolarisation causes the triggering threshold potential to be reached, voltage gated LTCC channels open, activating a largescale inward calcium current. Simultaneously, the outward potassium current (I_K) is deactivated which together permit the upstroke of phase 0 of the AP in pacemaker cells. Interestingly, the LTCC are activated more slowly in pacemaker cells if compared to the sodium channels in atrial and ventricular myocytes, producing a depolarization slope less steep than in the working myocardium. Phase 0 is followed by phase 3, during which delayed rectifier potassium channels (K_V) allow a slow repolarisation of the cell⁶⁸.

Therefore, the spontaneous AP potential produced by the pacemaker potential in pacemaker cells is reliant on the rhythmic cycling of calcium through the membrane bound ion channels and transporters, the membrane (M) clock, and the intracellular calcium handling mechanism, the calcium clock, which are functionally connected through the movement of calcium. Traditionally, both clocks were thought to be spatially and functionally separated, however, recent findings have shown that pacemaker cells are unable to generate a spontaneous action potential when the two clocks become experimentally uncoupled. Thus, the proper timing of membrane and intracellular events is of exquisite importance in order to produce a coupled-

clock system, which is required to drive automaticity of SAN pacemaker cells and to generate a stable heart rhythm (intrinsic entrainment)^{69–71}.

It is also noteworthy that in a recent study Unudurthi and colleagues have discovered that the two-pore potassium channel TWIK-related K⁺ channel (TREK-1) is highly expressed in the SAN. There it was shown to impact the diastolic depolarisation, spontaneous firing, and repolarization of the pacemaker AP. Thereby, it may play a role in cardiac pacemaking by regulating SAN excitability⁷².

It is evident from the processes described above that generating a heart rhythm is dependent on a harmonized flow of ionic currents through various ion channels that are embedded in the sarcolemma and cellular organelles. Alterations in their physiological function through acquired or genetic factors, collectively called channelopathies, can cause electrical heterogeneity and lead to cardiac arrhythmogenic disorders such as Brugada syndrome, long/short QT syndrome and sick sinus syndrome⁷³.

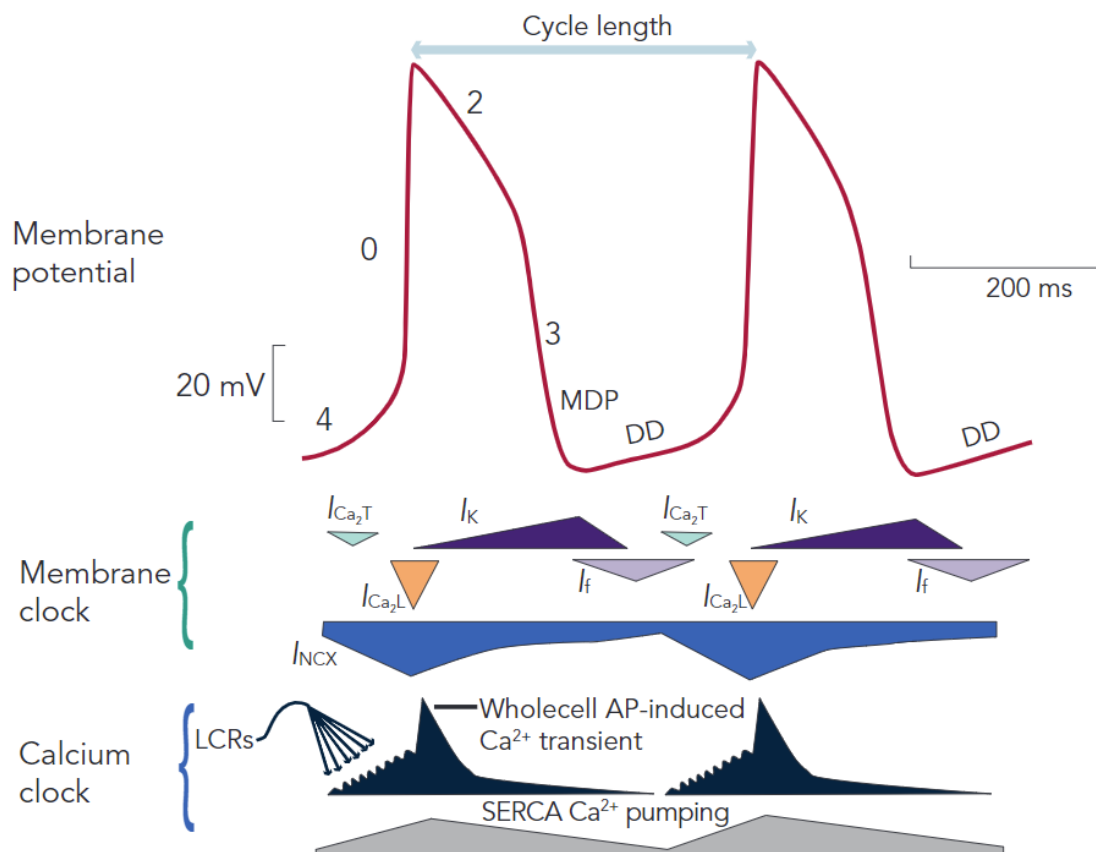


Figure 1.5. Sinoatrial node action potential and the corresponding timing of membrane clock and calcium clock components. SAN AP waveform and its individual phases (phase 4,0,2,3) are shown in red. Diastolic depolarization (DD) takes place during phase 4. MDP – maximum diastolic potential; DD – diastolic depolarisation; I_{Ca2T} – T-type voltage-dependent Ca^{2+} current; I_{Ca2L} – L-type voltage-dependent Ca^{2+} current; I_{NCX} – sodium-calcium exchange current; I_{K2} – delayed rectifier potassium current; I_f – funny current; SERCA – sarco-endoplasmic reticulum ATPase; LCRs – local Ca^{2+} releases (diagram was taken from⁷⁴).

1.1.7 The Cardiac Innervation

As discussed above the intrinsic heart rate is established by the SAN, however, the ability of the heart to adjust to physiological demands is achieved through innervation by the sympathetic (accelerants nerve) and the parasympathetic (vagus nerve) branches of the autonomic nervous system. In a simplistic description one could say, the sympathetic nervous system (SNS) drives physiological responses to stress (emotional, physical or pharmacological) and the parasympathetic nervous system (PSN) promotes homeostasis at rest. Three levels of control by the cardiac autonomic system can be described⁷⁵. Distally located are the medulla oblongata and the spinal cord and peripherally, cardiac activity is influenced by the intrathoracic extracardiac ganglia as well as the intrinsic cardiac nervous system (iCNS)⁷⁶. Cardiac function is modulated by the iCNS in a beat-to-beat fashion, and regulation persists

even in the absence of any influence by the higher levels. The iCNS is mainly composed of cardiac ganglia that play a significant role in the integration of cardiac electrophysiology⁷⁶. These ganglia reside in specific regions of the heart, mostly in the atria and particularly surrounding the sinus node, distribute subepicardially, and contain post-ganglionic sympathetic connections coming from the accelerans nerve and pre-ganglionic parasympathetic connections from the vagus nerve^{76,77}. The elements of the CCS, and particularly the SAN, are innervated extensively by the SNS and the PNS. The myocardium of the atria and the ventricles also receive innervation, albeit at a much lesser extent. The postganglionic fibres of the SNS synapse directly with the heart muscle and release norepinephrine (NE) as a primary neurotransmitter. NE acts on β -adrenergic receptors (β AR) to produce a positive chronotropic (increased heart rate), dromotropic (conduction velocity), inotropic (myocardial contraction) and lusitropic (relaxation) effects. The parasympathetic input is via the vagus nerve which forms synapses with postganglionic cells in the SAN and AV node. When stimulated, acetylcholine (ACh) is released to bind to muscarinic receptor 2 (M2) on nodal cells to slow conduction, decrease the contractility in the atria and ultimately reduce the heart rate. Therefore, to mediate the heart rate as necessary for a given physiological function, sympathetic and parasympathetic mechanisms work together to modulate the activity of many proteins associated with the membrane and calcium clock (neuronal entrainment). Disruption of this intricate molecular balance by myocardial disease and aging can induce lethal arrhythmias. Interestingly, the sympathetic innervation is not distributed evenly across the ventricle. The basal myocardium displays a higher density of innervation whereas the apical myocardium was shown to have a higher density of β ARs and obtaining sympathetic input from circulating plasma⁷⁸.

1.2 Cyclic nucleotide signalling

1.2.1 Second messenger signalling and cAMP

At any given time, cells receive a plethora of extracellular stimuli that must be transduced across the cell membrane in a controlled manner to initiate an appropriate and desired response. This signal transduction is generally based on an interaction between an extracellular ligand such as a neurotransmitter, small molecule or a hormone and a membrane bound receptor. Upon binding of the extracellular ligand, the receptor undergoes conformational change that initiates a signalling cascade to amplify the signal.

Part of such a cascade is a rapid, ubiquitous production of a second messenger that can easily diffuse through the intracellular space and relay this new information to a downstream pool of effector proteins, but which can also be restricted in a spatio-temporal manner to allow for a compartmentalised signal transduction that is both, effective and transient. The rapid rise of the second messenger is followed by its rapid decline via expulsion from the cell, reuptake into a cellular store or enzymatic degradation⁷⁹. There is several different second messengers that are active at different functional locations. For example, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) signal within lipid membranes, ions such as calcium signal within and between cellular compartments, and cyclic nucleotides signal within the cytosol. Of the cyclic nucleotides, there are two main forms: cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP)⁷⁹.

cAMP, first discovered by Earl W. Sutherland in 1958, is one of the most important second messengers found in eukaryotic cells that mediates a plethora of cellular responses. In the heart, the generation of cAMP is largely governed by β AR signalling which in itself is part of the sympathetically driven fight-or-flight response. Dysregulation of this signalling pathway has been described to give rise to many cardiac diseases. The classic cAMP signalling pathway involves activation of G-protein coupled receptor (GPCRs) such as β -adrenergic receptors (β AR) by ligand binding, G-proteins, which activate or inhibit adenylyl cyclases (AC), which convert adenosine triphosphate (ATP) into cAMP. The synthesis of cAMP results in a signal amplification and activates cAMP effector proteins, while phosphodiesterases (PDEs) ensure

cAMP degradation. All elements of this signalling cascade will be described and discussed throughout the rest of the chapter.

1.2.2 β -adrenergic receptors and their associated G-proteins

β ARs are part of the sympatho-adrenal signalling that transduce modifications of cellular physiology through the means of catecholamines. In the heart, β AR stimulation by catecholamines serves as the most powerful regulatory mechanism to enhance myocardial performance (heart rate, contractility, relaxation and speed of conduction) in response to stress or exercise by activating the classic cAMP signalling pathway.

Three β AR subtypes can be distinguished: β_1 -, β_2 -, and β_3 AR⁸⁰. They belong to the GPCR superfamily, currently consisting of 800 known receptors that occupy approximately 4% of the total protein coding genome. They have a widespread expression pattern and a huge range of physiological roles. GPCRs are sub-divided into 6 classes on the basis of their sequence homology, whereby β ARs are members of the Class A aka the rhodopsin-like class⁸¹. The general structure of GPCRs consists of a distinct extracellular N-terminal region which serves as the ligand binding site, seven trans-membrane domains which anchor the protein to the membrane and a cytosolic C-terminus which is involved in G-protein binding (cytosolic loop3, CL3) and signal specificity (cytosolic loop2, CL2)^{82,83,84}. CL2 is highly conserved between β_1 AR and β_2 AR but not with rhodopsin, which suggests that CL2 may be important for the function of β ARs⁸². β_1 AR and β_2 AR's trans-membrane regions show 67% of sequence homology, and the ligand binding regions seem also to be quite similar. This therefore suggests that the basis of the two sub-types vastly different binding of agonists and functional differences must come from regions outside of the binding and membrane spanning regions. Additionally, it has been suggested that binding affinity of β_1 AR and β_2 AR to different agonists may be the sum of very small differences within related structures⁸².

GPCRs bind an array of heterotrimeric G-proteins formed by α , β , γ subunits. There are about 21 types of different G-proteins that are defined by differences in their α subunits with the four main G_α subunits being: $G_{\alpha s}$, $G_{\alpha i}$, $G_{q/11}$ and $G_{12/13}$ ^{85,86}. $G_{\alpha s}$ and $G_{\alpha i}$ are generally referred to as G_s and G_i (Table 1.1).

Table 1.1. G-protein coupled receptor types

GPCR type	Effector pathway	Example
G _s - linked	increases cAMP levels	Beta-adrenoceptors
G _i – linked	decreases cAMP levels	Muscarinic receptors
G _{q/11} - linked	activates phospholipase C (IP3)	Alpha-adrenoceptors

Under physiological conditions, the G-protein that is associated with a β AR is activated through a conformational change within the receptor upon ligand binding. This causes the $G\alpha$ -subunit to release its attached guanosine diphosphate (GDP) and exchange it for a cytosolically much more abundant guanosine triphosphate (GTP). That leads to a dissociation of the $G\alpha$ subunit from the receptor and $G\beta\gamma$, allowing $G\alpha$ to bind to its target AC. The secondary effects of the $G\beta\gamma$ are not integral to the β AR signalling and is more influential in alternative signalling pathways. $G\alpha_s$ activates AC to catalyse the synthesis of cAMP from ATP, and $G\alpha_i$ has the contrary effect. Upon binding of AC, the GTP within $G\alpha$ is hydrolysed and $G\alpha$ is returning it to its basal conformation permitting the re-association with a free $G\beta\gamma$ and a GPCR.

The β_1 AR and β_2 AR sub-types predominantly bind to G_s and therefore stimulate an increase in cardiac contractility and heart rate, although studies have shown that β_2 AR does this sub-maximally in comparison to β_1 AR⁸⁷. This is due to a restricted ability of this receptor with respect to phosphorylation targets. Importantly, in response to chronic stimulation, the β_2 AR can also binds G_i and induce alternative downstream effects.

1.2.2.1 β -adrenergic receptor desensitisation

In favour of preservation of metabolic energy and to avoid potentially harmful effects of an excessive cell stimulation, once the signal amplification has been achieved, β AR is inactivated by heterologous and homologous desensitisation. Heterologous desensitisation is a non-agonist specific negative feedback loop that occurs through receptor phosphorylation by protein kinase A (PKA) which is activated by cAMP. Phosphorylation directly alters receptor conformation so that the interaction with a G-protein is impaired. Homologous desensitisation is accomplished by a conserved two-step mechanism: receptor

phosphorylation by GPCR kinases (GRKs) followed by β -arrestin binding⁸⁸. Put simply, free G $\beta\gamma$ subunit and a membrane phosphatidylinositol bisphosphate (PIP2) bind to the C-terminal pleckstrin homology domain of the GRK which seems to result in its translocation to the activated, membrane bound receptor. There, GRK interacts with and phosphorylates the receptor which increases its affinity for β -arrestin 10-30-fold. Binding of β -arrestin sterically interdicts signalling to the G protein as both engage the same inter-helical cavity in the cytoplasmic side of the receptor. Existence of and preferential affinity for different G $\beta\gamma$ subunit isoforms by GRK may present a basis for specificity in GRK-receptor interaction^{88,89}. Additionally, β -arrestins, due to their ability to interact with clathrin and its adaptor protein AP2, target the attached receptor to the clathrin-coated pits and lead to GPCR internalisation, further reducing cell responsiveness to β AR agonists. Internalised receptors can either be recycled back to the plasma membrane or degraded in lysosomes. β -arrestins also contribute to G-protein independent signalling (for example by activating the ERK-1/2 pathway)⁹⁰.

1.2.2.2 Downstream effects of β_1 AR and β_2 AR signalling

Interestingly, both β AR subtypes, initiate a rapid rise in cAMP levels, however the resulting downstream cellular responses are quite different. It has been shown that sustained β_1 AR activation is cardiotoxic and promotes apoptosis due to aberrant Ca^{2+} handling and β_2 AR has a cardio-protective character⁹¹. Nikolaev and colleagues argue that the difference in the observed physiological responses is due to a differential distribution of β_1 AR and β_2 AR receptors in cardiac myocytes. By combining scanning ion conductance microscopy (SICM) with Förster resonance energy transfer (FRET), they were able to show that a propagating cAMP signal across the entire cells was produced by β_1 ARs, whereby β_2 AR stimulation produced cAMP signalling in close proximity to the receptors and ion channels within the T-tubules which they are localised to^{92,93}. This may also explain the strict control of LTCC, but not other phosphorylation targets by β_2 AR, as LTCC are strictly localised to T-tubules as well⁹⁴. Notably, β_2 AR localisation is dependent on lipid rafts/caveolae as cholesterol depletion by methyl- β -cyclodextrin (M β CD) leads to redistribution of these receptors as observed in heart failure⁹².

Additionally, the difference in the pathological characteristics of the two receptor subtypes have been attributed to the ability of the β_2 AR to bind to Gs and Gi whereas β_1 AR exclusively binds to Gs proteins⁹⁵. β_2 AR coupling of Gi has an antagonistic effect on cAMP signalling as the activity of Gi recruits PDE4 (which degrades cAMP) to the β_2 AR complex causing a reduction in β_2 AR -Gs signalling. β_2 AR-Gi also appears to interact with and thereby enhance the inotropic effect of NCX in cardiomyocytes when NCX was selectively overexpressed⁹⁶.

However, arguably more significantly, $\beta\gamma$ subunit of Gi is able to control apoptosis by downstream activation of a novel set of proteins such as mitogen-activated protein kinase (MAPK) which signalling pathway can promote cell survival⁹⁷⁻⁹⁹. MAPKs are a well-studied family of proteins that play an integral role in signal transduction and regulation of events during cardiac development, physiological adaptation and pathological manifestation. Four primary MAPK subfamilies can be named: extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinases (JNK1, -2 and -3), p38 kinase and big MAPK (BMK or ERK5). MAPK signalling occurs via a well-conserved three-tiered kinase cascade in which a MAPK kinase kinase activates a MAPK kinase which in turn activates the MAPK through serial phosphorylation whereby specificity and efficiency is determined by an array of specific docking and binding partners. In case of β_2 AR-Gi, Daaka et al. have shown that MAPK is stimulated through the non-receptor tyrosine kinase c-Src and the G-protein Ras pathway upon heterologous receptor desensitisation¹⁰⁰. Application of pertussis toxin, which prevents Gi subunit dissociation, inhibited Gi-mediated MAPK signalling. The protective properties of activated MAPK signalling can be ascribed to its ability to actively oppose Gs activation and to prevent phospholamban (PLB) phosphorylation by PKA¹⁰¹. Phosphorylation of PLB increases SERCA activity and therefore the rate at which Ca^{2+} is loaded into the SR. Reduced SR loading translates to reduced cardiomyocyte contractility, which as a result, may provide a protective mechanism upon sympathetic overstimulation. Additionally, β_2 AR coupling to Gi is able to offset the toxicity produced through sustained β_1 AR signalling via ERK (and AKT) phosphorylation^{97,102}. The activation of p38 MAPK is shown to be negatively inotropic in adult cardiomyocytes, conversely, inhibition of p38 MAPK with SB203580 causes a strong but reversible increase in cardiomyocyte inotropy¹⁰³.

1.2.3 Adenylyl cyclases

10 AC isoforms are known, of which nine are membrane bound (AC1–AC9) and one that is soluble (sAC)¹⁰⁴. Five isoforms, AC1, AC5, AC6, AC8, and AC9, are mainly found in different regions of the heart. In cardiac ventricular myocytes AC5 and AC6 are recognised as the main isoforms. These isoforms appear to have approximately 65% protein sequence homology¹⁰⁵. Both proteins are activated through similar regulatory pathways and are inhibited by Ca^{2+} , but are divergent in their subcellular localization and function, i.e. AC5 is coupled to both $\beta_1\text{AR}$ and $\beta_2\text{AR}$ and is exclusively present in T-tubules only, whereas AC6 is coupled to $\beta_1\text{AR}$ in the plasma membrane outside of T-tubules¹⁰⁵.

AC5 and AC6 are also expressed at high levels alongside AC1 and AC8 in the SAN of the rabbit and guinea pig^{106,107}. Interestingly, AC8 is a neuronal-type AC that drives cAMP-PKA- Ca^{2+} signalling as part of the SAN automaticity¹⁰⁸. Both AC1 and AC8 are stimulated by Ca^{2+} through calmodulin and inhibited by $\text{G}\beta\gamma$ subunits¹⁰⁹. Transgenic mice overexpressing AC8 show an increased heart rate and a reduced heart rate variability¹¹⁰. Null mutants for either *Adcy1* or *Adcy8* display a normal heart rate, which may be due to a functional redundancy of these two isoforms¹⁰⁸. Studies with transgenic mice have shown that AC5 is largely associated with stress responses and AC6 is necessary for Ca^{2+} handling and contractility¹⁰⁴. In the SAN AC5, AC6 and AC8 are expressed in membrane rafts, where they respond to local changes in Ca^{2+} .

In the heart AC9, a unique isoform, which has a low sensitivity to the small molecule AC activator forskolin (FSK), forms a complex with the A kinase anchor protein (AKAP) Yotatio, potassium voltage-gated channel subfamily Q member 1 (KCNQ1), and protein phosphatase 1 (PP1)^{109,111}. AC9 is essential for cardiac pacemaking as null mutants for *Adcy9* present sinus bradycardia and a diastolic dysfunction with preserved ejection fraction¹¹¹.

1.2.4 cAMP effectors

A single activated βAR can activate many Gs proteins, that each can activate an AC to initiate a large-scale cAMP synthesis, that as a result greatly amplifies the impact of a single ligand. cAMP can activate a wide range of cellular processes through specific interaction with its effector proteins that contain a cyclic nucleotide-binding domain (CNBD). There are four

cAMP effectors, namely, PKA, exchange protein activated by cyclic AMP (EPAC1 and -2), cyclic nucleotide-gated ion channels (CNG), and POPDC proteins.

1.2.4.1 PKA

PKA, which is activated as part of a stress response, is the first effector protein that was discovered and is currently also the best characterised. PKA exists as a tetrameric enzyme consisting of two regulatory subunits, of which there are two classes: RI and RII, and two catalytic subunits of which there are three isoforms: C α , C β , and C γ , each encoded by a unique gene. RI is mainly found in the cytosol and RII in the particulate fraction. R α subunits are ubiquitously expressed; however, RI β and RII β are found mainly in the brain, and RII β is also found in fat, reproductive and endocrine tissues. The interaction between the R and C subunits has an inhibitory effect on the catalytic activity of PKA. Early in vitro experiments suggested that binding of cAMP to the R subunit initiates a conformational change that results in their dissociation from C subunits which allows for the activation of the catalytic activity of the enzyme^{112–116,117}. However, a more recent study by Isensee et al,¹¹⁸ suggested that under physiological conditions binding of cAMP induces an isoform-specific conformational change that instead merely opens the holoenzyme as even engineered fusion proteins of R- and C-subunits were able to reconstitute PKA functionality in cells. Therefore, at least for PKA-II, the model of full dissociation may not completely reflect the reality in cells. It remains to be clarified whether endogenous PKA-II is activated by full separation or partial dissociation only involving a conformational change leading to a more open conformation.

PKA is targeted to various subcellular domains by scaffolding proteins called AKAPs¹¹⁹. AKAPs are a family of structurally unrelated proteins that are unified by the presence of a 14-18 amino acid-long protein kinase-binding domain which interacts with the N-terminal D/D domain of the regulatory subunit of a PKA dimer via a conserved α -helix¹²⁰. In addition to PKA targeting, AKAPs also play a crucial role as protein hubs that allow cAMP signalling and other signalling complexes to form¹²¹. In the heart AKAPs can be grouped by the cardiovascular processes that they regulate, i.e. AKAP18 α , AKAP18 γ , AKAP79 are important in CICR; Yotatio and D-AKAP2 are important for repolarisation; AKAP-Lbc, mAKAP β , D-AKAP1 are important for stress response¹²².

In the heart, β AR induced activation of PKA is of great importance during ECC as it phosphorylates many key proteins that modulate the movement of Ca^{2+} in cardiac myocytes. Phosphorylation of 1) Rad G-Protein lifts its inhibitory function on LTCC which enhances their conductivity and therefore Ca^{2+} influx upon depolarisation. Phosphorylation of 2) phospholamban (PLB) increases SERCA activity and therefore the rate at which Ca^{2+} is loaded onto the SR (PLB in an unphosphorylated state inhibits SERCA2A activity). Phosphorylation of 3) RyR2 subunit calstabin2 leads to the dissociation of calstabin2 from the RyR2 complex, increasing its calcium sensitivity and in consequence increasing the amplitude of CICR which can lead to pro-arrhythmogenic SR leaks. Phosphorylation of 4) phospholemman removes its inhibitory effect on the Na^+/K^+ transporter, to enhance its responsiveness to depolarization. Phosphorylation of 5) myofilament proteins by PKA decreases their calcium sensitivity and enhances lusitropy as dissociation occurs more rapidly. Phosphorylation of 6) slow delayed rectified K^+ channel, can accelerate their activation kinetics and stabilize their open state^{123–129}. The combined action of these phosphorylated proteins can lead to numerous positive inotropic and lusitropic effects to help increased heart function in response to new physiological demands.

1.2.4.2 EPAC

A more recently identified cAMP effector protein is the exchange factor directly activated by cAMP – EPAC. There are two EPAC isoforms, which has one (EPAC1) or two cAMP-binding domains (EPAC2), which are homologous to those of the PKA R subunit¹³⁰. In mammals, both isoforms are the product of two independent genes that show extensive sequence homology and share the evolutionary conserved catalytic domain but have distinct N-terminal regions. In cardiac myocytes, both EPAC isoforms are expressed albeit at different subcellular localizations: EPAC1 is predominantly localized perinuclearly and EPAC2 is mostly present at the t-tubules¹³¹. EPAC1 was also shown to be present in mitochondria and its loss is associated with a reduced infarct size as well as reduced cardiomyocyte apoptosis following myocardial ischemia/reperfusion injury¹³². EPAC principally function as guanine nucleotide exchange factors through their GEF domain to stimulate Ras-like GTPases, Rap1 and Rap2, forming a cAMP response system alternative to that mediated by PKA. Nevertheless, PKA and EPAC2 show overlapping areas of regulation and synergistic effects as both can activate for example

the calmodulin-dependent protein kinase II (CaMKII). CaMKII can phosphorylate RyRs and PLB inducing pro-arrhythmic SR leaks¹³³. EPAC was also shown to have pro-arrhythmic effects by decreasing potassium currents in cardiac myocytes¹³⁴. Additionally, chronic EPAC activation by the specific EPAC agonist 8-CPT-cAMP triggered cardiac hypertrophy due to the activation of NFAT/calcineurin signalling¹³⁵.

1.2.4.3 HCN channels

In the adult heart, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are predominantly expressed in the SAN and the AV node where three isoforms (HCN1, HCN2, and HCN4) of the four HCN channels are expressed¹³⁶. These channels uniquely open at hyperpolarised membrane potential, although different HCN channels can vary in their characteristics. For example, HCN4 channels, compared to HCN1 channels, show the slowest activation and deactivation kinetics, and open at more negative potentials. Their gating is also facilitated by their ability to bind cAMP within the C-terminal CNBD with HCN4 showing the highest cAMP affinity whilst HCN1 shows only low cAMP sensitivity¹³⁷. As previously discussed, HCN4 channels, the main isoform present in the SAN, play a crucial role in the automaticity of the SAN through the generation of a slow diastolic depolarization during Phase 4 of the cardiac AP. The described characteristics of HCN4 channels allowed them to be put forward as the main effector of ANS-mediated chronotropic effect. HCN4-channel gating is affected by changes in cytosolic cAMP levels, which in turn are tightly controlled by the ANS: activation of the sympathetic nervous system leads to an increase in cAMP levels, increasing the I_f current, which results in accelerated slope of the slow diastolic depolarisation (SDD), producing a higher firing rate of SAN cells, and ultimately increasing the heart rate (HR). Conversely, activation of the parasympathetic nervous system reduces cAMP levels, which leads to a slowdown in HR.

A body of work utilising mouse knockout models and patient data produced results in support of this hypothesis. For instance, studies by Baruscotti et al. and Freske et al. revealed that null mutants for *Hcn1* and *Hcn4* show defective cardiac pacemaking^{138,139}. *Hcn1* null mutants present postnatal sinus bradycardia and *Hcn4* null mutants show embryonic lethality due to severe bradycardia. Additionally, reduced heart failure in heart failure patients was observed

after the application of ivabradine, which antagonizes I_f by binding to HCN channels in their closed state¹⁴⁰. Nevertheless, other work does not support this conclusion. For example, Hennis et al. revealed that HCN4-channel gating kinetics are too slow to mediate ANS-triggered changes in the firing rate of pacemaker cells and the successive change in sinus rhythm¹⁴¹. Kozasa et al. showed that overexpression of Hcn4 does not induce tachycardia, however, conditional knockdown of *Hcn4* results in bradycardia, sinus arrhythmia, and enhanced parasympathetic responses to cervical vagus nerve stimulation¹⁴². Moreover, Alig et al. were able to prove that adult mice with a SAN-specific loss of Hcn4 retain their ability to respond to sympathetic stimulation, despite progressively developing bradycardia and AV block¹⁴³. Based on these experiments, a new hypothesis has been put forward suggesting that instead of mediating the chronotropic response of the SAN, HCN4 channels' main role is to act as a depolarization reserve, which is important for attenuating parasympathetic responses by stabilizing spontaneous firing of the SAN¹⁴⁴.

HCN1 channels, on the other hand, are important during the phasic entrainment process. They suppress heart rate fluctuations by stabilizing the membrane potential in the subthreshold diastolic voltage range and thereby attenuate any interfering influences from neighbouring pacemaker and atrial cells during AP firing^{145,146}. It can be concluded that even though HCN1 and HCN4 are probably not essential for heart rate adaptation after adrenergic receptor stimulation, they are important in ensuring the robustness of cardiac pacemaking.

1.2.4.4 POPDC Proteins

POPDC are the newest class of cAMP effectors and will be discussed extensively in section 1.3.

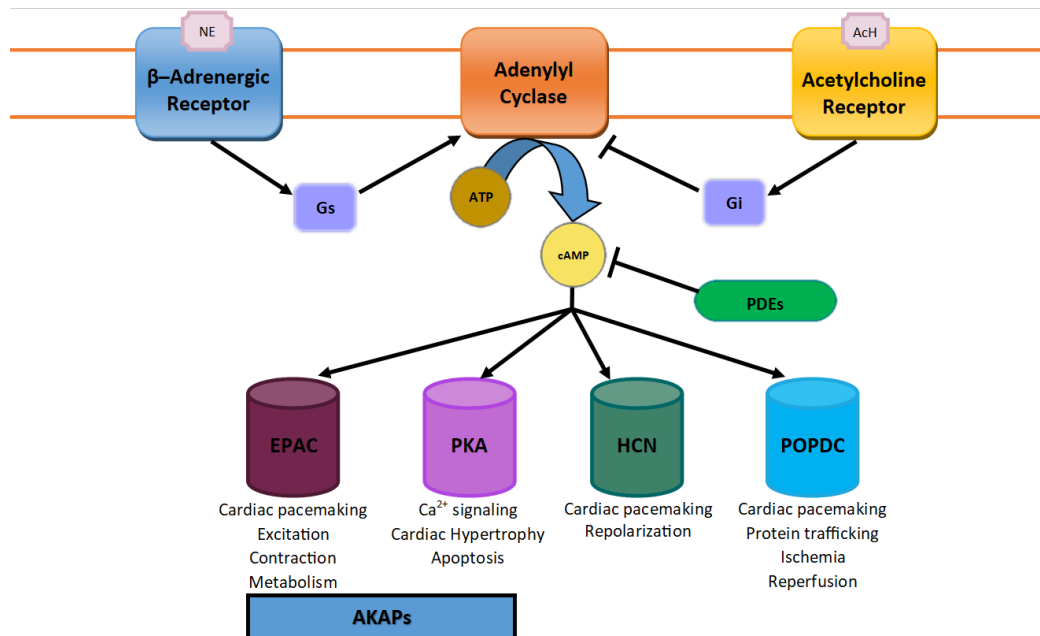


Figure 1.6. cAMP effector proteins, their signalling pathway and main functions within the heart. Norepinephrine (NE), a β -adrenergic receptor (β AR) agonist secreted by the sympathetic neurons, binds to the membrane bound β AR which results in the activation of Gs protein with the subsequent activation of an Adenylyl Cyclase (AC) and production of cAMP from ATP. Activation of Gi, through the binding of acetylcholine ligand to the muscarinic acetylcholine receptor, inhibits AC activity, resulting in a reduced cAMP production. cAMP, through direct binding, can alter the activity of its four effector proteins: exchange factor directly activated by cAMP (EPAC), protein kinase A (PKA), hyperpolarization activated cyclic nucleotide gated (HCN) channel, Popeye domain containing (POPDC) protein. cAMP is metabolised by the phosphodiesterase (PDE) enzymes. PKA and EPAC are often found tethered to the same A-kinase anchoring protein (AKAP). AKAPs target PKA to various subcellular localisations where PKA can participate in signalling complexes to generate downstream effects of cAMP signalling. HCN and POPDC proteins are membrane bound (Figure adapted from¹⁴⁷).

1.2.5 Compartmentalisation of cAMP signalling

The cAMP signalling pathway plays a critical role in the control of a wide variety of cellular responses whereby different receptors can produce distinct responses even though they act through this common signalling pathway. That signal specificity is achieved through cAMP compartmentation. The concept of cAMP compartmentation was originally proposed by the pioneering work of Buxton and Brunton^{148,149}. In essence, their work generated a shift in paradigm that specificity of hormone signalling was inherently based on the intrinsic properties of the receptor, to one involving cell structure.

Indeed, over the years the transformative development of optical probes based on FRET allowed for a real time visualisation of cAMP gradients in living cells that were spatiotemporally restricted to confined nanodomains¹⁵⁰. There, cAMP is locally generated and degraded thereby providing a mechanism by which cAMP can mediate many different cell type- and ligand-specific responses^{151,152}. Possibly the most dramatic result based on work utilising these techniques was the visualisation of discrete cAMP nanodomains in cardiac myocytes after β AR stimulation¹⁵³. Based on this work, the understanding has developed that effective cAMP compartmentation is based on two factors: the formation of protein complexes (signalosomes) at specific nanodomains, bringing PKA and a distinct and unique combination of its effectors and regulatory proteins near one another and a restricted diffusion of cAMP so it only activates PKA in its immediate proximity. Signalosomes generally form upon scaffolding proteins such as the previously described AKAPs, that target PKA to different locations within the cell¹⁵⁴. cAMP diffusion is restricted by phosphodiesterases (PDEs), enzymes that break down cAMP to below the activation threshold of cAMP effector proteins. Notably, PDEs, one or a subset of PDEs, form an integral part of signalosomes to which they are targeted to form cAMP sinks thereby shaping the local cAMP gradients^{155,156}. The activation of an effector can therefore only occur when, upon ligand dependent receptor activation, the levels of cAMP rise rapidly to overwhelm the tethered PDE. The formation of signalosomes permits a simultaneous formation of multiple cAMP gradients in different subcellular localisations¹⁵⁶.

1.2.6 Phosphodiesterases (PDE)

PDEs are the only mammalian enzyme that is known to catalyse the degradation of cyclic nucleotides such as cAMP and cGMP. There are approximately 100 isoforms, which are the product of 21 genes, generated by alternative mRNA splicing, and grouped into 11 PDE families. These isoforms have widespread distribution patterns and possess distinct functions within the cell. They can be categorised by the nucleotide specificity as shown in Table 2 with a varying spectrum of affinity^{157,158}.

Table 1.2. Specificities of PDE families

cAMP only	PDE4, PDE7, and PDE8
cGMP only	PDE5, PDE6, and PDE9
cAMP/cGMP dual specificity	PDE1, PDE2, PDE3, PDE10, and PDE11

Interestingly, despite the variability in their localisation, function and substrate specificity, PDE isoforms show a structure that is somewhat conserved. Generally, the PDE protein will have a NH₂-terminal regulatory (R) domain and a C-terminal catalytic (C) domain, with the exception of the PDE4 family which is known to have regulatory features in their C-domain¹⁵⁹. The PDE regulatory domains are connected to each other and other domains of a PDE protein by the so called linker regions, which serve to communicate changes in activity and structure between the different sections of a PDE enzyme¹⁶⁰. Regulatory control of PDEs is also achieved through other domains such as: calcium/calmodulin-binding domain (PDE1), GAF domains (PDE2, PDE5, PDE5, PDE10 and PDE11), PAS (Per-ARNT-Sim) domain (PDE8), the upstream conserved domains (UCR) (PDE4), and the autoinhibitory domains¹⁶⁰.

It has been shown that at least five isoforms, PDE1–PDE5 are expressed in the ventricles. In this context, PDE2, 3 and 4 appear to be the PDE families most intimately involved with cardiac cAMP compartmentation and thereby involved in the control of basal and adrenergic contractility. In the mouse heart, PDE4 provides 60% of cAMP hydrolytic activity, with PDE3 activity accounting to 30%¹⁶¹. On the other hand, in the human heart, PDE3 is the predominant PDE isoform, and, in combination with PDE1 and PDE2, they cover 90% of all PDE activity^{162,163}. PDE3 and PDE4 have been shown to localise to distinct compartments

which may underpin their different roles in that PDE4, rather than PDE3 appears to be responsible for modulating the amplitude and duration of the β AR induced cAMP response in rat ventricular myocytes. PDE2 does not appear to be responsible for more than 3% of the cAMP hydrolytic activity in neonatal rat cardiomyocytes although its inhibition greatly enhances physiologic activity of the β AR, possibly by regulation/termination of LTCC phosphorylation^{164,165}. In this context it is noteworthy that PDE integration into signalling complexes is isoform-specific and the relative abundance of a particular PDE isoform is therefore not always essentially reflective of its overall importance for cellular function. PDE2 has dual nucleotide specificity in that it binds both cAMP and cGMP at equal rates, however, binding of cGMP to the PDE2 GAF domain increases cAMP hydrolysis 6-fold which could function as a mechanism to blunt cAMP signalling after β AR stimulation¹⁶⁴.

Early PDE3 investigation suggested that they couple to ventricular T-tubules and SR¹⁶⁶ and more recent investigations utilising mouse knock out models have been able to discern different functions of various PDE3 isoforms. For example, Sun et al. identified that PDE3A is important for generation of chronotropic and inotropic effects¹⁶⁷ and Beca et al. showed that PDE3B isoform acutely protects the heart after biomechanical stress¹⁶⁸. They suggest that at baseline PDE3A impacts cAMP mediated Ca^{2+} reuptake into SR by controlling the activity of PLB without affecting L-Type Ca^{2+} currents. Collectively these findings may be clinically relevant as selective PDE3A inhibition could improve the contractile performance in heart failure patients¹⁶⁹.

It was shown that in cardiac myocytes PDE4 is localised to the sarcolemma where it has an integral role in the regulation of Ca^{2+} movement principally via LTCC and RyR2. Stimulation by either β ARs initiates hydrolytic activity of the PDE4 enzyme, although its hydrolytic activity in response to β_1 AR-induced cAMP was higher than that of β_2 AR in rat ventricular myocytes^{150,170}. In the heart, PDE4A, PDE4B and PDE4D isoforms are expressed. PDE4D has demonstrated the greatest impact on modulating cAMP levels and therefore contractility in response to β AR stimulation, by regulating the PLB/SERCA2A complex and the Ca^{2+} release from RyRs^{157,168,171}. Additionally, evidence has shown that PDE4D5 is specifically recruited to an agonist activated β AR in a complex with β -arrestin¹⁷². This PDE4D5/ β -arrestin interaction is particularly relevant for preventing PKA mediated phosphorylation of β_2 AR and its

subsequent stimulus trafficking to bind the Gi protein, which would dramatically change its downstream effects¹⁵⁵. Studies utilising PDE4D3 knock out mouse models identified a hypertrophy phenotype that is consistent with β AR overstimulation and heart failure. In these animals RyRs appeared to be 'leaky' due to hyperphosphorylation, resulting in an increased prevalence of arrhythmias and sudden cardiac death^{171,173}.

PDE3 and PDE4 activity in the SAN was also investigated. It was shown that inhibition of PDE3 activity resulted in an increase of basal SAN beating frequency in the mouse and human heart. However, neither PDE3 nor PDE4 blockers had an effect on cardiac pacemaking after β AR stimulation¹⁷⁴. From these findings it can be concluded that even though PDE3 and PDE4 may be important in basal pacemaking, they are probably not regulating upregulation of SAN function after β AR stimulation.

1.3 Popeye domain containing proteins

POPDC proteins participate in numerous pathways and show a broad expression pattern, with expression being notably enriched in cardiac and skeletal muscle. They have been identified as a novel cAMP effector protein, forming part of the β AR signalling pathway in the heart. POPDC proteins have been shown to have many interaction partners in different physiological settings. Through the interaction with electrogenic proteins they are involved in the regulations of the heart rate and rhythm whereby loss of POPDC proteins results in an arrhythmogenic phenotype in animal models. Findings derived from animal models are also reflected in the phenotypes discovered in patients with an ever-growing number of POPDC protein mutations. Additionally, POPDC proteins, and more extensively POPDC1, through its interaction partners, has been implicated in tight junction formation and maintenance of epithelial cells. Consistent with its role in epithelial cells, POPDC1 has also been identified as a tumour suppressor due to its ability to regulate epithelial-mesenchymal transition (EMT). The role of POPDC2 and POPDC3 in this context still needs to be clarified, although expression and function in breast epithelial cells has been reported^{185,206}. Some evidence suggests POPDC1 may also be implicated in plasma-membrane repair. The sections below will describe and discuss in further detail the POPDC proteins as well as their interaction partners and associated functions regarding the maintenance of homeostasis and in the progression of disease pathology.

1.3.1 Genomic Organization of POPDC genes

In the quest for finding novel genes with cardiac-restricted expression two independent research groups discovered the POPDC family consisting of three genes, giving rise to three proteins namely POPDC1 (also known as blood vessel epicardial substance (BVES)), POPDC2 and POPDC3^{175,176}. *POPDC* genes are highly conserved through evolution and are already present in *Hydra* at the base of metazoan evolution. Three genes are present in vertebrates whereas in lower chordates and echinoderms only two genes, probably *POPDC1* and *POPDC3* orthologues are present¹⁷⁷. Interestingly, *Drosophila* only contain one and in *C. elegans* no *POPDC* genes have been found¹⁴⁷. It is thought that *POPDC1* is the primordial gene as it shows only about 25% sequence homology at the protein level to either POPDC2 or POPDC3. Furthermore, *POPDC2* is only found in vertebrates and is thought to have arisen by gene

duplication from *POPDC3*, showing approximately 50% sequence homology at the protein level¹⁷⁶. Therefore, it can be speculated that three POPDC proteins arose through two separate gene duplication events: the first duplication probably occurred during early chordate evolution, producing *POPDC3* from *POPDC1*¹⁷⁸ and both genes are found in all vertebrates tandem organized. The second gene duplication of only *POPDC3* produced *POPDC2* and probably happened during early vertebrate evolution given that in teleosts, *POPDC2* is present, whereas in lower chordates such as tunicates, only *POPDC1* and *POPDC3* are found.

1.3.2 POPDC gene expression and regulation

POPDC genes have generally a broad expression pattern and have been detected in variety of smooth muscle containing organs (bladder, uterus, gastrointestinal tract, and the lung), neurons of the central and autonomic nervous system, and in several types of epithelial cells (epidermal cells, stomach, and cornea)^{179–183}. However, through an array of experimental procedures it has been shown that the tissues with the highest expression levels of *POPDC* genes are striated muscle cells of the heart and skeletal muscle^{176,181,183,184}. *POPDC1* is expressed equally high in skeletal muscle and the heart, whereby *POPDC2* and *POPDC3* are differentially expressed: *POPDC2* is predominantly found in the heart and *POPDC3* mainly in skeletal muscle. Interestingly, recent analysis of the LacZ expression pattern of *Popdc3* showed that in the mouse heart, it is expressed in a narrow domain in the subepicardial myocardium in both atrium and ventricle.

In man, *POPDC2* is localized to chromosome 3q13.33 whereas *POPDC1* and *POPDC3* are organized in tandem on chromosome 6q21. Transcription of *POPDC1* from its predicted bidirectional promoter also generates an antisense RNA from the short intergenic sequence between the two genes which seems to comprise a part of the *POPDC3* gene¹⁸⁵. Schindler et al. speculate that this antisense RNA may suppress *POPDC3* expression, which could explain the observed relatively lower expression levels of *POPDC3*. However, recent data suggest that the locus harbours a long non-coding RNA (BVES-AS9), which might encode a 50-amino acid long micro-peptide that possibly plays a role in controlling cell survival and migratory activity of colorectal cancer cells¹⁸⁶. The evolutionary preserved tandem configuration of *POPDC1* and

POPDC3 suggests importance for coordinated gene regulation¹⁷⁸. This hypothesis is supported by data on *POPDC* gene expression in heart failure and gastric cancer patients, which have shown co-regulation of both *POPDC1* and *POPDC3* and a concerted reduction in gene expression with a negative impact on the disease state^{187,188}. *POPDC2* is apparently regulated independently as neither of the conditions had an impact on the *POPDC2* expression levels.

Still, little is known about the exact mechanisms that govern *POPDC* gene expression and transcription. Nevertheless, it has been proposed that *Popdc1* in muscle cells is transcriptionally regulated by Pax3, during mouse embryogenesis and in alveolar rhabdomyosarcoma¹⁸⁹. Pax3 is a transcription factor which is important in muscle and neuronal development. *Popdc2* is highly expressed in cardiac muscle cells and investigation of its promoter revealed that it contains binding sites for both Meis homeobox protein 1 (Meis1) and NK homeobox protein 2.5 (Nkx2.5) in an overlapping manner¹⁹⁰. Both transcription factors are known to be essential in vertebrate heart development and by extension in the normal physiology of an adult heart. Utilising chromatin immunoprecipitation the differential binding of these transcription factors was further interrogated which revealed that Meis1 binds to the *Popdc2* promoter during anterior heart field formation and Nkx2.5 occupies the *Popdc2* promoter of differentiated cardiac myocytes¹⁹⁰.

In human hepatocellular carcinoma (HCC), and other cancer types, Netrin-1, a laminin-related neuronal guidance molecule, has been identified as a negative *POPDC1* expression regulator which acts through the modulation of the PI3-kinase/AKT signalling axis¹⁹¹. It was shown that increased Netrin-1 levels are associated with a decrease in *POPDC1* expression which in turn increases the risk of tumour progression¹⁹¹. Additionally, *POPDC1* and *POPDC3* promoter hypermethylation and histone modifications have been suggested as major mechanisms by which both genes are downregulated in gastric and colon cancer^{192,193}

It was also found that secretion of Gurken, an epidermal growth factor (EGF)-like signalling molecule, during *Drosophila* oogenesis, negatively regulates *popdc1* expression in dorsal follicle cells¹⁹⁴. The same form of regulation was also identified in breast cancer and gastric adenocarcinoma cells line SNU-216, which express all three POPDC isoforms, whereby

addition of EGF produced a decrease in *POPDC* gene expression levels^{192,195}. Moreover, neonatal rat cardiac myocytes cultured in serum, were shown to have reduced *POPDC1-3* levels; a phenotype that could be rescued by the addition of Tyrphostin, an EGF antagonist¹⁹⁶.

