Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognize the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
Multi-Scale Cardiomyocyte Organisation as a Determinant of Cardiac Function

Michelle L. Munro

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Physiology, the University of Auckland, 2016.
Abstract

Excitation-contraction (EC) coupling is a critical process underlying cardiac function, and for this to be effective, precise organisation of key proteins and structures is required. Transverse (t)-tubules and the sarcoplasmic reticulum (SR) form a close association known as the junction, where several EC coupling proteins localise, including the SR calcium release channels – the ryanodine receptors (RyR). In cardiomyocytes, formation of both junctions and t-tubules is promoted and maintained by junctophilin-2 (JPH2). Three mechanisms have been proposed through which JPH2 may impact cardiac function: 1) organisation of the junction, 2) t-tubule organisation, and 3) stabilisation of RyR. By investigating these mechanisms, this thesis aimed to examine aspects of the relationship between cardiomyocyte organisation and cardiac function in three studies. The first study aimed to determine the role of JPH2 in maintaining junctional protein and t-tubule organisation. Using JPH2 transgenic mice, super resolution imaging revealed an increased RyR cluster size in JPH2 over-expressing mice, in which smaller calcium sparks are observed, while JPH2 knockdown demonstrated no change to RyR cluster size, despite increased calcium spark size and frequency. Changes in density and co-localisation of junctional proteins provided an explanation for the apparent disparity between RyR cluster size and calcium handling properties. Increased longitudinal branching of the t-tubule network was identified following JPH2 over-expression, which overall, had no detrimental effect on EC coupling. The second study used a novel approach to directly correlate force development and cardiomyocyte structure in trabeculae from failing human hearts. Cardiomyocyte content of the myocardium was identified as a key indicator of contractile performance in these samples. Several changes in the microscopic organisation of cardiomyocytes were also confirmed as occurring in the failing human heart. The final study investigated the development of EC coupling structures, including t-tubules and junctions, in the large mammalian fetal heart. In the first known fluorescent study to examine this, t-tubules (which develop only postnatally in rodents) were identified as developing in utero in the sheep heart, and were associated with early co-localisation of junctional proteins. Combined, these findings support the three proposed mechanisms of JPH2, and provide new insights of implications for EC coupling.
Acknowledgements

I would like to thank:

My main supervisor Prof Christian Soeller, for the always constructive feedback on my work and enabling me to attend so many valuable conferences and spend time in the Exeter lab. Your support and encouragement has helped me enjoy my PhD experience and learn to trust myself in becoming an independent researcher.

My co-supervisor Dr David Crossman, for all the feedback and advice you have given over the course of my time in the lab. I have really valued your support over the last three and a half years. Also, thank you for allowing me to be part of the human transplant study; it was a great experience to have – even with the 2 am starts in the lab.

My co-supervisor Dr Marie Ward, for all the feedback, advice and help with interpreting the functional data. Thank you for supporting and encouraging me to apply for numerous conferences and awards.

My PhD advisor, Dr David Baddeley, for not only establishing the super resolution setup in our lab, but also for the numerous hours spent teaching me how to use the system and software for analysis.

Dr Isuru Jayasinghe, for help in pretty much every aspect of this thesis, including data analysis, proof-reading and teaching me many of these techniques. More importantly, thank you for all the conversions we had on academia, PhDs and life in general, and for always offering your support and advice. You have been a great friend to have through this process.

Dr Xin Shen for performing the dissection and functional experiments for the failing human trabeculae study, as well as making the transplant hours more enjoyable.

Dr Cherrie Kong for patiently teaching me how to isolate cells, and for being such a good friend from my early days in the lab.

Other past and present lab mates from the Biophysics group: Amelia Power, Yufeng Hou and Sarbjot Kaur for making my time in the lab more interesting. A special thanks to Lucy Goodman for listening and understanding my frustrations throughout the last few years.
Prof Xander Wehrens and his laboratory group for kindly supplying the isolated cells and hearts from the JPH2 transgenic mice for this study, as well as the corresponding functional and Western blot data.

Jacqui Ross and the BIRU staff for assistance and support with using the confocal imaging system.

Prof Peter Ruygrok and the cardiology staff at Auckland City Hospital for their kind assistance at all hours of the day and night in helping us obtain our transplant samples.

The Fetal Physiology and Neuroscience Lab, not only for kindly supplying me with tissue, but also for adopting me as an honorary member. You guys provided amusement that made this process thoroughly more entertaining.

Dr Joanne Davidson, for always willingly offering advice and encouragement which helped me get through this process. Thank you for being such a supportive friend and for rescuing me from some awkward situations!

My department partner in crime, Lotte van den Heuij, for all the ridiculous conversations, spontaneous cocktails and for being an amazing friend. Going through this process together has made it that much easier to manage, and I can’t wait for us both to be on the other side.

My parents and sister: Gill, Paul and Dale. Thank you all so much for supporting and encouraging me from day one, and all the way through this endeavour. For the reminders to take breaks, the evenings out (sorry I had to bail when transplants came up), and for keeping me smiling, I am beyond grateful.

My fiancé (now husband), Phillip Brandt, for your patience, understanding and unconditional support over the years. Thank you for putting up with my frustrations (sorry you bore the worst of it), helping keep me on track in any way you could, and for always being there for me. Thank you so very much.
This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

**Chapter 3**

<table>
<thead>
<tr>
<th>Nature of contribution by PhD candidate</th>
<th>Sample processing, data acquisition, data analysis, generation of all text and majority of figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of contribution by PhD candidate (%)</td>
<td>90</td>
</tr>
</tbody>
</table>

### CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Isuru Jayasinghe</td>
<td>Data analysis and generation of schematic diagram</td>
</tr>
<tr>
<td>Prof Christian Soeller</td>
<td>Data analysis and plot generation</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isuru Jayasinghe</td>
<td></td>
<td>11/01/2016</td>
</tr>
<tr>
<td>Christian Soeller</td>
<td></td>
<td>17/01/2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Last updated: 19 October 2015
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 5

<table>
<thead>
<tr>
<th>Nature of contribution by PhD candidate</th>
<th>Sample collection and preparation, structural experiments, data analysis, all text and figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of contribution by PhD candidate (%)</td>
<td>90</td>
</tr>
</tbody>
</table>

CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr David Crossman</td>
<td>Sample collection and processing</td>
</tr>
<tr>
<td>Dr Xin Shen</td>
<td>Trabeculae dissection and collection of functional data</td>
</tr>
</tbody>
</table>

Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xin Shen</td>
<td></td>
<td>07/01/2016</td>
</tr>
<tr>
<td>David Crossman</td>
<td></td>
<td>15/01/2016</td>
</tr>
</tbody>
</table>
Contents

Abstract ii
Acknowledgements iii
List of Figures ix
List of Tables xi
Abbreviations xii

Chapter 1. Introduction ...........................................................................................................1
  1.1 Cardiac Ultrastructure ........................................................................................................2
    1.1.1 Key structural components of the cardiomyocyte ................................................2
    1.1.2 Organisation of the transverse tubules .................................................................4
    1.1.3 Junctional organisation and the role of junctophilin .............................................6
  1.2 Cardiac Function .........................................................................................................7
    1.2.1 Overview of EC coupling and CICR mechanisms ................................................8
    1.2.2 RyR physiology and organisation ......................................................................10
    1.2.3 NCX1 physiology and organisation ....................................................................13
  1.3 Human Heart Disease and Failure ............................................................................14
    1.3.1 Dilated cardiomyopathy .....................................................................................14
    1.3.2 Hypertrophic cardiomyopathy ..........................................................................15
    1.3.3 Heart failure .......................................................................................................16
  1.4 Protein and Structural Changes in Human and Animal Models of Heart Failure ......17
    1.4.1 T-tubule changes ...............................................................................................18
    1.4.2 EC coupling protein changes .............................................................................20
    1.4.3 JPH2 in heart disease and failure ......................................................................21
  1.5 Project Aims ..............................................................................................................29

Chapter 2. General methods .................................................................................................32
  2.1 Sample Collection and Preparation ...........................................................................33
    2.1.1 Rat ventricular myocytes and myocardium ......................................................33
    2.1.2 Immunohistochemistry and immunocytochemistry ..........................................34
    2.1.3 Antibodies and stains .........................................................................................37
  2.2 Image Acquisition and Analysis ...................................................................................39
    2.2.1 Confocal microscopy ..........................................................................................39
Chapter 5. Structure-function relationship in human heart failure.................................................103

5.1 Background ...........................................................................................................................103
5.1.1 Structural and EC coupling changes in human heart failure ........................................103
5.1.2 JPH2 in human cardiomyopathy .....................................................................................105

5.2 Experimental Techniques and Analysis ..............................................................................106
5.2.1 Human heart failure sample preparation ........................................................................107
5.2.2 Trabeculae functional experiments ................................................................................108
5.2.3 Functional data processing and analysis .........................................................................110
5.2.4 Confocal image processing and analysis .........................................................................111
5.2.5 Statistical analysis ...........................................................................................................113