1.3.3 The POPDC Protein Family

All three POPDC isoforms are expressed in the heart in a somewhat overlapping manner, albeit with a distinct expression pattern: POPDC1 is expressed at higher levels in the atria than in the ventricles, whereas POPDC2 is expressed evenly across the whole heart and both proteins are expressed at elevated levels in the CCS¹⁸⁴. It has been shown that their expression level is confined to cardiac myocytes only, where they are localized to the plasma membrane, intercalated disk, t-tubules, caveolae, and costameres^{181,197}. Structurally, POPDC proteins are transmembrane proteins of medium size (POPDC1: 360aa, POPDC2: 364aa, POPDC3: 291aa), that have four distinct regions: amino terminus, three transmembrane domains, highly conserved cytosolic Popeye domain, and a carboxyl terminus as shown in Figure 1.7 A.

The hydrophobic transmembrane region of POPDC proteins consists of three alpha helices that anchor POPDC proteins to the plasma membrane. The extracellular amino terminus is short (27–39 residues) and harbours two conserved and functional N-glycosylation sites in POPDC1 and one site is present in POPDC2 and POPDC3^{198,182}. The level of glycosylation is quite extensive in POPDC proteins, which creates discrepancy between the predicted MW and the MW measured by Western blot analysis^{198,199}. The exact relevance of this extensive glycosylation is yet unclear; however, it has been proposed that it may have a protective role by preventing proteolytic decay or it might be implicated in the correct POPDC protein localisation^{177,182,198}.

The cytoplasmic region of POPDC proteins is evolutionary conserved and contains the 150 amino acid long Popeye domain which functions as a cAMP binding domain^{176,184} (Figure 1.7B). In general, the three members of the POPDC protein family share between 25 and 50% amino acid sequence similarity, with the sequence conservation reaching up to 60% in the Popeye domain^{176,184}. The sequence homology of the Popeye domain to other cAMP-binding domains is limited. Nevertheless, the bacterial catabolite activator protein (CAP), also known

as cAMP receptor protein (CRP) has shown a 25% sequence identity and 60% sequence similarity to the Popeye domain in POPDC proteins, which is the highest sequence similarity outside of POPDC orthologues^{176,184}. CRP is a transcription factor that controls the expression of enzymes involved in carbohydrate metabolism and its binding of cAMP increases the affinity of CRP for its target DNA sequence, therefore functioning as its effector²⁰⁰. Considering this information, it can be speculated that POPDC proteins might have evolved from a transcriptional regulator, gaining a transmembrane domain. Some support for this hypothesis comes from the work produced by Schindler et al. and others who showed POPDC1 localisation to the nuclear envelope and the nucleoplasm in myoblasts and cycling satellite cells, with an evident loss of the nucleoplasmic domain after the differentiation of myoblasts into myotubes²⁰¹. However, in an alternative hypothesis POPDC proteins could, in addition to their role at the plasma membrane, also have nuclear functions such as regulating gene expression in a cAMP-dependent manner.

Finally, distal to the Popeye domain is the carboxyl terminus of POPDC proteins. This region is variable in length and isoform specific. It is generated by alternative splicing in POPDC1 and POPDC2 and is predicted to have regions of low complexity and be structurally disorganised¹⁴⁷ (Figure 1.7A). Attributed to its carboxyterminal regions of low complexity, POPDC1 has recently been identified as a hub protein in the context of atrial fibrillation²⁰². Such disorganised regions with low complexity are an intrinsic part of hub-proteins as they mediate many protein-protein interactions due to their flexibility in attaining a specific secondary structure upon binding of an interaction partner. A feature that is essential to hub-proteins^{203,204}. The carboxyl terminus of POPDC proteins has also been shown to have clusters of serine/threonine residues, which form β AR-dependent phosphorylation sites²⁰⁵. Next to cAMP binding, phosphorylation may present an additional mode by which the biological activity of POPDC proteins can be modulated, with a possibly significant impact on their ability to mediate protein-protein interactions^{199,206–210}.

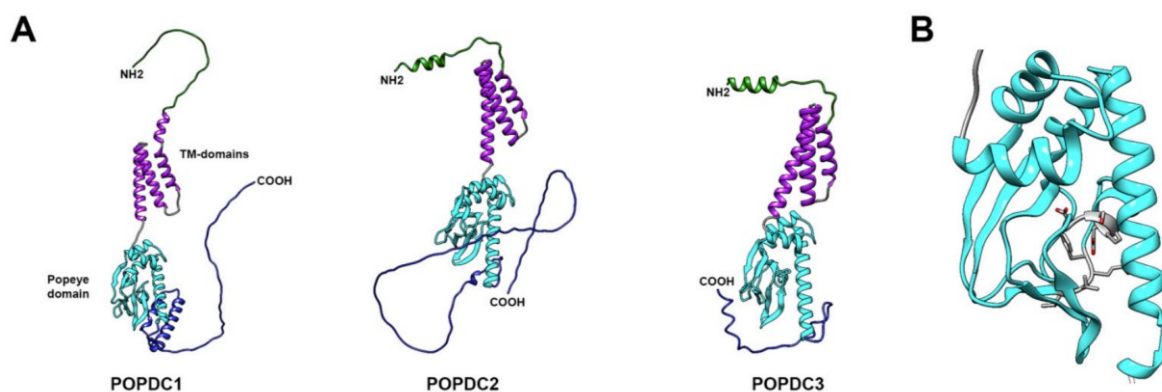


Figure 1.7. Structural models of human POPDC proteins generated by AlphaFold²¹¹. In (A) are depicted the structural models for POPDC1, POPDC2 and POPDC3. Each POPDC isoform is composed of four distinct regions: the extracellular amino-terminus (green), the three transmembrane domains (purple), the highly conserved, intracellular Popeye domain (sky blue), and a disorganised, low-complexity carboxyl-terminus (dark blue). In (B) is shown the structural model of the Popeye domain of POPDC1 protein. It serves as non-canonical cAMP binding domain composed of three alpha helices and an eight-stranded, antiparallel beta-barrel structure that harbour two ultra-conserved motifs, DSPE and FQVT (residues are labelled in grey) which are believed to form an atypical phosphate-binding cassette (PBC)^{184,212}. (Picture taken from²¹³)

1.3.4 POPDC proteins form homo and heterodimers

POPDC proteins can form homo- and heterodimers with other members of the POPDC protein family. For example, it was shown that POPDC1 proteins form homodimers that are stabilised by an intermolecular disulphide bond. In addition to the disulphide bond, further dimerization motifs were mapped to two conserved lysine residues in the carboxyl terminus of the Popeye domain of the POPDC1 protein^{198,214}. However, deletion of the said lysine residues did not completely prevent dimer formation, suggesting that multiple sites in the protein are probably involved in a stable dimer formation that can, to some degree, compensate for each other. The two lysine residues are not conserved between POPDC isoforms which is indicative of alternative sequences that allow for a heterodimer formation. In fact, in a recent study, Swan et al, have shown that POPDC1 - POPDC2 heterodimers form a heteromeric interface via their highly conserved hydrophobic residues located in α C-helices of the Popeye domains. A successful heterodimer formation was shown to be a requirement for correct heterodimer membrane trafficking, disruption of which was suggested to result in changes in POPDC1-POPDC2 interaction and subcellular expression which was observed in POPDC1 p.Q153X and the *POPDC2*^{W188X} mutants. It was further proposed that the heteromeric interaction interface between POPDC1 and POPDC2 α C-helices may mask ER

retention motifs, thereby allowing correct movement of the complex to the plasma membrane²¹⁵.

1.3.5 POPDC as cAMP effector proteins

POPDC proteins do not share any significant structural homology with any other established protein and thus it has been difficult to predict their function. Nevertheless, in the last two decades since their discovery they have been identified as the most recent class of cAMP effector proteins.

The difficulties in producing an empirical or crystal structure of the POPDC proteins and their cAMP binding Popeye domain led to the analysis of secondary structure prediction which revealed similarities to a CNBD as found in PKA and EPAC¹⁸⁴. From there, a homology based structural model of the Popeye domain was developed, whereby structures of CRP and PKA RII served as templates¹⁸⁴ (Figures 1.7B, Figure 3.3 page??). Based on this model, a non-canonical CNBD that harbours an atypical phosphate binding cassette (PBS) was predicted. The PBC is thought to be formed by the two ultra-conserved DSPE and FQVT motifs. A series of charge to alanine mutagenesis experiments confirmed the importance of these DSPE/FQVT motifs in cAMP binding¹⁸⁴. For example, mutation of aspartate 200 to alanine (D200A) in POPDC1 and a mutation of aspartate 184 to alanine (D184A) in POPDC2 both resulted in a severe loss of cAMP binding. These findings were corroborated further by the discovery of patients with a serine 201 to phenylalanine mutation (S201F) in POPDC1, displaying a 50% reduction in cAMP affinity compared to the wild type^{184,199}. These patients suffered from limb-girdle muscular dystrophy and AV-block. Collectively, these data presented strong empirical evidence that the Popeye domain functions as a cAMP-binding domain. Froese et al. were also able to show that POPDC1 can bind cAMP with a similar affinity as PKA and a much higher affinity than EPAC. At high concentrations, binding of cGMP by the Popeye domain has also been observed, albeit with an approximately 40-fold lower affinity compared to cAMP¹⁸⁴.

1.3.6 Working models of POPDC function

The investigation on how exactly cAMP affects POPDC protein function is still on-going, however, four models that are described below, have been developed. It is important to consider that different POPDC isoforms may follow different models and that the model that each POPDC isoform follows may also be tissue specific ²¹⁶.

The switch model proposes that POPDC proteins can activate or inhibit the activity of an interacting partner upon cAMP binding, possibly through a conformational change that extends to the binding partner. Some evidence for this model can be found in the POPDC1-TREK1 interaction, whereby TREK1 current at the membrane is only enhanced through the interaction with POPDC1 at basal cAMP levels; an effect that is lost at elevated cAMP levels ^{184,199}. Moreover, cAMP negative-feedback regulation of AC9 enzymatic activity has been recently predicted when characterizing the interaction of AC9 and POPDC1 ²¹⁷.

The shield model proposes that cAMP binding to a POPDC protein could induce a conformational change that would result in the substrate binding site of the interacting partner to be obscured and thereby protected from phosphorylation by PKA or other kinases. Recent evidence for this model can be derived from the findings that POPDC1, PDE4A and TREK1 are in a complex. PDE4A are keeping POPDC1 in an cAMP unbound state and thereby keeping TREK1 at the plasma membrane and thereby enhancing TREK1 current ²¹⁸.

The cargo model is based on the hypothesis that POPDC proteins play a role in membrane trafficking of their interacting partners and that this ability is modulated by cAMP binding. This has first become evident through the observation that TREK1 membrane trafficking was reduced in *Xenopus* oocytes in the presence of the POPDC1^{S201F} mutant, which binds cAMP with 50% less affinity. Additionally, *POPDC1* variants discovered in patients also show aberrant membrane localisation of POPDC2 ^{199,219}. Compromised POPDC1 expression has also been shown to result in an abnormal vesicular transport in epithelial cells ²¹⁰.

The sponge model proposes that POPDC proteins bind cAMP to limit its diffusion. cAMP is in theory in aqueous solution such as the cytoplasm predicted to be a highly diffusible molecule.

However, based on measurements of its actual diffusion rates in cells it seems that there must be mechanisms in place in cells, which constricts the free diffusion of cAMP. While cAMP diffusion is in part limited by PDEs, an enzyme family that degrade cAMP to form spatially restricted cAMP nanodomains, the hydrolytic activity of PDEs is calculated to be too slow to fully account for the limited diffusion of cAMP. However, it has been shown that PDEs enzyme activity is not fast enough to completely explain diffusion rate of cAMP in cells²²⁰. Therefore, cAMP is also thought to be restricted in its diffusion by binding to proteins in specialised nanodomains in which it is generated. Considering the high expression levels of POPDC1 and POPDC2 in cardiac myocytes and their high cAMP affinity, the sponge model was proposed suggesting that through binding of cAMP POPDC proteins may be contributing to restricting cAMP diffusion.

1.3.7 POPDC proteins in cancer, cell proliferation and cell motility

POPDC proteins are mis-regulated in many cancers in a tissue- and isoform-specific manner, whereby the role of POPDC1 in cancer is most extensively studied. POPDC1 is known to interact with several different molecules that regulate the cell cycle and cell proliferation, which when mis-regulated, promote malignant cell behaviour. Reduced *POPDC1* expression has been associated with enhanced tumorigenic cell migration, proliferation and metastasis, therefore POPDC1 is thought to function as a tumour suppressor^{191–193,195,221,222}. A study by Kim et al, 2010, investigated the relationship between *POPDC1* expression and survival rate and in gastric cancer patients¹⁹². In their study the authors showed that *POPDC1* expression is directly correlated with the survival length upon diagnosis. Specifically, high *POPDC1* expression was linked to an increased survival length with the opposite being true for a reduced *POPDC1* expression, especially in patients with early-stage tumours^{192,223}. This difference was particularly stark at the three-year survival mark, where survival was at 78.68% for patients with high vs. 12.18% for patients with low *POPDC1* expression¹⁹². Lost or reduced expression of *POPDC1* in gastric and non-small cell lung cancers were identified to be due to gene silencing by *POPDC1* promoter hypermethylation and histone deacetylation^{192,224} amongst other mechanism that were described in section 1.3.2.

Several mechanisms by which POPDC1 is linked to cancer progression have been put forward. POPDC1 was found to be localised to the nuclear envelope and nucleoplasm in myoblasts, cycling satellite cells but also in epithelial breast cancer cells^{225,226}. POPDC1 is also known to directly interact with zona occludens 1 (ZO-1), a tight junction adapter protein that interacts with actin, claudin, occludins and α -catenin and is implicated in controlling endothelial proliferation^{209,227}. It does that by binding and thereby sequestering of ZO-1 associated nucleic acid-binding protein/DNA binding protein A (ZONAB/DbpA) from the nucleus to the tight junctions¹⁹³. ZONAB/DbpA, is a transcription factor that regulates the expression of cell cycle and proliferation genes such as proliferating cell nuclear antigen (PCNA), cyclin D1 and HER2. Reduced ZONAB/DbpA results in a reduced transcription of cell cycle and proliferation genes. ZO-1 expression levels directly correlate with those of POPDC1. Thus, at reduced POPDC expression and therefore ZO-1 levels, there is more free ZONAB/DbpA to drive cell proliferation. This mechanism is corroborated by the findings of concomitant reduction of POPDC1 and ZO-1 expression in various cancer types, thereby confirming POPDC1 as a tumour suppressor.

POPDC1 has also been identified to interact with and /or influence members of the Wnt signalling pathway^{193,221,228,229}, which is an invaluable, evolutionary conserved pathway regulating many aspects of the cell including cell proliferation, apoptosis and cell cycle arrest^{230,231}. β -catenin is the central molecule of the Wnt signalling pathway that, which when activated, accumulates in the cytosol before translocating into the nucleus to drive transcription of Wnt target genes, of which some upregulate the cell cycle and cell proliferation^{231,232} thereby implicating Wnt signalling in tumorigenesis^{229,233}. POPDC1 was shown to downregulate the Wnt signalling mediated cell proliferation through regulation and localisation of β -catenin^{193,229}.

POPDC1 also regulates the transcription factor and proto-oncogene c-Myc²³⁴, which is often overexpressed in various cancer models²²¹. C-Myc regulates many cellular processes including cell cycle and proliferation^{235–237}. POPDC1, through its interaction with the PR61 α -containing PP2A phosphatase complex promotes proteasomal c-Myc degradation²²¹. In patients with ulcerative colitis POPDC1 was found to be expressed at lower levels due to promotor hypermethylation, resulting in elevated c-Myc protein levels^{193,221}.

There is also some evidence suggesting that POPDC1 may be able to inhibit breast cancer cell migration and proliferation through its interaction with cAMP²²². It is known that POPDC1 expression is reduced in many cancers such as gastric cancer, colorectal cancer and hepatocellular carcinoma. This is also true for aggressive breast cancers where its expression is mislocalised and significantly reduced which leads to a loss of tight junction integrity resulting in cell migration promoting malignant behaviour in cancer cells. Studies have shown that in breast cancer cells, elevation of intracellular cAMP is able to promote apoptosis and inhibit cell migration and invasion^{238,239}. Elevated cAMP levels also stabilise and up-regulate POPDC1 expression in breast cancer cells and overexpression of POPDC1 suppresses cell migration and proliferation in breast cancer cells. Thereby, these findings suggest that POPDC1 through its interaction with cAMP can mediate the inhibition of breast cancer cell migration.

Another proposed mechanism by which loss of POPDC1 drives a malignant cancer phenotype is within POPDC1s ability to regulate cell shape and motility. A study by Guo et al, 2003, showed that an overexpression of the Rho-family guanine nucleotide exchange factor (GEFT) leads to an alteration and re-organisation of the cytoskeleton giving rise to an altered cell morphology and by extension cell migratory activity. Additionally, overexpression of GEFT was associated with enhanced cell proliferation which is known to drive tumorigenesis²⁴⁰. GEFT has been identified as a POPDC1 interaction partner²⁰⁸. The exact mechanism by which POPDC1 proteins interact with and influence GEFT activity is largely unclear, however, it has been shown that an overexpression of POPDC1 reduces GEFT mediated activity of the Rho-family GTPases Rac1 and Cdc42 with the consequence of a reduced cell motility²⁰⁸. These findings are reminiscent of observations made in other cancer types where reduced POPDC1 expression generated cancer cells with an enhanced ability to migrate and proliferate^{191,193,222}.

Significantly less is known about the role of POPDC2 and POPDC3 in the context of tumorigenesis. Both *POPDC2* and *POPDC3* were found to be overexpressed in ductal breast cancer cells, albeit with expression levels varying between the early and late cancer stages¹⁹⁵. Additionally, Amunjela and Tucker were able to show correlation between *POPDC2* and

POPDC3 expression and HER2+, which is a marker for aggressive tumours^{195,241}. The role of *POPDC3* in tumorigenesis seems to be tissue dependent and aligned with its tissue specific roles, as it has been identified as a potential tumour enhancer in the head and neck squamous cell carcinoma²⁴² but its suppression in gastric tumours has been linked to oncogenesis²⁴³. However, no molecular mechanism is yet known regarding *POPDC3* in cancer and cell proliferation.

1.3.8 *POPDC1* and cell-cell contact formation

In epithelial and endothelial cells cell-cell contacts are governed by the formation of tight junctions. These multiprotein junctional complexes form a barrier, blocking the apical-basolateral diffusion of membrane proteins and regulating the intracellular passage of molecules²⁴⁴. Through the means of GST pull down assay it has been shown that in mature epithelial cells *POPDC1* proteins directly interact with ZO-1^{209,227}. The interaction between ZO-1 and *POPDC1* is important for the formation and maintenance of the epithelial monolayer. It is hypothesised that *POPDC1* combined with ZO-1 control and modulate RhoA signalling, because *POPDC1*, through its interaction with ZO-1, can sequester Rho guanine nucleotide exchange factor GEF-H1, which is an activator of RhoA²⁴⁵. This hypothesis is supported by the findings that *POPDC1* suppression, as unveiled by RhoA-GSTase activation assay, results in an increase in RhoA activity, prompting cell migration and invasion¹⁹¹. *POPDC1* overexpression, on the other hand, downregulates RhoA signalling which leads to an increase in tight junction formation and thereby a decrease in epithelial permeability^{245,246}. In zebrafish, as a model organism, *Popdc1* was shown to interact with the atypical protein kinase C (aPKC), which is known to recruit multiple members of the tight junction signalling complex and thereby *POPDC1* plays a role in maintaining of epithelial cell integrity^{247,248}. Overall, these finding assure that *POPDC1* has an essential role for tight junction and by extension epithelial function. Interestingly, ZO-1 is also known to interact with key components of adherens junctions such as N-cadherin and gap-junctions such as Connexin43²⁴⁹. From there, *POPDC1* have also been implicated in the regulation of adherens junctions through its interaction with elements of the WNT signalling pathway. Adherens junctions can regulate WNT signalling by sequestering β -catenin at the cell membrane²⁵⁰. *POPDC1* was shown to have an expression pattern that correlates and co-localises with that of E-cadherin and β -catenin^{193,251}. *POPDC1*

overexpression causes an increased β -catenin expression at the membrane with a decreased cytoplasmic expression, whereby loss of POPDC1 causes cytoplasmic and nuclear β -catenin expression. In light of the findings that POPDC1 is involved in mediating both tight junctions and adherens junctions, it has been proposed that POPDC1 has a role in regulating EMT^{193,251}. Further to this, EMT is often dysregulated in cancers and as discussed in section 1.3.5. it has been shown that loss of POPDC1 in cancer cells aggravates cell migration, invasion and metastasis^{191,195,222,251}.

1.3.9 POPDC proteins in vesicular transport

In a study by Hager et al, 2010, POPDC1 proteins in cardiomyocytes, skeletal muscle as well as epithelial cells, have been implicated in the process of vesicular transport through the interaction with the vesicle-associated membrane protein 3 (VAMP3)²¹⁰. This ubiquitously expressed vesicular SNAP Receptor (SNARE) protein regulates vesicle fusion and cell motility by mediating the trafficking of membrane proteins such as β -integrin and transferrin²⁵². Utilising a truncated dominant negative form of POPDC1 in MDCK cells, mimicking loss of POPDC1 proteins, resulted in a loss of VAMP3 mediated vesicular transport through a reduced β -integrin internalisation, a decreased transferrin uptake and exocytosis at the plasma membrane²¹⁰. These findings were further corroborated through *in vivo* studies utilising *Xenopus* embryos, where *Popdc1* knockdown produced morphological changes that were comparable with changes observed after integrin depletion²¹⁰.

Further to this, POPDC1 has been implicated in the mechanism underpinning directional cell motility in epicardial cells through its interaction with N-myc downstream regulated gene 4 (NDGR4)²⁵³. Directional cell movement is dependent on the recycling of the internalised soluble fibronectin, the motility substrate, to the cell membrane for re-secretion through the autocrine extracellular matrix recycling pathway. Interaction between POPDC1 and NDRG4 mediates the fusion of VAMP3 positive recycling endosomes with the cell membrane. Loss of this interaction produces randomised movement of epicardial cells and a loss in the ability to recycle fibronectin²⁵³. Collectively these findings solidify the importance of POPDC1 in cellular and physiological processes that require vesicular transport mediated by VAMP3, and cargo delivery through POPDC1 and NDRG4 mediated vesicle fusion.

1.3.10 POPDC in plasma membrane repair

POPDC1 also interacts with several proteins that are linked to the plasma membrane repair. To start with, POPDC1 appears to interact with annexin A5²⁰⁶, which together with other annexin subunits, is recruited to the injured plasma membrane, where they form a two-dimensional array in order to prevent an enlargement of the damaged area²⁵⁴. Anoctamin 5 (ANO5), which is associated with plasma membrane repair in skeletal muscle, has recently been identified as a novel POPDC1 interacting partner^{255,256}. Cav3 and dysferlin have long been known as POPDC1 interacting partners and have a link to plasma membrane repair in striated muscle^{197,199,257–259}. Additionally, the emerging role of POPDC1 in membrane repair can also be derived from existing patient data. For example, muscle biopsies of patients carrying the POPDC1^{S201F} mutation, revealed membrane discontinuities¹⁹⁹. Further to this, high serum creatine kinase levels, even in absence of an obvious skeletal muscle phenotype, has been recorded in many of the patients carrying POPDC mutations. This is suggestive of a leaky sarcolemma, which may be due to the plasma membrane repair mechanisms being impaired by the loss of POPDC. However, experimental evidence has not yet been generated to demonstrate directly that POPDC1 plays a role in plasma membrane repair. It is also noteworthy that membrane repair in other cell types requires elevated cAMP levels as was evidenced by Togo and colleagues²⁶⁰. Thus, it could be speculated that, POPDC1, being a cAMP effector, would modulate membrane repair in a cAMP dependent manner.

1.3.11 POPDC proteins in the heart

POPDC proteins are highly expressed in the heart and their role in cardiac function has been the subject of ongoing, extensive investigation. To study this important protein family mutant model organisms have been generated. In homozygous mice, loss of *Popdc1* and *Popdc2* produced an arrhythmogenic phenotype characterised by stress-induced sinus node bradycardia that was absent at baseline. Stress was induced either mentally by hot air jets, physically through swimming or pharmacologically through isoproterenol injections¹⁸⁴. Both mutants showed an increased number of sinus-pauses with episodes of tachybradycardia and an increased heart rate variability, overall resulting in a reduced mean heart rate¹⁸⁴. Similar to the cardiac arrhythmia phenotype observed in mice, *popdc1* and *popdc2* zebrafish morphants and the *popdc1*^{S191F} knock in mutant also presented a cardiac arrhythmia

phenotype, albeit in form of an AV-block^{199,261}. In both animal models the phenotype developed in an age dependent manner in homozygotes. In young mice, <3 month, no bradycardia phenotype was observed, but the severity of the arrhythmia developed with age. Interestingly, in very old *Popdc2* null mutant mice ventricular fibrillation after β AR stimulation was recorded. In zebrafish, cardiac arrhythmia was already present at the embryonic stage that became more severe with increasing age^{199,261}. Loss-of-function experiments showed that zebrafish larvae of three-to-four days post fertilization experienced AV-block type I, whilst larvae of older age developed complete heart block or occasionally a non-contracting heart^{199,261}. *Popdc1/Popdc2* double knock outs in mice were also generated. They presented atrial fibrillation, ventricular tachycardia, sinus pauses and AV block, as this is a more severe phenotype it infers that there is some redundancy between POPDC1 and POPDC2²⁶².

Alongside described electrophysiological abnormalities, altered SAN morphology and histology in five months and older *Popdc1* and *Popdc2* null mutant mice was also identified. Wholemount immunohistochemical analysis revealed a more compact SAN structure with fewer pacemaker myocytes and a reduced number of cellular extensions in the remaining SAN cells. Cellular extensions are essential for an effective electrical conduction from the electrically isolated SAN to the surrounding atrial cardiomyocytes¹⁸⁴. The described degeneration was particularly pronounced in the inferior part of the SAN, which is associated with cardiac pacemaking in response to adrenergic stimulation²⁶³. Based on these findings it can be speculated that POPDC proteins are necessary for pacemaker cell survival, however, at this stage, it is unclear whether the electrical changes are the consequence of the structural changes or vice versa.

Pathologies observed in the animal models were not limited to their hearts but have also been described in skeletal muscle. In *Popdc1* mutant mice an impaired regenerative capacity in their hind limb musculature with small immature myotubes was recorded after cardiotoxin injections¹⁷⁹. In zebrafish, *popdc2* morphants display aberrant myotomal segment boundaries and a malformed tail²⁶¹. Morpholino-mediated *popdc1* and *popdc2* morphants also presented myofiber ruptures, probably due to an impaired formation of the myotendinous junction (MTJ)^{199,261}. MTJ is a complex structure formed through the interaction between several extracellular matrix proteins and several plasma membrane proteins, collectively serving as a

basement membrane in skeletal muscle^{264,265}. It functions as a mediator for force transmission between the tendon and the muscle. Notably, in *popdc1*^{S191} knock in zebrafish mutant, mimicking the *POPDC1*^{S201F} mutation discovered in patients with muscular dystrophy and cardiac arrhythmia, lacked the electrogenic matrix proteins within the MTJ^{199,261,266}. POPDC1 was recently discovered to interact with dystrophin, an important MTJ formation protein, and through this interaction POPDC1 may play an important role in MTJ formation in zebrafish.

Table 1.3. Animal models for POPDC mutations and their associated heart and skeletal muscle phenotypes

Species	Mutation	Heart	Skeletal muscle	Reference
Mouse	Popdc1 ^{-/-}	Stress-induced bradycardia	Regenerative defect	184
		Ischemia-reperfusion damage	Not reported	197
	Popdc2 ^{-/-}	Stress-induced bradycardia	Not reported	184
	Popdc1 ^{-/-} /Popdc2 ^{-/-}	Arrhythmias	Not reported	262
Zebrafish	popdc1 morphant	AV-block	Muscular dystrophy	199
	popdc2 morphant	AV-block	Muscular dystrophy	261
	popdc1 S191F	AV-block	Muscular dystrophy	199

1.3.12 POPDC interact with electrogenic and structural proteins in the heart

The observed SAN abnormalities found in the *Popdc1* and *Popdc2* null mutants produce a phenotype like the one observed in patients with a sick sinus syndrome (SSS). Sick sinus syndrome is the disease of the elderly, with is a pronounced SAN dysfunction in absence of any other heart disease²⁶⁷. This led to the hypothesis that POPDC proteins might have a modulatory function on the pacemaker current *I_f*, an important pacemaking current that is uniquely generated by the HCN4 channels²⁶². However, the hypothesis was not supported by the data, which showed that there was no difference in the *I_f* current density or activation

time in the SAN myocytes isolated from *Popdc2* null mutants compared with their wild type counterparts at baseline conditions and after β AR stimulation simulation with the cAMP analogue 8-Br-cAMP¹⁸⁴. Nevertheless, these data allow the conclusion that POPDC proteins may regulate the activity of one or several other electrogenic proteins of the aforementioned membrane clock, that together with the proteins of the Ca^{2+} clock generate the oscillating pacemaker potential. In fact, just recently, the sodium calcium exchanger NCX1 has been identified as a POPDC2 interacting partner^{268,269}. As previously outlined NCX functionally couples the membrane clock to the Ca^{2+} clock and its loss was shown to cause SAN dysfunction with a phenotype similar to the one observed in POPDC2 mutants^{268–270}.

POPDC proteins also interact with other electrogenic proteins at the plasma membrane of cardiac myocytes. A functional interaction was identified between POPDC1, POPDC2 and POPDC3 and the two-pore potassium channel TWIK-related K⁺ channel 1 (TREK1)¹⁸⁴. Specific binding of TREK1 was identified to occur at the C-terminus of POPDC1. In the heart, TREK1 functions as a background channel that controls cell excitability to maintain the resting membrane potential below the threshold of depolarization²⁷¹. Its function is modulated by many stimuli including mechanical change to the membrane, pH and temperature. Work commenced in *Xenopus* oocytes, entailing co-expression of POPDC isoforms and TREK1, revealed a two-fold increase in the potassium current through the channel by the virtue of an increased channel trafficking to the membrane, if compared to TREK1 expression alone¹⁸⁴. Conversely, the interaction between POPDC proteins and TREK1 seems to be cAMP dependent, as it was shown that at elevated cAMP levels the stimulatory effect of POPDC proteins on TREK1 was lost¹⁸⁴. Data obtained from a bi-molecular FRET assay further corroborated these findings. It is therefore currently thought that POPDC proteins modulate TREK1 membrane trafficking, whilst their interaction at the membrane is regulated by cAMP. Mechanistically, it is thought that cAMP binding to POPDC protein initiates a conformational change in the Popeye domain, making the TREK1 binding domain of POPDC protein inaccessible¹⁸⁴. The cardiac-specific TREK1-deficient mice show stress-induced sinus bradycardia, which phenocopies the phenotype for *Popdc1* and *Popdc2* null mutants⁷². These findings suggest that POPDC proteins by modulating TREK-1, can affect the membrane potential and thereby the excitability of cardiac myocytes.

It is important to consider that sinus node pacemaking at baseline and after ANS stimulation is the result of a complex interplay between a network of proteins which makes it unlikely that the pacemaker phenotype of POPDC null mutants arises from a single aberrantly regulated ion channel. However, considering the previously discussed necessity for cAMP compartmentation and signalosome formation, within the bigger picture, POPDC1, TREK1, PDE4A and probably AKAP79/150 were shown to form a signalling complex. In this complex PDE4A serves as a protective measure, which prevents inappropriate cAMP binding to the Popeye domain under basal conditions. Disruption of PDE4A and POPDC1 interaction results in a decreased interaction between POPDC1 and TREK1, as well as causing a prolongation of AP duration in isolated ventricular myocytes²¹⁸. It can be speculated that in this setting POPDC1 may be balancing and fine tuning the effect of β -AR stimulation on TREK1 current.

1.3.13 POPDC proteins and membrane compartments in cardiac myocytes

As previously discussed, caveolae are important structures in cardiac myocytes which form membrane nanodomains that harbour important signalling proteins and allow crosstalk between signalling pathways. In line with that, caveolae have been reported to concentrate G-proteins and members of G-protein signalling pathways²⁷². For example, all components of the β_2 AR signalling: β_2 AR, G α s, G α i, AC5/6, LTCC and PKA-RII²⁷³ and other important ion channels and transporters such as SCN5A, and HCN4 were found to localise to the caveolae compartment²⁷⁴. In the heart caveolae are predominantly stabilised by the scaffolding protein Cav3. POPDC1 was shown to have a Cav3 binding site located in the carboxy-terminal end of the Popeye domain and thereby localise to the caveolae compartment²⁰⁷. Loss of POPDC1 from the caveolae compartment results in caveolae that are increased in size and reduced in number which translates to *Popdc1* null mutant hearts with an increased vulnerability to ischemia/reperfusion as well as larger infarcts and significantly reduced functional recovery after acute ischemic injury¹⁹⁷. Thus, it can be hypothesised that POPDC1 presence in caveolae is critical for cardiac protection and ischemic preconditioning¹⁹⁷. In addition to POPDC1, Cav3 also binds the LTCC CaV1.2, which initiates the recruitment of other elements of the cAMP signalling complex i.e., β_2 AR, PKA, and AC²⁰. Cardiac myocytes isolated from *Popdc1* null mutants were reported to present impaired calcium transients. It is therefore possible that

POPDC proteins, being an interaction partner of Cav3, are also part of this signalling complex which could explain the cardiac arrhythmia phenotypes to some degree.

The ID is a specialized subcellular region of cardiac myocytes which is important for mechanical and electrical coupling²⁷⁵. The integrity of the ID is modulated by the scaffolding protein XIRP1. XIRP1 is indirectly associated with N-cadherin in the adherens junction, by interacting with β -catenin and p120-catenin²⁷⁶. Likewise, XIRP1 modulates gap-junction function by modulating connexin 43 phosphorylation²⁷⁷. Missense mutations in XIRP1 have been associated with Brugada syndrome and nocturnal sudden cardiac death²⁷⁸. POPDC1 and POPDC2 are also present at the ID of cardiac myocytes in rat, mouse, human, and canine^{206,279} where they interact with XIRP1²⁰⁶ and show a significant functional overlap. However, it is currently unclear whether POPDC modulation of XIRP1 function is cAMP dependent.

1.3.14 POPDC mutations cause skeletal and cardiac disease in patients

Variants of *POPDC* genes were discovered in patients with heart and skeletal muscle disease^{199,219,266,280–282}. To date, seven variants in *POPDC1*, two in *POPDC2*, and three in *POPDC3* have been described (Table 4.). Generally, these variants give rise to disease in accordance with their predominant expression pattern: POPDC1 is expressed equally in the heart and skeletal muscle and patients with variants in *POPDC1* developed both limb-girdle muscular dystrophy (LGMD) and AV-block^{185,219,282}. POPDC2 is predominantly found in the heart and patients with *POPDC2* variants show AV-block of varying degrees (I-III), but no observable skeletal muscle phenotype²⁸⁰. POPDC3 is mostly expressed in skeletal muscle, and patients carrying variants in *POPDC3* display severe LGMD, but no observable cardiac defects²⁶⁶.

Patients that carry either of the *POPDC1* variants, commonly show mild to severe AV-block, muscle weakness, elevated creatine kinase (CK) levels and a wide spectrum of early and late onset of skeletal muscle disease. Most patients with *POPDC1* variants also develop LGMD. LGMD is highly diverse group of muscular dystrophies that arise due to abnormalities in proteins involved in processes such as glycosylation of dystroglycan, mechanical signalling (sarcoglycans) and in mitochondrial function²⁸³. Interestingly, three of the POPDC1 interacting

partners, namely dystrophin, anoctamin 5 and caveolin3 have also been linked to the development of LGMD^{197,199,255}. A *POPDC1* variants was also reported in a patient with Emery-Dreifuss muscular dystrophy (EDMD), which is phenotypically similar to LGMD but is caused by proteins that are part of the nuclear envelope and associated with contractures of the joints²⁸⁴. This is interesting considering the previously described findings that POPDC1 also localises to the nuclear envelope²⁰¹. There is also some evidence that POPDC1 proteins are implicated in long QT syndrome (LQTS), although the functional evidence between *POPDC1* variants and disease development affecting QT interval and QRS duration, seems to be model organism dependent^{218,285,286}.

Additionally, immunohistochemical staining of muscle biopsies from patients with *POPDC1* variants, revealed that both, the mutated POPDC1 and the physiological POPDC2, are mis-localised from the cell membrane. Based on these findings it can be hypothesised that the underlying cellular pathology of *POPDC1* variants lays in an aberrant membrane trafficking of the mutant protein^{199,219,281,282,287}. Conversely, muscle biopsies from patients carrying *POPDC3* variants did not show loss of membrane localisation of the other POPDC isoforms²⁶⁶.

Table 1.4. POPDC gene variants in patients suffering from heart and skeletal muscle disease.

Gene	Variant	Cardiac phenotype	Skeletal muscle phenotype	Reference
POPDC1	1A>G	1 st degree AV-block	LGMDR25	219
	R88X	1 st degree AV-block	LGMDR25	219
		Sinus tachycardia	LGMDR25	288
	D92G	None reported	EDMD	287
	P134L	None reported	LGMDR25	289
	R143X	1 st degree AV-block	LGMDR25	281
	Q153X	Sinus bradycardia	No	290
		2 nd degree AV-block	LGMD	
	V183F	None reported	LGMDR25	215
	I193S	Sinus bradycardia	LGMDR25	290
	S201F	2 nd degree AV-block	LGMDR25	199
POPDC2	Del56V217-K272	1 st /2 nd degree AV-block	LGMDR25	219
	S263X	1 st degree AV-block	LGMDR25	282
POPDC2	L245P	cJET	None reported	291
	W188X	1 st -3 rd AV-block	None reported	280
POPDC3	K154E	None reported	LGMDR26	292
	L155H	None reported	LGMDR26	266
	I163X	None reported	LGMDR26	293
	L217F	None reported	LGMDR26	266
	R261Q	None reported	LGMDR26	266
	c.486-1G>A	None reported	LGMDR26	294

Mutation in *POPDC1* and *POPDC3* genes are all recessive, which contrasts with the two known *POPDC2* variants, which both display a dominant trait. A novel *POPDC2*^{L245P} missense mutation was discovered in a family of four and has been linked to congenital junctional ectopic tachycardia (cJET), an extremely rare tachyarrhythmia in infants, that arises due to an abnormal automaticity in the AV node²⁹¹. The cJET pathology appears to represent a gain-of-function phenotype, although from a protein perspective, the mutant protein triggers loss of membrane localisation of both *POPDC1* and *POPDC2* when the mutant protein is co-expressed in HEK293 cells with wildtype *POPDC1*.

Another *POPDC2* variant was discovered in a group of patients that carry a heterozygous nonsense variant in *POPDC2* gene producing a cardiac phenotype characterised by AV-block I-III, with no apparent skeletal muscle phenotype²⁸⁰. The mutation gives rise to a premature stop codon at position 188 (*POPDC2*^{W188X}) which results in a truncated *POPDC2* protein²⁸⁰. The truncation causes a partial loss of the functionally important Popeye domain of the *POPDC2* protein. Surprisingly, it was shown that *POPDC2*^{W188X} can retain its ability to interact with TREK-1 and bind cAMP with similar affinity as its wild-type counterpart²⁸⁰. However, co-expression experiments in *Xenopus* oocytes identified an abnormal TREK-1 current modulation in presence of the mutant *POPDC2*^{W188X}. Knock-in mouse animal models were generated and revealed a stress-induced sinus bradycardia phenotype, similar to what was previously recorded in *Popdc2* null mutants²⁸⁰. It appears that the cardiac arrhythmia phenotype caused by *POPDC1* or *POPDC2* mutations manifests itself as sinus node dysfunction in mice, and as AV conduction abnormalities in zebrafish and humans, which is probably due to the vastly different heart rates between the species.

1.4 Hypothesis and aims

Over the last two decades since the discovery of POPDC proteins we have slowly been able to expand our understanding of their structure and function. They have an emerging role as tumour suppressors and have been implicated in skeletal muscle regeneration. They have also been identified as cAMP effectors and mutations of the *POPDC* genes in humans and animal models have shown to give rise to muscular dystrophy and cardiac arrhythmia phenotypes. An autosomal dominant *POPDC2*^{W188X} mutation was discovered in a group of patients of whom some individuals show a severe atrioventricular block. A *Popdc2*^{W188X} mouse mutant was generated, and an initial *in vivo* study has shown a stress induced bradycardia phenotype. However, no further characterisation of the mutant has been performed. Therefore, part of this work is dedicated to further characterize the homozygous *Popdc2*^{W188X} mutant mouse.

In the heart stress responses are driven by the β AR-cAMP-PKA signalling. cAMP is an important second messenger which is produced by adenylyl cyclases at the plasma membrane of cardiac myocytes in response to β -adrenergic receptor stimulation, to activate protein kinase A and induce protein phosphorylation of target proteins mediating the downstream

receptor effects. As cAMP is highly diffusible, its effectors and regulators are compartmentalised into signalling complexes to achieve signalling specificity. POPDC are thought to be important members and regulators of proteins found in these signalling complexes in cardiomyocytes. The second part of this thesis tests the hypothesis that the chronotropic incompetence of the *Popdc2*^{W188X} mutant is due to abnormal β -adrenergic signalling induced by the loss of POPDC1 and POPDC2 from the plasma membrane of *Popdc2*^{W188X} mutant cardiomyocytes.

In this project I have the following aims:

- To identify an antibody reactive with *Popdc2*^{W188X} mutant protein
- To characterise the *Popdc2*^{W188X} mutant mouse
- To investigate the cardiac expression Popdc1 and Popdc2 in the *Popdc2*^{W188X} mutant
- Utilising *ex vivo* preparations to characterize the electrophysiology of the *Popdc2*^{W188X} mutant heart in response to β -adrenergic stimulation
- To examine the effects of the *Popdc2*^{W188X} mutation on the β -adrenergic signalling in neonatal mouse ventricular cardiomyocytes

CHAPTER 2 Methods and materials

Most reagents used for this project were purchased from Sigma-Aldrich (Dorset, England) unless stated otherwise. All activities were performed according to general laboratory safety guidelines, as well as local regulations, using appropriate personal protective equipment where necessary. All locally produced buffers, media and various other solutions were prepared with water purified using the Milli-Q Integral 3 water purification system equipped with a Biopak Polisher ultrafilter. The purified water was sterilised by autoclaving if necessary, using the Priorclave Tactrol 2 autoclave.

2.1 Preparation and handling of plasmid DNA from competent *Escherichia (E.) coli* bacterial cells

2.1.1 Generation of chemically competent *E. coli*

To generate competent bacteria, 1 µl of *E. coli* glycerol stock (XL1-blue strain, Stratagene 200268) was diluted into 3ml of lysogeny broth (LB) medium (10g NaCl, 10g peptone, 5g yeast extract, in 1l of H₂O, adjusted to pH 7.5) and grown overnight at 37°C under vigorous shaking to allow for oxygenation. Next morning, 1ml of the bacterial suspension was inoculated in 100ml of fresh LB medium and grown further at 37°C and under vigorous shaking until an optical density of 0.5 at 600nm (OD₆₀₀) measured in the BioPhotometer plus (Eppendorf) was reached, resulting in the highest transformation competency. Subsequently, the *E. coli* suspension was placed on ice for 15 min to cool down and prevent further bacterial growth, before being centrifuged at 1500g for 5 min at 4°C. The supernatant was discarded, the pelleted cells resuspended in 40ml ice-cold transformation buffer I (100mM RbCl, 50mM MnCl₂, 30mM potassium acetate, 10mM CaCl₂, 15%(v/v) glycerol, adjusted to pH 5.8 by 0.1M acetic acid) and incubated on ice for a further 15 min before undergoing a second round of centrifugation as before. Once again, the supernatant was discarded and the pelleted cells resuspended in 4ml of ice-cold transformation buffer II (10mM RbCl, 10mM MOPS, 30mM potassium acetate, 75mM CaCl₂, 15%(v/v) glycerol, adjusted to pH 6.5 by 0.1M NaOH), incubated for another 15 min and aliquoted into sterile 1.5ml tubes at 100 µl per aliquot, before being snap frozen in liquid nitrogen and stored at -80°C until used for transformation.

2.1.2 Transformation of competent cells

All transformations were performed using a conventional heat shock protocol. 1µl of previously constructed and purified plasmid DNA, containing 50-100ng/µl of plasmid DNA was added to 100µl XL1 blue competent cells, which were thawed on ice and gently mixed for even distribution of the DNA followed by a 30 min incubation on ice to allow plasmid uptake. Bacteria were then heat shocked at 42°C for 45 sec and immediately cooled on ice for 2 min before 400µl LB media was added to the DNA-cell mix. In the following step, bacteria were incubated in an orbital shaker at 100 revolutions per min (rpm) for 1h at 37°C to allow for developing ampicillin (amp) resistance. Subsequently, cells were centrifuged at 1000g for 1 min, 400µl of the supernatant was removed and the cell pellet re-suspended in the remaining medium and the transformant spread onto amp containing agar plates. The agar plates were made by dissolving 15g agar-agar in 1l LB-medium, which was autoclaved and when cooled down supplemented with 100µg/ml of amp. The plates were incubated overnight at 37°C whilst kept upside-down to protect bacterial colonies from condensation.

Table 2.1. Plasmids used for the transfection of cell lines

Plasmid	Species	Tag	Antibiotic resistance	Vector	Source
Popdc1	mouse	flag	Ampicillin	pCDNA3.1 (+)	R.Schindler (Brand lab)
Popdc2	mouse	flag	Ampicillin	pCDNA3.1 (+)	R.Schindler (Brand lab)
Popdc3	mouse	flag	Ampicillin	pCDNA3.1 (+)	R.Schindler (Brand lab)
POPDC2 ^{W188X}	human	flag	Ampicillin	pCDNA3.1 (+)	R Schindler (Brand lab)
Popdc1	mouse	myc	Ampicillin	pCS2+MT	B. Andree/J.Kuthz (Brand lab)
Popdc2	mouse	myc	Ampicillin	pCS2+MT	B. Andree/J.Kuthz (Brand lab)
Popdc3	mouse	myc	Ampicillin	pCS2+MT	B. Andree/J.Kuthz (Brand lab)
AKAP79	mouse	flag	Ampicillin	pCDNA3.1 (+)	R. Efendiev (Dessauer lab)

2.1.3 Purification of plasmid DNA

To purify a large amount of plasmid DNA the Plasmid Midi Kit (QIAGEN, Hilden, order number: #12145) was used. To initiate purification a starter culture consisting of a single transformed bacterial colony was seeded into 3ml of LB media containing 100µg/ml amp and incubated in an orbital shaker (100 rpm) at 37°C for 8h. Subsequently, 1 ml of the starter culture was inoculated in 100ml of fresh ampicillin-selective LB medium and incubated at 37°C overnight in an orbital shaker (100 rpm) at 37°C. Next morning, cells were harvested by centrifugation at 1500g for 30 min at 4°C and the resulting pellet resuspended immediately in 4ml of resuspension buffer P1 (Tris-Cl, pH 8.0, 10mM EDTA, 100ug/mL RNase A). After 5 min incubation at RT, cells were lysed by adding lysis buffer P2 (200mM NaOH, 1% SDS) after careful mixing, avoiding to shearing the genomic DNA, the lysate was incubated for 5 min before 4ml of pre-cooled neutralization buffer P3 (3.0M potassium acetate, pH 5.5) was added. Following 15 min incubation on ice and 2x30-min centrifugation at 25,000g at 4°C, the supernatant containing the desired plasmid DNA was added to a QIAGEN-tip column where the plasmid DNA will bind to the resin. Prior to use the QIAGEN-tip column was equilibrated with 4ml of buffer QBT (750mM NaCl, 50mM MOPS, pH7.0, 15% isopropanol, 0.15% Triton X-100). Subsequently, the column was washed twice with 10ml of buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol) and the plasmid DNA eluted with 5ml of buffer QF (1.25M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol) into an appropriate glass centrifugation tube. To precipitate the eluted plasmid DNA 3.5ml of isopropanol was added, mixed well before being centrifuged for 30 min at 25,000g at 4°C to form a pellet. In the final steps, the supernatant was discarded, the plasmid DNA washed twice with chilled 75% ethanol before allowing the pellet to airdry for at least 10 min ensuring all traces of ethanol are evaporated. The final pellet was re-dissolved in 100µl of TE buffer (10mM Tris-HCl, pH8, 1mM EDTA). The plasmid DNA concentration was measured by Nanodrop spectrophotometer and stored at -20°C.

2.1.4 Sequencing

To verify the correct DNA sequence of constructs after cloning, DNA concentration was determined spectrophotometrically. 500-800ng of DNA per sequencing reaction as well 5µM dilutions of the appropriate sequencing primer were pipetted into separate tubes and sent to

GENEWIZ UK LTD (<https://www.genewiz.com/en-GB/Public/Service/Sanger-Sequencing>) for DNA sequencing. The resulting sequencing data were evaluated with the help of SnapGene software (www.snapgene.com).

2.1.5 Storage of Plasmid DNA and Glycerol stocks

650µl of a bacteria culture (grown overnight) were mixed with 300µl 80% (v/v) glycerol and stored at -80°C.

2.2 Mammalian Cell Line Cell Culture

2.2.1 Cell culture of Human Embryonic Kidney (HEK) and COS-7 cells

HEK293T (DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) and COS-7 cells (DSMZ) were cultured on 10 cm ø cell culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) (#91031101) substituted with 10% (v/v) foetal calf serum (FCS) (Gibco, 26140-079), and 1% (v/v) L-glutamine (#G7513) at 37°C in a 5% CO₂ humidified cell culture incubator. All cell culture procedures were undertaken under sterile conditions in a laminar flow safety cabinet.

2.2.2 Subculturing of cells

HEK293T and COS-7 cells were passaged at ~90% confluence by rinsing the culture plate with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄-2H₂O and 1.8 mM KH₂PO₄ adjusted to 1l with H₂O) and incubating the cell layer with trypsin-EDTA solution (5.0 g porcine trypsin and 2 g EDTA, 4Na/lof 0.9% sodium chloride; #T4174) for 5 min to initiate cell detachment after which the cells were centrifuged for 5 min at 500g and resuspended in fresh culture medium and plated at a 1:10 dilution

2.2.3 Transfection/ Co-Transfection of cells

Confluent HEK293T and COS-7 cell cultures were split one day before transfection and plated onto 10cm ø culture plates (company) to aim for 90% confluency at the time of transfection. In accordance with the manufacturer's protocol 1µl of Lipofectamine™2000 (Invitrogen, USA, #11668) was used per 0.4µg of plasmid DNA (plasmid isolation was done as described in 2.2.3

Purification of plasmid DNA). Appropriate amounts of LipofectamineTM2000 and plasmid DNA were diluted in serum free DMEM in separate tubes and incubated for 5 min at RT. In the case where cells were co-transfected with two different plasmid DNAs, the total plasmid DNA amount was diluted into DMEM containing tube at the appropriate ratio. Then LipofectamineTM2000 and plasmid DNA mixtures were combined, vortexed and incubated for a further 20 min at RT to allow DNA-Lipofectamine complexes to form before being added dropwise to the adherent cells. Cells were harvested 24h after transfection to allow maximum expression of the transfected construct.

2.3 Cell Lysates

2.3.1 Generation of cell lysates from cells cultures

Prior to harvest, cell cultures were transiently transfected (2.2.3.) Transfection/Co-Transfection of cells) and cultured for 24h with the appropriate plasmid DNA. To generate cell lysates, cells were washed with ice-cold PBS buffer three times and then incubated with 700µl of lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 2mM EDTA, pH 8.0, 1% (v/v) Triton X-100, 0.25% (w/v) gelatine, and 1 protease inhibitors tablet per 10 ml (Roche, Switzerland)) for 20 min at 4°C. Using a cell scraper, cells were scraped off the cell culture dish and the lysate transferred into a 1.5ml Eppendorf tube and passed several times through a 23G needle before being sonicated at 2mW three times for 15 sec at 4°C. Lysate samples were then centrifuged at 16,000g for 30 min at 4°C to remove cell debris. The supernatants were transferred into fresh, labelled tubes and stored at -20°C.

2.3.2 Determining protein concentration of cell lysates

Protein concentrations were determined using the Bio-Rad DCTM Protein microplate assay. This assay utilizes the Lowry method whereby proteins react with alkaline copper tartrate (solution A) and then are reduced by Folin (solution B) resulting in a blue-coloured solution with a maximum absorbance at wavelength of 750nm. The colour intensity is directly proportionate to the amount of protein in the sample. To quantify the amount of protein present in the samples, a standard curve was established by preparing dilutions of 0-1.2µg/µl of bovine serum albumin (BSA). Cell-lysates were diluted 1:10 and 1:100 in lysis buffer of

which 5µl were added in triplicates to a 96-well-plate alongside the standard curve dilutions. 25µl of the pre-prepared reagent A (20µl reagent S (Bio-Rad, #500-0115) in 1ml reagent A (Bio-Rad, #500-0113)) and 200µl reagent B (Bio-Rad, #500-0114) were then added to each well. The plate was incubated in the dark and at RT for 15 min, allowing the colour to develop, before absorbance was determined using a microplate spectrophotometer. The standard curve was generated using linear regression of calculated averages for the protein standards plotted against the known concentrations. SoftMax Pro® software then matched averages of the sample absorbance values to the standard curve producing an estimate protein concentration, which was multiplied by 10 or 100 to account for dilution.

2.4 Co-Immunoprecipitation

Co-IPs were performed using the ProFound Pierce c-myc-tag IP/Co-IP Kit (Thermo Scientific USA, #23620). To perform Co-IP experiments Cos-7 cells were cultured on ø 10 cm plastic dishes to 90% confluency, transiently transfected for 24h (2.2.3 Transfection/Co-transfection of cells) before being harvested for cell lysates (2.3.1. Generation of cell lysates from cell cultures). 30µl of the whole-cell lysate (WCL) was mixed with 10µl of sodium dodecyl sulphate (SDS) buffer (4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 40% (v/v) glycerol), 250mM Tris-HCl: (pH 6.8), 0.05% (w/v) bromphenol blue) and reserved as input control. The remainder of the lysate was used to conduct the Co-IP experiments as per the manufacturer's instructions. Lysates were incubated overnight with the anti-Myc agarose beads (MBL, USA, #M047-8) at 4°C in spin columns on a wheel rotator to allow the binding of antibodies to the antigens in the cell lysates. The following day, samples were centrifuged at full speed for 10 sec at 4°C to remove unbound lysates and washed 5 times in cold Tris-buffered saline solution before the non-reducing sample buffer was added. The lysate samples and the WCL input controls were then boiled at 96°C for 5 min and sample lysates were collected via centrifugation of the spin column into labelled collection tubes. To reduce the amount of antibody heavy and light chains in the sample 3µl of β-mercaptoethanol was added to each lysate sample. These prepared samples were then resolved by protein size using SDS gel electrophoresis (2.6. Sodium Dodecyl Sulphate Protein Electrophoresis) and analysed by western blot analysis (2.7. Western Blot).

2.5 Sodium Dodecyl Sulphate Protein Electrophoresis

2.5.1 Gel preparation

The Mini-PROTEAN® system from Bio-Rad was used for the SDS-polyacrylamide gel electrophoresis (PAGE). To this end, glass plates were cleaned with 70% alcohol and then assembled into plastic holders and mounted onto gel cast stands. The 10% SDS-PAGE gel solution (4ml H₂O, 3.3ml 30% (w/v) acrylamide solution, 2.5ml 1.5M Tris-Cl pH8.8, 100µl 10% (w/v) SDS, 100µl 10% (w/v) APS and 4µl TEMED) was poured between the glass plates and carefully overlaid with water for polymerisation and an even transition-edge for the stacking gel. Once the gel was polymerized the water was removed and the stacking gel (1.4ml H₂O, 330µl 30% (w/v) acrylamide solution (Bio-Rad, 1610154), 250µl 1.0M Tris-Cl pH6.8, 20µl 10% (w/v) SDS, 20µl 10% (w/v) APS and 2µl TEMED) was poured over the separation gel and an appropriately sized comb was inserted. Once the stacking gel had also polymerised, the gel cast stand was disassembled, the gel cast rinsed with water and the gel either used immediately for gel electrophoresis (2.5.2) or wrapped in a wet paper towel and stored in a tightly sealed plastic bag at 4°C until further use.

2.5.2 Gel Electrophoresis

To perform gel electrophoresis, the gel was inserted into the gel electrophoresis chamber and filled with running buffer (25mM Tris-Cl, pH8.0, 192mM glycine, 1% (w/v) SDS). The comb was removed, the wells briefly washed with running buffer to remove any un-polymerised residues that could interfere with the running of the samples. Boiled samples were then loaded onto the gel at predetermined protein concentrations alongside molecular weight markers (PageRuler™ Prestained Protein Ladder, Thermo Scientific, #26616) and resolved at 100V-150V until the bromphenol blue dye front from the sample buffer reached the bottom of the gel. The separation gel was then removed from the glass plates and the stacking gel discarded.

2.6 Western Blot

2.6.1 Transfer to nitrocellulose membrane

Once proteins from the cell lysate samples were separated based on their size via gel electrophoresis, they were transferred onto nitrocellulose membrane (BioTrace™ NT, Pall Corporations, USA, #66485) by a semi dry transfer method. To this end, gels were briefly incubated in blotting buffer (10% (v/v) methanol, 25mM Tris-Cl, pH8.5, 150mM glycine) and then placed onto the blotter (BioTrace™ NT, Pall Corporations, USA, #66485) in the following order: 3 pieces of blotting buffer soaked Whatman filter papers, followed by the separation gel, followed by the nitrocellulose membrane and topped by 3 more blotting buffer-soaked pieces of filter paper. Proteins were then electrophoretically transferred onto the nitrocellulose membrane at 70mA/gel for 1h.

2.6.2 Incubation with antibodies

To visualise specific proteins of interest from the cell lysates, the protein containing nitrocellulose membrane was washed with Tris-buffered saline with Tween (TBST) (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% (v/v) Tween-20) and blocked in blocking buffer (5% milk powder (w/v) in TBST) for 1h at RT before being incubated with the appropriate primary antibody (Table 6) over night at 4°C with gentle rotation. Next day, the membrane was washed with TBST three times for 10 min to remove any unbound primary antibody and then incubated with the appropriate secondary antibody (Table 6) for 1-2h on the rotator. Membranes were then washed in TBST 3-times as previously described and imaged in the syngene g:box imager using horseradish peroxidase substrate (Millipore, USA, #WBKLS0500).

2.6.3 List of antibodies

Table 2.2. Antibodies used for Western Blot analysis

Antibody	Species	Clonality	Source	Catalogue No
Popeye 2 (V-13)	goat	polyclonal	Santa Cruz	sc-66588
Anti-POPDC2	rabbit	polyclonal	Millipore	06-1007
Anti-POPDC2	rabbit	polyclonal	Atlas Antibodies	HPA024255
Anti-Popdc2 12G5	mouse	monoclonal	Dr Ian Holt, Keele University	N/a
Anti-Popdc2 12G12	mouse	monoclonal	Dr Ian Holt, Keele University	N/a
Anti-Popdc2 13C4	mouse	monoclonal	Dr Ian Holt, Keele University	N/a
Anti-Popdc2 9H10	mouse	monoclonal	Dr Ian Holt, Keele University	N/a
mNET3 11765	Rabbit	polyclonal	Prof. Eric Schirmer, University of Edinburgh ³⁰³	N/a
mNET3 11766	Rabbit	polyclonal	Prof. Eric Schirmer, University of Edinburgh ³⁰³	N/a
mNET3 11768	Rabbit	polyclonal	Prof. Eric Schirmer, University of Edinburgh ³⁰³	N/a
Anti-POPDC2	mouse	monoclonal	Sigma -Aldrich	SAB 1407852
Anti- POPDC2 ^{W188X}	human	monoclonal	Prof. Niels Decher, Philipps University of Marburg ²⁷⁵	N/a
Anti-Myc-Tag (9E10)	mouse	monoclonal	Abcam United Kingdom	Ab32
Anti-Flag-Tag (FLAG M2)	mouse	monoclonal	Sigma-Aldrich	F1804
Anti-Flag DYKDDDDK (FG4R)	mouse	monoclonal	Life technologies	MA1-91878
Anti GPF-tag Abcam, United Kingdom	rabbit	polyclonal	Abcam, United Kingdom	Ab290
HRP-conjugated anti-Rabbit	goat	polyclonal	Vector Laboratories, USA	PI-1000

HRP-conjugated anti-mouse	horse	polyclonal	Vector Laboratories, USA	PI-2000
Peroxidase labelled anti-goat IgG (H + L)	horse	polyclonal	Vector Laboratories	PI-9500

2.7 Mouse work

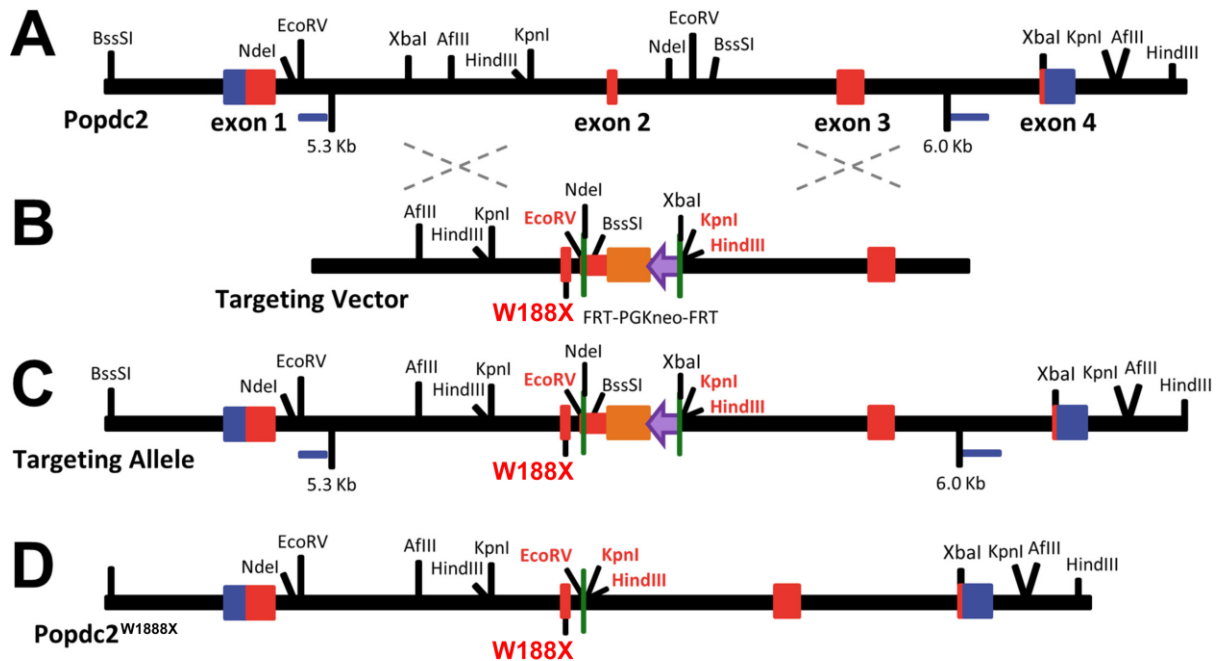


Figure 2.1. Targeting strategy to produce the *Popdc2*^{W188X} knock-in mutant by homologous recombination. Schematic depiction of (A) the wild-type *Popdc2* allele. The *Popdc2* gene consists of 4 exons (coding sequence (red) and 5'- and 3'-untranslated regions (blue)), (B) the targeting vector to introduce the *Popdc2*^{W188X} mutant allele by homologous recombination, (C) the targeted allele carrying the *Popdc2*^{W188X} mutation and (D) the *Popdc2*^{W188X} allele after removal of the neomycin selection cassette. The approximate positions of 5' and 3' probes, which were used for Southern blot analysis are depicted as horizontal blue lines. Figure was taken in modified form from S. Rinné, et al. *Journal of Molecular and Cellular Cardiology* 145 (2020) 74–83.²⁸⁰

2.7.1 Generation of *POPDC2*^{W188X} mutant

The *POPDC2*^{W188X} knock-in mouse model was generated by homologous recombination. First, the homology arms of 5.3kB and 6kB length, respectively were PCR amplified using a bacterial artificial chromosome (BAC) containing the genomic region of *POPDC2* as template (Fig.2.1). Subsequently the homology arms were subcloned into the pDTA-Amp plasmid. For selection purpose, a PGK-neo cassette was placed into exon 2 of *POPDC2* producing a deletion of the coding sequence for 5 amino acids (aa184-188). The *POPDC2*^{W188X} mutation was introduced using double stranded oligonucleotides, that substituted the selectable/counter selectable cassette. Additionally, a EcoRV-FRT-neoFRT-KpnI-HindIII cassette was inserted 300 bp downstream of exon 2 of the *POPDC2*^{W188X} mutation. Successful insertion of the targeting constructs was verified by sequencing. The targeting vector was linearised and introduced into a C57BL/6J ES cell line by electroporation. 14 positive clones were selected for expansion

and re-examination based on Southern blot analysis of genomic DNA of G418-resistant clones using 5'- and 3'-homology arm-specific DNA probes. Of the 14 probes, four clones were verified through sequencing to carry FRT sites and the point mutation and subsequently injected into mouse blastocysts and transferred into foster mothers. Of which one produced nine high-percentage chimeras. Three male chimeras were used for germ line transmission. Heterozygotes were bred for the generation of the homozygous *Popdc2*^{W188X} knockin mutant line.

2.7.2 Mouse husbandry

All animals were maintained and utilized in accordance with the Animals in Scientific Procedures Act 1986 (ASPA, 1986).

The wildtype C57Bl/6J0laHsd strain from Envigo, UK (abbreviated as WT) and homozygous *Popdc2*^{W188X} mutant mice (abbreviated as P2X) were fed standard mouse chow, to which they had unrestricted access. They were housed at a density of 4-6 animals per cage. Breeding cages were set up at a ratio of two females to one male to produce pups for neonatal work. Animals were adults from three months of age. Pups were collected at 0-2 days of age. Both female and male animals were used indiscriminately for all experiments unless stated differently. Mice were maintained on a 12h light-dark cycle at 21°C.

2.7.3 Isolation and culture of neonatal mouse ventricular cardiomyocytes

Single cell suspensions of structurally integral neonatal mouse ventricular cardiomyocytes (NMCM) were isolated using a neonatal heart dissociation kit for mouse and rat (Miltenyi Biotec, Germany, #130-105-420). Following schedule 1 killing of 1-2 days old mouse pups of WT and homozygous *POPDC2*^{W188X} mutant genotype, respectively, the hearts were excised, placed into cold, phenol red-free Hanks' Balanced Salt Solution (HBSS) (#14175053) and cleaned of excess tissue and blood. The aorta and atria were dissected and discarded and the ventricles cut into 1-mm³ cubes. The tissue samples were then incubated with enzymes 1&2 at 37°C for 15 min in a gentle MACS M tube (Miltenyi Biotec, Germany, #130-093-237) before being spun on the GentleMACS Dissociator (Miltenyi Biotec, Germany, #130-093-235) using the neonatal protocol. This process was repeated three times. The combined tissue digest

was then re-suspended in substituted 10% M199 (#M7653) medium (10% (v/v) foetal calf serum, 0.5% pen/strep antibiotics, 0.5% Vitamin B12 and 1% L-glutamine) filtered using MACS SmartStrainers (70 μm) (Miltenyi Biotec, Germany, #130-098-462) and centrifuged at 1000g for 5 min to collect isolated cells. To reduce fibroblast contamination, the cell suspension was resuspended in 20ml of 10% M199 medium and pre-plated in a T75 cell culture flask (#156800) in a humidified cell culture incubator at 37°C and 1% CO₂ for 1h before being strained through 70 μm filter. The NMCMs were seeded onto laminin I (Bio-Techne, #3446-005-01) (40 $\mu\text{g}/\text{ml}^{-1}$) coated dishes of a size and at a density appropriate for the experimental approach and cultured in 5% M199 medium (containing 5% (v/v) foetal calf serum and otherwise identical to 10% M199) in a humidified cell culture incubator at 37°C and 5% CO₂. All procedures following heart excision were undertaken under sterile conditions in a laminar flow safety cabinet.

2.7.4 Transfection of NMCM

NMCMs were isolated and cultured on laminin-coated 25mm glass coverslips at a density of 6×10^4 cells/coverslip for 24h before they were transfected with the cytosolic mTurquoiseD-Epac(CD, DDEP)-cp173- Venus-Venus (^TEpac^{VV} 295) sensor constructs (2.10.1. FRET biosensors) using LipofectamineTM 3000 (Life technologies, #L3000008) as per manufacturers instruction. Briefly, 1 μl of LipofectamineTM 3000 for each 1 μg of the reporter DNA was added to the appropriate amount of Opti-MEM (Life Technologies, #11058-021) medium. A master mix of plasmid DNA was prepared by diluting 1 μg of DNA/coverslip and 2 μl of P3000 reagent per 1 μg of DNA in the appropriate amount of Opti-MEM medium and incubated for 5 min. The Lipofectamine3000 mix and the DNA master mix were then combined and incubated at RT for 15 min before being added to the adherent cells. Cells were imaged 40-48h after transfection.

2.7.5 Isolation and culture of adult cardiomyocytes

The isolation of adult mouse ventricular cardiomyocytes (AMCM) was performed by Dr Laura Fedele and cells were gifted to me for use in the FRET experiments. To isolate AMCMs, 3 to 4-month-old mice were sacrificed by 5% isoflurane inhalation followed by cervical dislocation to confirm death. The heart was swiftly removed and placed in ice-cold Krebs-Henseleit solution (119mM NaCl, 4.7mM KCl, 0.94mM MgSO₄, 1mM CaCl₂, 1.2mM KH₂PO₄, 25mM

NaHCO₃, and 11.5mM glucose (pH 7.4)). The heart was cleaned of excess tissue to free the aorta, which was then cannulated, secured with a silk thread and attached to the Langendorff system. First, the heart was retrogradely perfused at 37°C with Krebs-Henseleit solution for 1 min, then the perfusate was switched to a low-Ca²⁺ solution (120mM NaCl, 5mM KCl, 5mM MgSO₄, 5mM Na pyruvate, 20mM glucose, 20mM taurine, 10mM HEPES, 5mM nitrilotriacetic acid and 35μM CaCl₂ (pH 6.96) for 4 min, followed by 1 min bacterial protease solution (3.6 mg of protease (Sigma, P8038-1G) in 10 ml of enzyme solution (120mM NaCl, 5mM KCl, 5mM MgSO₄, 5mM Na pyruvate, 20mM glucose, 20mM taurine, and 10mM HEPES, 35mM CaCl₂ (pH 7.4)) followed by 4-5 min of collagenase V solution (30mg of collagenase V (Sigma, C9263-1G) in 30 ml of enzyme solution). The hearts were then removed from the cannula, and the ventricles triturated in enzyme solution (without enzymes). The cell suspension was passed through a mesh to remove any undigested tissue.

The isolated adult cardiomyocytes were counted and seeded onto laminin coated MatTek dishes (MatTek Corporation, #P35G-1.5-14-C) at a density of approximately 400 cells per dish in a Minimum Essential Medium (MEM) (Thermo Fisher Scientific, #10370047)) substituted with 2.5% FBS, 100units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 9mM NaHCO₃. Cells were allowed to adhere at 37°C with 5% CO₂ for 2h after which the medium was replaced with FBS-free MEM with 20μM Blebbistatin (Bio-Techne, #1760).

2.7.6 Transduction of neonatal and adult cardiomyocytes

Following isolation, neonatal and adult myocytes were infected for 3h at a multiplicity of infection of 80-110 with adenovirus encoding for the CUTie or AKAP79-CUTie reporters (2.10.1. FRET biosensors). The medium was then replaced with fresh medium (according to cell type) containing 0.5μM cytochalasin D (Sigma-Aldrich, #C8273). Cells were imaged after 40-48h of culture.

2.8 Immunostaining

2.8.1 NMCM preparation for immunostaining

Immediately after isolation, NMCMs were counted and seeded onto laminin coated $\varnothing$ 13mm round glass coverslips placed in each well of a 24-well-plate. NMCM were seeded at a density of 2×10^4 cells/cover slip by pipetting 50 μ l of the cell suspension in 5% M199 onto the coverslip using water tension which allowed a media bubble to form without overflowing. NMCM were allowed 2h to adhere to the laminin coated glass cover slip before the culture dishes were flooded with 5% M199 media and incubated for a further 24h in a humidified cell culture incubator at 37°C and 5% CO₂. For fixation, NMCMs, were fixed for 10 min using 4% PFA at RT and then washed 3 times for 5 min with PBS to remove all traces of the fixative.

2.8.2 Incubation with antibodies

To assess the presence and localisation of proteins of interest in the isolated NMCM immunostaining was performed by incubating the fixed cell samples with primary and then secondary antibodies. To this end, fixed NMCM were first incubated in blocking buffer (2% FCS or donkey serum, 1%BSA and 0.25% Triton X-100 in PBS) for 1h at RT. The BSA and serum in the blocking solution prevent unspecific binding of the antibody and the triton X-100 is a detergent, which helps to permeabilise the cell to allow the antibodies to reach their specific antigens. Samples were then incubated overnight at 4°C with the appropriate dilution of primary antibody in blocking buffer. Next morning, NMCM samples were washed 3 times for 5 min in PBS and incubated with the appropriate secondary antibody (Table 6) also diluted in blocking buffer at RT for 1h in the dark before they were washed 3 times for 5 min in PBS. The coverslips were loaded with VECTASHIELD® antifade mounting medium (Vector laboratories, #H-1200) containing 4',6-Diamidino-2-Phenylindole (DAPI) and mounted onto microscope slides to be imaged. In the case of a co-immunostaining, both primary and the respective secondary antibodies were incubated at the same time if the primary antibodies were raised in different host animals, e.g., mouse and rabbit. In case they were raised in the same host the protocol described in 2.8.3 was followed.

2.8.3 Double staining with primary antibodies raised in the same host species

Immunostaining with primary antibodies that were raised in the same host species required the use of highly concentrated (1:50 dilution) Alexa flora 555-conjugated Fab fragments to saturate the binding site of the first primary antibody, effectively serving as the secondary antibody to the first primary antibody. The samples were incubated at RT for 2h and then washed with PBS three times for 5 min to remove all traces of the conjugated Fab fragments, before they were incubated with the second primary antibody over night at 4°C, from which point on standard protocol for secondary antibody was followed (2.8.2. Incubation with antibodies).

Table 2.3. Antibodies used for immunostaining

Antibody Name	Host Species	Manufacturer	Catalogue No
POPDC1 (anti-BVES)	Rabbit	Atlas	HPA018176
POPDC2	Rabbit	Sigma Aldrich	HPA024255
β -catenin	Mouse	Thermo Fisher Scientific	13-8400
Anti-rabbit Alexa flora 488	Donkey	Invitrogen	A21206
Anti- mouse Alexa flora 488	Donkey	Invitrogen	A21202
Anti- goat Alexa flora 488	Donkey	Invitrogen	A11055
Anti-rabbit Alexa flora 633	Goat	Invitrogen	A21071
Wheat germ agglutinin (WGA) tetramethyl rhodamine conjugate	n/a	Invitrogen	W849

2.8.4 Imaging and image analysis

Following the fluorescent immunostaining the samples were imaged using the Zeiss LSM-780 confocal microscope provided and managed by the Facility for Imaging by Light Microscopy (FILM) at Imperial College London. An argon laser was used to achieve fluorophore excitation and the images recorded using either 40x or 63x objectives. Images were recorded by sequential multi-channel scanning to reduce fluorophore bleed through and managed using the ZEN black edition (Zeiss, Germany) software.

2.8.5 Quantifying fluorescent images

Images were processed and quantified using Fiji is just ImageJ (Fiji) software, which was also used to generate immunostaining figures. To quantify the fluorescence intensity, regions of interest were marked, usually a single cell or a group of cells, the average pixel intensity calculated using the measurement function, a threshold set for a positive fluorophore signal and then the data measured against data from a negative control, which only showed unspecific Alexa Fluro binding. To ensure comparability between samples the threshold was maintained constant between all images of the same experiment.

2.9 Quantifying RNA levels

2.9.1 Treatment of cultured NMCM with cycloheximide

To compare expression levels for *Popdc1*, *Popdc2* and *Popdc3* in WT and *Popdc2*^{W188X} mutant mice, NMCM were isolated and cultured for 24 h (2.7.3. Isolation and culture of ventricular neonatal mouse cardiomyocytes) and then either left untreated or treated with 100µg/ml of cycloheximide in 5% M199 media for 4h to block protein synthesis.

2.9.2 Isolation of RNA from isolated NMCM

NMCM were treated with reagents where appropriate and their total RNA extracted using the RNeasy Mini Kit (Qiagen, #74104) according to manufacturer's instructions. Prior to lysis, cells were washed with PBS three times. To generate cell lysates suitable for RNA isolation, two-thirds of the total amount of the RLT lysis buffer substituted with 10µl/ml β-mercaptoethanol was pipetted directly onto the cells and incubated on ice under gentle movement for one minute. To ensure complete lysis, the cells were scraped using a cell scraper and the lysate was pipetted up and down three times before it was collected in a 1.5ml Eppendorf tube. The well was then washed with the remaining 1/3 volume of lysis buffer to ensure all cell material was collected. The total lysate was mixed at a 1:1 ratio with 70% ethanol before it was added to the RNeasy mini column placed in a 2ml collection tube and centrifuged for 15 sec at 8,000g. The silica gel membrane was washed with 350 µl of RW1 buffer and incubated with 10 µl RNase-free DNase I stock solution in 70 µl RDD buffer (Qiagen, #79254) for 15 min at RT, before it was again washed with RW1 buffer once and RPE buffer

twice. Any remaining RPE buffer was removed by centrifugating the sample for 1 minute at maximum speed at 4°C. The RNA was then eluted with 20µl RNAase-free water and RNA concentration, and purity were measured using a nanodrop spectrophotometer. Total RNA samples were snap-frozen and stored at -80°C.

2.9.3 Isolation of RNA from adult mouse ventricular tissue

The adult mouse ventricular tissue used in this set of experiments was collected from male and female mice between the ages of 3-6 months. Animals were sacrificed by inhalation of 5% isoflurane followed by cervical dislocation and decapitation to confirm death. The thoracic cavity was opened, and the heart was extracted promptly and immediately placed into ice cold HEPES buffered Tyrode's solution (130mM NaCl, 5.4mM KCl, 0.3mM NaH₂PO₄, 1mM MgCl₂, 10mM HEPES, pH 7.4). There it was cleaned from any excess tissue to reveal the aorta, which was cannulated, fixed with a silk thread and briefly perfused through the coronary circulation to remove any excess blood. The ventricles were then cut into several small tissue samples and snap frozen to be stored at -80°C until further use. To extract the total RNA from tissue, RNeasy Mini Kit (Qiagen, #74104) was used according to manufacturer's instructions. RLT lysis buffer was substituted with β-mercaptoethanol and the appropriate amount pipetted into a gentleMACS M Tube (Miltenyi Biotec, Germany, #130-093-237). 15mg of frozen ventricular tissue was carefully placed into the RLT lysis buffer, and homogenised using a gentleMACS Dissociator (Miltenyi Biotec, Germany, #130-093-235) using the gentleMACS Program RNA_02. The sample was then centrifuged at 2,000g for 1 minute at 4°C to pellet cell debris, the lysate transferred into labelled 1.5ml Eppendorf tubes and centrifuged at 4°C for a further 3 min at 16,000g. The supernatant containing RNA was removed and mixed 1:1 with 70% ethanol. The RNA was then isolated using the same protocol as described above.

2.9.4 Generating cDNA from RNA

Isolated RNA from NMCM and adult mouse ventricles was reverse transcribed and converted to cDNA using the iScript™ Synthesis kit (Bio-Rad, #1708890). 500ng of total RNA was added to 4µl of iScript reaction mix and 1µl of iScript reverse transcriptase and nuclease-free water was added to a total volume of 20µl per sample. The sample mix was then transferred to the Polymerase chain reaction (PCR) machine and run under the following programme: 5 min at

25°C, 35 min at 42°C and 5 min at 85°C. The finalized cDNA was then diluted 1:10 and stored at -20°C until further use.

2.9.5 Quantitative polymerase chain reaction

Quantitative polymerase chain reactions (qPCR) were set up in triplicates in a 384-well optical plate in accordance with the manufacturers protocol for PowerUp™ SYBR™ green (Applied Biosciences, A25742). The qPCR-mix per well consisted of 7.5ng of cDNA, 10mM forward and reverse primers (Table 2.5.), respectively and 10µl of SYBR green master mix reagent and nuclease-free water was added to a total volume of 20µl per sample. The plate was loaded and run on an Applied Bioscience Quanti studio 6 qPCR machine using the standard cycling mode for 40 repeats.

Table 2.4. qPCR thermal profile

Procedure	Temperature	Time
Hold Stage	50°C	2 min
	95°C	2 min
PCR stage 40 repeats	95°C	15 sec
	60°C	1 minute
Melt Curve	95°C	15 sec
	60°C	1 minute
	95°C	15 sec

2.9.6 Quantification of qPCR results

The results of the qPCR run were provided by the qPCR machine in form of CT values, with a higher CT value equating to a higher expression level in the sample. To quantify the results, the CT values of the triplicate reactions were averaged and normalised against the average CT value of the control samples by subtraction, which produced a Δ CT value. The actual relative expression was then calculated by taking 2 to the power of $-\Delta$ CT and multiplied by 100, which produced the final $\Delta\Delta$ CT analysis²⁹⁶.

2.10 CytoCypher

CytoCypher is a sophisticated software platform designed to quantify cellular morphology and mechanical properties, particularly in the context of high-throughput imaging and analysis. It integrates image analysis, machine learning, and computational techniques to measure and assess various characteristics of cells, such as shape, size, and structural organization. Although originally developed for broader cell analysis, CytoCypher can also be employed to measure the size and other features of isolated adult cardiomyocytes²⁹⁷. Here, CytoCypher was used to measure and compare the size of isolated AMCM from wild type and *Popdc2*^{W188X} mutant mice. AMCM were isolated as per protocol described in section 2.7.5 Isolation and culture of adult cardiomyocytes²⁹⁷. Isolated cells were plated on laminin-coated coverslips or Matek™ (Matek Corp, United States) dishes in culture media containing FBS (MEM salts 10.74 g/L, NaHCO₃ 9 mM, L-Glutamine 1%, and Penicillin/Streptomycin 1%). Cells were then allowed to attach for 1 h at 5%CO₂ at 37°C, before the dishes were supplemented with 1ml of MEM. For the measurements, dishes containing the attached cells were placed on a static observation platform and recorded by a high-resolution camera (IonOptix MyoCam S3 Video Camera, IonOptix LLC, United States) with a scanning microscope (CytoCypher Microscope Module with 35mm Dish Insert, CytoCypher BV, Netherlands). Several viable, individual cells were selected, oriented within the presented mosaic field, and optimised for sarcomere length detection, before scanning. To collect morphological data a segmentation mask was generated using an inhouse produced macro²⁹⁷ in FIJI in conjunction with the freeware image analysis program Cytospectre²⁹⁸.

2.11 Förster Resonance Energy Transfer

FRET²⁹⁹ employs non-radiation energy transfer by long-range dipole-dipole coupling between a fluorophore which serves as the donor and a chromophore which serves as the acceptor of energy. The energy transfer between them is proportional to the distance between the donor and acceptor and is therefore highly sensitive to any change in distance and/or geometric orientation of the fluorophores. Two parameters are paramount for FRET: overlapping donor spectrum emission and acceptor absorption spectrum (1-10nm) and acceptor-donor orientation being within the Förster distance^{300,301}.

2.11.1 FRET biosensors

To measure various live biological events different reporter molecules that use FRET were developed. For this body of work EPAC and PKA based FRET sensors were utilised to measure live cAMP levels in isolated neonatal and adult mouse cardiomyocytes at baseline and after treatment.

The ^TEpac^{vv} sensor²⁹⁵ is constructed based on the truncated form of the Epac1 protein which is stripped of the membrane-targeting DEP-sequence (DDEP) and made catalytically dead (CD), with only one cAMP binding domain flanked amino-terminally by the mTurquoise (ex. 434nm, em. 477nm) and carboxy-terminally by the Venus (ex. 515nm, em. 528nm) fluorophores. mTurquoise is a very bright and single-exponentially decaying CFP variant with a more-than-double quantum yield for an improved signal-to-noise and minimal excitation damage. In an unbound state, close proximity of the fluorophores results in high FRET, whilst cAMP binding induces conformational change that leads to a change in distance between the fluorophores and a proportionate reduction in FRET.

The cytosolic CUTie³⁰² (cAMP Universal Tag for imaging experiments) and the plasma membrane AKAP79-targetted CUTie³⁰² (AKAP79-CUTie) sensors (made available by Prof. Julia Gorelik lab, with permission to use by Prof. Manuela Zaccolo, Oxford University) are based on the cAMP-binding domain of the protein kinase A regulatory subunit type II β and use ECFP as the donor and EYFP as the acceptor. cAMP binding in these biosensors induces closer proximity between the fluorophores and an increased FRET.

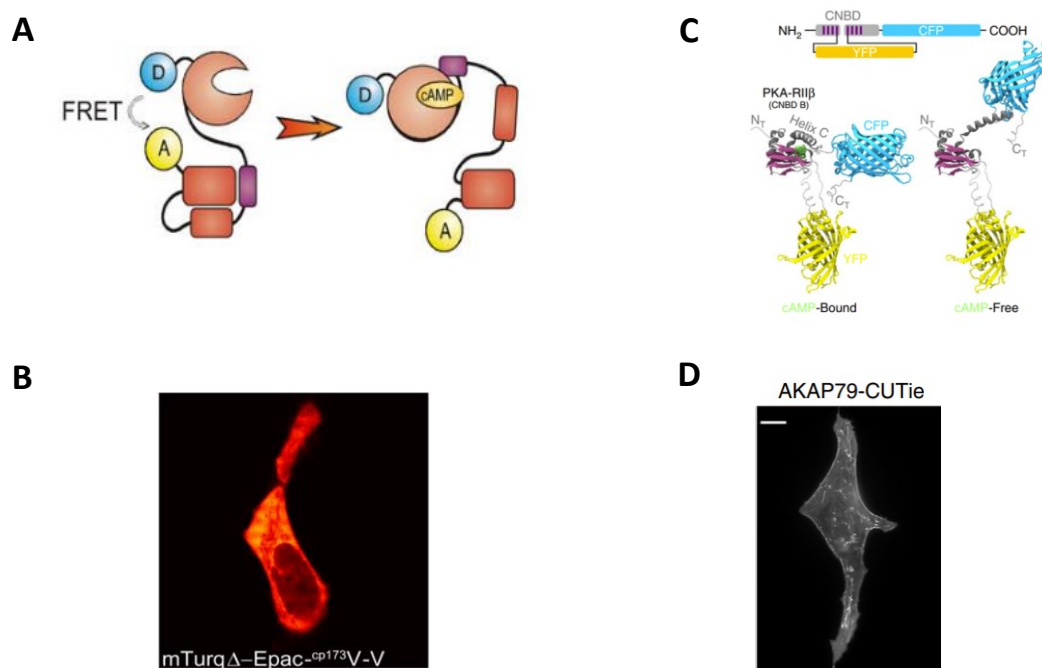


Figure 2.2. Graphic depiction of the TEpacVV and CUTie FRET biosensors for cAMP and a visual depiction of their cellular expression (taken from Klarenbeek et al. (2011)²⁹⁵, and Surdo et al. (2017)³⁰²). (A) Graphic of the TEpacVV sensor in a cAMP-bound and a cAMP-free states including their respective fluorophore positioning for the generation of FRET. (B) HEK293 cells expressing TEpacVV sensor showing cytosolic localisation 18h after transfection (C) Top: schematic of the CUTie biosensor, bottom: ribbon representation of the predicted molecular structure of CUTie biosensor in its cAMP-bound and cAMP-free forms, cAMP is shown in green. (D) Neonatal rat ventricular myocyte expressing the membrane targeted AKAP79-CUTie biosensor.

2.11.2 Pharmacological protocol for FRET microscopy

FRET imaging was performed 40-48h after transfection or transduction of neonatal or adult mouse cardiomyocytes (2.7.4. Transfection of NMCM and 2.7.6. Transduction of neonatal and adult cardiomyocytes) in physiological FRET buffer (144mM NaCl, 10nM HEPES, 1nM MgCl₂, 5mM KCl, and then equilibrated to pH7.4). To this end, cells were washed with FRET buffer, secured in the coverslip holder and flooded with an appropriate amount of FRET buffer before they were placed onto the microscope for imaging. Utilising the MultiFRET software cells of interest were selected as regions of interest (ROI) and cAMP levels were recorded at baseline and after pharmacological stimulation with different combinations of agonists and antagonists as described in Table 9. Where appropriate, cells were first exposed to a pre-treatment with an appropriate reagent (Table). After the final treatment, cells were stimulated with 100μM 3-isobutyl-1-methylxanthine (IBMX) (Abcam, #ab120840), a non-specific PDE inhibitor and 50μM Forskolin (FSK) (Sigma-Aldrich, #F6886), an adenylyl cyclase

activator, to produce the maximal cAMP release, which was used as the normalisation standard.

Table 2.5. Pharmacological protocol for FRET microscopy of neonatal and adult cardiomyocytes

	Cells	Biosensor	Pre-treatment	Treatment	Normalisation standard
Series 1	NMCM WT/ POPDC2 ^{W188X}	^T Epac ^{VV} CUTie AKAP79-CUTie	N/A	1nM, 10nM, 100nM ISO	100μM IBMX/ 50μM FSK
Series 2	NMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	N/A	50μM FSK	100μM IBMX/ 50μM FSK
Series 3	NMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	N/A	100nM PGR1 100nM ISO	100μM IBMX/ 50μM FSK
Series 4	NMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	50nM ICI, a selective β ₂ -AR antagonist or 100nM CGP, a selective β ₁ -AR antagonist	100nM ISO	100μM IBMX/ 50μM FSK
Series 5	NMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	3-4h with 1.5μg/ml Pertussis Toxin	100nM ISO	100μM IBMX/ 50μM FSK
Series 6	NMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	2h with either PDE4-POPDC1 disruptor peptide or PDE4-POPDC1 scrambled peptide	1nM, 100nM ISO 10μM Rolipram	100μM IBMX/ 50μM FSK
Series 7	AMCM WT/ POPDC2 ^{W188X}	AKAP79-CUTie	N/A	100nM ISO	100μM IBMX/ 50μM FSK

Isoproterenol (ISO) (Sigma-Aldrich, #I5627) a nonselective β₁-AR and β₂-AR agonist, Prostaglandin E₁ (PGR1) (Cambridge Bioscience, #CAY13010) a prostaglandin E₂ receptor agonist, Rolipram (Sigma-Aldrich, #R6520) a selective PDE4 inhibitor, ICI is short for ICI 118,551 a selective β₂-AR antagonist, CGP is short for CGP20712A a selective β₁-AR antagonist of which both were gifted to me by the Gorelik group, Imperial College London. PDE4-POPDC1 disruptor and PDE4-POPDC1 scrambled peptides²¹⁸ were a gift from Professor George Baillie, University of Glasgow.

2.11.3 Microscopy systems

To image NMCM transfected with The ^TEpac^{VV}, a Nikon TE2000 inverted microscope, equipped with mercury lamp (HB010303W12, Osram) a Nikon 60x/1.40 Plan Apo objective, a dual-view beam splitter (Optical Insights, Santa Fe, NM), EX436/20 excitation filters, a DM455 dichroic mirror and an ORCA-ER CCD camera (Hamamatsu Photonics, Welwyn Garden City, UK) were used. This method of signal capture allowed only single cell analysis.

A high-throughput real-time measurement tool developed by the Gorelik group, was used for neonatal and adult mouse cardiomyocytes carrying CUTie and AKAP79-CUTie biosensors. In this set up, to allow recording of multiple cells in one experiment, a mechanical sample stage and a focus drive were used (Märzhäuser Wetzlar, Wetzlar, Germany) in combination with the new MultiFRET software that runs in Icy imaging suit (open source, <http://icy.bioimageanalysis.org>). Cells are visualised using an inverted Nikon TE2000 microscope with a Nikon 60x/1.40 Plan Apo objective, a 430 nm LED light source (Cairn Research, Faversham, Kent UK) and a long-pass 455 nm dichroic mirror (DM455, Omega, Brattleboro, VT, USA). The reflected light passed through a beam-splitter QuadView (Photometrics, Tucson, AZ, USA) where it was split into four channels before it reached the ORCA-Flash 4.0 camera (Hamamatsu Photonics, Welwyn Garden City, UK). FRET signals were recorded and analysed using the Micro-Manager 1.4 software (open source, Vale Lab, University of California, San Francisco, CA, USA) that is integrated into MultiFRET. FRET signals were recorded every 6 sec and stacked as one image.