5.3 Structure-Function Relationship in Heart Failure ...............................................................113
5.3.1 Variability of functional performance in failing trabeculae ...........................................114
5.3.2 Altered tissue cardiomyocyte content .............................................................................115
5.3.3 Changes to EC coupling structural organisation .............................................................117
5.3.4 Microtubule and JPH2 alterations ..................................................................................121
5.3.5 Structural relationship with FFR and time course of contraction ....................................124
5.3.6 Structural comparisons between failing and non-failing wall ......................................125

5.4 Discussion ...........................................................................................................................129
5.4.1 Cardiomyocyte content and peak stress development ...................................................130
5.4.2 EC coupling protein organisation in failing trabeculae ..................................................131
5.4.3 Microtubules and JPH2 ..................................................................................................133
5.4.4 The FFR and temporal mechanics ................................................................................134
5.4.5 Alterations in organisation in the failing human heart and trabeculae as a model for failing myocardium .................................................................137
5.4.6 Significance, limitations and future directions ...............................................................140

Chapter 6. Development of t-tubule and EC coupling organisation in the fetal sheep 142

6.1 Background ...........................................................................................................................142
6.1.1 Development of EC coupling organisation in rodents ..................................................142
6.1.2 T-tubule formation in rodents ........................................................................................145
6.1.3 The role of JPH2 in cardiac development ......................................................................146
6.1.4 Development of EC coupling organisation in large mammals .........................................147

6.2 Experimental Techniques and Analysis ..............................................................................148
6.2.1 Fetal sheep myocardium samples ...................................................................................148
6.2.2 Image processing and analysis ......................................................................................149
6.2.3 Statistical analysis ..........................................................................................................150

6.3 Development of EC Coupling Organisation in Fetal Sheep .............................................150
6.3.1 Development of t-tubule network organisation ...............................................................150
6.3.2 RyR and JPH2 development ..........................................................................................153
6.3.3 NCX development ..........................................................................................................155
6.4 Discussion ...............................................................................................................156
6.4.1 T-tubule development in fetal sheep ...............................................................156
6.4.2 EC coupling protein maturation in fetal sheep .................................................158
6.4.3 Significance, limitations and future directions ..................................................159

Chapter 7. The relationship between cardiomyocyte organisation and cardiac function
..............................................................................................................................................161
7.1 JPH2 in the Cardiac Structure-Function Relationship .............................................162
7.1.1 Junctional organisation ....................................................................................163
7.1.2 Maintenance of t-tubule structure ....................................................................164
7.1.3 Evidence for stabilisation of RyR .....................................................................166
7.2 Implications in the Failing Heart ..........................................................................167
7.3 Implications in the Developing Heart ....................................................................168
7.4 Multi-scale Cardiomyocyte Organisation as a Determinant of Cardiac Function ...169

References 171
List of Figures

Figure 1.1 Schematic of mammalian cardiac myocyte organisation ........................................ 3
Figure 1.2 T-tubule labelling in mammalian cardiomyocytes ................................................... 5
Figure 1.3 Excitation-contraction coupling in mammalian cardiac myocytes .......................... 10
Figure 1.4 Super resolution RyR cluster imaging ................................................................... 12
Figure 1.5 T-tubule changes in failing cardiomyocytes .......................................................... 18
Figure 2.1 Detection of single molecule localisation events .................................................... 41
Figure 2.2 dSTORM acquisition properties ............................................................................ 43
Figure 2.3 Drift correction ........................................................................................................ 45
Figure 2.4 Co-localisation analysis process ............................................................................ 46
Figure 3.1 Calcium handling properties in control and JPH2-OE mice ................................. 49
Figure 3.2 RyR macro-cluster analysis steps .......................................................................... 52
Figure 3.3 RyR cluster longitudinal analysis .......................................................................... 54
Figure 3.4 JPH2 expression levels influence the pattern of localisation ................................. 57
Figure 3.5 JPH2 expression causes longitudinal reorganisation of the RyR clusters .......... 59
Figure 3.6 Changes to longitudinal component of RyR clusters due to JPH2 expression ...... 60
Figure 3.7 JPH2 expression causes transverse reorganisation of the RyR clusters ............... 61
Figure 3.8 RyR cluster distance analysis ............................................................................... 62
Figure 3.9 RyR labelling and cluster densities following JPH2 expression changes .......... 64
Figure 3.10 JPH2 knockdown reduces co-localisation of remaining JPH2 with RyR .......... 65
Figure 3.11 JPH2 over-expression alters junctional JPH2 and RyR densities ....................... 67
Figure 3.12 Junctional RyR stabilisation following JPH2 over-expression ......................... 76
Figure 4.1 FFT analysis process of t-tubule immunolabelling .................................................. 81
Figure 4.2 Steps for angle analysis of t-tubule labelling ......................................................... 82
Figure 4.3 Analysis of perimeter of myofibril contacting RyR ............................................... 84
Figure 4.4 Knockdown of JPH2 reduces t-tubule regularity .................................................. 86
Figure 4.5 JPH2 knockdown leads to t-tubule disorganisation .............................................. 87
Figure 4.6 Reduced co-localisation of JPH2 with t-tubules after knockdown ....................... 89
Figure 4.7 Junctional localisation of NCX is influenced by JPH2 expression ....................... 91
Figure 4.8 Changes in contact between RyR and myofibrils in JPH2 transgenic mice .......... 92
List of Tables

Table 2.1  Table of the primary antibodies used for immuno-histochemistry and cytochemistry with final dilutions used and source of the antibodies. ............................................................37

Table 5.1 Details of patients for human heart samples .........................................................106
Table 5.2 Variability of contractile performance in failing cardiac trabeculae......................114
Abbreviations

Cav-3 Caveolin-3
CICR Calcium-Induced Calcium Release
CRU Calcium Release Unit
CSA Cross-Sectional Area
DCM Dilated Cardiomyopathy
DHPR Dihydropyridine Receptor
dSTORM Direct Stochastic Optical Reconstruction Microscopy
EC coupling Excitation-Contraction coupling
ECM Extra-Cellular Matrix
EM Electron Microscopy
FFR Force Frequency Response
HCM Hypertrophic Cardiomyopathy
$\mathcal{I}_{Ca}$ DHPR-mediated inward calcium current
IDCM Idiopathic DCM
JPH Junctophilin
LSM Laser-Scanning Microscope
LV Left Ventricle
MORN Membrane Occupation and Recognition Nexus
NCX Sodium-Calcium Exchanger
PLB Phospholamban
PSF Point-Spread Function
RV Right Ventricle
RyR Ryanodine Receptor
SERCA Sarcoplasmic Endoplasmic Reticulum Calcium ATPase
SR Sarcoplasmic Reticulum
SR$_J$ Junctional Sarcoplasmic Reticulum
$T_{50\%}$ Time to 50% relaxation of force
TPS Time to Peak Stress
T-tubules Transverse Tubules
WGA Wheat Germ Agglutinin
Chapter 1. Introduction

From before we are born, our hearts begin to continuously pump blood around the body for the delivery of nutrients to all other tissues, making it arguably the most important organ in the human body. With an aging population and a major increase in the prevalence of lifestyle diseases, such as type 2 diabetes, cardiovascular disease is the leading cause of mortality and morbidity worldwide, and also produces significant healthcare costs (WHO, 2014). In many cases, this condition can also affect the young and otherwise healthy due to genetic factors, making heart disease a serious health concern. Heart failure is a frequent development in these patients, caused by the inability of the heart to pump a sufficient volume of blood to meet the demands of the body as a result of impaired cardiac function. Despite the impact this condition has on millions of individuals worldwide, relatively little is known about the cellular mechanisms underlying the development and progression of heart disease into heart failure. One of the key principles behind understanding these changes is the relationship between structure and function of cardiac tissue, which still remains relatively unclear.

A major step towards unravelling the mechanisms behind these changes is first understanding the structure and function in the healthy heart and cardiomyocytes. This includes the processes involved in excitation-contraction coupling (EC coupling) as well as the role of key structures in cardiomyocytes including the transverse-tubule (t-tubule) network and junctional spaces. Junctions, or dyads, are functional regions within cardiomyocytes which contain the majority of the proteins involved in handling of calcium ions, which is essential to cellular contraction, and therefore cardiac function. The organisation of many of these key structures is able to influence the processes required for cardiac function. One protein which has emerged as playing an important role in promoting and maintaining several aspects of cardiac organisation is junctophilin-2 (JPH2). The function of JPH2 has been associated with the development and maintenance of the t-tubule system, the formation of junctions, as well as being implicated in the development and progression of heart failure. Determining the role that JPH2 plays in the relationship between cardiac structure and function would provide valuable insight to potential mechanisms underlying cardiac disease and development.
1.1 Cardiac Ultrastructure

The heart is able to undergo extensive remodelling in response to changes in environment, which can be the precursors to heart disease. These changes occur at both the macroscopic and microscopic level, and it is therefore important to understand the structure of the healthy heart in order to recognise changes that occur during this cardiac remodelling process.