2.11.4 FRET calculations

The MultiFRET³⁰³ software automatically calculates the FRET ratio between the donor and the acceptor signals, minus the corresponding background bleed-through signals. These values are exported into Excel after data acquisition is completed. FRET response per stimulant can be calculated using these FRET ratios. First the mean of the baseline plateau (P_{base}) and the plateau for each stimulant (P_{stim}) are calculated by averaging FRET ratio values of the last 10 acquisitions. Baseline plateau is defined as FRET ratio values before stimulation. Stimulant plateau is defined as FRET ratio values after the curve plateaus following a pharmacological stimulation. The means are used to calculate the change (C) from the baseline caused by each stimulant. This shift is then normalised to the change calculated from baseline to the normalisation standard plateau (S_{max}) and the %FRET shift is calculated.

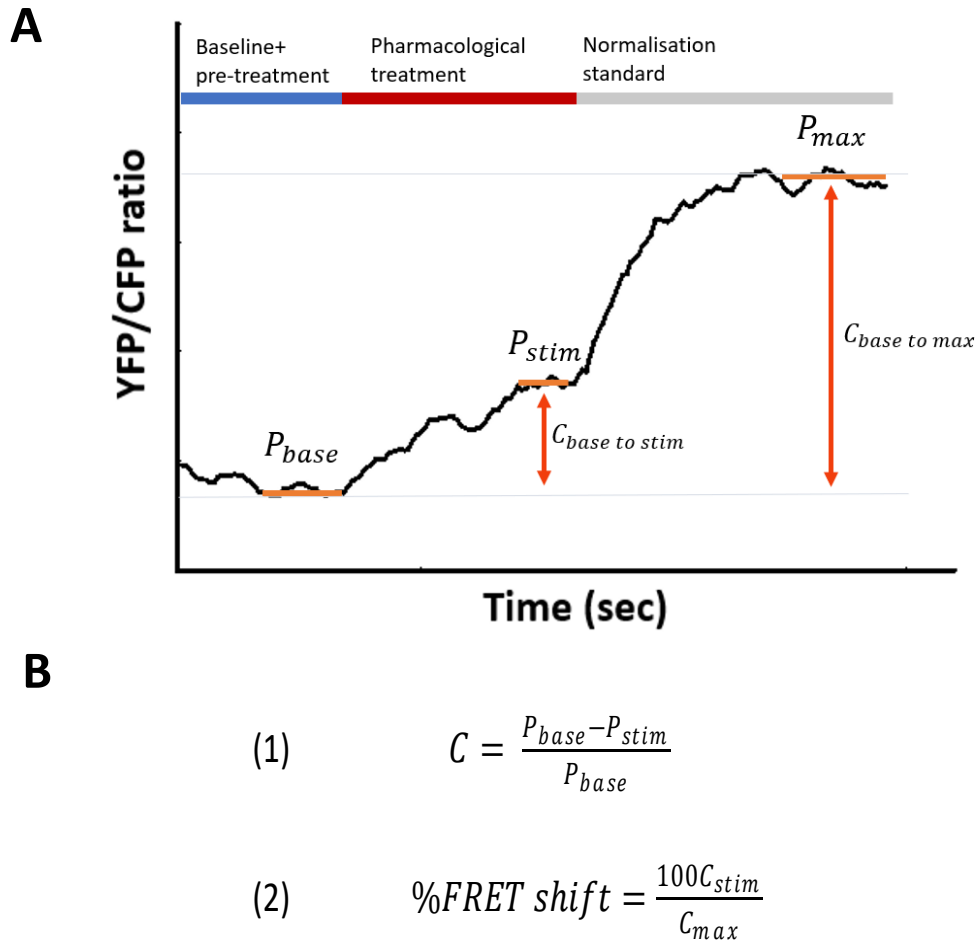


Figure 2.3. Visualisation of the pharmacological protocol on example traces showing kinetics of cAMP change (A) and equations used to examine the measurements generated by the protocol (B).

2.12 Langendorff-perfused heart model

The Langendorff perfused heart model²⁹⁹ was used to study the electrical activity of WT and *Popdc2*^{W188X} hearts in response to pharmacological intervention with 1nM, 10nM and 100nM isoproterenol in isolation of other organ systems and exocrine control, which may otherwise impact physiological measurements. To this end, prior to heart extraction the Langendorff system was set up. First, to avoid experimental failure due to microbial infection all tubing and chambers were washed with 2% acetic acid solution and thoroughly rinsed with boiled ddH₂O. Then the system was primed with oxygenated HEPES buffered Tyrode's solution which was pumped from the heated solution reservoir through the cleaned, heated tubing to the cannula attachment using a peristaltic pump at a constant flow rate of 1.8ml/min. Care was taken to remove all bubbles within the tubing using the bubble trap. Then a male or female 3-6 months old mouse was injected with heparin (100 I.U) and

sacrificed by inhalation of 5% isoflurane followed by cervical dislocation to confirm death. The heart was promptly extracted and immediately placed into ice cold oxygenated HEPES buffered Tyrode's solution. There it was trimmed of excess extra-cardiac tissue and the ascending aorta cannulated under illuminated and magnified visualization with a primed 21-gauge stainless steel murine cannula submerged in HEPES buffered Tyrode's solution to avoid air embolization of the coronaries. The aorta was fixed to the cannula with a silk thread, following which a small incision was made in the right ventricular outflow tract to allow free drainage of coronary effluent. The cannula was then connected to the primed transfusion rig. Two electrocardiogram electrodes were used to monitor electric activity of the heart of which one was carefully inserted into the wall of the right ventricle before organ bath chamber was attached to the rig and the heart was fully submerged in heated perfusate to promote a consistent heart temperature of 37°C. The temperature of the heart was monitored via a temperature electrode which was placed in close proximity of the heart. A consistent coronary perfusion pressure of 60-70 mm Hg was ensured using a pressure transducer placed above the cannula. Once the heart was attached to the Langendorff perfusion system and all parameters were set up, the heart was allowed 15 minutes to stabilize before it was perfused with 1nM, 10nM or 100nM Isoproterenol in HEPES buffered Tyrode's solution. The electrical activity of the heart was monitored and recorded using the LabChart Software (ADInstruments, New Zealand). The obtained data was assessed for the regularity of the heartbeat and the average heart rate before and after pharmacological stimulation.

2.13 Statistics

Statistical testing was performed with GraphPad Prism 9 (GraphPad Software Inc., La Jolla, California) and the data is displayed as mean± standard error of the mean. Number of biological repeats is indicated by an 'n' and each biological repeat has a multiplicity of technical repeats which collectively remove the effects of inter-individual variability or effects of isolation. The obtained p-value is denoted by asterisks: *=p<0.05, **=p<0.01 and ***=p<0.001 whereby p<0.05 is deemed significant throughout this thesis. Original data was tested for normality by Komogorov-Smirnov test. Data sets that had normal distribution were statistically tested by parametric tests such as student's t-test, One-Way, Two-Way Anova with Šídák post hoc analysis. The non-parametric Mann-Whitney test and Wilcoxon matched

pairs test were applied to data sets that were not deemed to have normal distribution. FRET data sets were tested with non-parametric tests even if the data passed the normality test as much of the FRET data is in form of a corrected ratio (fold change) and therefore it is uncertain how closely the data have a normal distribution.

CHAPTER 3 Characterisation of the *Popdc2*^{W188X} mouse mutant

3.1 Introduction

Since their discovery, a number of variants for each of the three *POPDC* genes have been identified in patients (Table 4, page 63)^{199,215,219,266,280–282,290,292}. The variants include missense and nonsense variants and deletions, which are predicted to have different effects on the structure of the resulting mutant protein, ranging from a presumably complete loss of the protein e.g., *POPDC1* c.1A>G, through protein truncations e.g., *POPDC1* p.R88X, to single amino acid substitutions e.g., *POPDC1* p.S201F. Clinically, *POPDC* variants have been associated with skeletal muscle pathologies, usually in form of a LGMD, and/or cardiac dysrhythmias, usually as an AV-block or sinus bradycardia (Table 4). In general, most *POPDC1* variants give rise to pathological phenotypes in both heart and skeletal muscle, patients carrying variants in *POPDC2* display only cardiac arrhythmia whereas variants identified in *POPDC3* are only associated with LGMD. These differences between *POPDC* isoforms are probably due to their isoform-specific differential expression pattern with *POPDC1* being equally expressed in both types of striated muscle, while *POPDC2* is preferentially expressed in the heart and *POPDC3* in skeletal muscle (Figure 3.1).

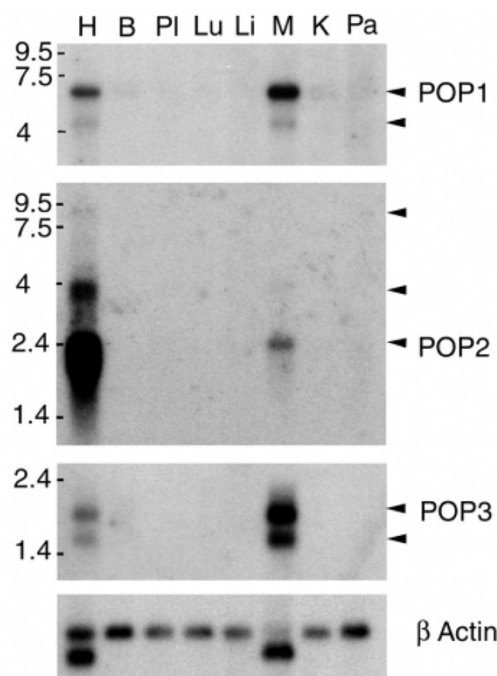


Figure 3.1. Northern blot analysis of *POPDC* gene expression in human tissues. H - heart, B – brain, PI – placenta, Lu – lung, Li – liver, M - skeletal muscle, K – kidney, Pa – pancreas. Figure taken from Andrée et al¹⁷⁶ (note that

in the provided reference abbreviations for POPDC proteins are: POPDC1-POP1, POPDC2-POP2 and POPDC3-POP3, but have since been renamed due to a name overlap with previously existing genes.)

POPDC2 variants are rare if compared with the variants in the other two family members, with only two *POPDC2* variants known to date (Table 4). Through whole exome sequencing (WES) one such *POPDC2* variant has been identified in two independent families with varying degrees of atrioventricular block (AVB I-III). The heterozygous nonsense variant (c. 563G>A) introduces a premature stop codon at amino acid (aa) position 188 (p.W188X), hereafter termed as *POPDC2*^{W188X} encoding a truncated protein lacking the carboxyterminal domain (CTD) and part of the atypical PBC of the Popeye domain downstream of the DSPE motif (Figure 3.2).

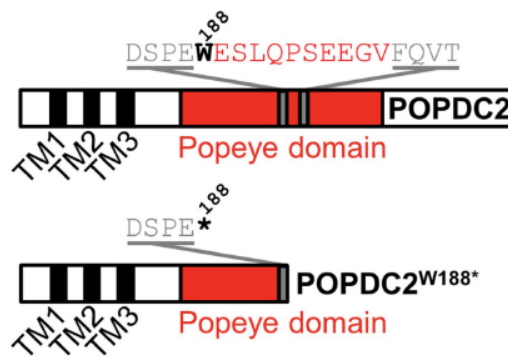


Figure 3.2. Schematic representation of the protein structure of POPDC2 (top) and *POPDC2*^{W188X} (bottom). The premature stop codon in the variant causes a truncation of part of PBC and the CTD. Figure taken from Rinné et al²⁸⁰.

The truncated *POPDC2*^{W188X} protein can be detected by Western blot in patient's leucocytes suggesting that it is not subject to nonsense-mediated decay (NMD)²⁸⁰. NMD is an endogenous surveillance pathway in eukaryotic cells, of which the main function is to recognise RNA carrying a premature termination codon (PTC) and label it for degradation to avoid deleterious gain-of-function or dominant-negative activity of the resulting truncated protein^{304,305}. Surprisingly, when the mutant protein was expressed in heterologous COS7 cells or in *Xenopus* oocytes, *POPDC2*^{W188X} mutant protein retained many of the currently established protein functions of POPDC proteins such as: high affinity binding of cAMP¹⁸⁴; the ability to dimerize with full length POPDC2 and to undergo heterodimer formation when co-expressed with POPDC1 and POPDC2^{215,306}; and to interact with the cardiac sodium channel SCN5A³⁰⁶ and the potassium channel TREK-1²⁸⁰. However, in contrast to wild type POPDC2, *POPDC2*^{W188X} produces an abnormal modulation of TREK1 current, which could indicate that

under physiological conditions the C-terminal part of POPDC2 has an important regulatory function. In fact, the CTDs of POPDC1 and POPDC2 carry phosphorylation sites, which are responsive to β AR stimulation³⁰⁷, a signalling pathway in the heart that enhances the sympathetic activity and is a potent stimulus for arrhythmogenicity^{308,309}. Importantly, the CTD of POPDC1 has an unusually high number of Ser/Thr residues (17/84, 20%), which are highly overrepresented and constitute potential phosphorylation sites and many of these residues are subjected to phosphorylation by PKA or other kinases³⁰⁷. Importantly, their presence is conserved in vertebrates, suggesting that they are of functional importance. Furthermore, the CTD is predicted to have an unfolded conformation (Figure 3.3) in its non-phosphorylated state, whilst phosphorylation could drive certain PPI which could change the nature of the interaction of POPDC protein and its interaction partner(s). POPDC proteins are transmembrane proteins and are normally found at the plasma membrane in striated muscle cells where they are known to possess many compartment-specific interaction partners³¹⁰. Therefore, it was initially hypothesised that the CTDs of POPDC1 and POPDC2 undergo a conformational change in response to β AR-mediated phosphorylation and that this protein modification might be important for their biological function. This hypothesis was supported by the fact that in patients and animal models the loss of this domain is arrhythmogenic²⁸⁰.

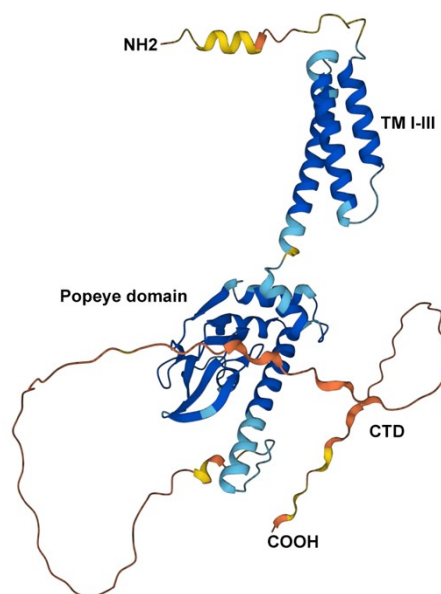


Figure 3.3. AlphaFold structure prediction of human POPDC2 (accession number AF-Q9HBU9-F1)²¹¹. The transmembrane domains (TM I-III) and the Popeye domain are indicated. Note the long unfolded CTD. Similar structural predictions can be found in the AlphaFold Structural database in the case of POPDC1 and POPDC3.

In contrast to *POPDC1* and *POPDC3* variants, which show a recessive trait, *POPDC2* variants seems to have an autosomal dominant trait (Both known variants (*POPDC2*^{W188X} and *POPDC2*^{L245P}) display a dominant trait). To study the biological consequences of the carboxyterminal truncation of *POPDC2* and to investigate the underlying pathogenic mechanism, a *Popdc2*^{W188X} allele was introduced into the murine *Popdc2* locus. The initial *in vivo* mouse studies revealed reduced heart rate at baseline and sinus bradycardia under sympathetic stimulation with stress induced sinus pauses, but an unaltered PR interval, P duration and QT interval²⁸⁰. Aside from these published *in vivo* investigations, the *Popdc2*^{W188X} mouse mutant has not been further characterised.

3.2 Outline of the work presented in this chapter and aims

3.2.1 Description of this work

On the quest to further our understanding of the role that POPDC proteins might have on the heart and especially the impact of the *Popdc2*^{W188X} mutation on the cardiac response of β AR activation, the opportunity presented itself to characterise some parameters of the *Popdc2*^{W188X} mouse mutant. Through work with these mutant animals, observations were made, that they produce whistling sounds in response to stress, and show a reduced fertility rate. An attempt was made to interpret these observations within the context of the current state of knowledge about the physiological role that POPDC proteins may have within different cell types and subcellular compartments in which they are expressed. Thereby also highlighting favourable areas of future research which should be pursued to uncover a possibly common mechanism by which POPDC proteins exert their functional role. Moreover, some parameters of the cardiac morphology, and, by virtue of the retrograde Langendorff perfusion technique, the electrophysiology of the *Popdc2*^{W188X} mouse mutant heart in response to β AR stimulation were assessed. And finally, at the protein level, the expression and localisation of POPDC1 was studied in cardiac tissue and cultured NMCM samples. Additionally, protein specific antibodies are an important tool in a research lab, which, with a high level of precision and specificity, can help uncovering the localisation, expression levels and interaction partners of the protein of interest within cells or tissue. Therefore, in the early stages of the project, an effort was made to identifying an antibody that was able to recognize the POPDC2^{W188X} mutant protein.

3.2.2 Aims

1. To test available POPDC2 antibodies for their reactivity to the POPDC2^{W188X} mutant protein.
2. To quantify observational data collected whilst working with the *Popdc2*^{W188X} mutant animals.
3. To characterise the impact of *Popdc2*^{W188X} mutation on cardiac size.
4. To prepare cardiac *Popdc2*^{W188X} samples for phosphoproteomic profiling following β AR stimulation and quantify the effects of the *Popdc2*^{W188X} mutation on the electrophysiological properties of the *Popdc2*^{W188X} mutant heart.
5. To study mRNA expression of *Popdc1-3* in homozygous *Popdc2*^{W188X} mutant and WT mice
6. To study the expression and localisation of the Popdc1 proteins in tissue and in isolated NMCM of the *Popdc2*^{W188X} mutant.

3.3 Results

3.3.1 Search for an antibody able to detect the POPDC2^{W188X} mutant protein

Several POPDC2 antibodies, summarized in Table 3.1., were assessed by epitope comparison with manufacturer's help and/or experimentally by western blot analysis (Figure 3.4) for their reactivity with the truncated POPDC2^{W188X} protein. To ensure isoform specificity antibodies were also tested for their reactivity with POPDC1, POPDC2 and POPDC3. Cell lysates were generated from HEK293 cells which were transfected with myc-tagged murine cDNA constructs encoding for Popdc1, Popdc2 and Popdc3 as well as human flag-tagged POPDC2^{W188X} to create an overexpression system for high protein yield. Lysates of untransfected HEK293 cells served as the negative control. The Sigma Atlas HPA024255 as an established POPDC2 antibody was used as the positive control. The antibody dilutions were produced at concentrations recommended by the manufacturer or higher (see Table 3.1). 20 μ g of total protein were loaded for every sample of each POPDC protein isoform on each 10% polyacrylamide gel.

None of the tested POPDC2 antibodies have shown reactivity with the POPDC2^{W188X} mutant protein (Figure 3.4). Of the commercially available POPDC2 antibodies only Sigma Atlas HPA024255 and Sigma-Aldrich SAB1407852 recognized the wild type murine Popdc2 protein, albeit at different molecular weight marks. The human POPDC2^{W188*} antibody²⁸⁰ and Santa Cruz, 9H10, 12G5 and 13C4 POPDC2 antibodies did not produce any immune-reactive bands, whilst the Millipore POPDC2 antibody only produced some faint unspecific bands at approximately 55kDa molecular weight mark. All the tested mNet3 POPDC2 antibodies produced numerous unspecific bands across various molecular weights. The 12G12 POPDC2 antibody²⁰⁶ was identified as a novel POPDC2 antibody to recognise the murine Popdc2 protein at a similar molecular weight as Sigma Atlas HPA024255.

Table 3.1 POPDC2 antibodies assessed for POPDC2^{W188X} reactivity

Antibody	Dilution	Source	Catalogue No	Reactivity with POPDC2 ^{W188X}
Popeye 2 (V-13)	1:500	Santa Cruz	sc-66588	No
POPDC2	1:500	Millipore	06-1007	No
POPDC2	1:250	Sigma Atlas	HPA024255	No
Popdc2 12G5	1:100	Dr Ian Holt, Keele University	N/a	No
Popdc2 12G12	1:500	Dr Ian Holt, Keele University	N/a	No
Popdc2 13C4	1:100	Dr Ian Holt, Keele University	N/a	No
Popdc2 9H10	1:100	Dr Ian Holt, Keele University	N/a	No
mNET3 11765	1:500	Prof. Eric Schirmer, University of Edinburgh ²⁰¹	N/a	No
mNET3 11766	1:500	Prof. Eric Schirmer, University of Edinburgh	N/a	No
mNET3 11768	1:500	Prof. Eric Schirmer, University of Edinburgh	N/a	No
POPDC2	5mg/ml	Sigma -Aldrich	SAB 1407852	No
human POPDC2 ^{W188X}	1:250	Prof. Niels Decher, Philipps University of Marburg ²⁸⁰	N/a	No

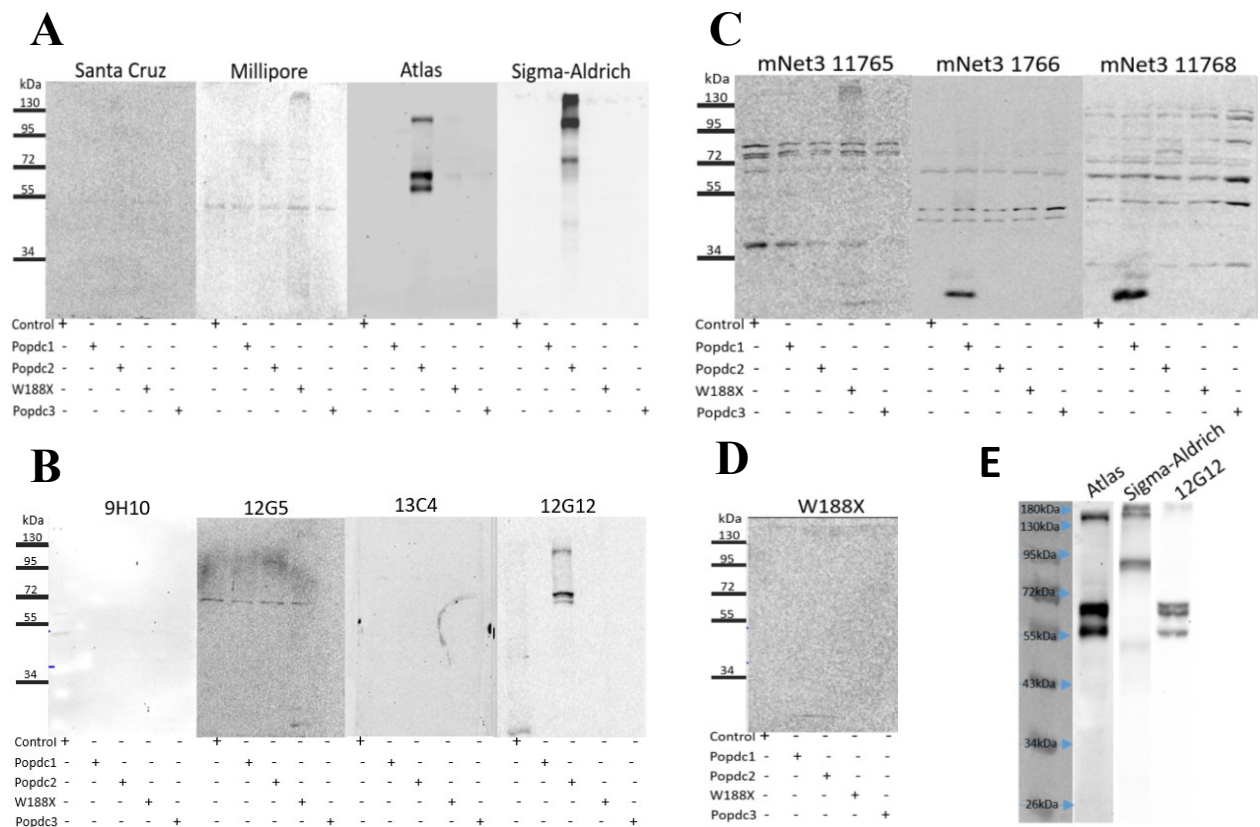


Figure 3.4. Western blot analysis of POPDC2 antibodies for reactivity with the *POPDC2*^{W188X} mutant protein. A Commercially available POPDC2 antibodies: Santa Cruz (sc-66588), Millipore (06-1007), Sigma (Atlas HPA024255) and Sigma-Aldrich (SAB 1407852). **B** Monoclonal POPDC2 antibodies developed and donated by Dr Ian Holt, Keele University. **C** Monoclonal POPDC2 antibodies received from Professor Eric Schirmer, University of Edinburgh²⁰¹, **D** Human POPDC2^{W188X} antibody²⁸⁰ donated by Professor Niels Decher, Philipps University of Marburg, **E** Summary of POPDC2 antibodies shown reactive with POPDC2 proteins in Western Blot preparations.

3.3.2 Implications of the *Popdc2*^{W188X} mutation beyond the heart of the *Popdc2*^{W188X} mouse mutant

Popdc2^{W188X} mutant mice were fertile and had a normal life span, however, under stress conditions such as handling, the homozygous mutant mice, showed signs of hyperexcitation; typically expressed as heavy breathing with a whistling sound. Heterozygous mutants showed the same hyperexcitation signs albeit at an older age. A similar phenotype has also been observed in *Popdc1* and *Popdc2* KO mice¹⁸⁴. At present, the reason for the observed phenotype is not fully understood, however, it is hypothesized that it might be due to abnormal changes at the neuromuscular junctions (NMJ) of the diaphragm (Prof. Thomas Brand, personal communication). Similar phenotypes were recorded for mouse mutants with gene ablations for neuromuscular junction proteins such as epsilon subunit of the nicotinic acetylcholine receptor, which remains the focus of ongoing research.

In addition to a normal life span, *Popdc2*^{W188X} breeders showed no significant difference in the average number of pups born compared to the wild type pairs (Figure 3.5 A), however, at further examination, it was revealed that *Popdc2*^{W188X} breeders produce litters less frequently than their wild-type counter parts (Figure 3.5 B). Litter frequency is determined as the number of days between each new litter to the same female.

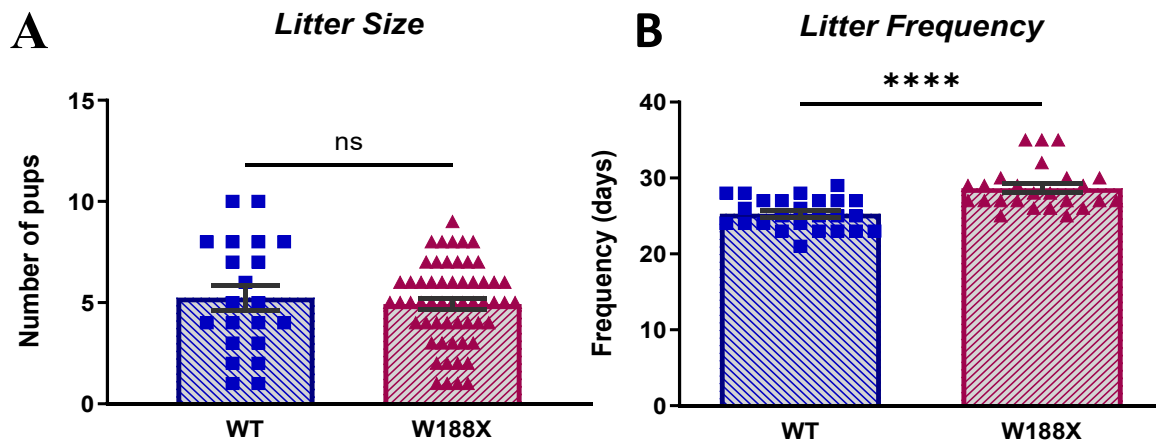


Figure 3.5. Litter size and frequency in wild type and *Popdc2*^{W188X} mutant breeders. (A) Number of pups born to and frequency of litters (B) from wild type and *Popdc2*^{W188X} mutant breeders were recorded and compared. Statistical analysis was performed using the student's t-test, ns $p > 0.05$, **** $p < 0.0001$. Error bars show SEM.

3.3.3 *Popdc2*^{W188X} mutants have smaller hearts compared to wild type mice

Transgenic mice carrying a homozygous *Popdc2*^{W188X} mutation were observed to have a reduced body weight compared to age-matched wild type animals (Brand group, personal communication), which was particularly pronounced in animals of increased age. To assess whether there are changes in the size of the heart of the *Popdc2*^{W188X} mutants independent of changes in the mutant's body weight, heart weight/tibia length ratios were calculated. The obtained data (Figure 3.6 A) show that the *Popdc2*^{W188X} mutant heart is significantly smaller than wild type hearts. To study this further it was decided to assess the size of isolated adult cardiomyocytes using the CytoCypher system^{297,298} (described in chapter 2 Methods and Materials section 2.10 CytoCypher). The CytoCypher MultiCell system is an inverted microscope system that has a mobile objective allowing it to move in a x-y-z position to take measurements of cells within the predetermined areas of interest selected either automatically by a software or manually. Surprisingly, the average cardiomyocyte size

between *Popdc2*^{W188X} mutants and their wild type counterparts did not differ significantly (Figure 3.6 B). Decreased heart size in the *Popdc2*^{W188X} mutants could also be caused by a reduced number of total cardiomyocytes in the heart. The presence of binucleated cardiomyocytes in neonatal hearts is considered a marker for cardiac maturation. During early postnatal development, a healthy heart typically undergoes some proliferation and terminal cardiomyocyte differentiation and an increase in binucleation signifies the completion of cell division and a transition to hypertrophic growth of individual cardiomyocytes³¹¹. An increased binucleation rate would be suggestive of more terminally differentiated cardiomyocytes during the neonatal development resulting in a smaller number of cells and smaller hearts. To this end, isolated neonatal ventricular cardiomyocytes were immunostained with a membrane marker, the nuclei labelled with DAPI and the cells imaged using a confocal microscope. The number of binucleated cells was calculated as a percentage of total number of cardiomyocytes. The data showed no significant difference in binucleation levels between the wild type and *Popdc2*^{W188X} mutants (Figure 3.6 C). Additionally, in a simple cell counting method, the number of isolated NMCM was compared between the *Popdc2*^{W188X} and wild type samples, which produced a significant difference in the number of cardiomyocytes (Figure 3.6 D) which suggests that the observed differences in the size of the *Popdc2*^{W188X} and wild type heart could possibly be due to a reduced number of cardiomyocytes.

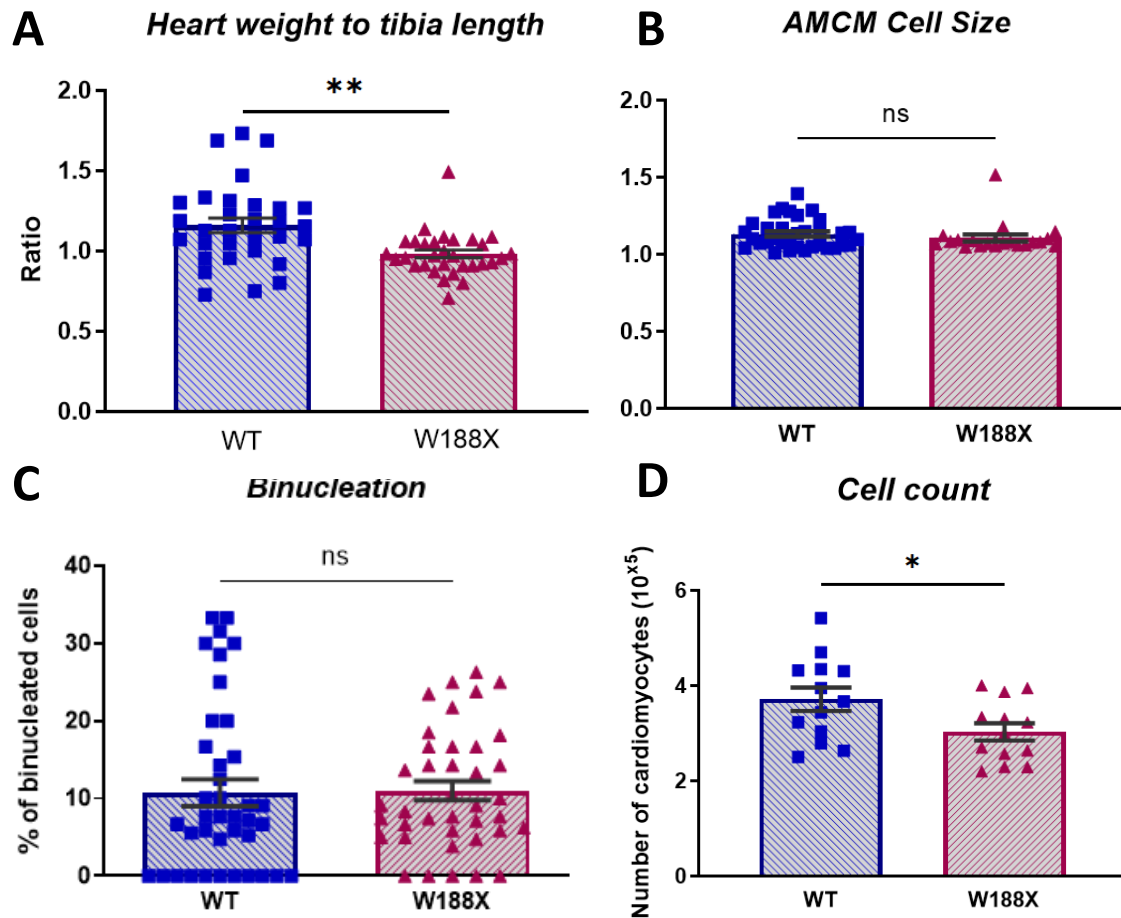


Figure 3.6. Assessment of difference in heart weight, number of adult cardiomyocyte size, binucleation levels and cell number between Popdc2W188X mutants and wild type samples. (A) Statistically significant difference in the heart weight-to-tibia length ratio of wild type and Popdc2^{W188X} mice (* $p < 0.001$; $n = 31$). **(B)** Ventricular cardiomyocytes isolated from wild type and Popdc2^{W188X} mutants have a similar cell size (20 cells from 4 isolations, $n = 4$). **(C)** Wild type and Popdc2^{W188X} mutants show similar levels of binucleation in isolated neonatal cardiomyocytes (43 cells from 5 different isolations, $n = 5$). **(D)** Number of isolated cardiomyocytes over 10 isolations was traced and assessed for statistically significant difference in the cell count. Statistical analysis was performed using the student's t-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$. Error bars show SEM.

3.3.4 Popdc2^{W188X} mutant mice have an abnormal cardiac electrophysiology

cAMP signalling plays a critical role in regulating cardiac function and electrophysiology. Dysregulation of cAMP signalling has been implicated in various cardiac disorders, including arrhythmias and heart failure³¹². In a healthy heart, cAMP regulates cardiac muscle contraction, relaxation, and heart rate by activating its key effector PKA which through substrate phosphorylation modulates Ca^{2+} dynamics during processes such as cardiac pacemaking and excitation–contraction coupling of the cardiac myocyte. As mentioned previously, POPDC proteins are also cAMP effectors and are highly expressed in the myocardium and the CCS¹⁸⁴. Loss-of-function mutations in mouse and zebrafish models

revealed that depletion of *Popdc1* and *Popdc2* leads to the development of cardiac arrhythmias and AV-block^{184,261}. Similarly, patients carrying *POPDC1*^{R88X}, *POPDC1*^{S201F}, and *POPDC2*^{W188X} mutation develop varying degrees of AV block^{199,219,280}. POPDC proteins were found to interact with sodium channels and Ankyrin-B and -G, which are essential for ion channel trafficking^{306,313}. Furthermore, POPDC have also been found to interact with TREK1, which likely plays a role in cardiac pace-making through regulation of SAN excitability⁷² and cAMP binding to POPDC proteins was shown to negatively modulate the TREK1 current¹⁹⁹. It has been proposed that the interaction between POPDC proteins and TREK1 might shield TREK1 from PKA mediated phosphorylation which is known to deactivate the TREK1 channel. This suggests that loss of POPDC proteins as has been identified in the *Popdc2*^{W188X} mutant (section 3.2.5.) may lead to an altered phosphorylation profile of the POPDC interacting partners. To assess whether there is an aberrant PKA mediated phosphorylation response to adrenergic stimulation in *Popdc2*^{W188X} mutants, a collaboration was established with the Proteomics facility of the University of Dundee. In preparation, hearts of 3-4 months old wild type and homozygous *Popdc2*^{W188X} mutant mice were isolated and immediately attached to a retrograde Langendorff perfusion system where they were stimulated with β AR agonist isoproterenol. Whilst the phosphoproteomic profiling is still outstanding, here I am presenting the analysis of the results for the *ex vivo* Langendorff-perfused hearts.

Hearts, which were isolated from 3-4 months old male and female animals were used for this set of experiments. Once the hearts were excised and perfusion through the retrograde Langendorff perfusion system was started, they were given at least 10 minutes stabilization time before application of the ISO treatment. For each heart, an electrocardiogram was recorded and analysed (Figure 3.7). At baseline, hearts of both genotypes were beating stably and consistently (Figure 3.7 A, B), however, the *Popdc2*^{W188X} mutant hearts had an elevated heart rate compared to the wild type hearts (Figure 3.7C). Upon treatment with isoproterenol, which induces a β -adrenergic stress response, an abnormal function of the mutant heart was detected (Figure 3.7 A, B). Whereas the wild type hearts were able to consistently increase and maintain an elevated heart rate in response to progressively higher concentrations of isoproterenol (Figure 3.7 A, C, D), the mean heart rate of the transgenic hearts failed to reach the same level of increase throughout all three drug concentrations (Figure 3.7C) and was significantly below of that of the wild type hearts (Figure 3.7D). Furthermore, there were

pronounced heart rate fluctuations between the maximum and minimum heart rate of the mutant hearts in response to isoproterenol stimulation, which was characterized by cyclical bradycardic episodes, with significant sinus pauses (Figure 3.7E). The cyclical heart rate fluctuations became more frequent with a greater range of heart rate min to heart rate max at higher stimulant concentrations (Figure 3.7B), features that were absent in the wild type samples. Overall, these data suggest that the *Popdc2*^{W188X} mutant hearts have an impaired ability to increase and maintain an elevated heart rate in response to β -adrenergic stimulation, resulting in a bradycardic profile. It would be extremely interesting to pair the results of these functional studies with the phosphoproteomic profiling of the *Popdc2*^{W188X} mutant hearts.

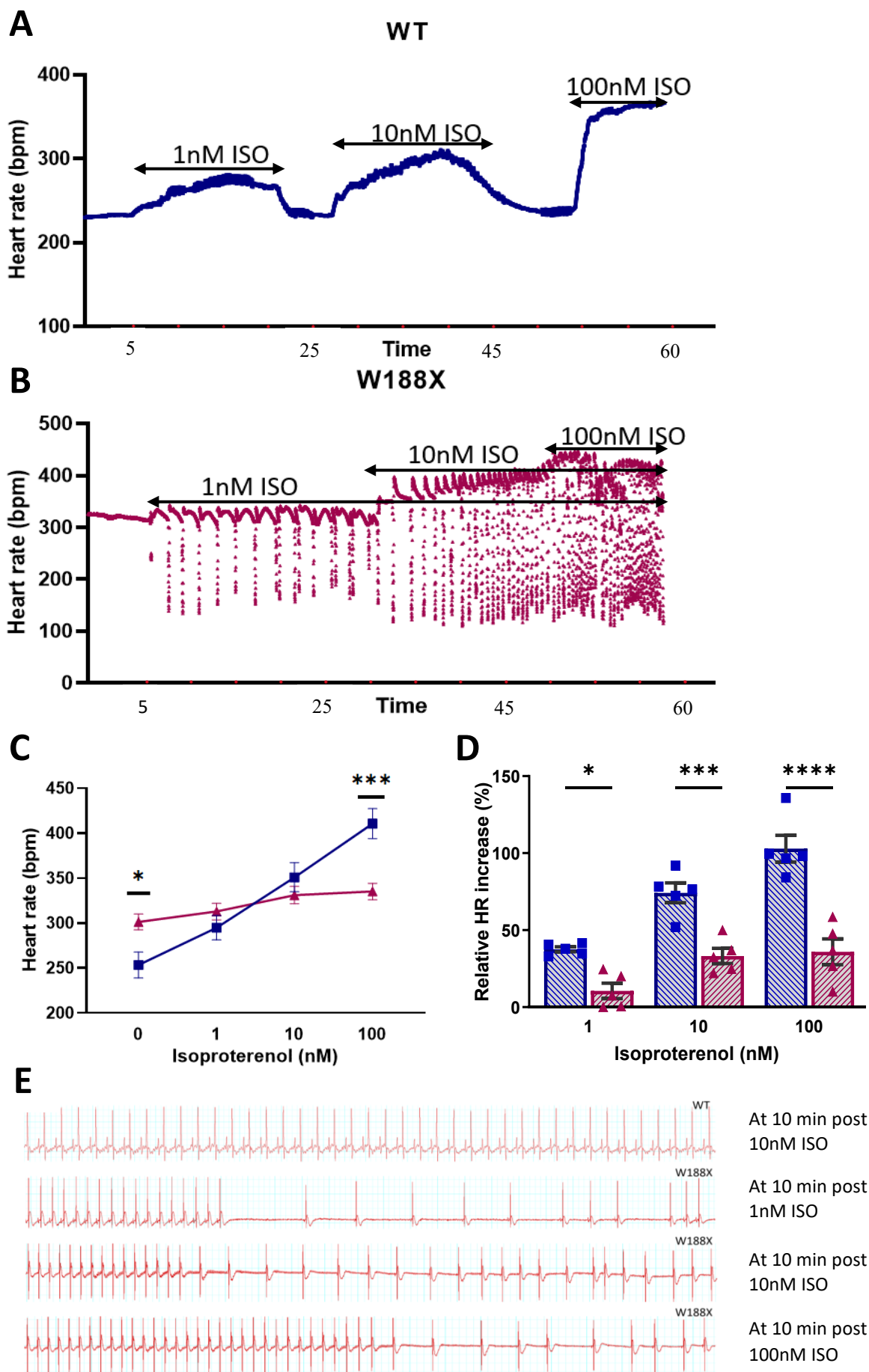


Figure 3.7. Heart rate analysis of Langendorff perfused hearts isolated from *Popdc2*^{W188X} mutant and wild type mice. (A, B) Representative traces of heart rates of (A) wild type and (B) *Popdc2*^{W188X} mutant ex vivo heart preparations in response to three different concentrations (1nM, 10nM and 100nM) of isoproterenol. (C) Changes in heart rate were quantified by calculating and plotting the average heart rate at the peak of the response after stimulation with 1nM, 10nM or 100nM isoproterenol (n=5). (D) Relative increase in heart rate in response to isoproterenol stimulation plotted as % increase relative to the average maximum response of wild type hearts at 100nM isoproterenol. (E) Representative examples of ECG curves of wild type and *Popdc2*^{W188X} mutant hearts after β -adrenergic stimulation. Statistical analysis was performed using two-way ANOVA, *p<0.05, **p<0.005, *** p<0.001 and ****p<0.0001. Error bars show SEM.

3.3.5 *Popdc2* expression is abolished in the *Popdc2*^{W188X} mutant hearts

In patients, the pathogenic *POPDC2*^{W188X} variant is carried as a heterozygous trait²⁸⁰. A transgenic homozygous mouse model was chosen to investigate this disease-causing loss-of-function mutation as heterozygotes did not show any phenotype. To commence the characterisation of this *Popdc2*^{W188X} knock-in mouse, genomic DNA was extracted from biological material collected during ear marking, exon 2 was PCR amplified from wildtype and homozygous mutants and a sequence analysis performed to confirm the presence of a premature termination codon (PTC) in the *Popdc2* gene at the aa position 188 and the results of this analysis are presented in Figure 3.8. A clear distinction can be made between the electropherograms of genomic DNA isolated from wild type and the *Popdc2*^{W188X} mutant mice. The change produces a PTC which would consequently translate to a POPDC2 protein with a truncation at position 188 (Figure 3.8C).

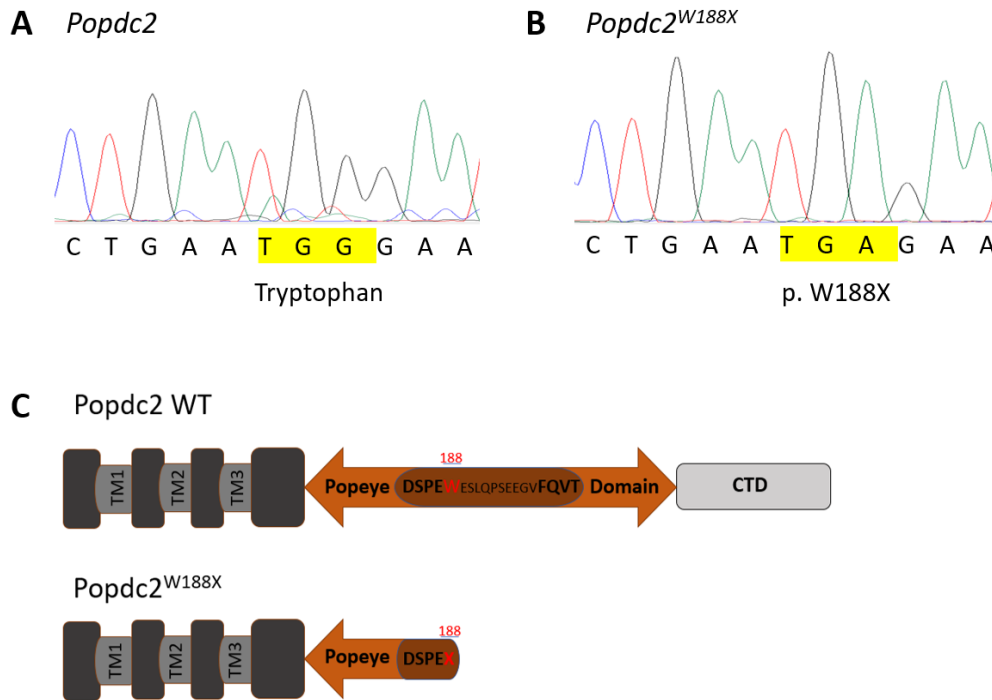


Figure 3.8. Popdc2^{W188X} mouse mutant. (A, B) Electropherograms of the sequencing results of genomic DNA of (A) wild type and (B) *Popdc2^{W188X}* mutants, which was amplified by PCR with primers flanking exon 2 of *Popdc2*. (C) Predicted protein architecture of wild type POPDC2 and POPDC2^{W188X} mutant proteins. Depicted are the three transmembrane domains, the intracellular Popeye domain and the CTD. Within the Popeye domain in dark orange are the DSPE and FQVT motifs, which are thought to be involved in cAMP binding. In red is tryptophan (W)188 labelled in wild type POPDC2. The mutant protein terminates after the DSPE motif due to the presence of a premature stop codon at this position in the mutant transcript.