1.1.1 Key structural components of the cardiomyocyte

The mammalian heart is made up of several cell types, but of particular interest are the striated cardiac muscle cells, the cardiomyocytes, which contribute to the majority of the volume (~75%) of adult heart tissue (Olivetti et al., 1980). Ventricular cardiomyocytes are excitable, multi-nucleated cells which are typically cylindrical in shape, being approximately 100 µm in length and 20 µm in diameter (Forbes and Sperelakis, 1984). Neighbouring cardiomyocytes form end-on-end connections with each other through structures known as intercalated disks, which contain gap junctions (Severs, 1990), facilitating intercellular communication to allow the synchronisation of electrical and chemical signals, as well as mechanically coupling them together (Sjöstrand et al., 1958). This is important for synchronous contraction of the ventricle walls during systole to produce an effective cardiac output. Due to the high energy demand of excitable cells, cardiomyocytes have a very high reliance on mitochondria, which occupy approximately 35% of the intracellular space (Bossen et al., 1978, Sommer and Waugh, 1978).

Cardiomyocytes also contain a modified endoplasmic reticulum known as the sarcoplasmic reticulum (SR), which extends throughout the cell and forms a network around other cellular structures, such as the contractile machinery (Sommer and Waugh, 1978). The SR contains many proteins which play an essential role in cardiac function, and is also the main intracellular store of calcium ions in cardiomyocytes, which is critical for cardiomyocyte contraction (Bers, 2001). The SR can be divided into free (non-junctional) and junctional (SRJ) regions, with the SRJ also known as the subsarcolemmal cisternae or terminal SR. The SR comes into close proximity to the plasma membrane (sarcolemma) to promote the functional coupling of sarcolemmal and SR proteins in specialised regions known as junctions, or dyads (see ‘Junctional organisation and the role of junctophilin’ below). Another important cardiomyocyte structure is the transverse tubule system (t-tubules), which is a network of invaginations of the sarcolemma (see section 1.1.2 for further details).

Myofibrils are another key structure within cardiomyocytes which contain the contractile
proteins, arranged into sarcomeres, forming the contractile unit of myocytes and occupying ~47% of the cross-sectional intracellular space (Soeller et al., 2007). Sarcomeres are highly organised and arranged regularly throughout the length of the myocyte, giving striated muscle cells their appearance. They contain two main contractile filaments: the 10-15 nm thick filaments made of myosin and the 6 nm thin filaments composed of F-actin, troponin and tropomyosin (Fawcett and McNutt, 1969). The interaction of these two filaments forms the molecular basis of cardiomyocyte contraction, enabling force production for ventricular ejection during systole. Upon release from the SR, calcium binds to the troponin (specifically troponin-C) on the thin filaments, causing a conformational change in the tropomyosin and revealing myosin-binding sites on the actin filament (Solaro, 2010). The binding of myosin to the actin filament leads to cross-bridge formation, with the heads of the myosin molecules exhibiting ATPase activity. This ATPase activity is required for the cycling of cross-bridges which slides the two filaments past each other, resulting in increased overlap, producing shortening of the sarcomere, and ultimately, cardiomyocyte contraction (Bers, 2001). Key ultrastructural components of the mammalian cardiomyocyte are summarised in Figure 1.1.

Figure 1.1 Schematic of mammalian cardiac myocyte organisation
Sarcomeres are connected end-on-end by z-disks, which are formed by multiple proteins important for structure and contractile function, including α-actinin, which aids in anchoring and maintaining the organisation of the thin filaments (Frank et al., 2006). In mammalian cardiomyocytes, z-disks are \( \sim 100 \) nm thick (Yamaguchi et al., 1985), with the distance between them used as a measure of sarcomere length and a typical relaxed sarcomere length of \( \sim 1.9 \) µm in cardiomyocytes (Scriven et al., 2005). The sarcomere length (and therefore resting tension), has a direct influence on the maximal force generated during cellular contraction due a combination of the degree of myofilament overlap and the stretch-dependent calcium sensitivity exhibited by troponin-C (Allen and Kurihara, 1982).

### 1.1.2 Organisation of the transverse tubules

Striated myocytes contain long invaginations of the plasma membrane, known as transverse tubules (t-tubules) which extend towards the interior of the cell, increasing the surface area to volume ratio (Bers, 2001). The continuation of the sarcolemma into the myocytes allows electrical signalling along the membrane to be propagated into the cell. This means that voltage-gated channels expressed along the t-tubule sarcolemma can generate responses faster than relying on diffusion of signalling ions, such as calcium, from surface sarcolemmal channels alone (Brette and Orchard, 2003). The t-tubules also allow the continuation of the extracellular constituents laterally into the cell, effectively reducing the distance that ions need to diffuse across the plasma membrane to reach the central cytosolic regions. The presence and pattern of cardiac t-tubules has been identified as being not only species-specific but also variable between the chambers of the heart within a species. Some species, including amphibians and reptiles, do not demonstrate t-tubule presence at all (Bossen and Sommer, 1984), while in mammals, t-tubules are predominantly observed in the ventricles and were considered to be virtually absent in the atria (Brette et al., 2002), although more recent studies have suggested otherwise (Dibb et al., 2009, Richards et al., 2011). Ventricular cardiomyocytes of small mammals such as rats and mice demonstrate a very extensive t-tubule network, with a high degree of axial branching, while larger mammals such as horses and humans possess more sparse, radial spoke-like arrangement of the t-tubules, with rabbits having an intermediate phenotype (Jayasinghe et al., 2012b, Jayasinghe et al., 2015, Soeller and Cannell, 1999). The average diameter of the t-tubule is also variable across species, with smaller mammals having narrower t-tubules than larger mammals (Guo et al., 2013). See Figure 1.2 for examples of t-tubule arrangement in different mammalian species. The progression of t-tubule development has also been identified as species-dependent, which is covered in detail in Chapter 6.
In mammalian ventricular cardiomyocytes, the majority of t-tubules are in line with the z-disks (Fawcett and McNutt, 1969); however, depending on the species, 40% of t-tubules can be located between z-disks, forming longitudinal extensions to create an inter-connecting network of membrane within the cell (Soeller and Cannell, 1999). The t-tubule network wraps around intracellular structures and organelles, such as the myofibrils and mitochondria, effectively allowing no region within the cell to be further than ~1.2 µm from the sarcolemma (Song et al., 2005). In the rat, the diameter of the cardiac ventricular myocyte t-tubule is on average ~200 nm, and accounts for an estimated 21-64% of the total plasma membrane and 0.8-3.6% of myocyte volume (Bosson et al., 1978, Page et al., 1971, Soeller and Cannell, 1999). The relatively small diameter of the t-tubule means that diffusion of extracellular compounds into (and out of) the network is restricted, effectively producing a 'micro-environment' within the t-tubules which may vary from the general extracellular fluid (Brette and Orchard, 2003). Further micro-domains are present along the t-tubule (and surface) membrane in the form of 50-100nm flask-shaped invaginations known as caveolae (Levin and Page, 1980), which are thought to be compartmentalised signalling domains, also known as signalosomes (Razani et al., 2002). Caveolin-3 (Cav-3) is a protein present along the membrane (Scriven et al., 2005) which is known to promote the formation of caveolae in cardiomyocytes, with knock-out of Cav-3 resulting in the disruption of t-tubules in striated muscle (Galbiati et al., 2001). Additional specialised regions along the t-tubules are junctions, which are discussed in Section 1.1.3.

As t-tubules are extensions of the sarcolemma, they possess many of the same biochemical features as the surface membrane. In cardiomyocytes, this includes the presence of glycocalyx, a network of polysaccharides rich in sialic acid residues, which are associated
with the outer layer of the sarcolemma (Fawcett and McNutt, 1969, Frank et al., 1977). T-tubules also contain many of the key proteins involved in EC coupling. It has been found that many of the calcium-handling proteins are more abundant in the t-tubules compared to the surface sarcolemma, including the voltage-gated L-type calcium channels (dihydropyridine receptors; DHPR) which is present in the t-tubules at several times the density of that found on the surface, based on both immunofluorescence labelling (Carl et al., 1995, Scriven et al., 2000) and calcium current determination (Kawai et al., 1999). In addition, it has been shown that the sodium-calcium exchanger (NCX) is expressed at high levels along the t-tubule system (Scriven et al., 2000, Frank et al., 1992, Thomas et al., 2003). There are also a large number of other ion channels and transporters present at high levels in the t-tubule membrane, including potassium channels (Takeuchi et al., 2000, Clark et al., 2001), the sodium-hydrogen exchanger (Petrecca et al., 1999), voltage-gated sodium channels (Maier et al., 2002), and the sodium-potassium ATPase (McDonough et al., 1996).

1.1.3 Junctional organisation and the role of junctophilin

In cardiomyocytes, the SR$_J$ comes in close proximity to sections of the sarcolemma at both the surface and t-tubules, to form functional regions called junctions (also known as dyads), with surface junctions also called peripheral couplings. The distance of the space spanning between the sarcolemma and SR membranes is approximately 10-12 nm in junctions, forming the junctional or dyadic cleft (Franzini-Armstrong et al., 1999). This close apposition of the two membranes allows for SR proteins to form close associations with those localised to the sarcolemma, as well as forming a micro-domain (often called ‘fuzzy space’) which can restrict the diffusion of ions from the junctional cleft, including calcium, enabling ion concentrations to differ from the rest of the cytoplasm (Scriven et al., 2000). It is estimated that 4-8% of the surface sarcolemma is associated with junctions, while 20-50% of the t-tubule membrane is junctional, depending on species (Page and Surdyk-Droske, 1979), with junctional regions extending ~400 nm laterally along the t-tubule membrane in mice (Takeshima et al., 2000).

The formation of junctions is believed to be promoted by a protein known as junctophilin (JPH), which contains domains that interact with both the surface sarcolemma and SR membranes, helping to anchor the two membranes in close proximity. In cardiomyocytes, it is the 696 residue JPH2 isoform which is predominantly expressed and found in the junctional regions (Takeshima et al., 2000) (although molecular weight estimates range from ~74 to 100 kDa (Golini et al., 2011, Takeshima et al., 2000, Landstrom et al., 2014)). The carboxyl end
of JPH2 is anchored into the SR membrane via a membrane spanning domain, with an alpha helix domain which spans the junctional cleft. The JPH2 sequence also contains eight membrane occupation and recognition nexus (MORN) motifs which facilitate the interaction with the plasma membrane (Takeshima et al., 2000), and promote junction formation. These functional domains within the protein sequence are highly conserved across different species, including human, monkeys and rodents (Garbino et al., 2009).

As mentioned, junctions are important functional regions within the cardiomyocyte as they allow the proteins located on the two opposing membranes to closely interact. This includes proteins involved in EC coupling, such as the DHPR and RyR which are localised to the t-tubule and SRJ membranes, respectively (Scriven et al., 2000, Franzini-Armstrong et al., 1999). It is thought that junctions play a role in controlling the gain of EC coupling, whereby the efficiency of RyR activity mediated via DHPR calcium influx ($I_{Ca}$) is related to the proximity of these two proteins, and therefore influenced by the distance across the junctional cleft (Stern, 1992). Due to the nanoscale nature of the junctional cleft, EC coupling is typically a ‘high gain’ mechanism, with a relatively small $I_{Ca}$ triggering a large release of calcium from the SR via RyR opening (Song et al., 2001, Wier et al., 1994). This is in part due to the previously mentioned restricted ion diffusion within the junctional cleft, meaning that a high calcium concentration can be established locally within the cleft to bind to the RyR (Lederer et al., 1990). In this way, junctions allow for ‘local control’ of the micro-domain ion concentrations within the cleft, making the organisation of the junctional proteins key for regulating EC coupling gain, and therefore having a major influence on overall cardiac function. Despite being discovered well over a decade ago, there is potentially more yet to be determined about the importance of JPH2 in cardiac function and development, and the role it may play in disease development and progression. An additional protein localised to the sarcolemma, bridging integrator 1 (BIN1), is a membrane scaffolding protein which has been implicated in both t-tubule formation (Lee et al., 2002) and anchoring DHPR within the junction (Hong et al., 2010), however this will not be the focus of this project.

1.2 Cardiac Function

Cardiac function involves the contraction of the myocardium to eject blood from the ventricles, to be pumped throughout the body efficiently during systole. For this to be effective, the myocardium must contract synchronously, which requires electrical and chemical signalling, both within the heart and individual cardiomyocytes, to be synchronised. This synchrony is
achieved through a number of mechanisms, with many of the key ultrastructural components described contributing to the efficiency of systolic function including the t-tubules and junctions.

1.2.1 Overview of EC coupling and CICR mechanisms

Excitation-contraction coupling (EC coupling) is the physiological mechanism in which electrical signalling is converted into mechanical cellular contraction (Bers, 2002). Cardiomyocytes are excitable cells, and are therefore sensitive to changes in sarcolemmal membrane potential. Electrical signalling, in the form of action potentials, causes the membrane potential of the myocytes to become more positive (depolarise). Depolarisation is initiated in the sino-atrial (SA) node, also known as the ‘pace-maker’ region of the heart (DiFrancesco et al., 2001). From there, the action potentials pass through the atrio-ventricular (AV) node to spread throughout the myocardium via Purkinje fibres, causing depolarisation of the ventricular (and atrial) cardiomyocyte sarcolemma, including the t-tubules (Bers, 2001). This depolarisation can modulate the function of voltage-sensitive proteins associated with the plasma membrane of cardiomyocytes, with the activation of these proteins being the initial cellular trigger for the molecular events responsible for contraction of cardiomyocytes, making cardiomyocyte depolarisation the key electrical event for EC coupling.

Calcium ions are vital signalling molecules for several cellular processes, including the contraction of myocytes. Calcium-induced calcium release (CICR) is accepted as the underlying mechanism of EC coupling in cardiomyocytes, in which calcium is able to induce greater intracellular calcium release and subsequent contraction of the cell (Bers, 2001). This involves a small influx of calcium into the cell \( I_{Ca} \), via the voltage-gated DHPR, which is essential to the CICR process. In the cardiomyocyte, it is the Ca\(_{1.2a}\) isoform splice variant which is expressed, and is unique to these cells (Triggle, 2003). The DHPR calcium channel is localised to the plasma membrane of the cardiomyocytes, in particular along the junctional regions of the t-tubules, as well as co-localised with caveolae near the openings of t-tubules on the surface sarcolemma (Scriven et al., 2000). The depolarisation of the membrane causes the opening of the DHPR, thereby initiating \( I_{Ca} \), which is the triggering calcium for CICR, and responsible for the plateau phase of the cardiac action potential (Bers, 2001). This \( I_{Ca} \) diffuses across the junctional cleft and binds to the SR calcium release channels, which are localised to the SR\(_i\) membrane, allowing the greater efflux of calcium from the SR into the cytosol (Fabiato, 1983). These SR channels were identified to be the ryanodine
1. Introduction

Receptors (RyR) in both cardiac (Inui et al., 1987a, Lai et al., 1987) and skeletal muscle (Lai et al., 1988, Inui et al., 1987b), with the binding of calcium causing opening of the RyR. RyR opening leads to a greater, temporary increase in cytosolic calcium, known as the calcium transient, which is able to diffuse away from the junctional space and bind to troponin-C on the thin filament of the myofibrils, enabling contraction, as previously described (section 1.1.1) (Bers, 2002). The synchronisation of these events both within and between cardiomyocytes enables the myocardium to contract in unison during systole to produce maximal contractile performance. The speed and strength of force production during contraction are dependent on the rate and amplitude of the calcium transient generated during CICR (Bers, 2001).

In order for cardiomyocytes to relax before the next cardiac cycle (by dissociation of calcium from troponin-C), the cytosolic calcium must be restored to its resting level. This is achieved through two main mechanisms: extrusion of calcium ions out of the cell and re-uptake into the SR (and to a lesser extent into the mitochondria), with the balance of the two being species-dependent (Bers, 2001). The latter of these processes is mediated by the sarcoplasmic endoplasmic reticular Ca\(^2+\) ATPase (SERCA), a pump which hydrolyses ATP in order to transport calcium across the SR membrane and accounts for the majority of calcium removal (Edes et al., 2001). In cardiomyocytes, SERCA2a is the dominant isoform which is localised to the membrane of the SR, predominantly along the longitudinal segments but also within the SRJ (Edes et al., 2001, Meissner, 1975). In addition to removal of excess cytosolic calcium, SERCA-mediated calcium re-uptake is essential for reloading SR during diastole to ensure stores are refilled prior to the next CICR event. The protein phospholamban (PLB) associates with cardiac SERCA and is able to regulate its activity via inhibitory mechanisms, with this interaction in turn modulated by phosphorylation (Inui et al., 1986).

The second main mechanism of cytosolic calcium removal is extrusion out of the cell, primarily via the sodium-calcium exchanger (NCX), and to a lesser extent the sarcolemmal calcium ATPase (Bers, 2001). In addition to removal of excess calcium from the cytosol, inactivation of \(I_{Ca}\) is required to allow relaxation of the contractile filaments during diastole. This occurs through a negative feedback mechanism involving calcium-dependent inactivation of the DHPR via the calcium binding protein, calmodulin (Peterson et al., 1999). In this way, the amount of calcium present in the cytosol at any one time is tightly regulated in order to maintain healthy cardiac function. A summary of EC coupling and the key proteins involved is presented in Figure 1.3.
1.2.2 RyR physiology and organisation

The discovery that the plant alkaloid ryanodine causes rigor in skeletal muscle and diminished cardiac twitches resulted in the identification of the subsequently named ryanodine receptor (RyR) (see (Jenden and Fairhurst, 1969) for review). The tetrameric RyR is a large protein found in striated muscle, made up of four ~565 kDa subunits, forming a calcium channel in both skeletal and cardiac tissue (Takeshima et al., 1989, Nakai et al., 1990). Identified as the SR calcium release channel, RyR plays a vital role in CICR (Bers, 2001). The RyR2 isoform is expressed in cardiac myocytes (Nakai et al., 1990), and is predominantly localised to the SRj membrane, arranged in clusters (Chen-Izu et al., 2006, Soeller et al., 2007). It has been shown that there is co-operative opening of RyRs within a cluster, in which the opening of one channel increases the open probability of the other.