It has been previously reported that the truncated mutant protein was found by western blot analysis to be expressed in leucocyte samples from patients carrying the *POPDC2^{W188X}* variant. Obviously in patients, the mRNA was not subjected to NMD as was confirmed by RNA sequence analysis²⁸⁰. It should however be mentioned that in my hands, the POPDC2 antibody utilized in this report did not show any reactivity with any of the POPDC isoforms (Figure 3.4D). Therefore, due to the lack of an antibody capable of POPDC2^{W188X} detection no western blot analysis could be performed, instead mutant transcript was assessed. To analyse the expression of the mutant transcript, RT-qPCR analysis was performed on mRNA samples extracted from ventricular tissue of homozygous and heterozygous *Popdc2^{W188X}* mutant and wild type mice (Figure 3.9). The data revealed an approx. 50% reduction of the *Popdc2* gene expression in the heterozygous samples and an approx. 95% reduction of the *Popdc2* gene expression in the homozygous samples in comparison to wild type samples (Figure 3.9A) which suggests that in the ventricular tissue of the *Popdc2^{W188X}* mouse the *Popdc2^{W188X}*

mutant allele may be subject to nonsense mediated Decay (NMD). The relative expressions of *Popdc1* and *Popdc3* transcripts in the three genotypes was also assessed (Figure 3.9 B, C) and revealed a significant increase of *Popdc1* and *Popdc3* transcripts in the ventricular tissue of homozygous but not heterozygous *Popdc2*^{W188X} mouse mutants.

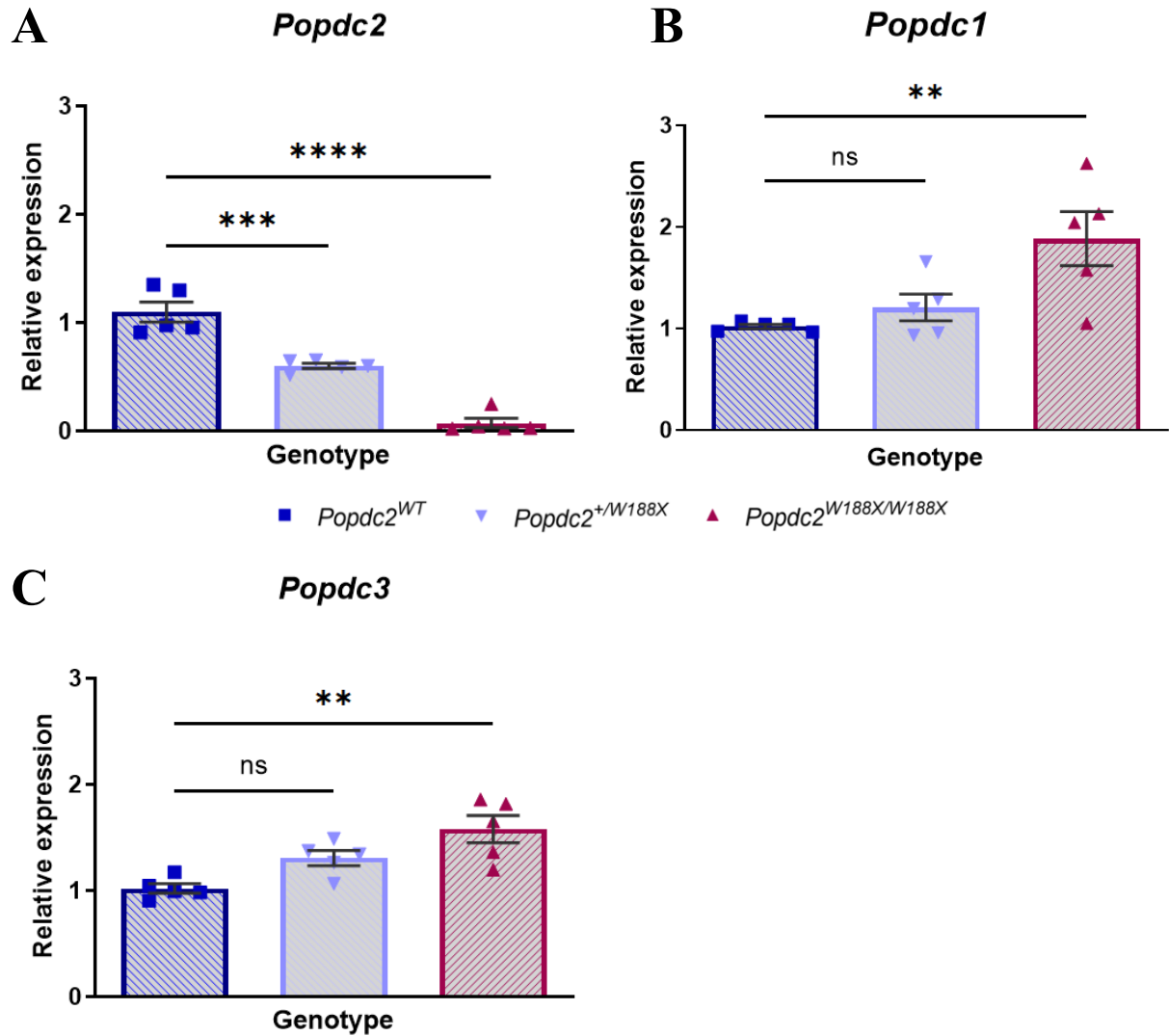


Figure 3.9. The *Popdc2*^{W188X} mutant transcript is subjected to NMD. Quantitative PCR analysis of (A) *Popdc2*, (B) *Popdc1* and (C) *Popdc3* expression in ventricular tissue of homozygous and heterozygous *Popdc2*^{W188X} mutants and wildtype mice. All values shown are normalised against GAPDH. Expression levels in wildtype mice are set as one. Statistical analysis was performed using one-way ANOVA, *p<0.05, **p<0.005, *** p<0.001 and ****p<0.0001, 5 independent samples were assessed in triplicates (n=5). Error bars show $\pm$ SEM.

The above-described significant reduction of the *Popdc2* mRNA in the ventricular tissue of the *Popdc2*^{W188X} mutant which carries a PTC in the *Popdc2* gene and no measurable loss of the other two POPDC family members, strongly suggests that this might be due to NMD. To

further investigate this hypothesis, ventricular NMCM were isolated from wild type and homozygous *Popdc2*^{W188X} mutant mice and treated with cycloheximide (CHX). CHX is a drug that inhibits protein synthesis by blocking the elongation phase of eukaryotic translation³¹⁴. NMD is an mRNA degradation pathway that eliminates transcripts containing premature termination codons during translation. Since CHX blocks translation and the NMD pathway gets co-translationally activated, it is expected that transcripts carrying a PTC and normally subjected to NMD will accumulate in the cell. Following CHX treatment, expression of the *Popdc2*^{W188X} mutant transcript showed a significant 8.6-fold increase compared to the non-treated control (C). In contrast no significant difference was seen for the normal *Popdc2* transcript between control and CHX-treated wild type cells (WT) (Figure 3.10). These results suggests that the *Popdc2*^{W188X} mutant transcript is subject to NMD.

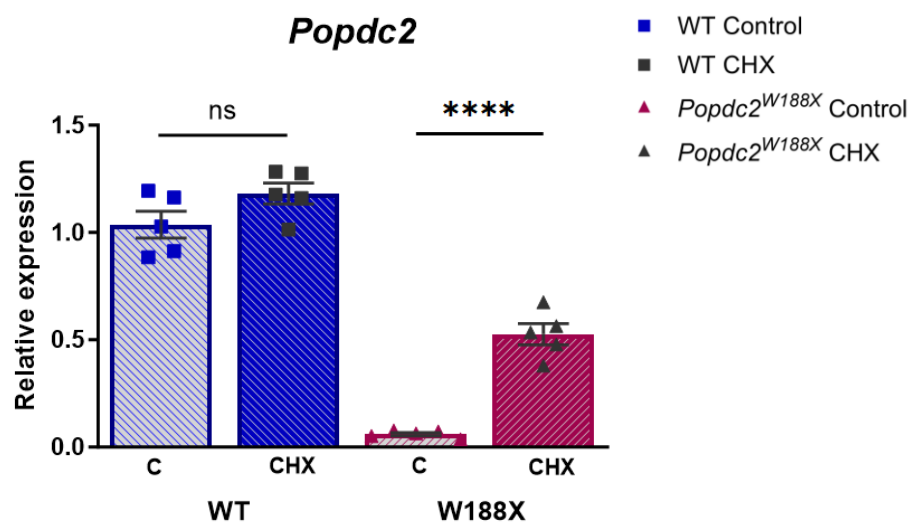


Figure 3.10. The *Popdc2*^{W188X} mutant transcript expression is rescued by CHX treatment. qPCR analysis of *Popdc2* expression in NMCC, isolated from wild type (blue) and *Popdc2*^{W188X} mutant (red) mice. Expression was analysed in cells before (C) and post (CHX) treatment. All values shown are normalised against GAPDH. Statistical analysis was performed using Student's t-test, ns: not significant, ****p<0.0001. Data are derived from 5 independent cell isolations (n=5) and measured in triplicates (N=3). Error bars show $\pm$ SEM.

3.3.6 *Popdc2*^{W188X} mutation causes loss of *Popdc2* and a mislocalisation of *Popdc1* in cardiomyocytes

Previous analysis of skeletal muscle biopsy material derived from patients carrying *POPDC* variants have reported disruptions to the plasma membrane localisation of the mutant protein and other family members^{199,219,280,215}. Similar findings have also been made for the

expression of POPDC proteins in *Popdc1* and *Popdc2* KO mutants and in knockin mouse and zebrafish models carrying variants found in patients. However, in case of some variants, expression and subcellular localisation was unaffected²¹⁵. Patients carrying POPDC2^{W188X} develop a cardiac arrhythmia phenotype and have slightly elevated creatinine kinase levels. However, no evidence for a functional involvement of skeletal muscle. Thus, in contrast to patients carrying *POPDC1* or *POPDC3* variants, no skeletal muscle biopsy material has been obtained and therefore no immunohistochemical analysis of POPDC1 or POPDC3 expression has been performed. Therefore, immunohistochemical staining for *Popdc1*, alongside β -catenin as a membrane marker, was performed on ventricular cryosections from *Popdc2*^{W188X} mutant and age- and sex-matched wild type mice (Figure 3.11 A, B). Furthermore, NMCM isolated from wild type and *Popdc2*^{W188X} mutant pups were immunocytochemically stained for POPDC1 (Figure 3.11 C, D) to investigate whether the loss and mislocalisation of POPDC1 is already present at the neonatal stage. Multiple images were recorded using a confocal microscope to visualize the subcellular expression of POPDC1 or POPDC2 and β -catenin. The findings are summarized in Figure 3.11. In both, immunostained tissue and isolated NMCM samples, the wild type NMCM showed strong POPDC1 expression that is mainly localized to the plasma membrane, which is also labelled by β -Catenin (Figure 3.11 A, C). The *Popdc2*^{W188X} mutant myocytes, on the contrary, showed almost no POPDC1 expression at the plasma membrane and only some weak perinuclear staining, which is vesicular and may correspond to intracellular vesicles (Figure 3.11 B, D). These findings suggest that the NMD-driven loss of POPDC2 in mutant cardiomyocytes also leads to a strongly reduced expression and mislocalisation of POPDC1. Recently it has been shown that POPDC proteins undergo heteromeric interaction and that interaction is required for proper membrane localisation²¹⁵. Consistent with the observation made here, regarding co-dependence of POPDC1 and POPDC2 for proper membrane trafficking, membrane localisation of both POPDC proteins were lost in case of a number of POPDC1 mutations¹⁹⁹. Additionally, isolated wild type and *Popdc2*^{W188X} NMCM were also immunocytochemically stained for POPDC2 expression using the anti POPDC2 antibody from Atlas, one of the most reliable POPDC2 antibody commercially available (Figure 3.11 E, F). The wild type samples displayed normal expression of POPDC2 in NMCM, which was predominantly localized to the plasma membrane. No POPDC2 staining is present in NMCM isolated from *Popdc2*^{W188X} mutants, which either is due to the lack of reactivity of the antibody with the carboxyterminal deleted mutant protein or alternatively

due to the absence of the mutant protein either because of protein degradation or because no protein is made due to NMD.

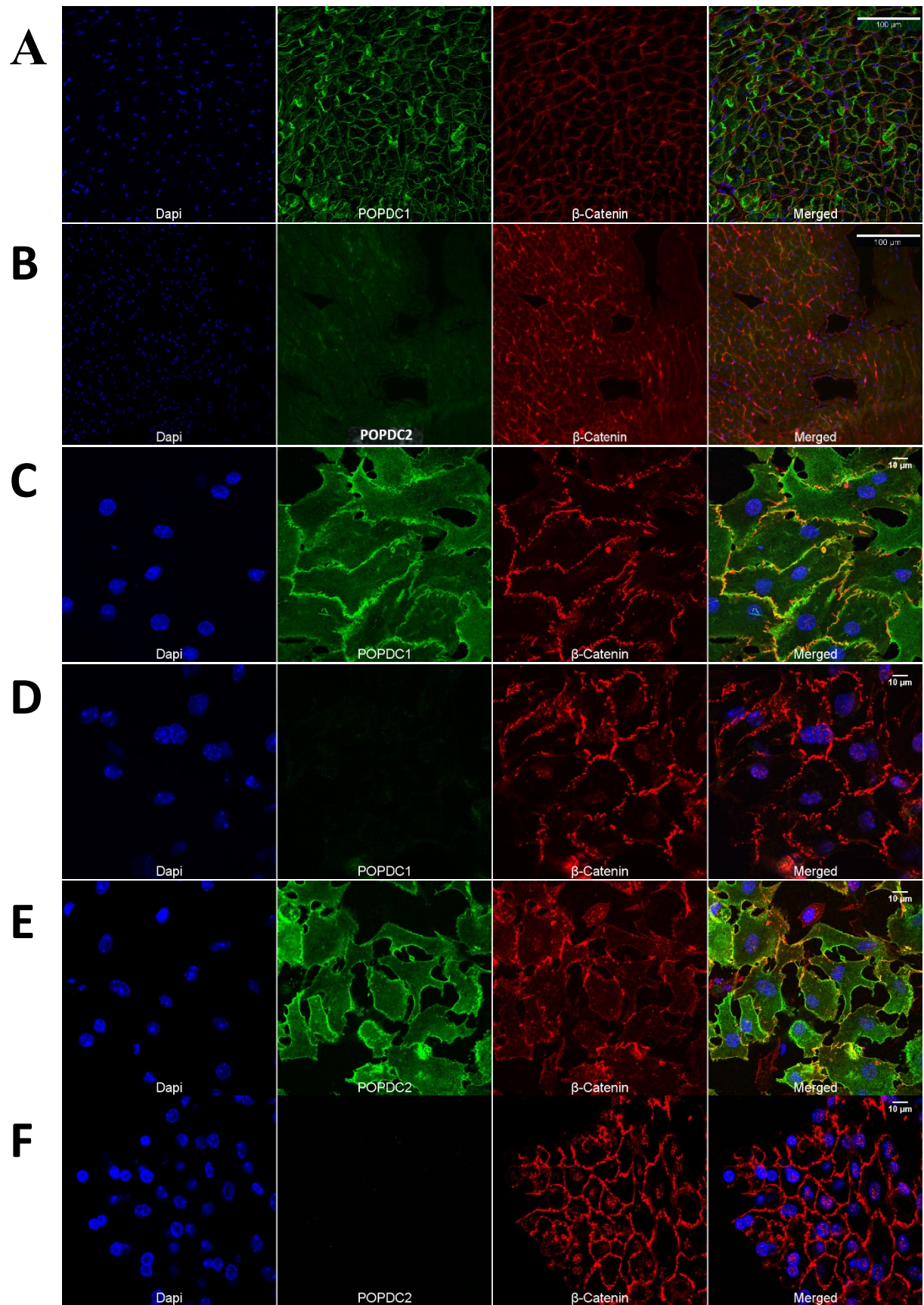


Figure 3.11. Immunostaining of POPDC1 in ventricular tissue and POPDC1 and POPDC2 in isolated NMCM of wild type and *Popdc2W188X* mutants. Immunostaining was performed with (A, C, D) anti-POPDC1 (green signal) and (B, E, F) anti-POPDC2 (green signal) antibodies on (A, B) cryosections of adult ventricular tissue from and (C-

F) cultured NMCM on (A, C, D) wild type and (B, D, F) Popdc2^{W188X} mutants. All samples were also stained with an β -catenin antibody (red), which served as a membrane marker and DAPI (blue) for nuclear staining.

3.4 Discussion

3.4.1 POPDC2^{W188X} antibody search

In their carboxyterminal domains (CTDs), POPDC1 and POPDC2 harbour PKA specific phosphorylation sites which may be of importance for protein-protein interaction (PPI) following β AR activation in cardiomyocytes. As previously elaborated a nonsense mutation within *POPDC2* introduces a premature stop codon resulting in a truncated POPDC2 protein i.e., POPDC2^{W188X}, which lacks the CTD. This presents a unique opportunity to study the role of the CTD during PPI, its interacting partners and how they change after phosphorylation. Therefore, efforts were made to identify a POPDC2^{W188X} reactive antibody out of the pool of the currently available POPDC2 antibodies to be utilised in subsequent proteomic and biochemical studies. In total 11 POPDC2 antibodies were tested by western blot analysis and epitope assessment for their reactivity and specificity to POPDC2^{W188X} protein. However, none of the tested POPDC2 antibodies showed successful POPDC2^{W188X} detection. Moreover, most did not recognise the wild type POPDC2 either. Only Sigma Atlas HPA024255 and 12G12 showed typical POPDC2 band pattern with one band appearing at ~130kDa and three bands appearing between 72kDa and 58kDa molecular weight marks. Interestingly, Sigma-Aldrich SAB 140752 also recognized the wild type POPDC2 protein, however, the band pattern deviated greatly from the typical pattern seen with the established POPDC2 antibodies with bands appearing at higher molecular weight marks, which is possibly due to its polyclonal nature. It is also possible that Sigma-Aldrich SAB 140752 antibody recognizes POPDC2 proteins that are either phosphorylated or more heavily glycosylated. Additionally, I tested a unique N-terminal reactive POPDC2^{W188X} antibody, which is reported to immuno-react with the human POPDC2^{W188X} protein isolated from patient leucocytes²⁸⁰. However, also this antibody did not show any reactivity with a POPDC2^{W188X} raised in HEK293 cells.

Despite the success in confirming the specificity of a monoclonal 12G12 POPDC2 antibody, the main objective to find an antibody reactive with the truncated POPDC2^{W188X} protein was not achieved. An epitope comparison of commercially available POPDC2 antibodies revealed

that their target region is downstream of amino acid 188, which is where POPDC2^{W188X} protein is truncated, and which provides a simple explanation for the lack of recognition. One could speculate that the sequence prior to the truncation is not easily accessible in the folded 3-D structure of the protein. It is also possible that epitopes are masked during glycosylation of the N-terminal region of the POPDC2 protein thereby prohibiting access by antibodies.

Consequently, due to the lack of a specific POPDC2^{W188X} antibody the originally planned proteomic and biochemical experiments could not be pursued and other ways to study the POPDC2^{W188X} were designed.

3.4.2 Possible implication of POPDC proteins in the orderly function of the neuromuscular junction

In this chapter the previously generated but not well characterized *Popdc2*^{W188X} mouse mutant was partially characterized. Among others, some parameters chosen were based on the observed differences between mutant and wild-type animals during animal handling and maintenance. It was noted that transgenic *Popdc2*^{W188X} mice displayed a typical whistling sound under stress conditions. This observation has also been made in the *Popdc1* and *Popdc2* KO mouse mutants¹⁸⁴. Whilst no histological analysis of organs which might be involved in this phenotype such as the lung, diaphragm, and the bronchi, was undertaken in the *Popdc2*^{W188X} mouse mutant, pathological alterations of the above-mentioned organs were previously investigated in the *Popdc1* and *Popdc2* KO mouse mutants and no pathologies were detected¹⁸⁴. Whilst it is appreciated that the *Popdc2*^{W188X} mutant must not necessarily phenocopy the *Popdc2* KO mutant, within the body of this work it has been shown that the *Popdc2*^{W188X} transcript, at least within the cardiac tissue, is subject to NMD (section 3.3.5 and 3.4.6), which strongly suggests that there is nearly a complete loss of the *Popdc2*^{W188X} mRNA and therefore of the mutant protein, and thereby likely phenocopies the *Popdc2* KO mutant at the protein level. However, residual mutant mRNA is still detectable by PCR and could be translated. Unfortunately, none of the available POPDC2 antibodies that we have tested were found to be suitable in our hands to investigate this question. Nevertheless, performing a histological analysis of the aforementioned tissues would be warranted to confirm the absence of histological abnormalities. Following the absence of pathological changes in the organs themselves, a hypothesis was formulated that this behavioural phenotype could be

driven by a neurological defect within the NMJ of the diaphragm, which deserves further studies.

Within the scope of this work, no experiments were performed to investigate the role of POPDC proteins in NMJ formation and function as the focus was predominantly to investigate the role of POPDC proteins in β AR signalling in the heart. Nevertheless, as part of the *Popdc2*^{W188X} mouse mutant characterization, below are outlined ways in which POPDC proteins may be involved in the processes governing NMJ with the potential consequence of giving rise to the whistling phenotype observed in the *Popdc1*, *Popdc2* KO and *Popdc2*^{W188X} mutant mice.

The diaphragm is a uniquely mammalian dome shaped structure, which in basic terms, is formed by two radial sheets of skeletal muscle that insert into a central tendon at the apex of the dome. The muscle tissue of the diaphragm is richly innervated by the phrenic nerves which form NMJs with different myofibers. During development, orderly primary branching is driven by the receptor protein tyrosine phosphatases σ and δ ³¹⁵, while *Hoxa5* and *Hoxc5* and interactions between the muscle and the nerve are required for proper secondary branching of the phrenic nerve³¹⁶. Muscle-derived signals regulate phrenic nerve development during secondary branching and arborization via β -catenin within the muscle fibre³¹⁷ whereby proper formation of phrenic nerve NMJ involves a complex interplay of muscle-nerve retrograde and anterograde signaling³¹⁸.

The NMJ is a structurally and functionally complex synaptic interface between the motor neuron nerve terminal and a specialised section of a muscle fibre where the electrical impulse of the motor neuron is converted into electrical activity of the muscle fibre that ultimately results in muscle contraction³¹⁸. NMJ can be defined pre-synoptically by the active zone, a highly specialized membrane enriched, amongst others, in SNARE complex proteins such as syntaxin, SNAP-25, and synaptobrevin (VAMP2), that facilitate the docking and fusion of synaptic vesicles with the presynaptic membrane in order to release the neurotransmitter ACh into the synaptic cleft³¹⁸. Post-synoptically the NMJ is defined by the presence of rich AChR clusters embedded into the postsynaptic membrane of a muscle fibre directly underneath the active zones of the motor neuron. The formation, maturation, and

maintenance of these AChR clusters and that of the NMJ at large is predominantly governed by the Agrin-LRP4-MuSK-Dok7 pathway³¹⁸. It is important to mention that alongside these core components, which function both in the synapse-specific transcriptional pathway and the post-translational anchoring pathway, there is a myriad of additional accessory proteins which ensure NMJ integrity.

POPDC protein expression in the central and the autonomic nervous systems has previously been recorded^{176,182,319} and some of protein interaction partners have been implicated in neuronal function^{184,199,210,253}. For example, synaptobrevin-2 aka VAMP2, as briefly outlined above, is a key component of the SNARE complex formed in the active zone of the presynaptic membrane, where it facilitates the fusion of synaptic vesicles with the presynaptic membrane and a Ca^{2+} -dependent synaptic vesicle exocytosis of the neurotransmitter³²⁰. POPDC proteins have been shown to interact with VAMP2 and VAMP3 in cardiomyocytes, skeletal muscle, and epithelia cells where they facilitate the process of VAMP-mediated vesicular transport which is disrupted after forced expression of a dominant-negative POPDC1 mutant²¹⁰. Although, the interaction of POPDC proteins with VAMP1 and VAMP2, which are enriched in the neuronal tissue, has never been directly studied in the neuronal context, parallels can be drawn, and POPDC interaction with VAMP2 may suggest a broader role of POPDC in vesicular transport in a variety of tissue types that utilise the SNARE system. Therefore, it would be interesting to study the interaction of POPDC proteins with VAMP1 and VAMP2 in neuronal tissue and determine any functional role of POPDC proteins at the NMJ.

POPDC1 and POPDC2 proteins have also been shown to interact with dystrophin¹⁹⁹, a sub-sarcolemmal cytoskeletal protein, which is part of the dystrophin-glycoprotein complex (DGC). It provides support to the muscle fibre by linking the actin cytoskeleton to the DGC, which consists of α - and β -dystroglycan, sarcoglycans, sarcospan, dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)³²¹. Interestingly the DGC is abundantly present at the postsynaptic membrane of the NMJ, where it interacts with Rapsyn, a highly expressed, but currently still poorly understood protein, that directly interacts with the AChRs³²². The association between Rapsyn and DGC acts as a linker between the AChRs and the postsynaptic cytoskeleton thereby anchoring AChRs and stabilising them³²². Interestingly, new Rapsyn-dependent partners have recently been identified, amongst which was Xin actin binding

repeat containing1 (XIRP1)³²³, another recently identified interaction partner of POPDC1 and POPDC2²⁰⁶. However, also in the case of dystrophin the interaction with POPDC proteins has not been studied within the NMJ context. However, bungarotoxin staining of the diaphragmic tissue from *Popdc1* and *Popdc2* KO mouse mutants, showed a reduced and malformed AChR density at the postsynaptic membrane (Thomas Brand – pers. communication). Therefore, it can be hypothesised that loss of POPDC proteins at the postsynaptic membrane of the NMJ may result in a destabilised DGC complex and a disruption of the mechanism by which AChRs are anchored to the postsynaptic membrane of an NMJ. Additional clues of a possible role of POPDC proteins in the NMJ might be derived from what is known about the role of POPDC in the MTJ. As mentioned above, POPDC interact with dystrophin, which as part of the DGC, which is abundantly expressed at the MTJ. DGC together with the integrin receptor complex, allows for proper force transmission between the tendon and the muscle by binding the basement membrane protein laminine2 and thereby anchoring the muscle cell to the MTJ³²⁴. Loss-of-function mutations in *popdc1* and *popdc2* in zebrafish induce severe muscular dystrophy phenotype, characterised by an impaired formation of the MTJ, causing myofiber raptures in the zebrafish mutants^{199,261}. Additionally, electron microscopy of the *popdc1*^{S191F} KI mutant revealed a lack of the electron-dense matrix proteins within the MTJ^{199,261}. In the NMJ, extracellular matrix proteins also interact with DGC and integrins on the muscle fibre surface, providing additional structural support and signalling cues for AChR aggregation. Interestingly, VAMP2/VAMP3, which were shown to interact with POPDC, are known to modulate the integrin receptor recycling^{325,326}. Considering the above it may be speculated that through the interaction with VAMP and/or dystrophin (and possibly other yet unknown proteins) POPDC contribute to a proper AChR anchoring at the postsynaptic membrane. Further research could uncover a common molecular pathway in which the interaction between POPDC and their NMJ relevant interaction partners may contribute to a proper AChR cluster formation at the postsynaptic membrane of a NMJ.

POPDC1 is also thought to be enriched in the membranes near the neuronal synapses of the hippocampus, another type of physiological junction, as it was preferentially found in the synaptosomal but not in the cytosolic fraction³²⁷. In the hippocampus, it was shown to fine tune the regulation of synaptic plasticity in a cAMP-PKA dependent manner by preventing PKA-driven phosphorylation of target proteins in response to subthreshold stimuli. There is

growing evidence that β AR signalling also plays a role in synaptic transmission at NMJ^{328,329} and of course, POPDC proteins as cAMP effectors, may be implicated. Immunostaining in murine skeletal muscle showed co-localisation of β_2 ARs with AChRs at the postsynaptic membrane^{329,330}. Assessment by selective adrenergic drug activity showed that β_2 AR agonists activate AChRs also predominantly at the postsynaptic membrane where the agonist mediated activation of the β_2 ARs leads to an increased AChR cluster formation³³⁰. cAMP release in response to β AR activation, allows for PKA activation, which has been shown to promote the recycling of internalized AChRs into the synaptic sites and anchoring of receptors at the postsynaptic membrane³³¹ through a pathway downstream from the Agrin-LPR4-MuSK-DOK7³³². POPDC proteins are well documented to be expressed in the sarcolemma and preliminary co-IP data produced from heterologous system demonstrated an interaction of POPDC protein with β_2 ARs (data not published, Thomas Brand personal communication). As mentioned already, Bungarotoxin staining of the diaphragm and different leg muscles from *Popdc1* KO mutants showed a reduced density and altered structure of the AChR clusters at NMJs (Thomas Brand, personal communication). Therefore, it is tempting to hypothesize, that POPDC proteins as cAMP effectors, may have a role in β_2 AR mediated regulation of AChRs cycling at the postsynaptic membrane of NMJs. The described above would warrant a dedicated investigation in the role of POPDC proteins in AChR cycling at the postsynaptic membrane of NMJs.

3.4.3 *Popdc2*^{W188X} mutant and reduced fertility

Most of what is known about the function of POPDC in embryogenesis is based on studies investigating POPDC1, with sparse literature available on the role of POPDC2 and POPDC3 in oogenesis and embryogenesis, even though their expression has been documented in the developing embryo^{176,181,183}. Nevertheless, POPDC proteins were shown to form homo and heterodimers as well as oligomers of higher order, which seems to be important for their proper function^{182,198,215,306}. Furthermore, it has become evident that mutation or loss of one POPDC protein isoform leads to a reduced expression and/or mislocalisation of its dimerization partner^{199,215,219,290}, a phenomenon also reported for the *Popdc2*^{W188X} mutant in this body of work (3.3.6 and 3.4.7). Therefore, the interpretation and discussion of the possible causes for the observed phenotype in the *Popdc2*^{W188X} mutant assumes that loss of

Popdc2^{W188X} consequently disrupts localisation and expression levels of Popdc1, leading (at least in part) to the observed phenotype.

Since their discovery, POPDC expression during embryogenesis has been studied in several organisms such as chick³³³, mouse³³⁴, *Xenopus laevis*³³⁵, and *Drosophila*¹⁹⁴. Within this body of work, it was observed that *Popdc2*^{W188X} mutant and wild type breeders on average produce comparable numbers of pups per litter, however, the litter frequency of the *Popdc2*^{W188X} mutants was significantly reduced compared to the wild type breeding pairs. A similar observation has previously been made for *Popdc1* KOs (paper in preparation by Lasman *et al*). In this study, it was shown by IHC and WB analysis that POPDC1-3 proteins are expressed in human and mouse ovarian cells, with the strongest expression in oocytes, where the POPDC proteins were localised in the cytosol as well as the nucleus. The presence of POPDC proteins, in the cell nucleus is an interesting finding, as they are normally known to be transmembrane proteins. A similar observation was made by Schindler *et al.* 2012, in myoblasts and cycling satellite cells, where POPDC1 protein localized to the nuclear envelop and the nucleoplasm, a localisation that was lost following myoblast to myotube differentiation¹⁸⁵. POPDC proteins share a 25% sequence identity and 60% sequence similarity in their Popeye domain, the highest sequence similarity for any protein not being a POPDC orthologues, with the bacterial transcription factor CRP^{176,184}. It can be hypothesized that POPDC proteins evolved from a transcriptional regulator and could possibly act as a transcription factor, which is involved in the regulation during oogenesis and oocyte maturation and possibly in a cAMP dependent manner. Alternatively, POPDC proteins could have a dual function; one as a signalling/scaffolding protein at the plasma membrane, and another in the nucleus as a gene expression regulator, possibly via the Wnt signalling pathway^{228,229}. Wnt signalling is an evolutionarily conserved pathway which has a significant role in malignancy, wound repair, tissue homeostasis as well as embryogenesis, with β -catenin being at the centre of it^{230,231}. POPDC1 was shown to downregulate the Wnt signalling by sequestering β -catenin to the plasma membrane probably via E-cadherins^{193,229}. It is possible that loss of POPDC protein function in the developing oocyte dysregulates orderly Wnt signalling mediated decisions related to proliferation, differentiation, and morphogenesis during various stages of ovarian folliculogenesis.

Moreover, cell migration and cell adhesion are fundamental processes during both oogenesis, and embryogenesis and Wnt signalling plays a significant role in their regulation. Cell adhesion is closely linked to cell migration and tissue remodelling processes during embryogenesis. Cells often need to detach from their original positions, migrate to new locations, and establish new adhesive contacts to shape and reshape structures such as neural tube, heart tubes, limb buds, and organ primordia. Adhesive interactions guide and regulate cell migration, allowing cells to move collectively or individually to specific destinations within the developing embryo. POPDC1 is thought to have a role in cell-cell adhesion^{209,214,319,336}. In this context, knockdown of *popdc1* in *Drosophila* with the help of a *popdc1* antisense construct produced an abnormal epithelial movement, inhibiting pole cell migration and a high percentage of early embryonic death, suggesting *popdc1* is required for cell migration and essential for embryonic development¹⁹⁴. As mentioned above, there is evidence that POPDC regulates the Wnt signalling. Cellular adhesion complexes including tight and adherens junctions are closely related to Wnt signaling^{337–339}. Adherens junctions are modulators of canonical Wnt signalling through sequestration of β -catenin by E-cadherins²⁵⁰ at the cell membrane and Popdc1 is an important regulator of intercellular connections, regulating the expression of E-cadherin in multiple cell lines and tumors^{193,251}. Popdc was shown to colocalize with E-cadherin and β -catenin at the cell membrane. Reduced Popdc expression as seen in *Drosophila*, therefore could result in more available cytoplasmic β -catenin which can be translocated into the nucleus initiating Wnt related transcription, thereby inhibiting cell migration properties.

Another pathway, which regulation is possibly disrupted in early embryogenesis by the loss of POPDC proteins is the GEFT/Rho signalling pathway. Rho signalling is involved in diverse cellular processes, including cell migration, cell adhesion, and cytoskeletal dynamics, including during early development³⁴⁰. Rac1, Cdc42, and RhoA are the main members of the Rho family of GTPases²⁴⁰. GEFT (Guanine nucleotide Exchange Factor for Rho family proteins) is a protein that acts as a guanine nucleotide exchange factor (GEF) for Rho GTPases and plays a crucial role in their activation and regulation²⁴⁰. GEFT was also the first protein identified to directly interact with POPDC1 at the plasma membrane²⁰⁸. During the early steps of embryonic development, the body axis (anterior-posterior) is specified by collective cell

migration of an extra-embryonic organizer before the onset of gastrulation³⁴¹. Whilst Wnt signalling is important for cell migration, Rho family GTPases are thought to have an important role in directional regulation of cell migration with Rac1 and Cdc42, which are crucial in actin cytoskeleton polymerization and filopodia formation, regulating cell-cell and cell-matrix adhesion³⁴². RhoA is less well studied during the early steps of embryogenesis, but its loss seems also to be embryonic lethal³⁴³. Since POPDC1 is known to be enriched at areas of cell-cell contact, it can be hypothesized that under physiological conditions, the membrane-bound POPDC1 through its interaction with GEF, localizes GEF proteins to the leading-edge membrane of motile cells, where Rac1 and Cdc42 have their highest activity²⁵⁰. This interaction brings these GTPases and their GEF together to allow for catabolic activity with a downstream effect of promoting cell motility. Loss of *Popdc1* consequently would prevent the formation of these active catabolic sites, thereby inhibiting cellular motility.

Rac1 and *Cdc42*-null mice are embryonic lethal during the early stages of development^{344,345}, however, *Popdc2*^{W188X} mutants do produce litters albeit at a reduced frequency. It is possible that mechanisms other than those described above are responsible for the observed reduction in fertility of mutant mice, for example disrupted mechanisms during oocyte development, sperm abnormalities or an abnormal uterine implantation, none of which have been investigated yet. Additionally, the *Popdc2*^{W188X} is not considered to be a fully penetrant mutation, which would possibly allow for offspring production at a reduced frequency.

There is also some evidence that *Popdc1* and *Popdc2* mRNA is present in testicular tissue and in the uterus of pregnant mice. Interestingly, in the uterus of mice that were not pregnant no expression of *Popdc1* and *Popdc2* mRNA was found¹⁷⁶. These results were produced by Northern blot analysis and the specific cell types expressing these transcripts within the analysed tissue, were unknown. However, it is plausible that *Popdc1* and *Popdc2* were in fact expressed in uterine smooth muscle cells. POPDC1 expression in smooth muscle cells has previously been reported by immunocytochemistry and LacZ staining in a variety of tissues^{175,176,181,336}. POPDC proteins have been shown to interact with TREK1 and promote trafficking of TREK1 channel to the plasma membrane in *Xenopus* oocytes, where POPDC proteins are able to modulate TREK1 current in a cAMP dependent manner. TREK1 channels have also been identified to be expressed and are thought to be important in uterine smooth

muscle function during pregnancy by promoting quiescence³⁴⁶. It is possible that POPDC proteins also interact with and regulate the activity of TREK1 in the uterine smooth muscle cells during pregnancy and their loss could contribute to a dysregulation of TREK1 channel function promoting a premature loss of pregnancy. Especially since *Popdc1* is only expressed in the uterus of pregnant mice, where TREK1 expression is also significantly upregulated. However, no evidence for preterm birth has been obtained in POPDC mutant mice.

No studies have investigated the function of POPDC in the testicular tissue, however, Rac1, Cdc42 and RhoA have been found to be upregulated in testicular tumors³⁴⁷. As previously described, there is evidence suggesting that POPDC1 through its interaction with GEFT and ZO-1 inhibits Rho activity^{245,348}. Suppression of POPDC1 in cell lines resulted in increased RhoA activity promoting cell migration and invasion. Loss of POPDC proteins could disrupt mechanisms governing orderly spermatogenesis, reducing fertility.

3.4.4 *Popdc2*^{W188X} mutants have significantly smaller hearts than wild-type mice

Popdc2^{W188X} mutants have a lower body weight if compared to wild type mice³⁴⁹. To understand whether the *Popdc2*^{W188X} mutation has an impact on the heart size of the mutant mice independently of the reduced body weight, heart weight-to-tibia length ratios were measured and compared to wild-type animals. Using tibia length to normalize the heart weight relative to body size minimizes the influence of body weight variations and facilitates a better comparison between *Popdc2*^{W188X} mutant and wild-type animals. The heart weight normalized to tibia length was significantly reduced in the *Popdc2*^{W188X} mutant. Similarly, a reduced body weight was recorded for the *Popdc2* null mutants^{184,349}, albeit with a preserved heart weight-to-tibia length ratio. This is an important distinction as the *Popdc2*^{W188X} mutant shows NMD driven loss of *Popdc2* expression which one could argue mimics the *Popdc2* KO phenotype (described in 3.3.5 and 3.3.6). However, since there is some residual mRNA, which could be translated into protein, it is possible that the truncated protein might have a more severe phenotype than the null mutant. This will need to be investigated further.

Reduced heart weight can result from impaired cardiomyocyte growth, through reduced cell proliferation and premature terminal differentiation. Of course, the reduced heart size could be due to a loss of other cell types such as fibroblasts or endothelial cells or due to a reduced volume of the extracellular matrix. However, there are at least two main reasons speaking against non-myocyte involvement: firstly, cardiomyocytes make up approximately 70% to 85% of the volume of a mammalian heart³⁵⁰, so a significant reduction in the size of the heart would be expected to be predominantly driven by changes in cardiomyocyte size or number. Secondly, POPDC proteins are known to be expressed in cardiomyocytes and not in other cell types within the heart, therefore heart size alterations recorded in the *Popdc2*^{W188X} mutation carriers would be expected to be likely due to changes within the cardiomyocyte population.

In the *Popdc2*^{W188X} mutant impaired cardiomyocyte growth would result in a smaller cardiomyocyte size, however, no significant difference in the size distribution of AMCM was recorded between the *Popdc2*^{W188X} and wild-type genotypes, i.e., isolated AMCM from both genotypes were on average of a comparable size. Cellular differentiation is often associated with decreased cell proliferation and differentiated cardiomyocytes acquire a binucleated state. Rodent cardiomyocyte binucleation begins and ends within the first two weeks after birth³¹¹, with a detectable increase in cell volume and binucleation only occurring from day four post-partum³⁵¹. Prior to that cardiomyocytes undergo cell proliferation. For this set of experiments, binucleation levels were compared in isolated NMCMs of pups that were 0-2 days old to identify whether the *Popdc2*^{W188X} mutation induces premature terminal differentiation in the NMCM, thereby reducing the cell number and the overall size of the heart. However, the data shows no significant increase in the number of binucleated myocytes in the *Popdc2*^{W188X} mutants. On the other hand, assessing the total number of cardiomyocytes after each NMCM isolation, collectively showed a significantly reduced yield of cardiomyocytes from *Popdc2*^{W188X} mutant hearts if compared to the wild type samples. These findings would suggest that the *Popdc2*^{W188X} mutation might negatively impact on cell proliferation of NMCM. It would be of advantage to confirm these differences between the mutant and wild type neonatal hearts with a more sophisticated method, such as flow cytometry or by staining tissue sections immunohistochemically with mitosis markers such as phospho-histone 3, which would label proliferating cells, before comparing the outcomes between the mutant and wild type samples.

The role of POPDC as modulators of cell proliferation has not been extensively studied and what is known is predominantly centred around POPDC1 within the context of cancer studies^{191,193,221,222,245}. POPDC1 has been shown to interact with multiple molecules in pathways regulating cell proliferation and loss of *Popdc1*, or suppression of its function are generally associated with a pro-malignant cell behaviour such as increased cell proliferation, migration, and invasion^{195,208,221,245,352}. Therefore, it is thought to function as a tumour suppressor in various cancer types. In the cardiac myocytes of the *Popdc2*^{W188X} mutant, POPDC1 expression is strongly reduced at the plasma membrane and the remaining protein is mislocalised, often found in the cytoplasm or in perinuclear vesicles (section 3.3.6 and 3.4.7). The level of expression often varies between individual cardiomyocytes. The role of POPDC1 as a tumour suppressor seems to be at odds with the current findings in NMCM, which show that its loss/mislocalisation may be preventing cell proliferation rather than promoting it. Even less is known about the role of POPDC2 as modulator of cell proliferation. One study however, identified POPDC2 to have oncogenic function in ductal breast carcinoma, where it was significantly overexpressed in the breast cancer tissue at all clinical stages of the disease¹⁹⁵. Additionally, authors suggested a possibly synergistic link between POPDC2 and HER2 (Human Epidermal Growth Factor Receptor 2 aka ErbB2 in mice). HER2 is known to synergize with factors which drive tumorigenesis such as mitogen-activated protein kinase p38 (p38 MAPK), as leptin/Ob-Rb/signal transducer and activator of transcription 3 (STAT3) and transforming growth factor β (TGF β)^{353–356}. In the murine heart ErbB2 is widely expressed during prenatal development followed by a rapid decline in expression in the neonatal heart³⁵⁷. During the narrow period of neonatal proliferative growth, ablation of ErbB2 results in an upregulation of negative cell cycle regulators and a reduced cardiomyocyte proliferation. It is possible that, under physiological conditions POPDC2, which is known to be widely expressed throughout the developing heart, with or via ErbB2 is implicated in promoting proliferation of neonatal cardiomyocytes. Additionally, POPDC proteins were reported to display nuclear expression in cardiac myocytes, myoblasts, and epithelial breast cancer cells^{225,226,358}. The functional implications of this localisation and the mechanisms that facilitate the transport of POPDC proteins to and into the nucleus are currently unknown, however, it may be indicative of a role that POPDC proteins play in regulating cell cycle gene expression, or they could interact with proteins that regulate cell cycle and proliferation. The lack of empirical data makes the above-mentioned mechanisms however highly speculative,

and more studies are needed to investigate the role POPDC2 might have in the regulation of cell proliferation in neonatal cardiomyocytes. This, for example, could have relevance regarding cardiac regeneration, which is maintained in the neonatal period and lost rapidly postnatally. If POPDC proteins might be involved in modulating postnatal proliferation a way to modulate their activity could foster cardiac regeneration after myocardial infarction.

Lastly, in cancer studies it is emerging that the three POPDC proteins can have opposite effects potentially through the interaction with different protein partners and signalling cascades that they are part of. Additionally, the individual function of each POPDC protein isoform is showing to be tissue specific. This can possibly explain the contradictory observation in the *Popdc2*^{W188X} mutant, which shows a reduced cell proliferation despite the apparent dysregulation of Popdc1 localisation and expression. More work is clearly required to untangle the role of POPDC proteins in cardiomyocyte cell cycle control.

3.4.4.1 *Popdc2*^{W188X} mutants show significant loss of abdominal fat

Over the duration of this project, whilst working with *Popdc2*^{W188X} mutant mice, it was observed that these mutants are leaner, with a significant loss of abdominal fat (Prof. Thomas Brand, personal communication). While the topic of metabolic control is outside of the scope of this thesis, it is noteworthy that through α - and β ARs, cAMP signalling has an enormous impact on metabolic pathways at the cellular level. cAMP signalling is involved in regulating basal metabolic rate, glucose disposal, and lipid partitioning and there is a growing body of work which links dysregulation of adrenergic receptor signalling to metabolic disorders³⁵⁰. In light of the observation that *Popdc2*^{W188X} mutants (as do POPDC1 and POPDC2 null mutants) show a significant loss of abdominal fat, and that POPDC proteins are cAMP effectors which have an important role in cAMP signalling in cardiac myocytes¹⁸⁴ (also discussed in chapter 4), it would be warranted to investigate the possible role of POPDC proteins in fat metabolism.

3.4.5 *Popdc2*^{W188X} mutants have an abnormal cardiac electrophysiology

Cardiac arrhythmia is characterized by irregular rhythm of the heartbeat which could be either bradyarrhythmic (too slow) or tachyarrhythmic (too fast)^{9,359}. The irregularity of the heartbeat can be initiated through damage from disease and injury (non-hereditary

arrhythmia) or genetics (hereditary arrhythmia) causing alterations in the complex electrophysiological properties of cardiomyocytes and their underlying ionic mechanisms regulating calcium cycling. Hereditary forms of arrhythmia are predominantly caused by mutations in genes encoding ion channels or their modulators. Mutations in POPDC genes have been shown to generate an arrhythmogenic phenotype in animal models. For example, *Popdc1* and *Popdc2* KO mouse mutants display stress induced sinoatrial node dysfunction, *popdc2* morphant zebrafish show AVB and *Popdc1/Popdc2* double KO mice displayed a sinoatrial node dysfunction and AVB^{184,307}. Similarly, patients carrying mutations in *POPDC1* and *POPDC2* present sinus bradycardia and an AV block of varying severity^{199,219,281,282,287}. The differences in cardiac pathologies observed between humans, mouse and zebrafish are most likely related to species-dependent differences in heart rate as well as ion channel distribution and utilisation for AP generation. Here, the novel *Popdc2*^{W188X} mouse mutant has been assessed for its electrophysiological phenotype. The *Popdc2*^{W188X} mouse mutant was generated upon a WES-based discovery of a heterozygous *POPDC2*^{W188X} variant in a group of patients with a mild to severe forms of AV block displaying autosomal dominant inheritance trait, which contrasts the recessive inheritance traits observed in patients carrying either *POPDC1* or *POPDC3* variants^{213,280}. A homozygous instead of a heterozygous animal model was chosen because the heterozygous mutant does not show a pathological phenotype. The *ex vivo* hearts of the *Popdc2*^{W188X} mouse mutant show a stress-induced bradyarrhythmic phenotype. ECG recordings have revealed an inability of the mutants to appropriately elevate and maintain their heart rate in response to different levels of β -adrenergic stimulation, showing significant beat-to-beat heart rate fluctuations and sinus pauses generated. These findings affirm further the important role of POPDC proteins in regulating cardiac electrophysiology, although the underlying mechanisms are not yet fully understood. POPDC proteins have been identified as cAMP effector proteins and are probably mediating the stress response via the cAMP signalling pathway. Under physiological conditions, a regular, spontaneous heart beat is by the molecular mechanisms that drive SAN automaticity and heart rate adaptation is the result of the net outcome of the antagonism between sympathetic activation of the β AR-cAMP-PKA signalling pathway and the parasympathetic activation of the muscarinic ACh receptor signalling, before the signal is propagated to the AV node, followed by the impulse propagation to the ventricular conduction system, which orchestrates synchronous ventricular myocyte contraction. Disorders, which may lead to

bradyarrhythmia generally originate in the SAN or in the AV node. The use of retrograde Langendorff perfusion as the experimental set up allowed to study the effects of the *POPDC2*^{W188X} mutation on the cardiac electrophysiology in isolation from the influences by the autonomic nervous system, confirming that the observed pathology is originating from within the intrinsic cardiac conduction system.