Figure 1.3 Excitation-contraction coupling in mammalian cardiac myocytes
Schematic of the key proteins involved in EC coupling and CICR localised to the t-tubule and junctional sarcoplasmic reticulum (SRj), including the dihydropyridine receptor (DHPR), ryanodine receptor (RyR), sarcoplasmic reticulum calcium ATPase (SERCA), phospholamban (PLB) and sodium-calcium exchanger (NCX) along with the myofibrils and structural protein junctophilin-2 (JPH2). Blue arrows indicate Ca^{2+} movement; red arrows indicate Na^{+} movement.
channels within that cluster (Stern et al., 1999). This has led to RyR clusters often being referred to as calcium release units (CRUs), with the brief spike of release of calcium from a single CRU giving rise to a local release called a ‘calcium spark’ (Cheng et al., 1993, Bers, 2001). The temporal and spatial summation of calcium sparks from multiple CRUs results in the generation of the calcium transient throughout the cardiomyocyte, producing the increased cytosolic calcium concentrations required for initiation of contraction (Cheng et al., 1996).

Structural studies have identified the predominant cellular localisation of RyR clusters within the cardiomyocyte, with early electron microscopy (EM) studies locating them in close proximity to the t-tubules (Frank, 1990). This is further supported by the observation of calcium sparks originating at t-tubules (Cheng et al., 1996), which are in alignment with the z-disks. EM studies first revealed the RyR appearing as ‘feet’ within the dyadic regions, with the channels partially spanning the junctional cleft (Franzini-Armstrong et al., 1999). Detailed work by several groups revealed the morphology of individual RyR channels, each being ~30 nm wide x 12 nm tall and display a 4-fold symmetry with a central pore formed by the four subunits (Inui et al., 1987a, Lai et al., 1988, Franzini-Armstrong and Protasi, 1997). Estimates made from EM studies indicated that the average mammalian junctional CRU, or RyR cluster, contain between ~90-270 channels, with 300-400 nm between neighbouring clusters, depending on species (Franzini-Armstrong et al., 1999). However, EM studies are typically limited by the exact orientation of the thin sample being unknown, such that reported estimates of RyR cluster size may not be accurate (Franzini-Armstrong, 2010).

The use of immunofluorescence in combination with recent imaging advancements have enabled increased resolution to be achieved, which has provided further levels of detail into the organisation of RyR clusters within mammalian cardiomyocytes. One of the first studies of this kind by Baddeley et al. (2009) revealed that cluster organisation is more complicated than initially thought, with variations in the packing density of the receptors within clusters, as well as the observation that several smaller clusters can contribute to what would have traditionally been detected as a single cluster (see Figure 1.4) (Baddeley et al., 2009b). This increased optical resolution resulted in the average cluster size of ~14 RyR detected in rat peripheral couplings, with many of these clusters exhibiting non-uniform shape (Baddeley et al., 2009b), compared to the previously described rounded shape (Franzini-Armstrong et al., 1999). Similar findings have been reported in an EM tomography study in mice (Hayashi et al., 2009). A follow-up study examining rat intracellular junctions revealed a similar appearance of cluster morphology, with the average cluster containing ~63 RyR channels (although ~10% of clusters contained >200 RyR) and ~140 nm separating neighbouring
1. Introduction

junctons (Hou et al., 2015). The increased optical resolution achieved in these studies has been able to provide measurements of RyR cluster properties with greater accuracy than those obtained with conventional microscopy techniques (Baddeley et al., 2009b), which is a great advantage given the nanoscale nature of CRUs.

Obtaining accurate measurements of cluster parameters is essential for determining calcium release properties, particularly in models of EC coupling. Along with the restricted diffusion of calcium from $I_{\text{Ca}}$ due to the narrow junctional cleft, the organisation of channels within the RyR cluster determines the likelihood of CICR occurring within the cluster due to a single channel opening, also known as ‘local control’ of CICR (Stern, 1992). Furthermore, the spacing between clusters contributes to ‘local control’ within the cardiomyocyte, due to the observation that CRUs usually function independently of each other due to the spatial separation between them (Bers, 2001). This means that a single calcium spark is unlikely to activate neighbouring CRUs, reducing the occurrence of spontaneous calcium transients under normal conditions (Parker et al., 1996). Calcium transients are typically achieved by the synchronised activation of the DHPR along the t-tubules throughout length of the myocyte, which allows for the near-simultaneous activation and opening of RyR clusters (Wier and Balke, 1999), which is necessary for the synchronous contraction of the cardiac

Figure 1.4 Super resolution RyR cluster imaging
Detection of RyR clusters in rat peripheral couplings showing super resolution imaging (green) with overlaid corresponding diffraction-limited image (red) of RyR antibody labelling. Arrows indicate smaller clusters not identified with conventional imaging. Scale bar: 1 µm. Reproduced from Baddeley et al. 2009. © Baddeley et al., 2009b.
myocyte (Bers, 2001). The functional coupling of a CRU with DHPR is often termed a 'couplon', which includes both intracellular junctions and peripheral couplings (Franzini-Armstrong et al., 1999). In addition to forming couplons with DHPR, there are many other proteins identified as associating and interacting with RyR. One such protein is calsequestrin (CSQ), which is localised to the SR, within the SR lumen, and functions as a buffer of intra-SR calcium (MacLennan and Wong, 1971). The association of CSQ with RyR has been suggested to regulate calcium release (Scriven et al., 2013), with additional proteins junctin and triadin involved in the CSQ-RyR interaction (Franzini-Armstrong and Protasi, 1997). Studies have also shown a high degree of co-localisation between RyR and JPH2 in both peripheral couplings (Jayasinghe et al., 2012a) and junctions (Hou et al., 2015). There is also evidence that JPH2 may bind to and directly interact with RyR by stabilising the channel, which would provide a physical basis for JPH2 regulation of RyR mediated calcium release (Van Oort et al., 2011).

1.2.3 NCX1 physiology and organisation

The sodium-calcium exchanger (NCX) is a counter-transporter responsible for the main mechanism of extruding calcium from the myocyte cytoplasm, although it is capable of bi-directional transport (Bers, 2001). NCX1 is the primary isoform in cardiomyocytes which exchanges one Ca$^{2+}$ ion for 3 Na$^+$ ions and is able to create an influx or efflux of calcium based on the relative sodium and calcium gradients across the sarcolemma (Kimura, 2001). Since the movement of ions by NCX is electrogenic, the direction of movement is also determined by the plasma membrane potential (Scriven and Moore, 2013). The contribution of NCX (versus SR re-uptake) for removal of cytoplasmic calcium for cardiomyocyte relaxation is species-dependent (Bers, 2001). In rats it has been shown that NCX contributes to ~7% of calcium removal (Bassani et al., 1994), while in humans it is closer to 25% (Weber et al., 2003). For cardiomyocytes to maintain calcium homeostasis, the amount of calcium brought into the cell during the action potential must equal the amount extruded from the cell during diastole, with NCX calcium efflux typically balancing DHPR-mediated \( I_{Ca} \) (Ottolia et al., 2013). The ‘reverse’ mode of NCX results in calcium influx in exchange for the extrusion of sodium to the cytosol, and in animals with high resting intracellular sodium, such as rats, this has been shown to contribute to loading the SR during diastole (Shattock and Bers, 1989).

Functional studies have suggested that the reverse mode of NCX may play a role in the entry of calcium into the myocyte during the early phase of the action potential, supplementing that brought in via the DHPR (Sobie et al., 2008), and work by LeBlanc and Hume showed that it
could even potentially generate calcium influx capable of triggering CICR (Leblanc and Hume, 1990). This supports the idea that NCX is located within the ‘fuzzy space’ of restricted diffusion associated with the junctional cleft (Scriven and Moore, 2013). While there is some discrepancy on the exact location of NCX within the cardiomyocyte, the majority of studies are in agreement that it is localised to the t-tubules and outer surface sarcolemma (Frank et al., 1992, Kieval et al., 1992, Thomas et al., 2003). There have also been reports of a junctional sub-population of NCX1, which is associated with couplons (Scriven and Moore, 2013), and supports the early functional findings. The proportion of NCX reported as localised to junctions has been inconsistent, with some studies reporting minimal (~6%) co-localisation with RyR (Scriven et al., 2000), while more detailed confocal imaging revealed up to ~27% of NCX1 co-localised with RyR in adult rat ventricle, with ~42% of RyR clusters exhibiting a degree of association with NCX labelling (Jayasinghe et al., 2009).