During the initial *in vivo* studies in the *Popdc2*^{W188X} mutant mouse, performed by Rinne et al., (2020), using non-invasive ECG in conscious young adult mice, the authors have not seen alterations in the PR interval, P duration and QT interval²⁸⁰. The PR interval on an ECG provides information about the conduction time from the atria to the ventricles and any prolongation of the PR interval would be indicative of a delay or blockage in the AV node. Since the PR intervals seem unaltered, it suggests that there is no abnormal AV node function in the *Popdc2*^{W188X} mouse mutant, which is in line with previous observations in *Popdc1* and *Popdc2* null mutants¹⁸⁴.

In contrast, the SAN of the mouse mutant shows abnormalities as it is unable to raise the heart rate upon β AR stimulation. The pacemaker activity of the SAN and the frequency with which the pacemaker potential is generated, depend on the open probability of the HCN4 channels, which are responsible for the *I_f* current and drive the spontaneous depolarization of the pacemaker cells. The open probability of HCN4 channels is increased in response to cAMP binding, upon β -adrenergic activation. However, when Froese et al. (2012), measured the *I_f* current density and activation time in isolated SAN myocytes of the *Popdc2* null mutant, no differences were present when compared with wild type¹⁸⁴, suggesting POPDC proteins may regulate the SAN activity through the interaction with other proteins involved in cardiac pacemaking.

SAN automaticity and the generation of the pacemaker potential relies on a complex interplay of multiple voltage-gated ion channels and transporters at the plasma membrane of the SAN myocytes, collectively called the membrane clock and the intracellular Ca²⁺-clock, which is formed by the proteins involved in the orderly Ca²⁺-cycling in cardiomyocytes (i.e., LTCC, SERCA2a, PLN, CASQ2, RYR2, NCX etc.). The two clocks are inextricably linked and disruption to the orderly function or regulation of any of its constituents can result in an abnormal SAN

automaticity. It is becoming apparent that POPDC proteins interact with several ion channels and other electrogenic regulators of cardiac pacemaking. The following section discusses these interactions and how they might contribute to the phenotype recorded in the *Popdc2*^{W188X} mutant.

Previous in vivo assessment of the *Popdc2*^{W188X} mutant showed no significant difference in their heart rate at baseline if compared to the heart rate of the wild type animals²⁸⁰. However, in absence of autonomic nervous system influences, such as during the retrograde Langendorff experimental set up, a significantly elevated heart rate prior to β AR stimulation was indeed recorded in the mutant hearts, followed by a failure to further adapt the heart rate appropriately in line with the strength of the β AR stimulus. This would suggest that loss of POPDC proteins at the plasma membrane in the *Popdc2*^{W188X} mutant permits cAMP effector activation and substrate phosphorylation by PKA even at low cAMP levels during baseline conditions.

cAMP compartmentalization is a well-established paradigm^{149,360–364}. cAMP is a highly diffusible second messenger employed in many cellular responses and is therefore ubiquitously present in the cardiac myocytes. Its activity within a specific and desired signalling cascade is achieved by spatially organizing its effectors and regulators into specialized signalling complexes or signalosomes. Further to this, activation of cAMP effectors depends on their proximity to PDEs, which are the only known enzymes with the ability to hydrolyse cAMP and thereby forming cAMP gradients around their effectors^{156,161,365,366}. Additionally, PDEs themselves are translocated to the membrane or a variety of scaffolds by virtue of special sequences which promote protein-lipid or protein-protein interaction³⁶⁵. There is emerging evidence that alongside the PKA substrate protein, PKA, AKAP and PDE, POPDC proteins are also found in these signalling complexes or alternatively are part of a protein complex adjacent to these signalling domains and bind some of these proteins. This is important as POPDC proteins are known to function by way of protein-protein interaction and their loss from within the signalosome would result in a dysregulation of the associated protein(s) within the signalling complex. In fact, a recent study by Tibbo et al. (2022), reported that POPDC proteins exist in complexes with members of the PDE4 family in cardiac myocytes. It was shown that, POPDC1 can bind several of the PDE4 long isoforms, but has a preference

for PDE4A. Significantly, disruption of the POPDC1-PDE4 interaction by a membrane permeable disruptor peptide in murine SAN explants, resulted in an acceleration of the Ca^{2+} -transient frequency, an effect which was lost after isoproterenol stimulation²¹⁸. These findings suggest that the POPDC1-PDE4 interaction is functionally relevant in the SAN under basal cAMP levels. The frequency of Ca^{2+} transients, corresponds to the pacemaker rate as the calcium and membrane clock are tightly linked. A faster frequency of Ca^{2+} transients, leads to a higher heart rate. Therefore, it is conceivable, that in the *Popdc2*^{W188X} mutant, the observed elevated heart rate during basal conditions is driven at least in part by the loss of the POPDC1-PDE4 interaction in relevant signalling complexes in the SAN myocytes.

In the SAN of the murine heart PDE4A, B and D are the predominant isoforms^{367,368}, whereby PDE4B controls the intrinsic SAN automaticity and PDE4D seems to be important for autonomic nervous system-mediated regulation of the heart rate and conduction. The specific role of PDE4A in cardiac pacemaking is not fully defined yet. However, as described above it forms a strong interaction with POPDC1 and has functional relevance in the SAN explants during basal conditions. It may also have relevance in binding with other members of the POPDC protein family. The core sequence at which the POPDC1-PDE4A interaction was identified, is also present in the POPDC2 and POPDC3 with some conservative substitutions²¹⁸. More work is required to determine which PDE isoform POPDC2 may interact with and its exact functional relevance, particularly regarding the phenotype observed in the *Popdc2*^{W188X} mutant.

POPDC proteins have also been shown to directly interact and directly or indirectly regulate the activity of electrogenic proteins important in generation of SAN automaticity such as TREK1 and NCX1, which could be disrupted in the *Popdc2*^{W188X} mutant thereby resulting in chronotropic incompetence of the mutant heart^{184,199,268,280}.

TREK1 is a potassium channel that is thought to be involved in regulation of pacemaking activity in the heart by controlling cell excitability and maintenance of the membrane potential below the threshold of depolarization⁷². TREK1 is highly expressed in the SAN of mouse and rabbit and its downregulation is associated with sinus node dysfunction where loss of TREK1 promotes deregulation of excitability of the SAN myocytes and pacemaker

defects. Thereby, cardiac specific ablation of TREK1 in these model animals produces bradycardia with episodes of sinus pauses following stress, which is comparable to the phenotype observed in the *Popdc2*^{W188X} mouse mutant. TREK1 is known to display functional interaction with POPDC2 proteins. Froese et al. in a series of experiments involving co-expression, GST-pull-down and FRET analysis, were demonstrated that the interaction of TREK1 and POPDC2 proteins produces an increased outward current at the plasma membrane through an enhanced surface expression of TREK1, suggesting that POPDC proteins promote trafficking of TREK1 to the plasma membrane¹⁸⁴. The enhanced TREK1 current flow is negatively modulated in presence of elevated levels of cAMP such as after β AR stimulation. It is thought that cAMP binding to POPDC proteins induces structural rearrangement which disrupts the interaction between POPDC and TREK1. This is further supported by the finding in the human *POPDC1*^{S201F} mutant, which shows a lack of membrane localisation, and a reduced cAMP binding affinity, where during increased cAMP levels there is a distinct reduction in membrane localisation of TREK1¹⁹⁹. These findings suggest that POPDC proteins modulate TREK1 channel activity and TREK1 membrane trafficking. Thereby, POPDC proteins may modulate the resting membrane potential and the excitability of cardiac myocytes. As mentioned previously, the human *POPDC2*^{W188X} variant presents an AVB phenotype instead of the SAN dysfunction as seen in the mouse mutants and in the study by Rinne et al, it was shown that TREK1 had a very strong and predominant expression in the AV node where POPDC2 also has a prominent expression pattern²⁸⁰. Additionally, authors have shown TREK1 activity to be directly modulated by POPDC2. This expression profile might be the basis for a TREK1 modulation via POPDC2 in the human AV node, whereby in the mouse it is more relevant in the SAN. Mechanistically, it has been shown that under physiological conditions, TREK1 binding site within the POPDC1 Popeye domain is in proximity or overlaps with the binding site of PDE4, suggesting they may exist in a signalling complex³⁶⁹. Furthermore, TREK1 in its C-terminus harbours a binding site for PKA and AKAP79/150 complex. Collectively this suggests that POPDC1 forms a complex with TREK1 and PDE4, and the PKA-AKAP79/150 complex is either part of or located adjacent to the POPDC-TREK1-PDE4 signalling complex. Moreover, aside from the described role that POPDC play in TREK1 membrane trafficking and channel gating, an interaction of sodium channel SCN5A and POPDC proteins has also been shown³⁰⁶. It is therefore conceivable that POPDC proteins may have the potential to be involved with ion channel trafficking in general and this may be essential in particular in SAN

myocytes and maybe involved in pacemaker adaptation to stress, presently the full spectrum of protein-protein interactions involved has not yet been fully characterized. Experiments involving proximity labelling using Turbo-ID or Apex2 might be helpful in this regard as in particular protein-protein interactions of membrane proteins are difficult to characterise with more conventional approaches such as immunoprecipitation coupled to mass spectrometry³⁷⁰.

Recently, a proteomic study has demonstrated by affinity purification coupled with mass spectrometry a protein-protein interaction between POPDC2 and NCX1²⁶⁸. Their interaction was confirmed by co-immunoprecipitation. NCX1 has an important role in maintaining cytosolic Ca^{2+} homeostasis in cardiomyocytes and is the primary mechanism for Ca^{2+} extrusion during diastolic relaxation by the virtue of the depolarizing I_{NCX} current. Its loss in the NCX KO mouse model was shown to cause SAN dysfunction, which the authors suggested is due to elevated cytoplasmic calcium levels, and the resulting phenotype is similar, although more severe, to the one observed in *Popdc2*^{W188X} mutants²⁶⁹. At the plasma membrane, NCX1 is found in a macromolecular complex with kinases, phosphatases, and adaptor proteins. The exact mechanism of how POPDC2 and NCX1 interact is not fully understood, but it is possible that under physiological conditions, POPDC2 is part of the NCX1 signalling complex where POPDC2 regulates NCX1 membrane trafficking and gating directly or via the cAMP-PKA pathway. Since POPDC2 has a high cAMP binding affinity it could function as a cAMP sink to spatially regulate cAMP diffusion and thereby limiting other cAMP effector activation, such as PKA, or POPDC2 could regulate post-translational modification of the NCX1 molecule by altering PDE activity within the complex. In either case, the presence of POPDC2 would contribute to the regulation of the phosphorylation state of NCX1 thereby controlling its membrane trafficking and Ca^{2+} sensitivity. Conversely, loss of POPDC2 from the signalling complex would result in a dysregulation of the NCX1 phosphorylation state and activity in response to βAR activation. Further studies are needed to fully understand the mechanism of interaction between POPDC2 and NCX1.

Overall, pacemaking at baseline and after βAR stimulation is reliant on a complex interplay between a network of electrogenic and regulatory molecules and POPDC proteins play a role in fine tuning the intricately balanced interactions and regulation of enzyme activity. It is

unlikely that the recorded phenotype of the *Popdc2*^{W188X} mutant would arise from a single aberrantly regulated protein within the pacemaker network, however, loss of POPDC1 and POPDC2 from the plasma membrane as seen in the *Popdc2*^{W188X} mutant may lead to a dysregulation within several signalling complexes leading to an aberrant phosphorylation profile and precocious activation of various electrogenic proteins.

3.4.6 *Popdc2*^{W188X} is subject to nonsense mediated decay in the mouse mutant

As previously described in this chapter, the nonsense *POPDC2*^{W188X} variant, produces a truncated POPDC2 protein with a partial loss of the functionally important Popeye domain and the carboxy-terminus²⁸⁰. Surprisingly, the *POPDC2*^{W188X} mRNA transcript does not seem to be subject to NMD in patients' leucocytes despite the presence of a PTC. Rinne and colleagues were able to detect the *POPDC2*^{W188X} transcript by cDNA sequencing and the corresponding truncated protein was detectable by western blot analysis²⁸⁰. This is particularly interesting as by in vitro analysis the truncated POPDC2^{W188X} was shown to retain its ability to bind cAMP with similar affinity as its wild-type counterpart, form homodimers and interact with the wild type POPDC1 and POPDC2 and also with TREK1, which results in an aberrant current modulation^{280,306}. Based on these findings it was tempting to hypothesize that the phenotype observed in patients may be due to the presence of a dominant-negative POPDC2^{W188X} protein. However, in this body of work, I have been able to show that in ventricular tissue and in isolated NMCM of the *Popdc2*^{W188X} mutant mouse the mutant transcript is subject to NMD, which makes it highly unlikely that any mutant protein is being expressed. The extraction and sequencing of the genomic material from the *Popdc2*^{W188X} mutant mice, confirmed the presence of a PTC at aa position 188 within exon 2 of the *Popdc2* gene. However, almost no *Popdc2*^{W188X} mRNA was detectable in ventricular tissue of homozygous adult animals, and a 50% reduction of *Popdc2* expression was recorded in heterozygotes compared to wild type. NMD is a translation-dependent mechanism and arresting translation in isolated NMCM by cycloheximide, resulted in a nearly 9-fold increase of *Popdc2*^{W188X} transcript levels. These results strongly suggest that the *Popdc2*^{W188X} transcript is largely eliminated by the NMD mechanism in the *Popdc2*^{W188X} mouse mutant.

What could be the reason for the different results in patients' leucocytes versus ventricular cardiomyocytes? In mammalian cells, the mechanism by which the PTC is recognized and discriminated from natural stop codons is currently explained by two models³⁰⁵. One suggests that a PTC within an mRNA can be recognized as such during all cycles of translation and is based on the distance between the PTC and to the poly (A) tail due to the competitive binding of the major NMD regulator, up-frameshift factor 1 (UPF1), and cytoplasmic poly (A) binding protein 1 (PABPC1) with eukaryotic polypeptide chain release factor 3 (eRF3), which interacts with eRF1³⁷¹. An alternative and more widely supported model in mammals^{372,373} suggests that a PTC carrying mRNA is targeted for NMD based on the position of the exon-junction complexes (EJC) whereby the presence of an EJC more than 50-55 nucleotides downstream of the PTC defines the mRNA as a NMD substrate³⁷⁴. It is therefore a splicing dependent pathway that triggers PTC recognition, as EJC, a multiprotein complex, is bound to the mRNA near the exon-exon junctions during the pre-mRNA processing before the mRNA leaves the nucleus. In this model NMD can only be activated during the pioneer round of translation when the first ribosome reads the mRNA and removes all EJCs present on its path until it reaches a stop codon. If any EJCs remain on the mRNA after the ribosome encounters a stop codon, NMD factors are recruited by that EJC, and mRNA degradation is triggered. More recently, a combinatory model has been proposed, suggesting PTC can be recognized at two successive steps, incorporating both, the PTC recognition during the pioneer round of translation and recognition of any remaining PTC during later rounds of translation^{375–377}. Still, neither of the independent models nor the combinatory model are able to explain all cases of NMD activation, indicating that NMD activation remains incompletely understood³⁷⁸. While at their core these basic principles of PTC recognition and NMD activation are shared across different cell types, there can be significant regulatory differences. NMD has a central role in cell homeostasis and can interact bi- or unidirectionally with many cellular pathways which can influence its efficiency. It has been shown that NMD efficiency can be influenced by the position of the PTC³⁷⁹, tissue type and tissue-specific factors^{372,373,380} as well as stress conditions^{381–383} and NMD escape is not uncommon even though little is known about the exact mechanisms that permit NMD escape to take place.

It is likely that the efficiency of the NMD mechanism in leucocytes vs cardiomyocytes varies. Cardiomyocytes have an extremely limited capacity for regeneration with nuclear DNA

synthesis events estimated to be as low as 0.5-2% per year³⁸⁴. Hence, sophisticated, and efficient surveillance systems such as for example for protein quality control, are in place to preserve the cardiomyocyte homeostasis^{385,386}. POPDC proteins are highly expressed in the myocardium and the CCS, therefore, it is possible that due to their elevated levels of expression, surveillance by NMD is more efficient. However, it also needs to be stressed that in patients, the POPDC2^{W188X} is dominant and therefore patients are heterozygous, which may also play a role for the NMD mechanism to be triggered.

A nonsense mutation, which affects translation should phenocopy a null allele. However, in case of a nonsense mutation, the cell retains the genetic information, and down regulation may occur at the transcriptional and posttranscriptional level, whilst in the case of a knockout mutation, the gene sequence is fully deleted. Nonsense mutations can be pharmacologically rescued by a readthrough activator. A readthrough activator induces the incorporation of a near cognate codon at the PTC position and thereby preventing NMD activation resulting possibly in a rescue of the nonsense phenotype^{375,387,388}. Ataluren and gentamycin are two known readthrough activators, which induce a readthrough of PTCs but not of any natural stop codon^{388–390}. Attempts to induce a readthrough using these compounds in *Popdc2*^{W188X} NMCM (data not shown, experiments performed by a visiting student) did not rescue the expression of POPDC2, likely because PTC-readthrough substrates are first NMD substrates, so that the number of PTC-containing mRNAs available for readthrough is extremely low. PTC readthrough is a highly promising therapeutic approach for genetic diseases caused by a nonsense mutation, therefore, should there be capacity to investigate this further in the future, a combinatory approach of NMD inhibitors with readthrough activators could possibly yield more favourable results.

Of course, it cannot be excluded that some of the truncated POPDC2^{W188X} protein, albeit possibly at exceptionally low quantities, does escape the NMD and might have deleterious effects in cardiomyocytes, or other cell types where POPDC2 is expressed. Raising antibodies, which are suitable to recognize the truncated POPDC2^{W188X} protein would help to uncover, whether the phenotype observed is solely due to the loss of the POPDC2 protein and the consequential downregulation and mislocalisation of POPDC1, or whether the pathological phenotype is also due to the expression of a deleterious POPDC2^{W188X} protein.

3.4.6.1 Increased levels of *Popdc1* and *Popdc3* transcript in the *Popdc2*^{W188X} mutant

In addition to measuring the levels of the mutant transcript, the expression of *Popdc1* and *Popdc3* were also assessed in the *Popdc2*^{W188X} mutant mouse to identify the impact of the mutation on the expression of the other two POPDC genes. Interestingly, whilst *Popdc2*^{W188X} levels were nearly abolished, the transcript levels of *Popdc1* and *Popdc3* were significantly increased in the *Popdc2*^{W188X} mutant cardiomyocytes. The transcript levels for both, *Popdc1* and *Popdc3*, were also increased in the heterozygous animals, but the difference reached no significance. POPDC1 and POPDC2 are known to form dimers, the formation of which is essential for proper membrane transport of these transmembrane proteins²¹⁵. And dimer formation is dependent on an appropriate expression level of both proteins. The closest related protein to POPDC is the bacterial transcription regulator CAP, from which POPDC proteins might possibly have evolved. POPDC1 proteins are known to accumulate in the nuclear membrane and the nuclear envelope and interact with some transcriptional regulators²¹⁶. It is possible, although experimentally no data in support of this hypothesis has been obtained, that POPDC1 and POPDC2 could be regulating each other's expression. Therefore, when the expression of POPDC2 is lost due to NMD the result is an upregulated *Popdc1* expression assuming that POPDC2 is involved in suppressing POPDC1 expression levels. POPDC1 and POPDC3 are organized in tandem on chromosome 6q21. The tandem configuration of POPDC1 and POPDC3 is preserved down the evolutionary tree to lower chordates, which suggests importance for coordinated gene regulation. Additionally, strong correlation between a reduction in the transcript levels of POPDC1 and POPDC3 was observed in patients with gastric cancer. Therefore, the upregulation of *Popdc1* in the *Popdc2*^{W188X} mutant could also have a similar effect on *Popdc3* because of a co-regulation of both genes. Furthermore, the increase in *Popdc1* and *Popdc3* transcript was particularly present in the animals of younger age. Due to the low sample number, it is hard to draw any definitive conclusion about the significance of this observation, however, it could be considered an adaptive mechanism to compensate for the loss of POPDC2 which becomes less efficient with persistent and accumulative dysregulation in absence of a functional *Popdc2* at an increasing age. (Possible since the pathological phenotype in *Popdc1* and *Popdc2* null mutants worsens with age.

3.4.7 Loss of POPDC2 affects the membrane expression of POPDC1 in the *Popdc2*^{W188X} mutant heart

POPDC proteins are transmembrane proteins, which are normally found at the plasma membrane of cardiomyocytes, with a particular enrichment at the cell-cell contact sites, where they are thought to interact with other proteins in order to mediate proper cellular function^{206,279,349}. In section 3.4.6 I discussed the fact that POPDC2 expression is probably lost from the plasma membrane due to NMD in the *Popdc2*^{W188X} mutant mouse. Evidence has accumulated over the years that POPDC1 and POPDC2 are dependent on each other for proper membrane transport and in most cases a mislocalisation or loss of both proteins occurs if either POPDC1 or POPDC2 carries a mutation, regardless whether the observation has been made in patients or in animal models and was in most cases independent of the variant or the impact of the mutation on protein structure^{184,199,215,219,290}. The loss of POPDC1 and POPDC2 at the plasma membrane is assumed to be related to the pathologies observed in patients and animal models.

Also, in the *Popdc2*^{W188X} mouse mutant the subcellular expression and expression levels of POPDC1 were assessed by immunostaining. In wild type cardiomyocytes, POPDC1 is expressed at the plasma membrane, while in the *Popdc2*^{W188X} mouse mutant, a significant reduction and mislocalisation of POPDC1 is observed. Similarly, in cultured NMCM of wild-type animals, a strong POPDC1 signal can be detected at the plasma membrane, with additional signal enrichment at sites of cell-cell contact. This expression pattern is largely lost in NMCM of the *Popdc2*^{W188X} mutant, where only a faint intracellular or perinuclear expression of POPDC1 was detected. It appears, that the *Popdc2*^{W188X} mutation is another example of a POPDC mutation resulting in a loss of both POPDC1 and POPDC2 expression at the plasma membrane. The reason for the loss of both POPDC proteins at the plasma membrane is still subject of ongoing investigations. However, it has been demonstrated that when co-expressed in HEK293 cells at the appropriate levels, POPDC1 and POPDC2 are transported to the plasma membrane^{215,306}.

As more POPDC mutations are being uncovered, in the future, it will probably be possible to make predictions about the pathogenicity of a particular POPDC mutation based on the level

of mislocalisation present in transfected cells, where stronger impairment of plasma membrane localisation is more likely detrimental to protein function and giving rise to cardiac arrhythmias and muscular dystrophy.

The presence of multiple interaction sites between POPDC1 and POPDC2 and their ability to form heterodimers as well as oligomers of higher order, has previously been shown^{182,198,306}. Additionally, in a recent study by Swan et al. (2023), by means of mutagenesis, *in vitro* experiments and the use of the protein structure predictions such as AlphaFold²¹¹, it was shown that the interaction between POPDC1 and POPDC2 via their Popeye domain and specifically via some ultra-conserved hydrophobic residues within the core of the α C-helix of the Popeye domain is essential for the correct membrane trafficking of the POPDC protein complex to the plasma membrane²¹⁵. Whereby the previously reported interaction sites within the N-termini and/or transmembrane domains as well as the cytoplasmic regions are proposed to be necessary for the preferential heteromeric interactions, which have been shown to be the favoured dimer constellation between POPDC proteins²¹⁵. Therefore, it is plausible that a mutation of residues necessary for POPDC protein heterodimer formation or a complete loss of one of the dimerization partners, as in case of the *Popdc2*^{W188X} mutant, would result in the mislocalisation of both POPDC proteins.

However, in *Popdc2*^{W188X} mutants, we not only see a mislocalisation but also a severe reduction in expression of POPDC1, despite the increased expression of *Popdc1* mRNA. This suggests that loss of *Popdc2* not only prevents proper subcellular localisation but also promotes enhanced degradation of the orphan POPDC1 in the *Popdc2*^{W188X} mutant. The specific mechanism by which this occurs is currently not known. However, one clue might lie in the observation made after forced expression in cell lines of either *Popdc1* or *Popdc2* alone, an aggregation in the ER of the corresponding protein is observed. Thus, in absence of the dimerization partner, the orphan POPDC proteins might not be properly trafficked to the plasma membrane and instead is targeted for degradation. Amino acid motifs, usually within the cytoplasmic domain of the protein, often play a role in subcellular targeting of transmembrane proteins. Proteins can be prevented from leaving the ER by the action of so-called ER retention motifs within the amino acid sequence of the protein. By sequence inspection, putative motifs, which may function as ER retention motifs have been identified

in the three POPDC isoforms²¹⁵. Moreover, the monomeric AlphaFold models of POPDC1 and POPDC2 predicts proximal localisation of the putative ER retention motifs to the proposed Popeye domain dimerization helical interface. It has been proposed that under physiological conditions the formation of the aforementioned α C-helix interface between the Popeye domains of POPDC1 and POPDC2 produces a shielding effect of the ER retention motifs within the cytoplasmic region of both proteins, an effect that may be lost in the absence of the POPDC dimerization partner²¹⁵. It is important to stress that the presence of these ER retention motifs and their functional involvement in membrane targeting of POPDC proteins have not yet been verified empirically. Nevertheless, a transmembrane protein retained in the ER is ultimately degraded by the ubiquitin ligase complexes, which are present in the ER. Alternatively, orphan Popdc1 proteins, which are mislocalised into the cytoplasm would be degraded by the ubiquitin-proteasome system due to the recognition of its transmembrane domains³⁸⁵, with the final outcome being that both POPDC1 and POPDC2 proteins are not expressed at the plasma membrane where they have their main function.

3.5 Conclusion and future work

In the initial stages of this project, it was hypothesized that the POPDC2^{W188X} protein is expressed in cardiomyocytes and that the phenotype observed may be due to the loss of the functionally important CTD. However, in this chapter I have shown that the *Popdc2*^{W188X} transcript is subject to NMD in the heart. Therefore, the mutant POPDC2^{W188X} protein is probably not expressed. Moreover, in the *Popdc2*^{W188X} mutant, a strongly reduced expression and mislocalisation of POPDC1 was observed, suggesting that like in case of numerous *POPDC1* variants, a *POPDC2* mutant variant affects the expression and subcellular localisation of both POPDC isoforms. A complex formation between POPDC1 and POPDC2 seems to be necessary for the correct plasma membrane trafficking of both proteins and based on the current state of knowledge, I have provided a possible explanation for this phenomenon. However, it must be taken into consideration that it is a field of ongoing research. The conclusion of a presumptive loss of POPDC2 at the mRNA and protein level is exclusively based on the results obtained by quantitative PCR, and even though the reduction in transcript level was at 95%, without further confirmation by immunohistochemistry or western blot analysis it cannot be excluded that some of the truncated protein is still expressed in the *Popdc2*^{W188X}

mouse mutant. Therefore, as new POPDC2 antibodies are being developed, these should be tested for their reactivity with the mutant Popdc2^{W188X} protein as even at small quantities the truncated POPDC2^{W188X} protein could have deleterious effects, if, for example, it was trafficked to the plasma membrane as a homodimer where it would impair an orderly regulation of the POPDC interacting partners within specific signalosomes.

Irrespective of the question whether some truncated protein is expressed in the *Popdc2*^{W188X} mouse mutant, I have been able to demonstrate that the *Popdc2*^{W188X} mutant hearts have an abnormal electrophysiology, probably by experiencing a dysregulation of the electrogenic proteins involved in generation of the pacemaker potential. Moreover, in hindsight, the truncation of the Popdc2^{W188X} may be of a lesser significance in the context of this project where the Popdc2^{W188X} protein is presumed to be lost. However, this does not diminish the putative role of the CTD of POPDC proteins in the regulation of POPDC protein activity during β AR stimulation. Therefore, defining the role of the CTD would still be warranted to further our understanding of the biological activity of POPDC proteins.

Additionally, in this chapter, I have commenced the characterization of the structure of the *Popdc2*^{W188X} mutant heart. *Popdc2*^{W188X} mutants have smaller hearts, probably due to a reduced cell proliferation. Still, in absence of any evidence for a premature terminal differentiation of neonatal cardiomyocytes and in the absence of changes in the size of AMCM of the *Popdc2*^{W188X} mouse mutant, the hypothesis of an impaired cardiomyocyte proliferation is largely based on a simple cell counting method and it would be warranted to confirm these finding with a more sophisticated cell counting methods such as flow cytometry or by BrdU labelling experiments. I have attempted to provide some suggestions for the molecular pathways by which these changes may be occurring; however, the ideas are highly hypothetical and derived from what is known about the role of POPDC proteins in cell proliferation, which has mainly been studied in the context of cancer studies from which parallels might be drawn, it still remains an exciting vast subject to be investigated, many gripping and tantalizing mysteries to be solved.

Finally, whilst working with the *Popdc2*^{W188X} mouse mutant, observations were made that the phenotypic consequences of this mutation reach beyond the heart. In the context of the

Popdc2^{W188X} mouse mutant characterization these have been included and I have attempted to provide some explanations for the observed changes, based on what is known about the role of POPDC proteins in different tissues and their interaction partners.

CHAPTER 4 β -adrenergic signalling in the *Popdc2*^{W188X} mutant

4.1 Introduction

The sympathetic stimulation of the heart is regulated via the activation of the β AR-cAMP-PKA signalling pathway. β_1 AR and β_2 AR are the two main β AR family isoforms in cardiomyocytes, of which both are activated by the sympathetic hormones epinephrine and norepinephrine to regulate cardiac contractility (inotropy), heart rate (chronotropy), and relaxation (lusitropy) in response to physical or emotional stress. Simplistically, in a canonical β AR signalling pathway, ligand dependent activation of the β AR induces a dissociation of the associated heterotrimeric $G\alpha\beta\gamma$ -protein complex into $G\alpha$ and $\beta\gamma$ which can activate an AC isoform to stimulate cAMP synthesis and cAMP is able to propagate sympathetic signalling through its effector proteins PKA, EPAC, HCN, and POPDC. (Detailed description in Chapter 1.2.).

Of the four cAMP effectors, PKA is the main effector to drive substrate phosphorylation during cardiac contractility and heart rate regulation. Phosphorylation is a quick and extremely sensitive and reversible post-translational modification (PTM), in which a phosphate group is added to a side chain of certain amino acids, most commonly serine (S), threonine (T), or tyrosine to change the active state of a protein, change its catalytic activity or even drive the change of its subcellular localisation^{391,392}. In cardiomyocytes, cAMP-activated PKA can phosphorylate many target proteins involved in ECC and automaticity including Rad for augmentation of the LTCC at the plasma membrane, PBL, and RyR2 at the SR as well as proteins of the contractile apparatus such as troponin I (TPNI) and myosin binding protein C (MyBPC) on the myofilaments. Significantly, β ARs themselves can be phosphorylated by PKA in a process called heterologous desensitization which is a negative feedback loop aiming to reduce the effectiveness of β AR signalling and consequently reduce cAMP levels to prevent overstimulation of cells and apoptosis.

As previously described, cAMP is a ubiquitous second messenger utilised by a plethora of GPCRs which regulate many distinct aspects of cardiac myocyte function including cell metabolism, gene transcription, ECC and automaticity. Therefore, establishing specificity of the β AR-cAMP-PKA signalling pathway becomes essential to achieve the desired downstream response. Signalling specificity is achieved through the spatiotemporal compartmentalisation

of the cAMP effectors, regulators and cAMP signalling associated proteins into specialised signalling complexes or signalosomes. Dysregulation of cAMP compartmentalization has been observed in cardiovascular diseases, highlighting the importance of appropriate control of local cAMP signalling. Importantly, cAMP-PKA signalling within the different signalosomes, can be modulated by restricting or enhancing AC activity and through recruitment of specific PDEs that generate discrete and distinct cAMP gradients around cAMP effectors to modulate their activity within the aforementioned signalosomes. Recent research has shown that POPDC proteins can form functional complexes with some cAMP regulators, e.g. AC9 and PDE4^{218,393}. Although a full catalogue of protein-protein interaction partners is still missing, the fact that POPDC proteins, which bind cAMP with high affinity also interact with other elements of the cAMP signalling pathway suggest an intricate network of protein-protein interactions, which probably is modulated by cAMP binding to the Popeye domain to achieve fine-tuning of cAMP signalling in response to sympathetic stimulation.

POPDC proteins are expressed at high levels in working cardiac myocytes, nodal myocytes and myocytes of the ventricular CCS. Their loss from the plasma membrane of cardiac myocytes has been associated with an arrhythmogenic phenotype in knockout animal models and patients carrying *POPDC1* and *POPDC2* variants^{184,199,218,280}. This also includes the *Popdc2*^{W188X} mouse mutant. A functional assessment of the *Popdc2*^{W188X} mutant hearts, both in vivo²⁸⁰ and as ex vivo preparations (see Figure 3.7), has shown a sinus bradycardia phenotype with significant number of sinus pauses resulting in an impaired chronotropic response to sympathetic stimulation producing significant beat-to-beat heart rate variability.

Out of the four known cAMP effectors, POPDC proteins are the most recently established cAMP effectors and their specific role within the β AR-cAMP-PKA signalling pathway in cardiomyocytes is an excitingly new area of investigation. In this Chapter, I present the results of my experimental work, uncovering the role that POPDC proteins have in regulating β AR signalling in the *Popdc2*^{W188X} mutant cardiomyocytes, which to my knowledge has not yet been done.

4.2 Hypothesis and aims

4.2.1 Hypothesis

In Chapter 3, I have shown that the Langendorff perfused *Popdc2*^{W188X} mutant hearts experience stress induced chronotropic incompetence in response to β AR stimulation. A phenotype likely arising because of an NMD-driven loss of POPDC2 and mislocalised/reduced POPDC1 expression in cardiomyocytes²¹⁵. Therefore, as cAMP is an important second messenger for β AR signal transduction and POPDC proteins are cAMP effectors which can complex with cAMP regulators, and the loss of which causes chronotropic incompetence in mutant hearts, a hypothesis was formulated that in *Popdc2*^{W188X} mutant cardiomyocytes, loss of POPDC1 and POPDC2 from the plasma membrane induces abnormal cAMP dynamics due to a dysregulation of cAMP signalling intermediates following β AR activation. The hypothesis was later refined to include that the aforementioned dysregulation may be due to an aberrant β 2AR - G α i coupling, triggered by the loss of POPDC1-PDE4A complex formation.

4.2.2 Aims

1. To study cAMP dynamics in cultured NMCM and AMCM isolated from wild type and *Popdc2*^{W188X} mutants in response to β AR stimulation

Objectives:

- a. I will utilise the cytosolic EPAC FRET reporter to establish whether there is a difference in cAMP dynamics between the mutant and wild type NMCM following β AR activation.
 - b. I will use the cytosolic CUTie FRET reporter to confirm results produced in (1), and to improve the FRET reporter expression levels in NMCM.
 - c. I will use the plasma membrane localised AKAP79-CUTie FRET reporter to measure cAMP dynamics in the mutant and wild type NMCM and AMCM at the plasma membrane.
2. To study the selective β_1 AR or β_2 AR signalling in cultured wildtype and *Popdc2*^{W188X} mutant NMCM.
 3. Using a synthetic disruptor peptide, to study the significance of the recently identified POPDC1-PDE4 complex formation at the plasma membrane compartment for β AR induced cAMP release in cultured NMCM.
 4. To study whether POPDC1 or POPDC2 proteins form a complex with AKAP79.

4.3 Results

4.3.1 Investigating cAMP dynamics in cultured *Popdc2*^{W188X} mutant NMCM

It is known that under physiological conditions agonist-induced activation of β 1AR and β 2AR in cardiomyocytes results in a G α s-mediated activation of AC and a rapid increase in cAMP levels. To develop an understanding of the cAMP dynamics following β AR stimulation in real time in individual NMCM isolated from wild type and *Popdc2*^{W188X} mutants, FRET microscopy (described in Methods 2.10.) was performed in cultured NMCM expressing cellular compartment-specific FRET biosensors.

4.3.1.1 *Popdc2*^{W188X} mutant NMCM show reduced cytosolic cAMP levels following β AR stimulation

The initial investigations assessing cAMP levels in cultured NMCM were commenced using the cytosolic ^TEpac^{VV} FRET biosensor (Figure 4.1 A) which was readily available as an established sensor for FRET microscopy in the research group of my second supervisor Professor Julia Gorelik. For the assay, NMCM were isolated from *Popdc2*^{W188X} mutant and wild type neonatal hearts, transiently transfected with equal amounts of the cytosolic ^TEpac^{VV} FRET biosensor construct and cultured for 40-48h to permit sufficient expression of the FRET biosensor. NMCM expressing the cytosolic ^TEpac^{VV} FRET biosensor were selected based on their fluorescent signal and imaged using an inverted microscope equipped with a camera, a lamp illuminator pillar and a Dual View beam splitter which allowed the fluorescence emanating from the cells to be split into YFP and CFP channels and thereby, using a PC and the Micro-Manager software, to record and quantify the relative ratio of YFP and CFP channels and hence monitor FRET output. The FRET output recorded using the ^TEpac^{VV} FRET biosensor is inversely proportional to the cAMP concentration in the cell due to the structural arrangement of the cAMP binding domain and the fluorophores within the 3D structure of the folded EPAC reporter molecule; in an unbound state the fluorophores are in close proximity resulting in high FRET, whilst cAMP binding induces a conformational change of the reporter molecule, moving the fluorophores further apart, resulting in a lower FRET signal (Figure 4.1 B). The cAMP concentration within the cytosolic compartment was recorded at baseline, and after successive treatments of the same NMCM of interest with the global β AR agonist isoproterenol (ISO) at 1nM, 10nM and 100nM concentrations. The recorded cAMP

levels were then calculated as a percent of the total cAMP release induced by the full activation of ACs with 50 μ M FSK and complete block of PDEs by 100 μ M IBMX. FRET sensors for cAMP are most commonly read out as ratio metric time-lapse recordings; therefore, every 6 seconds one image was recorded, resulting in a time-lapse recording of 10 images per minute. In NMCM of the *Popdc2*^{W188X} mutant, cAMP levels are significantly lower at all three β AR agonist concentrations if compared to the wild type NMCM (Figure 4.1 A, B).

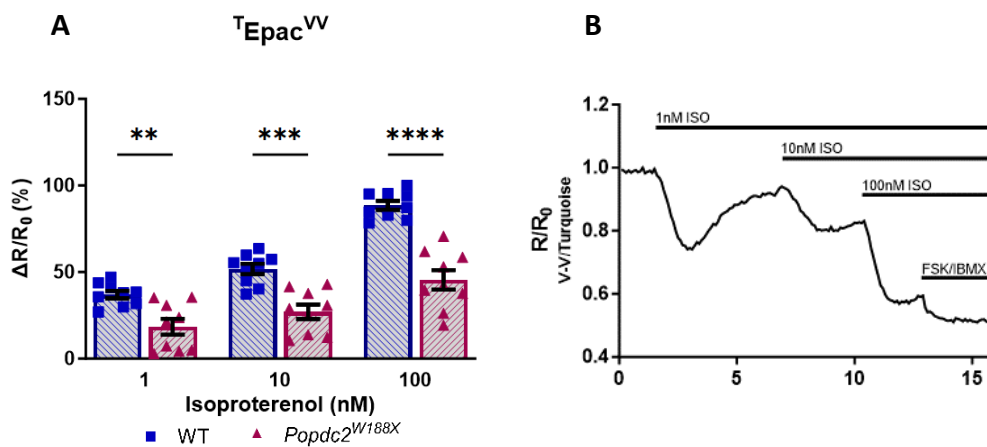


Figure 4.1. Cytosolic cAMP release induced by β AR stimulation in wild type and *Popdc2*^{W188X} mutant NMCM expressing the ^{TEpacVv} FRET reporter. (A) Summary data shown as % change in FRET of the saturating stimulus (FSK+IBMX) recorded after each successive stimulation with 1nM, 10nM and 100nM β AR agonist isoproterenol (ISO). (B) Representative kinetics of FRET change at baseline and after ISO treatment. Statistical analysis was performed using two-way ANOVA with Šídák post hoc analysis, n=3, **p<0.01, *p<0.001 ****p<0.0001. Error bars show $\pm$ SEM.**

A reduction in the cAMP response after ISO stimulation was also observed with another cytoplasmic FRET biosensor (Figure 4.2) developed by Professor Manuela Zaccolo and colleagues at the University of Oxford, which was termed cAMP Universal Tag for imaging experiments (CUTie)³⁰². The widely used Epac1 based biosensors generally feature a variation of the YFP-CFP fluorophores fused amino- and carboxyterminally of the CNBD. When fused with components of various multiprotein complexes for specific nanodomain targeting, these were found to produce variable effects on the maximal FRET change when stimulated with a saturating stimulus (FSK+IBMX) which made them unsuitable for comparing cAMP signals at different target cellular sites. CUTie was designed based on a novel topology to serve as a universal FRET moiety to tag proteins of interest without affecting FRET sensor properties. The CUTie FRET biosensor utilises the second CNBD of the regulatory subunit type II β of the PKA as the cAMP-sensing moiety. CFP has been placed carboxyterminally, however YFP was

moved from the amino terminus and inserted at the 4-5 loop within the CNBD, which minimises the potential interference of the fluorophore with the appropriate targeting of the FRET sensor whilst simultaneously showing minimal risk of perturbing the folding of either CNBD or YFP. Additionally, fusion of CUTie to targeting domains such as for example AKAP79 for plasma membrane localisation, did not significantly alter the kinetics or maximal amplitude of FRET change, which is important for the studies presented later in this Chapter. FRET output produced using the CUTie reporter is directly proportional to the cAMP levels as cAMP binding to the CNBD brings the fluorophores in close proximity allowing for high FRET. Conversely, when CUTie is not bound by cAMP, the YFP-CFP fluorophores are found at a greater distance from each other resulting in low FRET.

Significantly, CUTie FRET biosensors were available as adenoviral preparations which allowed for a consistently increased transduction efficiency of NMCM as well as AMCM (presented later in this Chapter) at a reduced loss of viable cells in each preparation. Additionally, for further studies an increased number of NMCM robustly expressing the CUTie FRET reporter was combined with an updated imaging system using the high-throughput real-time measurement tool called MultiFRET, developed by the Gorelik group at Imperial College London³⁰³. In addition to some hardware updates, the main advantage of the new system was the permissibility to record FRET from multiple FRET reporter expressing cells within one preparation, making it a much more time efficient and cost-effective tool, which allowed to produce a higher volume of consistent data. For this set of experiments, the underlying principle of FRET microscopy remained the same, however, as mentioned above, an updated hardware and software were used, a more detailed description of which can be found in the methods and materials section 2.10.

NMCM expressing the cytosolic CUTie FRET biosensor, were not treated with successively increasing concentrations of β AR agonist, as was the case for the NMCM expressing the $T\text{E}^{\text{pac}}^{\text{VV}}$ FRET biosensor. Instead, each NMCM was stimulated with one ISO concentration which was then quantified against the FRET response after saturating stimulation (50 μ M FSK + 100 μ M IBMX). This set-up shortened the duration of the experiment, preserved the integrity of the imaged cells and importantly, avoided false results due to β AR desensitisation after prolonged stimulation. Nevertheless, the cultured NMCM of the *Popdc2*^{W188X} mutant

expressing the CUTie FRET biosensor also showed significantly reduced cytosolic cAMP levels in response to ISO stimulation. The level remained consistently below 20% of the saturating stimulus across all three ISO concentrations (Figure 4.2), thereby confirming the results obtained with a slightly different experimental protocol and the ^TEpac^{VV} FRET biosensor. In Figure 4.2 B, C are also shown representative kinetics for the CUTie reporter expressing wild type and *Popdc2*^{W188X} mutant NMCM, respectively, stimulated with 100nM ISO. There is no apparent difference in the kinetics between the NMCM of the two genotypes, and the predominant difference is in the amplitude of the cAMP release after β AR stimulation (Figure 4.2 B, C).

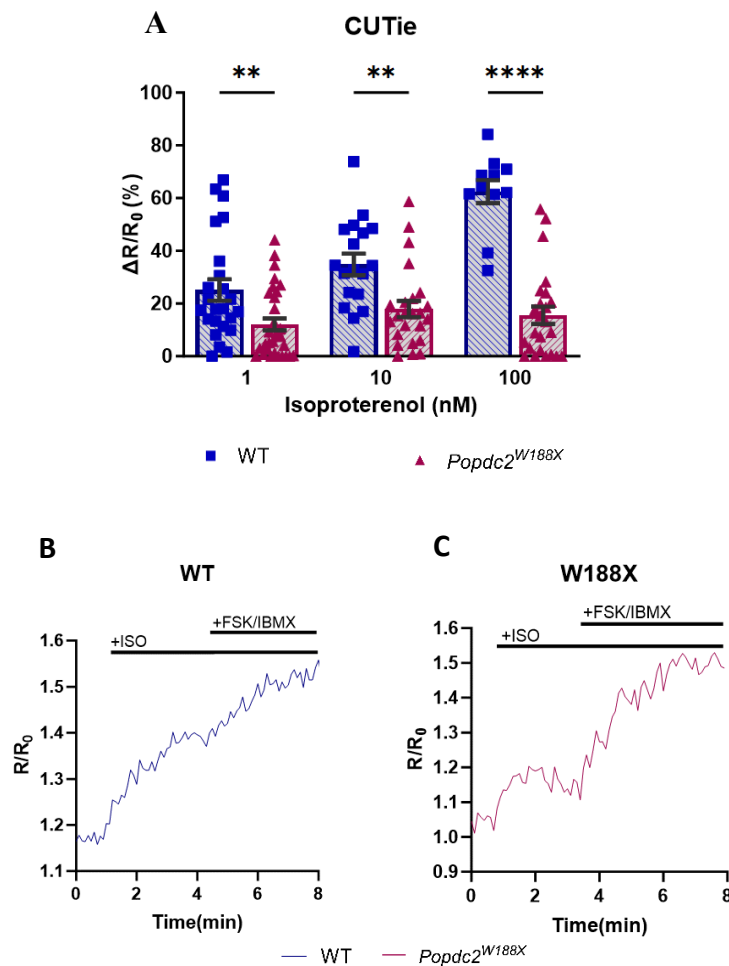


Figure 4.2. Cytosolic cAMP release induced by β AR stimulation in wild type and *Popdc2*^{W188X} mutant NMCM expressing the CUTie FRET reporter. (A) Summary data shown as % change in FRET of the saturating stimulus (50 μ M FSK+100 μ M IBMX) recorded after stimulation with either 1nM, 10nM or 100nM concentration of β AR agonist ISO. (B, C) Representative kinetics of FRET change at 100nM ISO in (B) wild type and (C) *Popdc2*^{W188X} mutant NMCM. Statistical analysis was performed using two-way ANOVA with Šídák post hoc analysis, n=4 **p<0.01 and ****p<0.0001. Error bars show $\pm$ SEM.