1.3 Human Heart Disease and Failure

As previously mentioned, cardiovascular disease is the leading cause of mortality and morbidity worldwide, and is also the leading cause of non-communicable disease deaths (WHO, 2014), generating significant healthcare costs. Disease of the heart muscle, also called cardiomyopathy, can exist in several different forms and commonly progresses into heart failure. There are four main types of cardiomyopathy: restrictive, arrhythmogenic, dilated and hypertrophic. For the purpose of this thesis, these latter two will be the primarily focus. In both cardiomyopathy and heart failure, there is an alteration in the function of the myocardium, whereby affected regions may exhibit diastolic dysfunction, such as impaired relaxation of the ventricle wall, systolic dysfunction (reduced ejection fraction), or both (Marian and Roberts, 2001). In a healthy human heart, the ejection fraction is 55-70% (AHA, 2015). As the left ventricle (LV) is responsible for the ejection of oxygenated blood for circulation around the body, LV function is commonly used as a marker for cardiac function, although many forms of cardiomyopathy affect the right side or both (Segers et al., 2012).

1.3.1 Dilated cardiomyopathy

The most common form of heart disease is dilated cardiomyopathy (DCM) (Mestroni et al., 2014) which is characterised by the dilation of one or several of the chambers, often with all four chambers affected as the disease progresses (McNally et al., 2013). In DCM, the lumen of the ventricles (typically the left) is greatly enlarged, while the thickness of the ventricle wall
remains relatively unchanged (Mestroni et al., 2014). While one or both ventricles can be affected, clinically, DCM is typically diagnosed based on presentation of impaired LV function or systolic dysfunction, such as reduced (<45%) ejection fraction, coupled with an enlarged end-diastolic LV diameter (Friedrich and Carrier, 2012). Due to the increased lumen volume, it becomes difficult for the heart to sustain cardiac output, with the ejection fraction of blood decreasing to ~15% in the left ventricle in some patients (Sugrue et al., 1992). DCM commonly progresses into heart failure which is the leading underlying cause of heart transplant procedures (Hong et al., 2012). Some of the contributing factors to the development of DCM can include coronary artery disease and hypertension, with myocardial infarction accounting for approximately half of all cases (McNally et al., 2013).

While there is a high incidence of idiopathic DCM, there are also a significant proportion of cases associated with a genetic basis for the disease (Marian and Roberts, 2001). There have been many genes associated with the development of DCM, including cytoskeletal proteins such as dystrophin, troponin and the heavy chain of myosin, and including some which are localised to the z-disk such as α-actinin and α-cardiac actin (for review see (Mestroni et al., 2014) or (Friedrich and Carrier, 2012)). The majority of cases are due to autosomal dominant inheritance, although autosomal recessive and X-linked inheritance can also occur (Marian and Roberts, 2001). While the exact trigger and progression of DCM remains unclear, one of the proposed mechanisms suggests that the high prevalence of mutations in contractile and cytoskeletal proteins points towards impairment of cytoskeletal integrity and transmission of force being responsible (Friedrich and Carrier, 2012). It is thought that force transmission at the z-disk is commonly impaired, resulting in an abnormal stretch response and subsequent lengthening of the cardiomyocytes (dilating the ventricle) (Marian and Roberts, 2001). However, in many patients the identified mutation is associated with ion channels or the nuclear membrane, suggesting that there are potentially multiple pathological mechanisms possible (Friedrich and Carrier, 2012). Histologically, DCM presents with mild to moderate hypertrophy of degenerating cardiomyocytes, which maintain cell-to-cell alignment, in addition to variable degrees of interstitial fibrosis (Schönberger and Seidman, 2001).

1.3.2 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is characterised by a thickening of the wall of the ventricle, resulting in a smaller lumen space in the chamber. This is usually associated with hypertrophy of the inter-ventricular septal wall, which is typically asymmetrical in presentation,
leading to reduction of the LV cavity with reduced diastolic function due to impaired ventricle relaxation (Friedrich and Carrier, 2012). This essentially means that there is less capacity for blood in the chamber, and therefore there is a smaller volume of blood pumped out during each cardiac cycle, despite often maintaining a normal ejection fraction (Marian and Roberts, 2001). Clinically, HCM is diagnosed by the presence of a thickened LV wall of >15 mm (Varma and Neema, 2014). While many patients can reach a normal life expectancy, approximately half of HCM patients develop heart failure to some degree, typically presenting with preserved systolic function but breathlessness brought on by exercise (Maron et al., 2014). In some cases, heart failure presents as either end-stage systolic dysfunction or LV outflow obstruction (Melacini et al., 2010). As heart failure progresses, dilation of the ventricles can be observed in some cases, particularly in those with systolic dysfunction (Melacini et al., 2010). Undiagnosed HCM patients often suffer sudden cardiac death, particularly in competitive athletes (Friedrich and Carrier, 2012).

While there are a proportion of patients with sporadic HCM, the majority of cases are due to hereditary conditions, with HCM considered to be the most common inheritable cardiac disease (Maron et al., 2014). There are a large number of genes implicated in the inheritance of HCM, with the majority of mutations associated with sarcomeric proteins, including the troponins and myosin (for review see (Maron et al., 2012)), mostly due to autosomal dominant inheritance (Marian and Roberts, 2001). A large number of the mutations identified to date are associated with regions of the sarcomeric proteins involved in mechanical contraction of the cardiomyocyte, leading to impaired force generation (as opposed to impaired transmission of force in DCM) (Marian and Roberts, 2001). At the cellular level, in HCM there is hypertrophy and disorganisation of the cardiomyocytes, with interstitial fibrosis throughout the myocardium (Landstrom and Ackerman, 2012). It is thought that cardiomyocyte hypertrophy is a compensatory mechanism in response to impaired contractile function (Marian and Roberts, 2001). This adaptive response may become insufficient to compensate for reduced function, becoming ‘decompensatory’ hypertrophy which can contribute towards heart failure development (Bers, 2001).

### 1.3.3 Heart failure

Heart failure is a condition in which the heart is no longer able to pump sufficient blood around the body to meet metabolic demands (Forbes and Sperelakis, 1984). The loss of cardiac function is often coupled with shortness of breath due to fluid build-up in the lungs (known as pulmonary oedema) as a result of distension and congestion of blood in the veins,
leading to oedema in the extremities, and is also referred to as congestive heart failure (Kannel et al., 1972). In addition to progressive cardiomyopathy, heart failure can also develop due to coronary heart disease and ischemic episodes (heart attacks), as well as pressure overload (Downey and Heusch, 2001), with coronary artery disease indicated to be the most common cause (McMurray and Stewart, 2000). Heart failure affects ~4 people in every 1000 in the general population, with the prevalence greatly increasing in the elderly (28/1000 people over 65 years of age) (McMurray and Stewart, 2000). The prognosis of heart failure is typically very poor, with ~50% mortality within 5 years of diagnosis, which may be due to insufficient cardiac function or arrhythmia induced sudden cardiac death (Bers, 2001).

While heart failure is characterised by a reduction in contractility of the heart muscle, typically with a reduced ejection fraction or fractional shortening of the myocardium, it has more recently been recognised that a normal or preserved ejection fraction can also occur (McMurray and Stewart, 2000). This has led to heart failure patients as being categorised as those with either reduced ejection fraction (HFrEF) or preserved ejection fraction (HFpEF), with approximately 1:1 occurrence in each category (Vasan et al., 1999). HFpEF occurs most commonly in patients with diastolic dysfunction, such as those with HCM in which relaxation of the myocardium is impaired (Garg et al., 2013). For the purpose of this thesis, HFrEF will be focussed on, and from here on simply referred to as heart failure. Hearts which are in end-stage failure typically have regions with varying degrees of diminished contractile function throughout the ventricular walls, including the septum (Crossman et al., 2015). It is common for one side of the heart to be affected more than the other, resulting in the two sides no longer functioning as a unit (Downey and Heusch, 2001). There are several molecular mechanisms suggested to contribute to the reduced contractile function observed in heart failure, which will be discussed below in Section 1.4.

1.4 Protein and Structural Changes in Human and Animal Models of Heart Failure

Both human samples and animal models have been widely studied to determine the underlying changes that occur in the myocardium during the development and progression of heart failure. Several significant changes have been found, including altered expression and distribution of many key proteins and structures in the cardiomyocytes. These include the t-tubules as well as several calcium-handling proteins involved in EC coupling such as RyR,
1. Introduction

SERCA and NCX. Despite being widely studied, exactly whether many of these changes are the cause or effect of the pathological mechanism remains unclear.

1.4.1 T-tubule changes

One of the most characterised ultrastructural changes within diseased and failing cardiomyocytes is the disorganisation, or remodelling, of the t-tubule system. These changes have been observed in both human heart failure (Cannell et al., 2006, Crossman et al., 2011, Lyon et al., 2009) and several animal models of heart failure progression (Wei et al., 2010, Song et al., 2006, Louch et al., 2013). The primary changes observed include an overall reduction in t-tubule density, the misalignment of the t-tubules from the z-disks, along with increasing numbers of oblique or longitudinal extensions of the network (Guo et al., 2013). In diseased human hearts, the t-system becomes less regular throughout the cardiomyocytes, with regions showing a decrease in the transverse component in favour of an increasing

![Figure 1.5 T-tubule changes in failing cardiomyocytes](image)

WGA labelling of t-tubules in ventricular cardiomyocytes from A,B) non-failing and C,D) failing human hearts. A) Longitudinal view of a healthy cardiomyocyte with regular, uniform t-tubules; B) magnified view of the indicated region in panel A. C) Diseased cardiomyocyte showing loss of organisation and regularity of the t-tubules; D) magnified view of indicated region in panel C. Scale bars: 10 µm in panels A and C; 2 µm in panels B and D. Modified from Crossman et al. (2011).
number of longitudinal projections (Crossman et al., 2011). While the diameter of the t-tubules can be widely varied throughout healthy cardiomyocytes (Soeller and Cannell, 1999), overall there is an increase in the average diameter in heart disease (Cannell et al., 2006), often observed as ‘dilation’ of the t-tubules with increased membrane area (Louch et al., 2004, Maron et al., 1975). These changes have also been observed in animal models of pressure overload (Wei et al., 2010, Page and McCallister, 1973) and myocardial infarction, which is also associated with extensive remodelling of t-tubule orientation (Wagner et al., 2012). In some instances, failing cardiomyocytes demonstrate a loss of t-tubule density or even t-tubule openings (Maron et al., 1975, Lyon et al., 2009). These studies have all shown t-tubule reorganisation in a variety of species resulting from different pathological causes, indicating that it is a common structural alteration in the failing heart. Examples of the t-tubule changes observed in dilated human heart failure are shown in Figure 1.5.

While the exact mechanism(s) contributing to pathological remodelling of the t-tubules still remains uncertain, it has been recognised that the t-system is able to undergo significant adaptive remodelling, both during development and in response to changes in environmental conditions (Brette and Orchard, 2003). It has been proposed that pathological t-tubule remodelling is a cellular response during the disease process, rather than part of the initial trigger or cause (Lyon et al., 2009). However, exactly whether remodelling is a cause or an effect of heart failure pathology is difficult to determine, although changes identified prior to the onset of failure (Wei et al., 2010), suggest that it is causative. Recent research has also shown that variability in t-tubule disorganisation in failing human hearts is correlated with the degree of mechanical dysfunction (Crossman et al., 2015). It is possible that during the progression of heart failure, alterations in mechanical performance of the myocardium may be transferred to the t-tubule structures, as it has been shown that increased strain on cardiomyocytes can lead to altered shape and volume of t-tubules (McNary et al., 2011).

The loss and displacement of the t-tubules from the z-disks reduces their regularity throughout the cell, so there is reduced synchronisation of signalling in these regions. Aberrations of the t-tubules are associated with a reduction in their close apposition to the SRJ, resulting in a decreased number of junctions observed in human heart failure, and remaining junctions being smaller overall in size (Zhang et al., 2013). Furthermore, animal models of heart failure with loss or disorganisation of the t-tubules also show increased calcium spark frequency with reduced homogeneity of calcium release during the transient (Lyon et al., 2009). This indicates that there is increased ‘leak’ but reduced synchrony of the CRUs activity, with recent imaging advances indicating that these sparks are occurring at the t-tubules (Sikkel et al., 2015). Similar functional findings have also been observed following
detubulation treatment in cardiomyocytes, with dysynchrony of the calcium transient observed (Brette et al., 2002). The loss of t-tubule connections with the surface sarcolemma means that there are membrane regions no longer activated by the action potential, leading to higher dependence on the diffusion of calcium from the surface membrane DHPRs to activate central RyR. The resulting transients are therefore reduced in synchronisation and amplitude, with slower kinetics, as is characteristic in heart failure (Guo et al., 2013). Overall, the remodelling of the t-tubules in heart disease and failure causes impaired EC coupling, with many of the underlying mechanisms a result of additional changes to the associated proteins.

1.4.2 EC coupling protein changes

In addition to the t-tubule changes observed in heart disease and failure, there are also changes involving several of the key EC coupling proteins. The re-organisation of the t-tubules will also affect the cellular distribution of proteins normally localised to the t-tubule membrane, including the DHPR and NCX. While this can greatly influence EC coupling due to displacement from the z-disk and SRj regions, it is a secondary effect of the t-tubule remodelling (Song et al., 2006) and is additional to mechanistic EC protein changes associated with pathophysiology of heart failure.

Due to the characteristic impairment of the calcium transient observed in heart failure, many studies have focussed on identifying various changes to RyR. While some groups have provided evidence for reduced mRNA and protein expression of the RyR in human and animal model failing hearts (Go et al., 1995, Vatner et al., 1994, Yano et al., 2000), other studies did not support these findings (Gomez et al., 1997, Sainte Beuve et al., 1997). Functional studies have indicated that, while RyR expression levels may be unchanged, the sensitivity of the channel is potentially altered through hyperphosphorylation and altered FKBP12.6 binding (Marx et al., 2000) such that there is increased leak but reduced coordination of opening (Yano et al., 2000, McCall et al., 1996). It has also been reported that there is a reduction in the number or RyR clusters present per volume of myofibrils (Crossman et al., 2011). Furthermore, as previously mentioned, one of the secondary consequences of t-tubule remodelling is their displacement from the z-disk and therefore from the SRj. This has led to the identification of ‘orphaned’ RyR present in heart failure, whereby a large proportion of channels are no longer associated with the t-tubules, and as a result have reduced co-localisation with the DHPR (Song et al., 2006). These findings were confirmed by Crossman et al (2011) who showed that in human heart failure there is an apparent reduction in DHPR present in line with the z-disk, leading to reduced co-localisation.
with RyR. Functionally, this results in impaired EC coupling due to the increased distance between DHPR and RyR for CICR to occur across. Also, the increased distance (or complete loss) of the junctional cleft reduces the ability for local control of calcium diffusion and concentrations, and as a result, the calcium released by the RyR has a reduced ability to inactivate $I_{Ca}$ via a calcium-dependent mechanism (Song et al., 2006).

One of the most consistent findings across human and animal studies of heart failure is the loss of SERCA activity (Pieske et al., 1995, Gwathmey et al., 1987), which can be due to reduced expression levels (de la Bastie et al., 1990, Takahashi et al., 1992, Hasenfuss et al., 1994, Arai et al., 1993). In addition, some studies have shown that while PLB protein expression is also reduced, it maintains a similar (or slightly increased) expression ratio with SERCA compared to normal hearts (Arai et al., 1993, Meyer et al., 1995). However, studies indicate that there is a decrease in the extent of PLB phosphorylation (Huang et al., 1999, Schwinger et al., 1999), resulting in reduced activity of SERCA. This results in impaired SR calcium re-uptake, associated with prolonged relaxation of the cardiomyocytes due to intracellular calcium levels remaining elevated for longer, which are characteristic kinetics of heart failure (Bers, 2001).

NCX expression has been found to be increased ~2-fold in nearly all studied cases of human and animal models of heart failure, which is associated with increased exchanger activity as well (Hasenfuss et al., 1999, Pogwizd et al., 1999, Sipido et al., 2000). When coupled with the reported decrease in SERCA expression, there is a shift in the balance of calcium removal from the cytoplasm towards extrusion from the cell, as opposed to SR re-uptake, resulting in decreased SR calcium content in the absence of PLB phosphorylation (Bers, 2001). Given the bidirectional ability of NCX to exchange ions, its increased expression alongside the observed decrease in the calcium transient in heart failure (Pieske et al., 1995, Sipido et al., 1998) results in an increased calcium influx via the NCX during the action potential, contributing to the characteristic slow transient decay in heart failure (Dipla et al., 1999). The extent of this altered calcium handling is frequency-dependent, indicating an important role of increased NCX expression in calcium dynamics in heart failure (Bers, 2001).

### 1.4.3 JPH2 in heart disease and failure

One of the key proteins of interest in this study was JPH2 and the influence it has in the development and progression of heart failure. As previously mentioned, JPH2 is thought to be the protein responsible for promoting the association of the SR$_j$ and t-tubule membranes to form junctions, or dyads (Takeshima et al., 2000). Since junctions are the structures
responsible for bringing key proteins involved in CICR and EC coupling in close proximity, alterations to JPH2 in heart disease and failure may have a major impact on the efficiency of these two processes. Several studies using human or models of heart disease and failure have identified changes in JPH2 in disease, as well as cellular changes induced by genetic modification of JPH2 expression.

1.4.3.1 JPH2 mutations in human heart disease

In addition to the mutations found in sarcomeric and contractile proteins, several mutations have also been identified in the JPH2 protein in human cardiomyopathy. In genetic studies, five separate JPH2 mutations were identified in patients with HCM (Landstrom et al., 2007, Matsushita et al., 2007). Three of the identified mutations are relatively close together along the JPH2 sequence, at amino acid positions 101, 141 and 165 (out of a total of 696 residues), with two of the mutations within regions encoding MORN motifs (Garbino et al., 2009). This means that there could potentially be a reduced affinity of the mutant JPH2 isoforms to bind to the plasma membrane, and therefore there may be an increase in the size of the junctional space, reducing the efficiency of EC coupling. Two of these mutations involved the substitution of serine residues for a different amino acid (arginine or phenylalanine), while the third substituted a tyrosine for a histidine (Landstrom et al., 2007). Interestingly, these involve the substitution of residues which could potentially be phosphorylated by protein kinases, with S165 suggested as a putative phosphorylation target (Landstrom et al., 2007). In a study by Woo et al (2010), it was shown that the S165F mutation does in fact lead to reduced levels of protein kinase C-mediated phosphorylation of JPH2 in skeletal tissue, indicating that this residue is able to be phosphorylated (Woo et al., 2010).