4.3.1.2 *Popdc2*^{W188X} mutant NMCM show reduced cAMP following β AR stimulation levels using an AKAP-79 sensor

POPDC proteins are normally expressed at the plasma membrane of cardiomyocytes, therefore the next logical step was to investigate cAMP dynamics of the *Popdc2*^{W188X} mutant NMCM using a FRET sensor, which is localized to the plasma membrane. To this end the plasma membrane-targeted AKAP79-CUTie FRET biosensor was expressed in cultured NMCM using the same protocol as previously described for the cytosolic FRET sensor. Identical to what was seen for the cytoplasmic sensor, β AR-induced cAMP release was significantly reduced at the plasma membrane of *Popdc2*^{W188X} mutant NMCM for all three agonist concentrations, whilst the kinetics were alike (Figure 4.3). Interestingly, the mean reduction at all agonist concentrations is consistently 2-2.5-fold lower in the *Popdc2*^{W188X} mutant compared to wild type NMCM. Thus, although the cAMP level is reduced in mutant cells, the response appears to be proportionally increased at all three agonist concentrations.

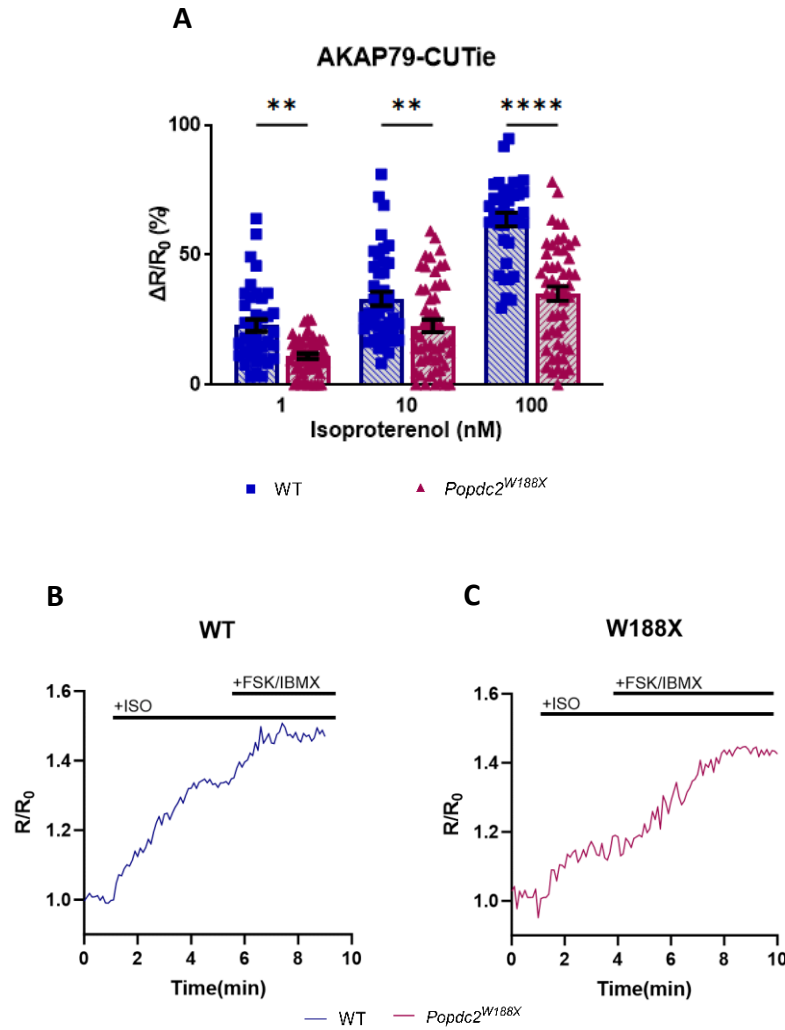


Figure 4.3. β AR induced release of cAMP at the plasma membrane compartment in cultured wild type and $Popdc2^{W188X}$ mutant NMCM. (A) Summary data showing % change in FRET in response to β AR stimulation using 1, 10 and 100 nM ISO recorded in cultured wild type and $Popdc2^{W188X}$ mutant NMCM expressing the AKAP79-CUTie reporter. The % change in FRET was calculated of the saturating stimulus (50 μ M FSK+100 μ M IBMX). (B, C) Representative kinetics of the FRET change in (B) wild type and (C) $Popdc2^{W188X}$ mutant NMCM. Statistical analysis was performed using two-way ANOVA with Šídák post hoc analysis, data are based on 6 myocyte isolations and a total of 39, 42 and 37 cells (WT) and 45, 47 and 51 cells ($Popdc2^{W188X}$) at 1, 10 and 100 nM ISO stimulation, respectively were investigated. ** $p < 0.01$ and **** $p < 0.0001$. Error bars show $\pm$ SEM.

4.3.2 cAMP dynamics in the $Popdc2^{W188X}$ mutant NMCM are perturbed by dysregulated β ARs

Given that cAMP is an important second messenger for β AR signalling in cardiomyocytes and important for proper heart rate regulation, which in Chapter 3 has been shown to be pathologically impaired in $Popdc2^{W188X}$ mutants, it was hypothesised that the reduction in cAMP levels in $Popdc2^{W188X}$ mutant NMCM may be driven by an abnormal regulation of the protein intermediates within the β AR signalling pathway upon stimulation.

4.3.2.1 *Popdc2*^{W188X} mutant NMCM show normal cAMP dynamics at baseline and in response to maximum β AR stimulation

To further study the above formulated hypothesis, I first aimed to determine whether there are any differences in cAMP concentration between wild type and *Popdc2*^{W188X} mutant NMCM at baseline (Figure 4.4A) i.e., prior to stimulation, and after treatment with the saturating stimulus of 50 μ M FSK with 100 μ M IBMX (Figure 4.4B) i.e., measuring the total capacity of a given NMCM to generate cAMP. Neither value showed a significant difference. Together, these data suggest that on average the cultured NMCM of both genotypes have the capacity to generate similar cAMP dynamics at the plasma membrane at baseline and after being challenged with a saturating stimulus and the above-described differences in cAMP levels between the wild type and mutant NMCM only arise following an agonist-induced β AR activation.

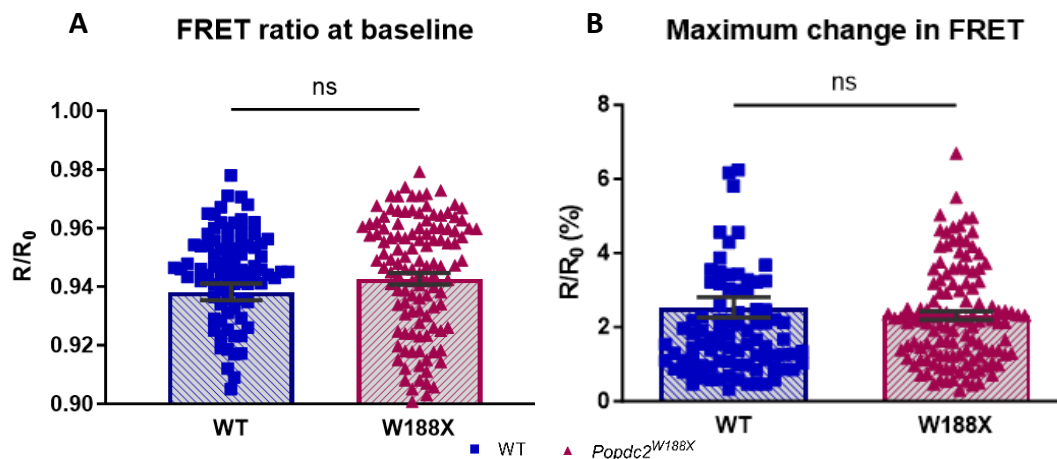


Figure 4.4. FRET at baseline and maximum change in FRET in cultured wild type and *Popdc2*^{W188X} mutant NMCM. (A) FRET ratio at baseline in the absence of any pharmacological stimulation and (B) maximum change in FRET induced by 50 μ M FSK with 100 μ M IBMX in cultured wild type and *Popdc2*^{W188X} mutant NMCM expressing the AKAP79-CUTie reporter. Statistical analysis was performed using the Mann-Whitney test, ns: not significant. Data are derived from 7 independent NMCM isolations (n=7). Error bars show $\pm$ SEM.

4.3.2.2 Abnormal cAMP dynamics in *Popdc2*^{W188X} mutant NMCM are recorded after β AR activation but not in response to activation of prostaglandin receptors

cAMP is a ubiquitous second messenger for a broad range of biological processes and is used to transduce intracellular signals from a variety of Gs-coupled receptors. For example, E-type prostaglandin receptors (EPs) act via production of cAMP, but do not stimulate in cardiac contractility^{394,395}. Prostaglandin E1 (PGE1) binds to EP2 and EP4 receptors in cardiac

myocytes and both receptors are excluded from caveolae and therefore signal through a different cAMP nanodomain than β ARs³⁹⁶. To assess whether the aberrant cAMP dynamics of the *Popdc2*^{W188X} mutant NMCM are also present in response to activation of other GPCRs or specific to β ARs, cultured wild type and *Popdc2*^{W188X} mutant NMCM expressing the plasma membrane-localised AKAP79-CUTie FRET biosensor were treated with either 100nM PGE1 agonist or 100nM ISO following which the cAMP levels were measured by FRET microscopy. As seen in Fig. 4.5. A and B, stimulation of the PGE1 receptor induces a measurable increase in cAMP at the plasma membrane of both the wild type and the *Popdc2*^{W188X} NMCM. However, in contrast to the ISO-stimulated cells (Fig 4.5 A and C), no significant difference in cAMP kinetics or amplitude of the cAMP response between the genotypes was observed after treatment (Figure 4.5). These findings suggest that the observed dysregulation of cAMP signalling in *Popdc2*^{W188X} mutant NMCM is specific to β AR-dependent signalling.

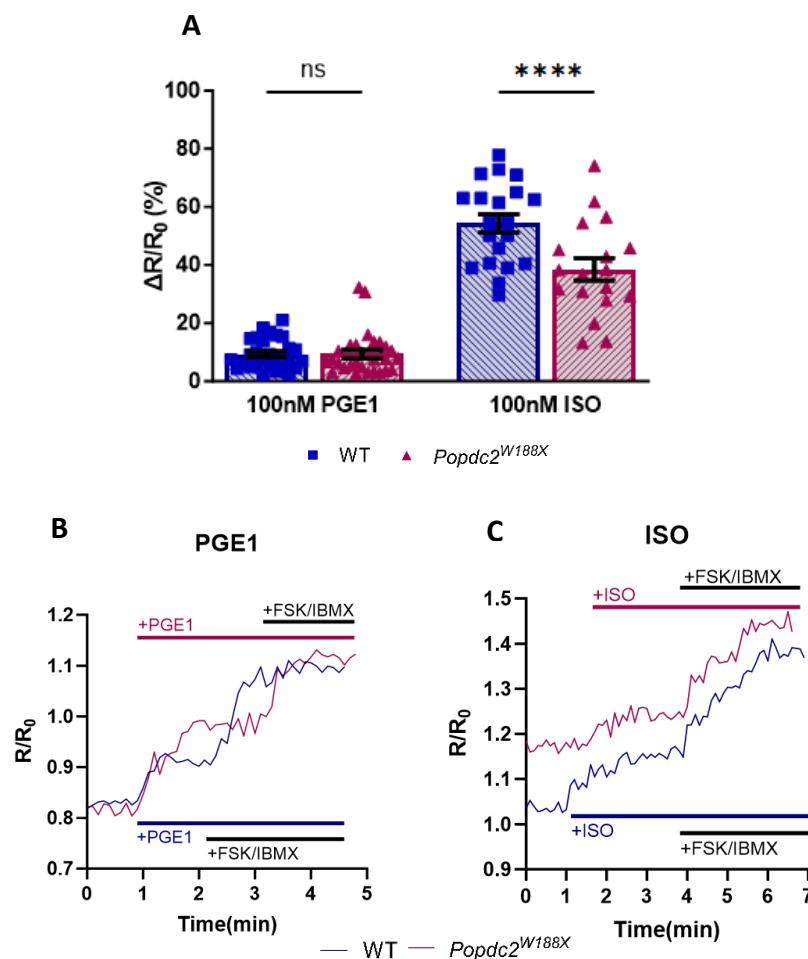


Figure 4.5. β AR or PGE1 induced plasma membrane localised cAMP release in cultured wild type and *Popdc2*^{W188X} mutant NMCM. (A) Quantification of the FRET change [%] relative to a saturating stimulus (FSK+IBMX) in wild type and *Popdc2*^{W188X} mutant NMCM expressing the plasma membrane-specific AKAP79-CUTie reporter after treatment with 100nM PGE1 or 100nM ISO. (B, C) Representative kinetics of FRET change

for (B) 100nM PGE1 or (C) 100nM ISO. Statistical analysis was performed using two-way ANOVA with Šídák post hoc analysis, n=4 for PGE1 and n=3 for ISO, ns=not significant, ***p<0.0001. Error bars show $\pm$ SEM.

4.3.2.3 The enzyme activity of AC is not impaired in *Popdc2*^{W188X} mutant NMCM

The concentration of cAMP available for effector activation in response to β AR activation in cardiomyocytes is tightly controlled and a balance of AC-catalysed cAMP synthesis and PDE-mediated cAMP degradation within the nanodomain. Using a host of cell biological and biochemical methods, it has been shown that POPDC1 and POPDC2 can form a complex with AC9.³⁹³ In Chapter 3, it was shown that POPDC1 and POPDC2 are lost from the plasma membrane in the *Popdc2*^{W188X} mutant and in this chapter, it has been uncovered that NMCM of the *Popdc2*^{W188X} mutant have significantly reduced cAMP concentrations following β AR activation. To determine whether the loss of POPDC1 and POPDC2 has an aberrant effect on the enzyme activity of AC at the plasma membrane, cultured NMCM of the wild type and *Popdc2*^{W188X} mutant expressing the AKAP79-CUTie FRET reporter, were treated with 25 μ M FSK and the amplitude and kinetics of the cAMP release were measured using FRET microscopy (Figure 4.6). There is no significant difference between genotypes in the cAMP response initiated by direct activation of AC. This suggest that the loss of POPDC proteins has no direct effect on cAMP synthesis and does not explain the observed reduction in cAMP concentration following β AR stimulation.

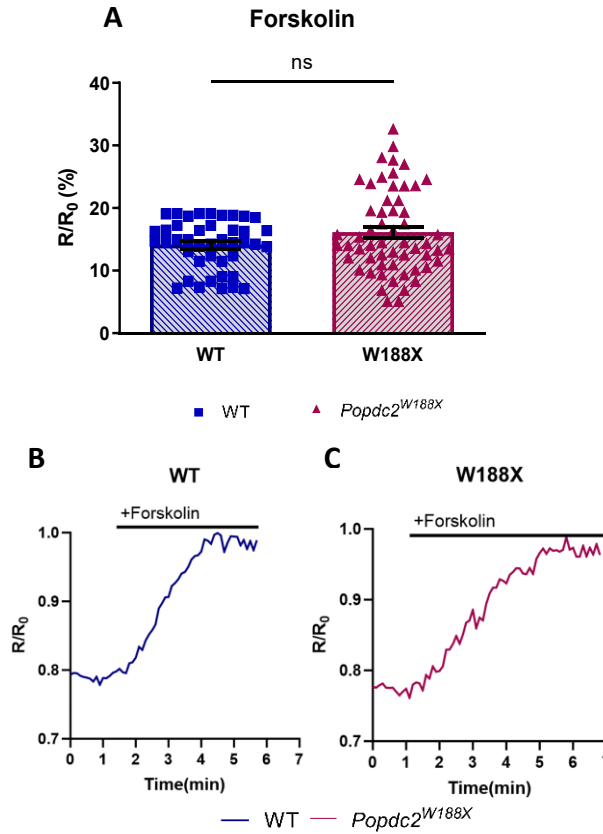


Figure 4.6. cAMP release induced by the direct AC activation in cultured wild type and *Popdc2*^{W188X} mutant NMCM. (A) Quantification of the FRET change [%] in response to direct AC stimulation with 25 μ M FSK in cultured wild type and *Popdc2*^{W188X} mutant NMCM expressing the AKAP79-CUTie reporter. (B, C) Representative kinetics of FRET in (B) wild type and (C) *Popdc2*^{W188X} mutant NMCM. Statistical analysis was performed using the Mann-Whitney test, ns: not significant. Data are derived from 4 independent NMCM isolations n=4. Error bars show $\pm$ SEM.

4.3.2.4 Enhanced coupling of β AR to the inhibitory Gai protein in *Popdc2*^{W188X} mutant NMCM and rescue of the mutant phenotype with pertussis toxin

Upstream of AC are the β_1 - or β_2 AR and we therefore tested the signalling capacity of these receptor isoforms in wild type and *Popdc2*^{W188X} mutant NMCM. To this end, prior to stimulation with 100nM ISO, NMCM were pre-treated with selective β_1 AR (100nM CGP), or β_2 AR antagonists (50nM ICI). These concentrations were chosen as previous work in the Gorelik laboratory demonstrated that these are the IC50 concentrations. Otherwise, cultured NMCM were treated and FRET recordings taken as previously described. A summary of all data points is presented in Figure 4.7A, where the results for wild type NMCM are grouped on the left and the results for the *Popdc2*^{W188X} mutant are shown on the right. Global (β_1 AR/ β_2 AR) response in the wild type samples produced a strong cAMP signal, which is comparable with the signal obtained from selective β_1 AR stimulation. Expectedly, selective β_2 AR induced

cAMP signal in wild type samples was significantly below the global and selective β_1 AR response, due to the strongly compartmentalised β_2 AR signalling capacity. Conversely, the global response of the *Popdc2*^{W188X} mutant NMCM was significantly reduced, similarly to previous observations. However, selective activation of β_1 AR resulted in significantly increased cAMP production, while the cAMP signal obtained from β_2 AR alone was on par with the global response previously observed in the mutant NMCM. These data suggest that in the *Popdc2*^{W188X} mutant NMCM activation of the β_2 AR suppresses the proper activation of β_1 AR thereby reducing the overall cAMP production during a dual stimulation of both receptor subtypes with ISO. Additionally, selective β_2 AR induced cAMP release appears on average to be elevated, compared to the wild type recordings, which could be suggestive of a breakdown of the tight β_2 AR compartmentalisation.

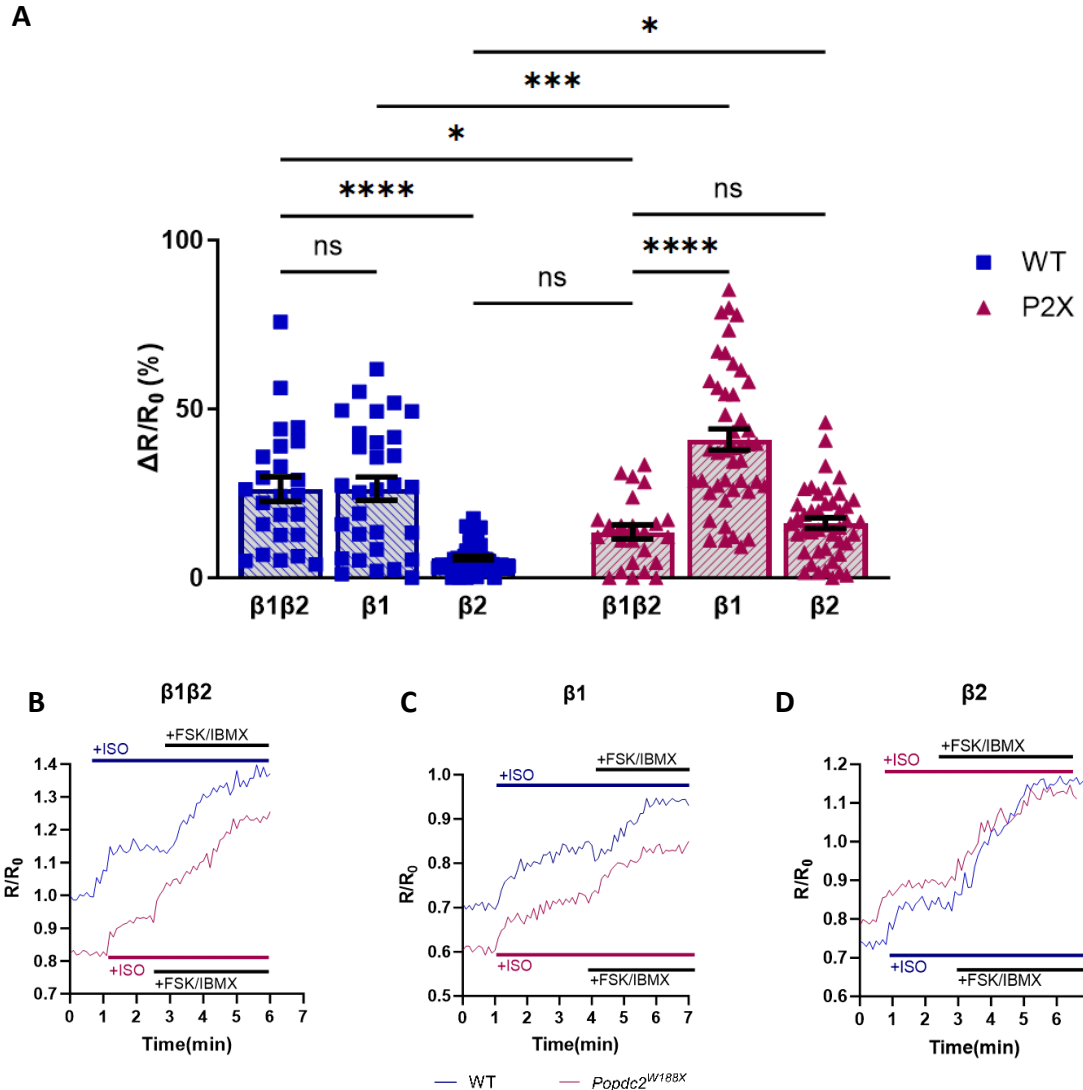


Figure 4.7. Plasma membrane localised cAMP release induced by the selective stimulation of β_1 AR or β_2 AR in cultured wild type or *Popdc2*^{W188X} mutant NMCM. Summary data and example traces showing change in FRET under (A and B) unspecific β AR stimulation, in response to the selective (A and C) β_1 AR or (A and D) β_2 AR stimulation, recorded in cultured wild type and *Popdc2*^{W188X} mutant NMCM expressing the AKAP79-CUTie reporter. For (A, B, and C) % change in FRET was calculated of the saturating stimulus (50 μ M FSK+100 μ M IBMX). Statistical analysis was performed using the Mann-Whitney test, * p < 0.05, ** p < 0.01 and **** p < 0.0001, n =3 for (A) and n =4 for (B) and (C). Error bars show $\pm$ SEM.

Under physiological conditions both β_1 AR and β_2 AR receptor sub-types preferentially couple to the stimulatory Gs which activates AC. However, at elevated agonist levels β_2 ARs can switch to the inhibitory Gi, which inhibits AC activity and cAMP formation. The reduction in cAMP levels resulting from β_2 AR-Gi coupling can have also an attenuating effect on β_1 AR signalling since both receptors are coupled to the same AC. Therefore, Gi coupling of the β_2 AR can lead to a decreased cAMP response of ligand activated β_1 ARs. Based on my data shown in Figure 4.7, I hypothesised that in *Popdc2*^{W188X} mutant NMCM, the β_2 AR is aberrantly coupled to Gi,

resulting in a suppressed cAMP response upon receptor activation. To test this hypothesis, NMCM expressing the plasma membrane localised AKAP79-CUTie FRET biosensor were treated with Pertussis toxin (Ptx) prior to ISO-induced β AR activation and cAMP release. Ptx is a toxin produced by the bacterium *Bordetella pertussis*, which is responsible for causing whooping cough. Ptx specifically targets and modifies the activity of the inhibitory Gi protein, by ADP-ribosylation and thereby inhibiting the interaction with AC. Treatment of *Popdc2*^{W188X} mutant NMCM with Ptx, rescued the β AR-induced cAMP response after ISO stimulation (Figure 4.8), which is a significant effect representing almost a doubling of the cAMP concentration produced if compared to untreated mutant cells. These results suggest that the reduced cAMP levels in the NMCM of the *Popdc2*^{W188X} mutant following β AR activation are probably largely due to an aberrant coupling of β_2 AR to the inhibitory Gi proteins.

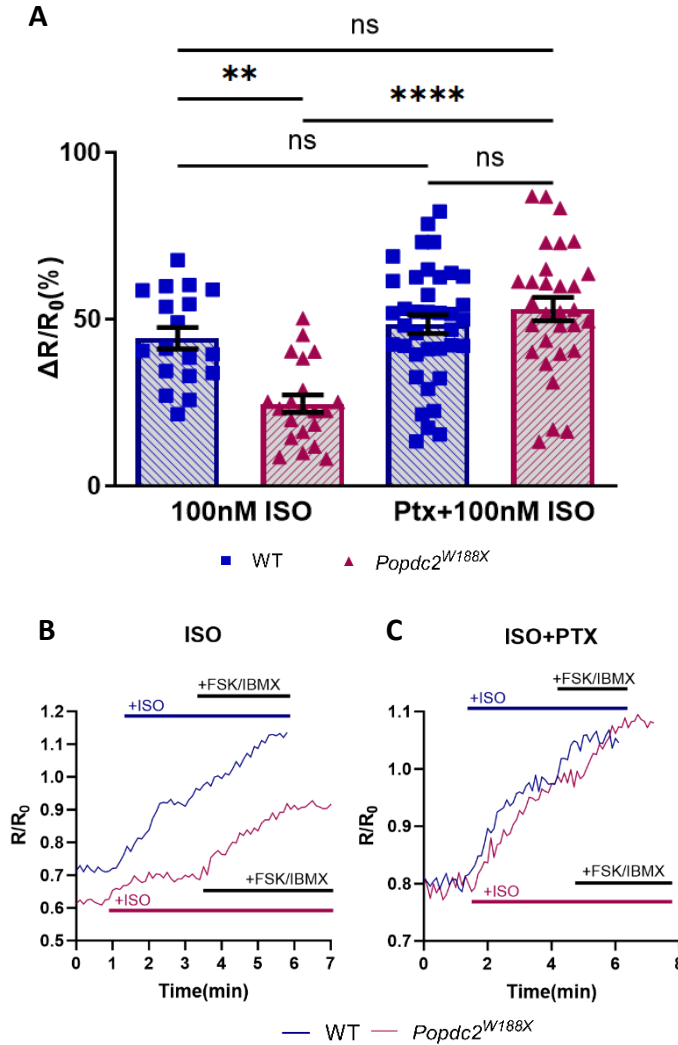


Figure 4.8. Inhibition of β_2 AR-Gi coupling normalises β AR-induced cAMP release in the *Popdc2*^{W188X} mutant NMCM. (A) Summary data and example traces showing change in FRET under (A left and B) untreated NMCM or (A right and C) pretreated NMCM with 1.5 μ g/ml of Ptx for approximately 4h. For (A, B and C) % change in FRET was calculated of the saturating stimulus (50 μ M FSK+100 μ M IBMX). Statistical analysis was performed using two-way ANOVA with Šídák post hoc analysis, n=4, ns=not significant, **p<0.01 and, ****p<0.0001. Error bars show $\pm$ SEM.

4.3.3 At the plasma membrane POPDC proteins may be in signalling complexes with β AR, AC and AKAP79

Tethering of the individual cAMP regulators and effectors into signalling complexes is achieved through the application of scaffolding proteins such as AKAPs. AKAP79 also known as AKAP150 or AKAP5 in murine cardiomyocytes anchors a multiprotein complex with β AR and AC at the plasma membrane of cardiomyocytes^{104,397}. The CUTie FRET biosensor used here is targeted to the plasma membrane by a tethered AKAP79 (AKAP79-CUTie) and thus, monitoring the nanodomain in which β AR and AC are localised. Using Co-IP technique, it was

investigated whether POPDC1 or POPDC2 are in a complex with AKAP79. The very first preliminary data shown in Figure 4.9 A, B, suggests that Popdc1-myc (B) or Popdc2-myc (A) Co-IP AKAP79-flag when co-expressed in Cos7 cells. These first findings if confirmed through further experimental assessment would suggest that POPDC proteins indeed might be forming complexes with AKAP79 in Cos7 cells when co-expressed. Additional experiments in cardiomyocytes should also be performed to investigate if this interaction occurs in native cells. (Images of full blots are found in the Appendix Figure 6.1)

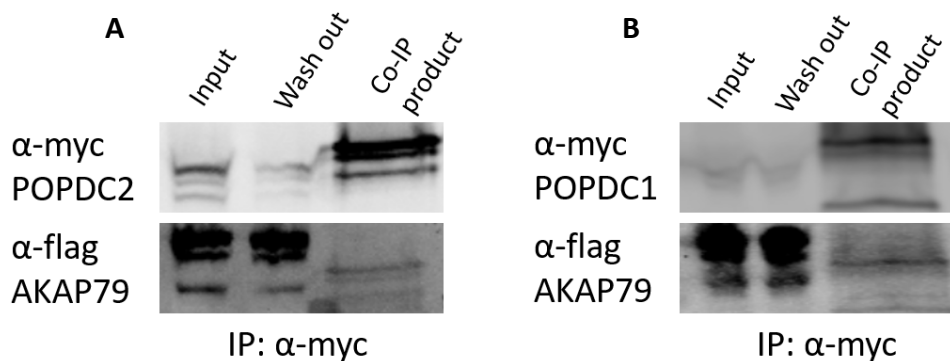


Figure 4.9. Co-Immunoprecipitation of POPDC1 or POPDC2 with AKAP79 from Cos-7 cells. FLAG-tagged AKAP79 was co-transfected with either Myc-tagged Popdc1 or Popdc2 in Cos7 cells and cell lysates were subjected to co-immunoprecipitation analysis. Results for lysates of cells co-transfected with AKAP79 and (A) Popdc2 or (B) Popdc1, respectively. Input = cell lysate, wash out = supernatant, co-IP product = precipitated POPDC1 or POPDC2 or AKAP79. Figure displayed is representative of an n=2 for (A) and n=1 for (B). Images of the full blots can be found in the Appendix Figure 6.1.

4.3.4 AMCM of the *Popdc2*^{W188X} mutant show reduced cAMP levels at the plasma membrane following β AR activation

β AR-induced cAMP dynamics in *Popdc2*^{W188X} mutants were so far assessed in neonatal cardiomyocytes. Since there are extensive differences in compartmentation between adult versus neonatal cardiomyocytes. Towards the end of my project, an opportunity presented itself to perform preliminary FRET microscopy experiments in isolated AMCM of the *Popdc2*^{W188X} mutant. To this end, isolated AMCM expressing the plasma membrane localised AKAP79-CUTie FRET biosensor were stimulated with the global β AR agonist ISO at 100nM concentration. The resulting β AR-induced cAMP release was normalised against the saturating stimulus (50 μ M FSK and 100 μ M IBMX) and presented as % change in FRET (Figure 4.10). It was found that in response to 100nM ISO stimulation, *Popdc2*^{W188X} mutant AMCM produce reduced cAMP levels if compared to their wild type counterparts, which reflects the findings described for NMCM.

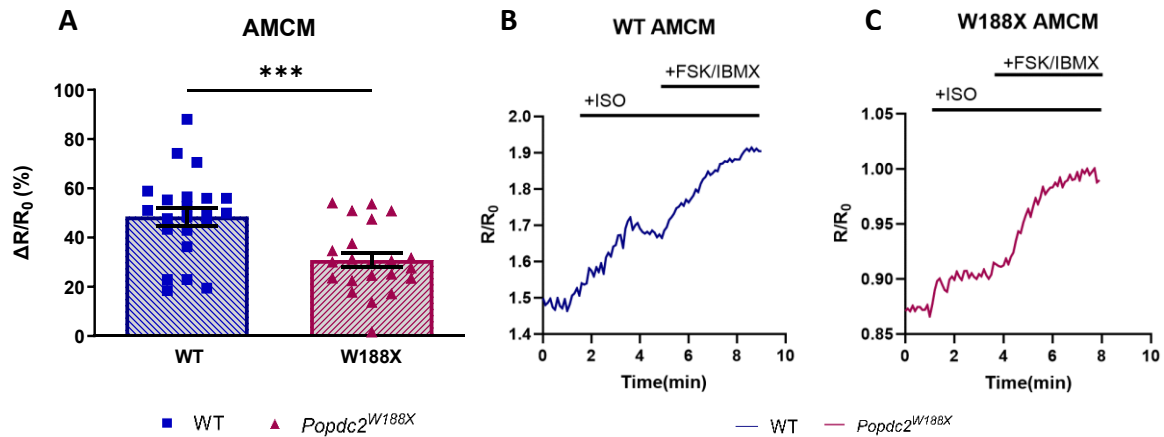


Figure 4.10. β AR induced cAMP release at the plasma membrane of cultured wild type and *Popdc2*^{W188X} mutant AMCM. (A) Summary data showing % change in FRET in response to 100nM ISO induced β AR activation measured against a saturating stimulus of 50 μ M FSK+100 μ M IBMX. Representative kinetics of FRET change in (B) wild type and (C) *Popdc2*^{W188X} mutant AMCM. Statistical analysis was performed using the Mann-Whitney test. Data are from 3 cell isolations n=22 (WT) and n=20 (*Popdc2*^{W188X}), respectively. ***p<0.001. Error bars show $\pm$ SEM.

4.3.5 Disrupting the interaction between POPDC1 and PDE4A reduces cAMP release at the plasma membrane of the NMCM following β AR stimulation

Classically, the ability of the β_2 AR to switch from Gs- to Gi-coupling requires PKA-dependent phosphorylation of the β_2 AR in a process termed heterologous desensitisation³⁹⁴. The cAMP-dependent PKA-activation within the macromolecular receptor complex, on the other hand, is regulated by PDE4 which is known to be recruited to the ligand-loaded β_2 AR in a complex with β -arrestin following GRK-mediated receptor phosphorylation (homologous desensitisation). Blocking of PDE4 activity with rolipram results in enhanced Gi activity³⁹⁸. POPDC1 proteins have recently been shown to form a complex with PDE4A in cardiomyocytes²¹⁸. PDE4A tethered to POPDC1 regulates the interaction of POPDC1 and TREK1 by modulating the cAMP levels in the vicinity of POPDC proteins. In this context it was decided to investigate whether the established interaction between POPDC1 and PDE4A may be of relevance in the observations made for the *Popdc2*^{W188X} mutant NMCM where β AR stimulation produces reduced cAMP levels relative to the wild type samples. To this end a cell-penetrating disruptor peptide was utilised, which when employed, can, with a high degree of specificity, disrupt the interaction of PDE4A and POPDC1. Conceptually, it would mimic the loss of the interaction of POPDC1. This seems to be the case in *Popdc2*^{W188X} mutant NMCM where POPDC1 and POPDC2 are lost from the plasma membrane of cardiomyocytes, as described in Chapter 3. The disruptor peptide was developed and kindly donated to me by

the Baillie group at the University of Glasgow. This cell permeable disruptor peptide was based on the 25mer POPDC1 sequence identified from POPDC1 peptide arrays to which PDE4A is bound and contains the sequence: TLKKGQTYAAEDKTSVDDRLSILLK. Additionally, a control scrambled peptide was also developed and contained the same amino acid residues but in a random order: TTLYTDSSVLKKGRLQDKEKALADI. The sequence of the scrambled peptide was checked by BLAST to prevent presence in any another protein and both peptides had stearate group conjugated at the N-terminal to permit cell penetration. To study the effects of the disruptor peptide in blocking the interaction between POPDC1 and PDE4A, cultured wild type NMCM, expressing the membrane specific AKAP79-CUTie FRET biosensor were incubated with the disruptor peptide or control scrambled peptide for 2h. Following this treatment, cAMP production at baseline and after maximum stimulus of 50 μ M FSK and 100 μ M IBMX were measured by FRET microscopy (Figure 4.11). At baseline there is no difference in FRET signal and therefore cAMP levels do not differ between scrambled and the disruptor peptide-treated NMCM. Likewise, both disruptor and the scrambled peptide containing NMCM have on average comparable capacity for total cAMP production. Together these data confirm that the introduction of the disruptor peptide on itself does not introduce any changes in cAMP dynamics in absence of β AR stimulation.

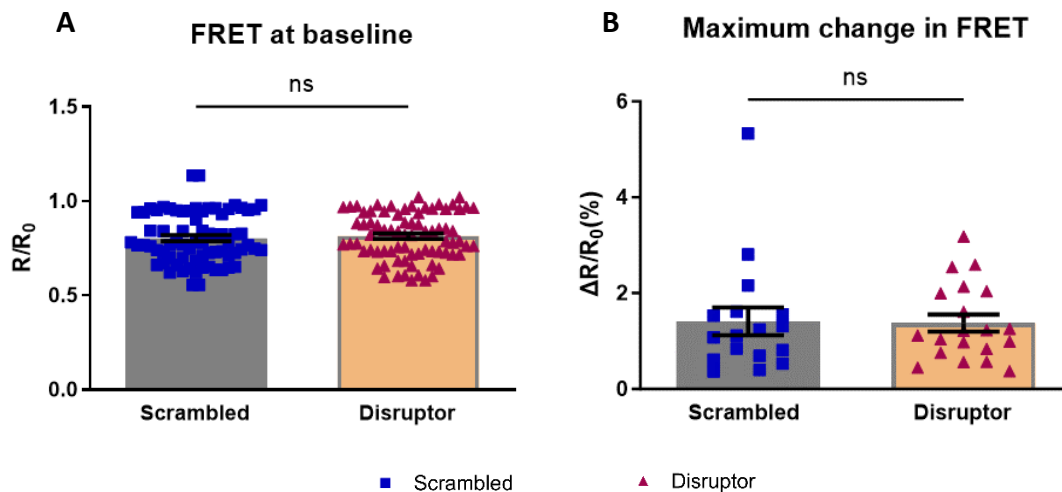


Figure 4.11. FRET at baseline and maximum change in FRET in cultured wild type NMCM carrying a POPDC1-PDE4A disruptor or scrambled peptides. Summary data showing (A) FRET at baseline and (B) maximum change in FRET induced by 50 μ M FSK with 100 μ M IBMX in cultured wild type NMCM expressing the AKAP79-CUTie FRET reporter after 2h incubation with 10 μ M POPDC1-PDE4A disruptor or scrambled peptides. Statistical analysis was performed using the Mann-Whitney test, ns: not significant. Data are derived from 3 independent NMCM isolations (n=3). Error bars show $\pm$ SEM.

Secondly, following the above-described control experiments, using FRET microscopy, I measured whether the introduction of the disruptor peptide impacts on the cAMP dynamics at the plasma membrane, presumably within the AKAP79 complex, in cultured wild-type NMCM in response to β AR stimulation. Interestingly, β AR activation with 100nM ISO did not show any significant difference in cAMP release or cAMP kinetics between NMCM containing the disruptor or the scrambled peptides (Figure 4.12 A, D). However, at 1nM ISO concentration a significant reduction in cAMP levels is present in the cultured NMCM holding the disruptor peptide if compared to the cAMP signal in myocytes treated with the scrambled peptide (Figure 4.12 B, E). These data suggest that the disruption of PDE4A from the vicinity of POPDC1 in wild type NMCM results in reduced cAMP levels following low levels of β AR stimulation which mimics the phenotype observed in the *Popdc2*^{W188X} mutant NMCM at any ISO concentration. It is possible that at higher concentrations of the β AR agonist the resulting high levels of receptor activation and consequential high amounts of cAMP release mask the effects of the disruptor peptide which likely acts within very specific nanodomains where the interaction between PDE4A and POPDC1 occurs. This hypothesis is further supported by the data presented in Figure 4.12 C, F which shows that when NMCM are treated with the PDE4 inhibitor rolipram which blocks all PDE4 sub-isoforms the significant difference in cAMP release present between the NMCM holding either the disruptor or the scrambled peptides, is lost, further demonstrating that the differences in cAMP concentrations are specific to the POPDC1-PDE4A complexes following β AR activation.

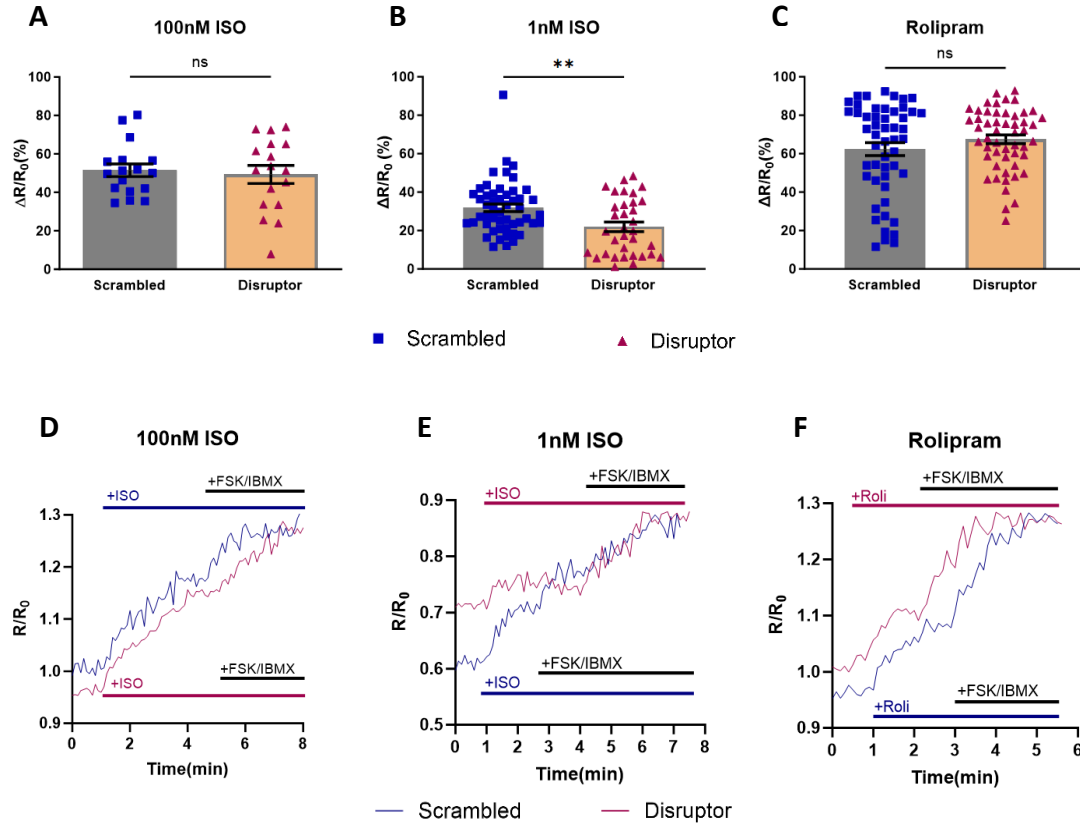


Figure 4.12. Plasma membrane localised cAMP release in wild type NMCM in presence of a POPDC1-PDE4A disruptor or scrambled peptides following different treatments. Summary data and example traces showing change in FRET following (A and D) 100nM ISO (B and E) 1nM ISO and (C and F) 10 μ M Rolipram treatments, recorded in wild type NMCM expressing AKAP79-CUTie FRET reporter after 2h incubation with 10 μ M POPDC1-PDE4A disruptor or scrambled peptides. For (A, B, and C) % change in FRET was calculated of the saturating stimulus (50 μ M FSK+100 μ M IBMX). Statistical analysis was performed using the Mann-Whitney test, ns=not significant, **p<0.01, n=3 for (A) and n=6 for (B) and (C). Error bars show $\pm$ SEM.

4.4 Discussion

β AR-cAMP signalling stands as a pivotal pathway in the cardiac stress response, influencing contractility, heart rate, and calcium handling. Many of the POPDC mutants, as well as human POPDC variants present with a defective stress response, resulting in abnormalities in cardiac pacemaking and conduction^{184,199,219,280,348}.

For example, in the study by Froese et al. (2012), both *Popdc1* and *Popdc2* null mutants showed a defective β AR signalling characterised by stress induced bradycardia in response to sympathetic activation¹⁸⁴. The phenotype was not present in young mice (3 months) but increased in severity with age; characteristics which are reminiscent to those associated with sinus node dysfunction¹⁸⁴. In a subsequent study, Dr Navneet Bhogal studied atrial preparations of *Popdc1* and *Popdc2* null mutants which revealed changes in automaticity. In that investigation, the heart rate in response to escalating concentrations of β AR-agonist was scrutinised and revealed hypersensitivity of *Popdc2* null mutants to low levels of β AR agonist. Notably, the mutant demonstrated a propensity to attain near-maximum heart rates at low agonist concentrations, with subsequent elevations in β AR agonist levels yielding only marginal increases in heart rate.

Previously four working models of POPDC function were introduced in section 1.3.6, of which the sponge model, in short, proposes that POPDC proteins which are abundantly expressed in cardiomyocytes and cells of the CCS may buffer cAMP levels to provide a way to fine tune the system when subtle responses are required. In cardiomyocytes, it is generally understood that the extent of PKA activity is directly proportional to the level of cAMP release within the relevant signalling complex which can bind to and initiate PKA activity. Upon cAMP-mediated activation, PKA can phosphorylate key proteins involved in myocyte calcium handling to regulate cardiac contraction, relaxation and heart rate generation. β AR mediated increase in cAMP, therefore, results in an elevated PKA activity and protein phosphorylation to alter cardiac output. In line with the sponge model, it was hypothesised that in the *Popdc2* null mutants, loss of POPDC1 and POPDC2 from the plasma membrane permits high levels of free cAMP, which can generate maximum PKA mediated phosphorylation of effector proteins thus producing maximally accelerated heart rate even at low β AR agonis concentration.

Popdc2^{W188X} mutants show a similar phenotype characterised by chronotropic incompetence following β AR activation²⁸⁰ (and chapter 3, section 3.2.6). In analogy, at the time, it was hypothesised that *POPDC2*^{W188X} mutation causes elevated cAMP levels. In fact, the impact of the *POPDC2*^{W188X} mutation on cAMP dynamics following β AR activation was initially assessed by FRET microscopy using the ^TEpac^{VV} cytosolic sensor in HEK293 cells transiently expressing *Popdc2* WT or *POPDC2*^{W188X} (Appendix Figure 6.2). The *POPDC2*^{W188X} transfected HEK293 cells displayed a statistically significant increase in cAMP upon β AR activation compared to the *Popdc2* WT expressing HEK293 cells. Based on the data presented by Rinne et al., which shows *POPDC2*^{W188X} expression in human leucocytes detected by western blot analysis²⁸⁰, it was inferred that the *POPDC2*^{W188X} nonsense mutant is not subjected to NMD and the truncated *POPDC2*^{W188X} protein is expressed, including in cardiomyocytes. The suggestion was that the truncated *POPDC2*^{W188X} protein probably loses its ability to buffer the elevated cAMP levels during a stress response, ultimately leading to dysregulation of cardiac function. In this thesis, of course, I have shown that in mouse cardiomyocytes *Popdc2*^{W188X} is subjected to NMD and therefore not expressed at the plasma membrane, rendering the above findings somewhat redundant, however, possibly still relevant within the context of a deleterious *POPDC2*^{W188X} protein escaping NMD (discussed in chapter 3.4.6).

Contrary to the hypothesis that loss of *POPDC1* and *POPDC2* proteins results in elevated levels of cAMP production following β AR activation, here, I have provided evidence that in fact, *Popdc2*^{W188X} mutant NMCM generate low cAMP levels following β AR activation. Furthermore, I show that this is not achieved through direct regulation of AC activity and with that the production of cAMP in response to sympathetic activation. By selective activation of β_1 AR or β_2 AR in NMCM it was revealed that in the *Popdc2*^{W188X} mutant, due to an aberrant β_2 AR -Gi coupling, stimulation of β_2 AR results in the suppression of β_1 AR activity by inhibiting AC activity, thereby indirectly suppressing AC-mediated cAMP production upon sympathetic activation. It was also observed that artificially blocking the interaction between *POPDC1* and *PDE4A* mimics the *Popdc2*^{W188X} phenotype in wild type NMCM at low levels of ISO. The suggestion is therefore that in the *Popdc2*^{W188X} NMCM loss of *POPDC1* proteins abolishes recruitment of *PDE4A* to the β_2 AR allowing for premature receptor phosphorylation by PKA within the signalosome and Gi-coupling.

Collectively, these data led to the conclusion, that at the plasma membrane of NMCM, POPDC proteins are unlikely to function as cAMP sinks and that the role of POPDC proteins exhibits complexity beyond the initial expectation. Below I discuss the results of this chapter in detail and attempt to provide some explanations for the made observations.

4.4.1 *Popdc2*^{W188X} mutant NMCM produce low cAMP levels in response to β AR activation

The analysis of the first three FRET experiments in this chapter, for the first time has shown that β AR agonist induced cAMP release in *Popdc2*^{W188X} mutant NMCM generates low cAMP signal. Following on from the initial FRET experiments conducted in *Popdc2*^{WT} and *Popdc2*^{W188X} transiently expressing HEK cells (described above), results produced using the same cytosolic ^TEpac^{VV} sensor²⁹⁵ in cultured NMCM were highly unexpected, albeit reproducible using two further FRET sensors: the cytosolic CUTie, and the plasma membrane localised AKAP79-CUTie³⁰². PKA type II β based CUTie confirmed the initial ^TEpac^{VV} reporter-based observations of low β AR-mediated cAMP production in NMCM of the *Popdc2*^{W188X} mutant. The AKAP79-CUTie, by virtue of its AKAP79 sequence, localised the low cAMP signal at the plasma membrane where POPDC proteins and β AR receptors naturally reside. Therefore, all three FRET sensor at both the cytosolic and at the plasma membrane localisations, show low cAMP signal in the NMCM of the *Popdc2*^{W188X} mutant following β AR activation, which suggest that β AR-mediated signal transduction is dysregulated in the *Popdc2*^{W188X} mutant myocytes. Additionally, the observed differences between the mutant and the wild type NMCM persisted across low (1nM), medium (10nM) and high (100nM) levels of β AR activation where wild type NMCM show an exponential increase in cAMP signal whilst the *Popdc2*^{W188X} mutant response is linear at best, indicating a premature termination of the signal amplification mechanism in the mutant myocytes. Particularly, the CUTie-measured cAMP response shows no notable elevation in cAMP signal between the three levels of stimulation, which stands in stark contrast to the exponential increase in cAMP signal recorded in the wild type counterparts. The reasons underlying the observed effect are currently not fully understood, however, this phenomenon is specific to the cytosolic CUTie reporter as cAMP data collected using the cytosolic ^TEpac^{VV} reporter reflects an increase in cAMP between the increasing levels of β AR activation. Proteins involved in cAMP

compartmentation and β AR-cAMP signalling are not dispersed freely at the plasma membrane or the cytosol but are anchored to scaffolding proteins that via PPI organise these signalling complexes. For cAMP diffusion to occur from the plasma membrane where cAMP is generated by AC, cAMP levels must be elevated well above the number of free binding sites. Therefore, it is possible that in the *Popdc2*^{W188X} mutant NMCM levels of cAMP generated are not substantial enough to diffuse into the cytosol in the vicinity of the cytosolic CUTie reporter, whilst the ^TEpac^{VV} could be localised more accessibly or the localisation of the CUTie reporter within the complex cellular context of a cardiomyocyte might be more susceptible to rapid cAMP degradation.

As previously discussed, cAMP is a second messenger to a plethora of GPCRs. However, the phenomenon of low cAMP signal upon receptor stimulation in the *Popdc2*^{W188X} mutant NMCM was confirmed to be specific to β AR activation, as control experiments in which prostaglandin receptors EP2/EP4 were activated, did not generate significant differences in the cAMP signal between the mutant and wild type NMCM. Prostaglandin receptors EP2 and EP4 also signal via the production of cAMP albeit preferentially activate type I PKA which is anchored via a distinct pool of AKAPs to phosphorylate a different set of effectors which are preferentially localised outside of caveolae¹⁵¹. As previously described, in cardiomyocytes GPCR-cAMP signalling is compartmentalised to produce downstream signal specificity. Compartmentation depends on several factors but is in part achieved through the organising of specific cAMP signalling intermediates, effectors and regulators into macromolecular signalling complexes within distinct cellular micro-architectures such as T-tubules, caveolae, plasma membrane³⁹⁹. These GPCR-cAMP signalling complexes are most often assembled upon differentially targeted AKAP proteins for spatial and temporal regulation. Consequently, stimulation of β AR promotes change in cardiac inotropy, lusitropy and chronotropy, but stimulation of prostaglandin receptors does not, even for similar global cAMP levels⁴⁰⁰.

Interestingly, even though there was no difference in the cAMP dynamics between the mutant and wild type NMCM upon stimulation of the prostaglandin receptor, the strength of the cAMP response was approximately 4-fold smaller if compared to that induced by the β AR activation. This can possibly be explained by the localisation of the AKAP79-CUTie sensor which is targeted to the microdomains near β ARs such as T-tubules and caveolae by virtue of

the AKAP79 sequence. As mentioned above, PGE1 stimulated prostaglandin receptors localise outside of caveolae. It is possible, that due to the nature of cAMP compartmentation within cardiomyocytes, the signal produced by the prostaglandin receptor stimulation was generated relatively farther from the AKAP79-CUTie sensor than the β AR induced cAMP signal so that due to processes such as cAMP buffering and molecular crowding only a reduced fraction of the cAMP signal generated via prostaglandin receptor signalosomes reached the AKAP79-CUTie sensor.

In any case, the recorded low cAMP levels following β AR activation, are in line with the hypothesis that loss of POPDC proteins in the *Popdc2*^{W188X} mutant NMCM results in abnormal cAMP dynamics due to dysregulation of the cAMP signalling intermediates following β AR activation. These findings are further supported by the evidence that there was no significant difference in gross cAMP levels between the wild type and *Popdc2*^{W188X} NMCM at basal activity (section 4.3.2.1); nor was there any disruption in the mutant NMCM capacity to produce total cAMP release stimulated independently of β AR activation (section 4.3.2.1).

Additionally, POPDC1 and POPDC2 proteins were shown to co-IP with AKAP79. As scaffold and PKA targeting proteins, AKAPs can couple regulatory proteins to downstream effectors to facilitate temporal and spatial cAMP signalling. AKAP79 form complexes with multiple regulatory molecules of the cAMP signalling pathway such as PKA, PKC, PP2B and possibly PDEs and in cardiomyocytes AKAP79 also facilitate β ARs-AC5/6 complex formation at the membrane to regulate cAMP synthesis in an agonist dependent manner. It must be noted that the complex formation between POPDC1 or POPDC2 and AKAP79 was tested in an overexpression system by a co-IP assay of which the results for POPDC2-AKAP79 complex formation were conclusive and the results for POPDC1-AKAP79 complex formation could be interpreted as not fully conclusive and could benefit from a repeat of the assay. However, Swan et al, has shown that POPDC1 and POPDC2 proteins at the plasma membrane predominantly exist as heterodimers from which it can be inferred that the existence of a POPDC2-AKAP79 complex by necessity also places POPDC1 within the same complex. As it stands the suggestion is that POPDC proteins may be a part of β AR signalling complexes that are scaffolded by AKAP79 at the membrane of cardiomyocytes.

4.4.2 *Popdc2*^{W188X} changes β AR signalling

The β AR-cAMP complexes are spatiotemporally orchestrated and are formed by the dynamic interaction of β ARs with G-proteins, AC, PKA, AKAPs, PDEs and various effector molecules at different subcellular sites to coordinate and regulate cAMP-dependent signalling pathway in response to sympathetic stimulation. As previously described, the ligand-mediated activation of a membrane bound β ARAR allows for it to function as a GEF and catalyse the exchange of GDP for GTP on the Gs protein α subunit, leading to the dissociation of the Gs protein into G α s-GTP and G β y subunits. The liberated G α s (Gs) subunit activates the adjacent AC enzyme to initiate cAMP production resulting in an increase in the intracellular cAMP levels and a proportionate activation of cAMP effectors, e.g. HCN, EPAC, PKA, POPDC and a fraction of PDEs, of which all are important for various sympathetic downstream effects. The signal is terminated by PDEs that degrade cAMP to AMP. Levels of cAMP following β AR activation can be crudely described as the balance between β AR induced cAMP production by ACs and cAMP degradation by PDEs.

4.4.2.1 AC activity in *Popdc2*^{W188X} NMCM

In cardiomyocytes AC5 and AC6 are considered the major and most studied isoforms¹⁰⁴. AC5 and AC6 have similar regulatory mechanisms, however, are differentially localised in cardiomyocytes¹⁰⁵ and physiologically appear to play distinct roles in cardiac function^{104,105}. Several overexpression and deletion studies have allowed the conclusion that AC5 is largely associated with the stress response while AC6 is necessary for calcium handling and contractility¹⁰⁴, which in light of the *Popdc2*^{W188X} mutant stress induced phenotype would make AC5 the more relevant isoform. Furthermore, studies of AC KO models revealed that loss of AC5 in cardiac membranes and isolated cardiomyocytes decreases the total cAMP activity under both the basal and stimulated conditions^{401,402}, which contrasts the findings in the *Popdc2*^{W188X} mutant NMCM which show unchanged basal but low stimulated conditions; a phenotype more similar to the observations made in the AC6 KOs, which show no significant change to basal cAMP production with reduced stimulated cAMP dynamics⁴⁰². Therefore, both AC5 and AC6 phenotypes show some similarities with the *Popdc2*^{W188X} NMCM phenotype. However, when AC activity was assessed by forskolin in the *Popdc2*^{W188X} NMCM no difference between the mutant and the wild type samples could be recorded. This suggests

that in the *Popdc2*^{W188X} mutant NMCM, POPDC proteins do not regulate cAMP production by regulating the activity of AC5 or AC6 and the recorded low levels of cAMP following β AR activation are not due to a dysregulated AC5 and AC6 activity in the *Popdc2*^{W188X} mutant NMCM. These findings are not completely unexpected as there has so far been not evidence in literature that POPDC proteins form functional interactions with either AC5 or AC6 in cardiomyocytes.

Lower levels of AC2, AC4 and AC9 are reported in cardiomyocytes¹⁵⁴ and AC1 in the sinoatrial node¹⁰⁶. AC1 is a calcium-stimulated AC which modulates the *I_f* pacemaker current^{106,107} however, AC1 KO mice are not reported to have a dysregulated heart rate. Additionally, through RNA sequencing, higher expression of AC1 was detected in the right atrium versus the sinoatrial node¹⁰⁴. The role of AC4 in the heart is currently not known due to the lack of a knockout model. AC2 KO mice, on the other hand, show no cardiac phenotype¹⁰⁴. Finally, at around 3% of the total cardiac membrane AC activity the global contribution of AC9 can be considered negligible, however, it was shown to be important for targeted downstream signalling in local cAMP levels in macromolecular complexes¹⁰⁴. In the heart, AC9 is involved in the slow delayed rectifier (IKs) current through its interaction with the AKAP Yotiao and KCNQ1/KCNE1 channels⁴⁰¹; KCNQ1 being a critical component for the late phase repolarisation of the cardiac action potential in humans⁴⁰³. AC9 has also been suggested to have a cardioprotective role in the heart through its interaction with Hsp20⁴⁰³. In mice AC9 is important for resting HR control; deletion of AC9 results in a bradycardic phenotype in AC9KO mice at rest as well as heart rate variability during the recovery period from high stress which suggests a role for AC9 in the sinoatrial node¹⁰⁴.

As of the current understanding there is no evidence for a functional interaction between POPDC and the above-mentioned AC1, AC2 and AC4 isoforms in the heart or otherwise. In contrast, AC9 and POPDC have been shown to form functional interactions in skeletal muscle²²⁰ and in cardiomyocytes³⁹³.

Li et al. unveiled that POPDC1 functions as a cAMP-dependent negative regulator of AC9 activity in skeletal muscle where AC9 is a major adenylyl cyclase responsible for cAMP

biosynthesis²²⁰. Skeletal muscle of the POPDC1 KO mice showed consistently elevated cAMP/PKA levels, leading to an elevated activation of the ubiquitination-proteasome degradation system (UPS), reduced muscle mass and impaired muscle performance compared to the wild type samples. Although not explored in this thesis, it would be interesting and relevant in the future to investigate whether *Popdc2*^{W188X} mediated loss of POPDC1 in the *Popdc2*^{W188X} mutant skeletal muscle also has a similar function. Swan et al, have shown loss of POPDC1 and POPDC2 expression at the sarcolemma of stained *gastrocnemius* sections of the *Popdc2*^{W188X} mutant. *Popdc2*^{W188X} mutant mice have been recorded to be smaller and slimmer than the wild type mice, albeit without an apparent muscular dystrophy phenotype²¹⁵.

Conversely, in cardiomyocytes, Baldwin et al, have shown that POPDC1 acts as a scaffold protein that facilitates the assembly of a functional macromolecular complex involving AC9 and TREK-1. Binding of AC9 and POPDC was shown to be independent of cAMP production, however AC9 association with TREK-1 was reduced in an isoproterenol (ISO)-dependent manner, requiring an intact Popeye domain and local production of cAMP within the complex. Depletion of either of the three proteins has been associated with dysregulation of heart rate control suggesting interaction between TREK-1, AC9, and POPDC1 plays a crucial role in regulating cardiac pacemaking and conduction by influencing ion channel activity, membrane potential, adrenergic responsiveness, and cAMP signalling¹⁰⁴. Interestingly, in the study the authors observe a clear interaction between POPDC2 and AC9, albeit one that is lacking the ability to produce Gs stimulated activity. The authors suggest that POPDC2 may have an inhibitory role for AC9 with more the research still being conducted.

Whilst I have been able to clearly show that the recorded low cAMP levels in the *Popdc2*^{W188X} mutant NMCM are not due to dysregulated AC5/AC6 activity, AC9 activity was not assessed. Global AC activation in cardiomyocytes is regularly achieved through stimulation with forskolin. However, AC9 is known to be forskolin insensitive¹⁰⁴. Therefore, my FRET results do not include a measurement of AC9 activity in the *Popdc2*^{W188X} NMCM. Whilst POPDC/AC9 interactions are certainly of greater importance than the AC9 expression levels in cardiomyocytes may suggest, in the context of the globally low cAMP levels following β AR activation present in the *Popdc2*^{W188X} mutant NMCM, AC9 at 3% signal contribution simply

has a negligible role. Nevertheless, in the future AC9 activity can be explored by blocking AC5 and AC6 with the P-site inhibitor, SQ 22,536, which displays more than 100-fold selectivity for AC5/AC6 over AC9³⁷⁰.

These results suggest that in the NMCM of the *Popdc2*^{W188X} mutant AC5 and AC6 are not dysregulated in their capacity to produce cAMP on par with the wild type samples when activated directly with forskolin. Lack of POPDC proteins does not have a negative impact and their function is not dependent on regulation by POPDC proteins.

4.4.2.2 Dysregulation of β_2 AR-G-protein coupling in *Popdc2*^{W188X} NMCM

Taking into account that enzyme activity of the two main AC isoforms in cardiomyocytes, AC5 and AC6, has not shown to be directly regulated by POPDC proteins in NMCM, the suggestion was that the recorded low cAMP signal arises due to a dysregulation of the β AR signalling upstream of ACs, with upstream being defined as the signal transduction from the ligand-activated β AR to an adjacent AC via the Gs protein. Some preliminary data produced by our collaborators in the Dessauer group at the University of Texas Health Science Center Houston, has shown that POPDC1 and POPDC2 co-IP with β AR.

Both β_1 AR and β_2 AR receptors are expressed in cardiomyocytes, although it has been shown that they follow a divergent expression pattern with β_1 AR being expressed all over the cell and β_2 AR being confined within the T-tubules and caveolae of adult ventricular myocytes, in NMCM which do not have T-tubules they are predominantly known to be localised within the caveolae⁹³. In line with their restrictive localisation, it was shown that β_1 AR upon stimulation can produce a global and an overall stronger and a more far-reaching cAMP signal, affecting many different processes, with β_2 AR activation resulting in cAMP production only within the caveolae compartments near the LTCC in cardiomyocytes^{92,93}. It is therefore plausible that a global cAMP response initiated by ISO stimulation when both receptors are activated, is on average expected to be of a similar amplitude as β_1 AR alone, as during a global response β_2 AR mediated cAMP production is somewhat negligible. This is exactly what was recorded for the wild type NMCM: a robust cAMP signal at the plasma membrane produced by the NMCM following ISO stimulation, which is of a similar amplitude generated by the selective β_1 AR

stimulation alone, while the cAMP signal produced by the selective β_2 AR stimulation, in line with expectation, was significantly reduced if compared to β_1 AR/ β_2 AR or selective β_1 AR response as measured by the cAMP signal. Conversely, global β_1 AR/ β_2 AR stimulation of the mutant NMCM yielded low cAMP signal which is in line with my previous findings discussed above. However, selective β_1 AR activation results in a significantly elevated cAMP signal if compared to both the global β AR activation of the mutant and selective β_1 AR activation in the wild type, which suggests that in absence of β_2 AR in the mutant NMCM β_1 AR has a heightened activity evident by the significantly increased magnitude of cAMP production.

We know that β_2 AR can couple the inhibitory G_i at higher levels of agonist which in turn inhibits AC activity, thereby dampening the β_1 AR response by blocking AC activity as a measure of preventing overstimulation and β_1 AR toxicity. Therefore, it would be plausible that by pharmacologically inhibiting β_2 AR activity β_1 AR-mediated cAMP signal could be elevated above the signal produced by global β AR stimulation. However, in that case it would be expected to see a similar response to the selective wild type β_1 AR response, whilst the mutant produces significantly increased β_1 AR mediated cAMP signal. Elevated selective β_1 AR cAMP signal is not observed in the wild type, which indicates that this condition is specific to the *Popdc2*^{W188X} phenotype. Further studies would need to be performed to identify the underlying cause; however, a possible explanation could be an upregulated expression of β_1 AR receptors to compensate for reduced cAMP production in the *Popdc2*^{W188X} NMCM overall in a compensatory feedback mechanism aimed at restoring cellular homeostasis. It cannot be excluded that a chronic alteration in cAMP levels or loss of POPDC proteins can influence processes involved in receptor trafficking and membrane insertion- low cAMP may favour the recruitment of β_1 AR to the plasma membrane or inhibit receptor internalisation.

In addition to the elevated cAMP response upon selective β_1 AR activation, NMCM of the *Popdc2*^{W188X} mutant also show an overall increased β_2 AR-dependent activity relative to the wild type β_2 AR response, whilst the cAMP response amplitudes between the global activation and β_2 AR alone of the *Popdc2*^{W188X} mutant NMCM only are not statistically different. This suggests that in the mutant NMCM during a global β AR activation β_2 AR activity determines the final cAMP signal, whilst β_1 AR response is quenched. This stands in direct contrast to the observation made in the wild type samples. As discussed above, β_2 AR can switch agonism

away from the Gs mediated control of cAMP production to a Gi pathway to inhibit AC activity and reduced cAMP levels. Ablating the Gi effects by pre-treating the NMCM with pertussis toxin normalised cAMP levels between the mutant and wild type NMCM following global β_2 AR stimulation. Pertussis toxin ADP-ribosylates the alpha subunit, preventing the dissociation of the heterotrimeric G proteins in mammalian cells, which inhibits activation of these G proteins by ligand-bound GPCR³⁹⁸. This suggests that in the *Popdc2*^{W188X} NMCM β_2 AR aberrantly couple to Gi which inhibits AC activity thereby also quenching β_1 AR response ultimately resulting in diminished cAMP production. It was hypothesised that loss of POPDC proteins from the plasma membrane creates conditions permissible of rapid β_2 AR stimulus trafficking to Gi.

The ability of β_2 AR to switch from signalling through Gs to signalling through Gi is part of a complex series of regulatory feedback mechanisms which govern orderly β_2 AR signalling in a measure to protect the myocardium from overstimulation^{97–99,398}. β_2 AR induced Gi activation then leads to signalling thorough pathways such as MAPK and also PI3-kinase and Akt pathways which is meant to be somewhat cardioprotective if compared to β_1 AR signalling^{98,99}. Ligand activated β_2 AR initiates Gs-dependent activation of AC and subsequent cAMP production by the β_2 AR signalosome, resulting in PKA mediated phosphorylation of the β_2 AR itself. This receptor modification reprograms the receptor-G-protein specificity, with the PKA-phosphorylated receptor conformation favouring Gs coupling a little bit less which allows β_2 AR to couple Gi^{172,404,405}. In a negative feedback loop, Gi inhibits AC activity and cAMP production diminishes. Indeed, it has been shown that the β_2 AR can interact with PKA anchor proteins able to specifically recruit PKA and thereby facilitate PKA phosphorylation of the β_2 AR⁴⁰⁶. Gs- β_2 AR coupling is also rapidly regulated by G $\beta\gamma$ -recruited GRK2 (or GRK 5 in humans⁴⁰⁷), which phosphorylates ligand bound β_2 AR, exposing it to recruitment and binding by the cytosolic β -arrestin which 1) sterically prevents further Gs- β_2 AR coupling and 2) recruits PDE4D5 to the β_2 AR forming a signalling complex of AKAP79/ β_2 -arrestin/PDE/ PKA⁴⁰⁸ to aid the regulation of the local pool of cAMP activated PKA and phosphorylation status of the β_2 AR itself. Therefore, it can be concluded that β -arrestin-recruited PDE4 regulates the local pool of PKA which serves to phosphorylate the β_2 AR and thereby controls the switching of β_2 AR from coupling to Gs and the activation of AC to coupling to Gi and inhibiting of AC activity.

PDEs are the only known enzyme able to hydrolyse cAMP and are therefore the primary mechanism for regulation of PKA activity. In the context of the *Popdc2*^{W188X} mutant, where, as discussed above, β_2 AR-Gi coupling is dysregulated, it can be hypothesised that loss of POPDC proteins causes loss of PDE-mediated regulation of PKA activity at the β_2 AR resulting in rapid PKA-mediated receptor phosphorylation and receptor-Gi coupling. In line with the above hypothesis, POPDC1 proteins have recently been shown to form complexes with the PDE4 long isoforms via the PDE4 UCR1 region, with a particular affinity for PDE4A via an additional, PDE4A specific docking domain localised within UCR1. Supportive of the prevailing understanding, PDE4A function, either through its activation by PKA or its hydrolytic activity, are not regulated by POPDC proteins. On the contrary, it was put forward that PDE4A by altering the cAMP gradient in the vicinity of POPDC1 modulates its function²¹⁸. Functionally, dislocation of PDE4A from POPDC1 decreased interaction between POPDC1 and TREK1, caused prolongation of the action potential duration and increased frequency of spontaneous Ca²⁺ sparks in pacemaker cells from murine SAN explants. POPDC-TREK1 interaction has a positive modulatory effect on TREK1 activity at the plasma membrane under basal conditions, while elevation of cAMP levels such as during β AR stimulation downregulates TREK1 activity. cAMP binding to POPDC causes structural rearrangements leading to a dissociation of TREK1 and POPDC, leaving TREK1 exposed to PKA mediated phosphorylation which is potentially inhibitory on TREK1 activity. Tibbo et al, propose that recruitment of PDE4 to the POPDC1-TREK1 complex which is either in a complex with or in the vicinity of AKAP79-PKA complex²⁸⁹, allows for the regulation of TREK1 phosphorylation status and with that POPDC could be involved in fine-tuning the effect of β AR activation on TREK1 current to regulate membrane excitability²¹⁸. Therefore, the interaction between POPDC1 and PDE4A may represent a novel control mechanism by which local cAMP levels are modulated within effector signalosomes to regulate their activity. It is therefore tempting to speculate that through interaction with PDE4, POPDC1 might also regulate post-translational modification of other proteins, including β_2 AR, and thereby at least in part explain the dysregulated β_2 AR-Gi coupling and subsequent low cAMP signal occurring in the *Popdc2*^{W188X} mutant. It was decided that experiments should be performed to assess whether pharmacological disruption of the POPDC1-PDE4A interaction by a membrane permeable disruptor peptide can induce similar physiological effects in wild type NMCM as observed in the *Popdc2*^{W188X} mutant. The disruptor peptide was designed to exclusively displace native PDE4A from the vicinity of POPDC1. Indeed, at low

(1nM) agonist concentration NMCM carrying the disruptor peptide showed a significantly reduced cAMP signal compared to the scrambled peptide control, thereby phenocopying the *Popdc2*^{W188X} mutant. While selective blocking of PDE4 activity with rolipram effectively removed the observed differences in cAMP signal between scrambled and disruptor peptide treated myocytes. These data confirm that complex formation between POPDC1 and PDE4A has an essential role and is at the centre of regulation of cAMP levels in NMCM upon sympathetic activation.

Interestingly, at higher agonist concentration of 100nM ISO, both the control and the disruptor peptide treated wild type NMCM did not present with any difference in cAMP signal, whilst in the original *Popdc2*^{W188X} NMCM measurements reduced cAMP levels were prevalent across any agonist concentration. It is possible that in otherwise physiologically normal wild type NMCM at higher concentrations of the β AR agonist the resulting high levels of receptor activation and consequential high amounts of cAMP release mask the effects of the disruptor peptide which likely acts within very specific nanodomains where the interaction between PDE4A and POPDC1 occurs.

CHAPTER 5 General discussion

5.1 Background and rational for the findings of this work

This study was one of the first studies to characterise the *Popdc2*^{W188X} mutant mouse and using cultured ventricular myocytes of this mouse to investigate the role of POPDC proteins in the regulation of β AR-cAMP signalling.

POPDC are predominantly membrane bound signalling proteins defined and unified by the presence of a highly conserved unique Popeye domain. They are known to function through PPI and have an expanding list of interaction partners across different tissues. Their expression (predominantly POPDC1) in a number of epithelial cells, has been implicated in a variety of cancers, where POPDC function to control proliferation, migration, invasion and metastasis^{191–193,209,221,222,245}. However, they have the highest expression levels in striated muscle in an overlapping but isoform specific expression pattern. In skeletal muscle they were shown to be important for the maintenance of the skeletal muscle fibre integrity and muscle regeneration following injury^{179,199,261}. While in the heart it was shown that *Popdc1* and *Popdc2* have an essential role in cardiac heart rate regulation and stress response¹⁸⁴, which is in alignment with their elevated expression levels in the AV, SAN and other parts of the CCS. A small number of patients carrying *POPDC* variants has also been identified, and in humans POPDC mutations lead to limb-girdle muscular dystrophy and cardiac arrhythmias^{213,280}, which is largely corroborated by loss of function or missense mutation generated in mice and zebrafish models. Recently, POPDC proteins have also been identified as cAMP effectors, and are emerging as an important mediator within the cAMP signalling^{213,268,393}. Despite their importance, the precise molecular mechanisms by which POPDC proteins regulate cellular function remain incompletely understood. Therefore, there is a lot of interest in furthering the understanding of the underlying regulatory mechanism(s). Phosphorylation is a critical mechanism for regulating protein function through PPI. The CTDs of POPDC1 and POPDC2 which are conserved in vertebrates, harbour several β AR activation sensitive phosphorylation sites which are thought to be important for PPI. This post-translational modification can modulate protein conformation, dynamic regulation, integration of signalling pathways, and cellular responses, playing a central role in cellular signalling networks and disease pathogenesis⁴⁰⁹. The human *POPDC2*^{W188X} variant produces a truncated form of POPDC2

lacking the CTD²⁸⁰, therefore, a *Popdc2*^{W188X} mouse mutant was engineered to help, amongst other research questions, unravel the regulatory mechanism(s) governing POPDC PPI, at baseline and after sympathetic activation, to understand how functions of POPDC1 are controlled. Additionally, characterizing engineered mouse mutants provides a powerful tool for understanding mutations found in human patients and elucidating the function of the protein affected by the mutation. Moreover, comparing the phenotypic and molecular impact of various known POPDC mutations in animal models and patients where possible, provides a valuable tool to further our understanding of the function of POPDC proteins. On this basis the *Popdc2*^{W188X} mutant served as a valuable model organism to study the underlying dysregulation of the β AR signalling pathway which gives rise to stress induced bradycardia observed in the *Popdc2*^{W188X} mutant. Whilst also laying bare the possible role of POPDC proteins in the underlying mechanism governing this signalling pathway.

5.2 Summary of the main findings of this work

5.2.1 *Popdc2*^{W188X} mutation abolishes the expression of *Popdc1* and *Popdc2* at the plasma membrane

The first and original aim of this project was to study the role of POPDC2 CTD in PPI at baseline and following β AR activation and thereby elucidate the underlying mechanism(s) by which POPDC may be regulating the function of their interaction partners during different states of cardiac activation. However, lack of appropriate antibodies reactive with the *Popdc2*^{W188X} mutant presented the first immediate and limiting obstacle in this pursuit. Probably more importantly, the study of the *Popdc2*^{W188X} mouse mutant unexpectedly unveiled that *Popdc2*^{W188X} is subject to NMD in the mouse, which is contrasting the findings provided by Rinne et al. The authors were able to identify the *POPDC2*^{W188X} transcript by cDNA sequencing as well as the human *POPDC2*^{W188X} protein by Western Blot analysis, when extracted from patient leucocyte samples. NMD is a highly regulated and complex mechanism which can vary greatly between different cell types and is strongly influenced in its efficiency through bi- or unidirectional interaction with other cellular pathways. Interestingly, the human *POPDC2*^{W188X} variant is dominant and therefore patients are heterozygous, while the mutant mice are homozygous, which may have an additional, important regulatory role for the highly sensitive NMD mechanism in cardiomyocytes.

Despite the set-back regarding the study of the POPDC2 CTD, the discovery that *Popdc2*^{W188X} is subject to NMD in *Popdc2*^{W188X} mutant mice, aligns with the observations made in samples collected from patients suffering from muscular dystrophy and/or heart problems that carry mutations in the *POPDC* genes. These patients to varying degrees display changes in the plasma membrane expression of both POPDC1 and POPDC2 regardless of which of the genes carries the mutation, resulting in varying degrees of loss of expression and/or mislocalisation of both proteins. Swan et al, have recently shown that the interaction between POPDC1 and POPDC2 is an essential prerequisite for the correct membrane trafficking of both proteins. Therefore, in the *Popdc2*^{W188X} mutant presumed lack of *Popdc2* expression results in reduced expression and mislocalisation of *Popdc1*, phenocopying the *Popdc1* and *Popdc2* KO mutants as was confirmed by immunostainings. It is possible that as more POPDC mutations are analysed and characterised a pathogenicity catalogue will be created to help predict the severity of a particular POPDC mutation based on the level of disruption to POPDC localisation and expression levels.

The discovery that POPDC1 and POPDC2 expression in the *Popdc2*^{W188X} mutant animal hearts is predominantly lost, allows for a new angle at which results describing the cardiac electrophysiology of the *Popdc2*^{W188X} mutant are interpreted. From this new perspective, the *Popdc2*^{W188X} mutants should phenocopy *Popdc1* and *Popdc2* KO mutants. However, this is not entirely the case. For example, *Popdc1* and *Popdc2* KO do not show the stress induced phenotype at a young age, while the *Popdc2*^{W188X} mutants' phenotype is present all through their life, worsening as they age. Additionally, the ECG recordings in live *Popdc2*^{W188X} animals, revealed that they have a reduced heart rate at baseline, whilst the null mutants, show normal heart rate prior to β AR activation. More research is needed to understand how the presence of the genetic material is used and relevant for the nuanced expression of a particular phenotype prior to protein expression, for example during development.

5.2.2 Loss of POPDC in the *Popdc2*^{W188X} mutant may permit aberrant β_2 AR-Gi coupling

In the heart, compartmentalised cAMP signalling is essential for many processes, including the fine control of β AR function during sympathetic activation. The cAMP signalling system has been repeatedly shown to coordinate precise and directed cellular responses that are

underpinned by numerous PPI³⁹⁹. Based on the knowledge that POPDC are cAMP effectors which function by means of PPI, and are known to interact with proteins of the cAMP signalling pathway^{218,393}, it was hypothesised that their loss results in dysregulation of the β AR-cAMP signalling intermediates. Using predominantly FRET microscopy in combination with the CUTie FRET reporters, excitingly it was revealed that loss of POPDC indeed negatively impacts the cAMP production at the plasma membrane and in the bulk cytosol of NMCM as well as AMCM, which probably underpins the strongly dysregulated heart rate of the *Popdc2*^{W188X} in response to stress, when cAMP levels are dramatically increased to propagate the sympathetic signal for elevated heart rate and contractility. Interestingly, the data showed that reduced cAMP levels are not the consequence of reduced AC activity in absence of POPDC. The two major AC in ventricular myocytes AC5 and AC6 remained unchanged in their ability to synthesise cAMP even in the *Popdc2*^{W188X} mutant when stimulated with forskolin. This is in line with previous observations which identified no effect on cardiac contractility in *Popdc1* null mutants¹⁸⁴. On the other hand, literature has shown that POPDC1 has an important role in regulating AC9 activity in skeletal muscle and in the myocytes of the SAN. AC9 is forskolin insensitive and its function has not been assessed in the *Popdc2*^{W188X} mutant. However, it only accounts for about 3% of total cAMP activity in a ventricular myocyte, making it an unlikely candidate when dysregulated, to generate loss in cAMP of the magnitude observed in the mutant NMCM. It can be concluded that the underlying mechanism resulting in low cAMP signal in the *Popdc2*^{W188X} mutant, has a higher level of regulatory complexity. While here ventricular neonatal and adult cardiomyocytes have been utilised as a cell model to study β AR signalling in the *Popdc2*^{W188X} mouse mutant, the predominant phenotype caused by loss of POPDC is of dysregulation of pacemaker activity in mice and therefore resides within the myocytes of the SAN. Interestingly, as previously discussed, in the SAN the interaction between AC9 and POPDC1 regulates TREK1 activity in response to β AR activation. Deletion of either of the three genes produces phenotypes that are similar but of varying severity³⁹³. Crucially, the phenotype is characterised by bradycardia at rest and stress induced heart rate variability. This is in line with the observations made for the in vivo studies of the *Popdc2*^{W188X} mice²⁸⁰, but contradicts the observation made here for the ex vivo heart preparations where at rest, the heart rate is elevated (Langendorff data, page 108). These observations suggest that loss of POPDC proteins has a negative effect on the sympathetic regulation of the heart rate in an intact animal, resulting in a bradycardic phenotype at rest. Since in the absence of

sympathetic stimulation the heart rate of the *Popdc2*^{W188X} mutant is increased, it is possible to speculate that, the bradycardic phenotype is an overcompensation for the tachycardiac phenotype that is caused by the loss of POPDC. A recent article has shown that in hippocampal slices from the *Popdc1* null mutant, loss of *Popdc1* seemed to reduce the threshold for long term potentiation induction³²⁷. These results can be interpreted as evidence for a buffering function of POPDC proteins to prevent abnormal levels of cAMP accumulation in cells and thereby ensuring a graded response to different cAMP levels under physiological conditions.

As it was established that in the NMCM of the *Popdc2*^{W188X} mutant, AC activity is not measurably impacted by the loss of POPDC expression, β ARs themselves were selectively assessed in their function. Both β_1 AR and β_2 AR upon activation couple with the Gs proteins to initiate cAMP driven amplification of the sympathetic signal to the downstream effectors. β_2 AR also can couple to Gi at higher levels of agonist, through PKA mediated phosphorylation which in turn inhibits AC and diminishes the overall cAMP production for both β_1 AR and β_2 AR. The data has shown that in the *Popdc2*^{W188X} mutant, β_2 AR prematurely couples to Gi, thereby preventing AC activity and rise in cAMP levels upon stimulation with β AR agonist. Additionally, a novel interaction between POPDC and PDE4A was shown to be important as a regulatory mechanism to mediate PPI between POPDC and its interaction partners in a cAMP dependent manner. Interestingly, disruption of this interaction in wild type NMCM mimicked low cAMP signal produced in the mutant NMCM. PDEs have been established to have an essential role in regulation of the phosphorylation status of β_2 AR during sympathetic activation to prevent premature Gi coupling, as PKA mediated phosphorylation aids the shift of β_2 AR-Gs to β_2 AR-Gi coupling. Based on the data produced in this thesis, I propose a role of POPDC in regulation of the phosphorylation status of the β_2 AR receptor.

5.2.3 Conclusion and Proposed mechanism

In the heart, mutations in *POPDC* genes give rise to stress induced disorders of cardiac pacemaking and conduction, while no effect on cardiac contractility has been reported. Nevertheless, low cAMP levels have been recorded in NMCM of the *Popdc2*^{W188X} mutant following activation of β ARs with ISO. POPDC proteins have numerous interaction partners important for the pacemaker mechanism of the SAN and cardiac electrophysiology as a

whole. However, β ARs have so far not been part of the POPDC protein interactome in the heart, albeit some preliminary data suggest that both POPDC1 and POPDC2 do co-IP with β ARs in a heterologous system. Here I have shown that loss of POPDC1 and POPDC2 in the *Popdc2*^{W188X} mutant NMCM generates low cAMP concentration following β AR activation, due to premature β_2 AR coupling to the inhibitory G_i , probably caused by the loss of PDE4A regulation of PKA activity at the β_2 AR signalosome. At present, it is not possible to determine by which model of action POPDC proteins regulate β AR signalling in NMCM and more experimental work would need to be performed to validate and expand findings presented in this chapter. However, in light of the here presented results, parallels that can be drawn from relevant POPDC interactions and other known mechanisms regulating β AR signalling in the heart a working model for the role of POPDC in β AR signalling can be proposed.

In wild type NMCM, possibly via AKAP79, β_2 ARs are in a complex with POPDC1, which recruit and bind PDE4A long isoforms through their UCR1 region. The POPDC1 recruited PDE4A functions as a cAMP sink around the receptor complex to prevent PKA mediated receptor phosphorylation from low level stimuli from for example heterologous cAMP production. Additionally, the interaction of PDE4A with POPDC1 may keep the enzyme in its more catalytically active monomeric state (Burgin et al., 2010) as the binding site of POPDC1 on PDE4A lies within the dimerization motif of PDE4 (Bolger et al., 2015). Therefore, absence of POPDC1 in the *Popdc2*^{W188X} mutant has a twofold effect: loss of precise β_2 AR signalosome localisation of PDE4A and a reduced catalytic activity of PDE4A near the receptor due to PDE4A dimer formation. These changes leave the β_2 AR signalosome exposed permitting a rapid PKA activation and receptor phosphorylation already at low cAMP activity. As previously discussed, PKA mediated phosphorylation of β_2 AR promotes receptor – G_i coupling and inhibition of AC activity.

5.2.4 Graphic depiction of the proposed mechanism

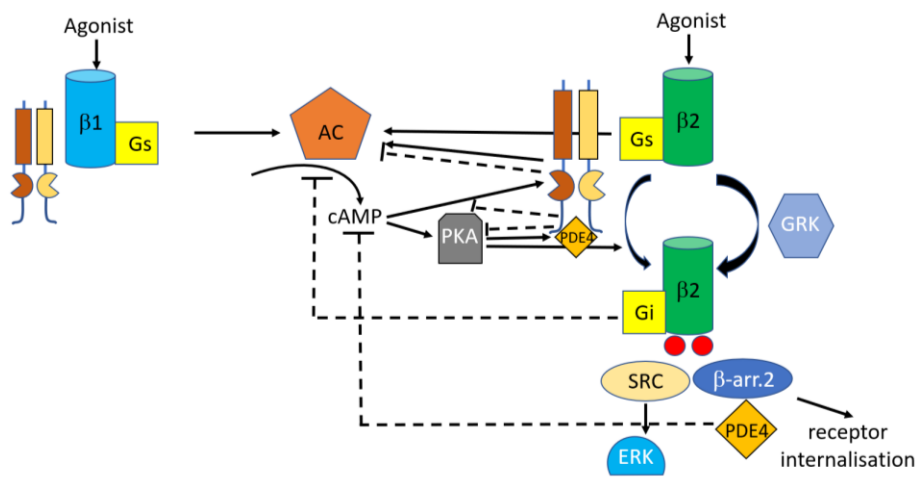


Figure 5.1. Graphic depiction of the proposed mechanism by which POPDC proteins play a role in β AR signalling regulation

CHAPTER 6 Appendix

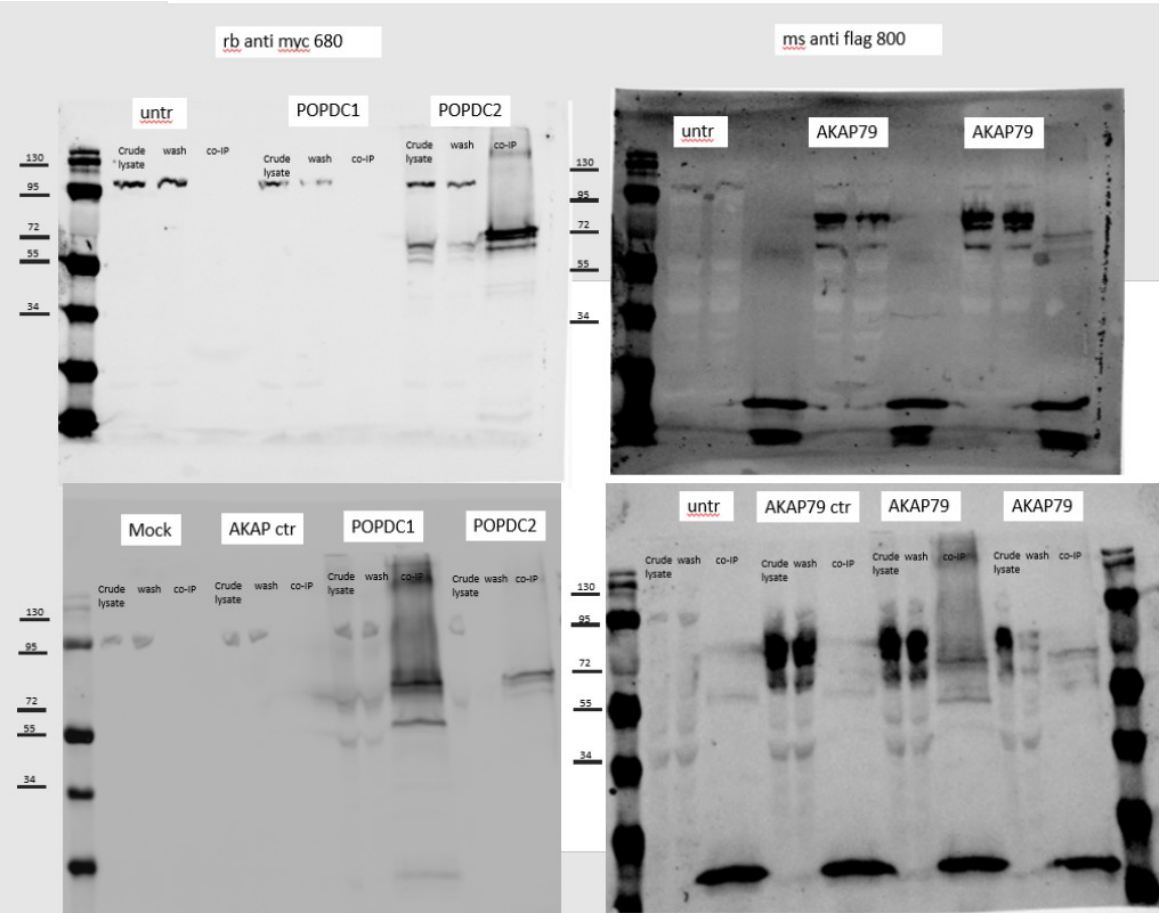


Figure 6.1. Appendix Western blots for Co-IP experiments

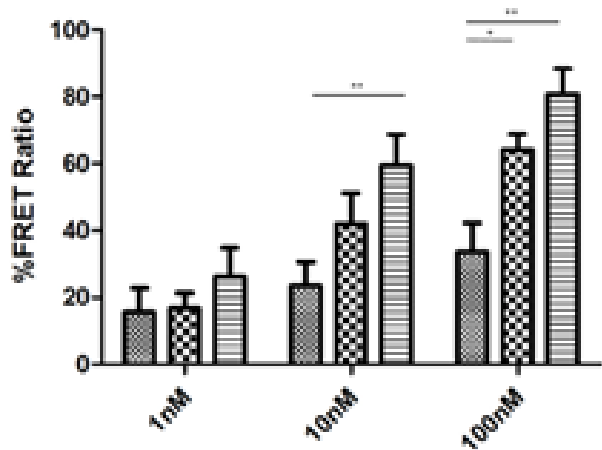


Figure 6.2. Appendix FRET in transfected HEK cells

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