Cultured cell lines, including cardiomyocytes, expressing mouse JPH2 with these three mutations revealed that they produce altered calcium signalling and cellular hypertrophy which was associated with abnormal distributions of JPH2, compared to wild-type JPH2 (Landstrom et al., 2007). In addition, the S165F mutation was found to induce hypertrophy in developing muscle fibres which was coupled with abnormal calcium signalling and increased resting intracellular calcium levels, despite normal expression of DHPR and RyR (Woo et al., 2010). These studies suggest that while calcium handling protein expression is maintained, mutations in JPH2 can still affect calcium signalling. A fourth mutation associated with the divergent, cytosolic region of the JPH2 protein (Garbino et al., 2009) involved the substitution of a glycine for a serine residue (G505S). Since the G505S mutation that was identified is localised to a region of the sequence not associated with MORN motifs, it is unlikely that this mutation affects the interaction of JPH2 with the plasma membrane (Matsushita et al., 2007).
While data to date suggest that this mutation has minimal influence on JPH2 structure and interaction, this mutation does also involve a serine residue. In this instance, a serine residue is being added to the protein, rather than removed as in S165F. While it is unknown if this mutation could produce a potential phosphorylation target, it is interesting that several of the JPH2 mutations associated with HCM identified to date involve either serine or tyrosine residues which may be modified through phosphorylation. The effect of phosphorylation of JPH2 on its function is still unclear; however it may be a potential factor in the development of heart disease.

One of the recently identified JPH2 mutations in patients with HCM was a mis-sense mutation in which amino acid 169, normally a glutamic acid, was substituted for a lysine (Beavers et al., 2013). This E169K mutation occurs within the joining region between the sixth and seventh MORN motifs of the protein, with E169 highly conserved across species (Garbino et al., 2009). By introducing this mutation into mice via knock-in, it was found that the mice were more prone to atrial fibrillation induction through spontaneous calcium release from the SR via RyR, and that there was a reduction in the JPH2 binding affinity for RyR (Beavers et al., 2013). These findings suggest that JPH2, in particular the region containing this point mutation, is involved in stabilising RyR calcium release from the SR (Beavers et al., 2013).

1.4.3.2 JPH2 expression in disease and failure

Since JPH2 mutations occur only in a subpopulation of patients with cardiomyopathies, the majority of heart failure patients express normal JPH2 (or potentially may contain mutations which have not yet been identified). However, it has been shown that compared to healthy individuals, some HCM and DCM patients have significantly decreased levels of JPH2 cardiac expression (Landstrom et al., 2011, Zhang et al., 2013). There have also been several studies which have used HCM or myocardial infarction animal models to examine the changes that occur with the progression towards heart failure. Many of these studies have found that there is a reduction in the expression of JPH2, which may occur at the mRNA or protein level of expression (Woo et al., 2010, Xu et al., 2007, Minamisawa et al., 2004, Wagner et al., 2012). In addition, in a model of DCM, it was seen that while there were normal JPH2 mRNA levels, there was decreased expression of the protein (Minamisawa et al., 2004). This suggests that there may be post-transcriptional regulation or increased protein degradation which is causing a reduction in the amount of protein expressed.

One potential mechanism for post-transcriptional regulation of JPH2 mRNA has been recently suggested. This involves microRNAs, which are non-encoding short (~22) nucleotide
sequences that can bind to mRNA and potentially influence their transcription into functional proteins (Nelson et al., 2003). Recent studies have found that the microRNA 24 (miR-24) can bind to specific regions of JPH2 full-length mRNA, and that there is an increase in miR-24 levels in a model of HCM and heart failure (Xu et al., 2012). These models also had reduced JPH2 protein expression levels, and it was shown that over-expression of miR-24 can produce down-regulation of JPH2 (Xu et al., 2012). While the suppression of increased miR-24 activity in a heart failure model did not influence compensated hypertrophy development, it did prevent the transition to decompensated hypertrophy, with JPH2 protein levels maintained at normal levels (Li et al., 2013). These studies also examined the influence of miR-24 expression on cardiomyocyte function, and showed that increased miR-24 produced reduced calcium transient amplitudes with prolonged transient times (Xu et al., 2012). This reduced and desynchronised calcium release was associated with a reduction in the area of t-tubule membrane coupled to the SR, with normal DHPR calcium influx, indicating there has been a loss in EC coupling gain (Xu et al., 2012). Suppressing miR-24 in an HCM model protected from the loss of EC coupling gain, and restored the reduction in t-tubule-SR association usually observed with this model (Li et al., 2013). It has also recently been found that human DCM and ischemic cardiomyopathy patients have an up-regulation in miR24 levels, with an associated down-regulation in JPH2 protein levels (Zhang et al., 2013). In addition to miR-24, a potential mechanism by which the amount of full-length wild-type JPH2 protein could be reduced is proteolytic cleavage. Recent findings have shown that JPH2 can be cleaved by a calcium-dependent protease, potentially µ-calpain, in both skeletal and cardiac myocytes (Murphy et al., 2013). Further studies have revealed that there may be several calpain-specific cleavage sites along JPH2 (Wu et al., 2014, Guo et al., 2015).

1.4.3.3 JPH2 knockdown/knockout models

In addition to the role of miR-24, the influence of JPH2 expression on cardiomyocyte structure and function has been examined by many groups using direct JPH2 expression manipulations in both cell and animal models.

Structural Changes

Several studies have used JPH2 knockdown in cultured cell lines to determine the effects of reduced JPH2 expression on cell structure and function. One such study involved adenoviral mediated knockdown of JPH2 in cardiac protein expressing HL-1 cells (Landstrom et al., 2011). Findings from this study showed that reduced JPH2 expression in this model led to increased cell size (hypertrophy), which is matched with up-regulation of pro-hypertrophic
1. Introduction

factors (Landstrom et al., 2011). Additional studies in cultured cardiomyocytes showed that knockdown of JPH2 leads to remodelling and decreased regularity of the t-tubule network (Wei et al., 2010).

While cell cultures are a useful tool for controlling protein expression and monitoring cell behaviours, a more powerful tool (in terms of translation to human disease) is the use of animal models in which JPH2 expression has been knocked out or down-regulated. Homozygous JPH2 knockout animal strains resulted in embryonic lethality, with very few embryos surviving past E9.5, which is that at which JPH2 expression can be detected in control animals (Takeshima et al., 2000). Of the surviving embryos, weak and irregular heartbeats are observed, along with over half of the surviving E10.5 knockout animals developing cardiac arrest and peripheral tissue congestion (Takeshima et al., 2000). In addition, the peripheral couplings (cell surface ‘junctons’ in embryonic cells) in the knockout mouse strain were altered such that there was an overall increase of the gap distance in peripheral couplings, which were also reduced in length compared to control animals (Takeshima et al., 2000). These changes were observed before cardiac arrest occurred, providing further evidence of the importance of JPH2 in the formation of functional couplings, which are essential for proper cardiac function.

To circumvent embryonic lethality and study the effect of JPH2 knockdown in adult animals, one research group developed an inducible, cardiac-specific knockdown of JPH2 in adult mice (Van Oort et al., 2011). These animals show changes in the gross morphology of the heart, including severe dilation of the chambers which is associated with the development of acute heart failure within one week of JPH2 knockdown (Van Oort et al., 2011). Acute JPH2 knockdown resulted in increased end-diastolic and end-systolic diameters of the ventricles along with a reduction in ejection fraction. The knockdown animals also present with an increase in relative lung weight compared to tibia length which is indicative of pulmonary congestion (Van Oort et al., 2011). At the cellular level, these animals show disorganisation of the t-system, which was coupled with a relative decrease in the number of junctions per sarcomere (Van Oort et al., 2011, Wu et al., 2012). While loss of junctions is not surprising in JPH2 knockdown, the remaining junctions in these animals were altered compared to controls, whereby there was greater variability of the junctional space distance as well as increased variability of the width of the junctions (Van Oort et al., 2011).

Cardiomyocytes from neonatal rodents do not contain t-tubules (Seki et al., 2003, Haddock et al., 1999), with recent work showing that, in mice, the developmental age at which t-tubules begin to form is ~P10 (and equivalent age of ~P15 in rats), and that knockdown of JPH2
significantly impairs t-tubule formation and leads to heart failure development (Chen et al., 2013, Reynolds et al., 2013). In contrast, when JPH2 is over-expressed, the maturation of the t-tubules is enhanced, occurring earlier in post-natal mice (Reynolds et al., 2013). These, along with previous findings indicate that JPH2 plays a pivotal role in the formation and organisation of, not only the essential EC coupling domains (peripheral couplings), but also the t-tubules along which junctions form with further postnatal development (Chen et al., 2013, Han et al., 2013, Reynolds et al., 2013).

Functional Changes

As a potential consequence of these structural changes, calcium signalling is also found to be abnormal in JPH2 knockdown models. JPH2 knockdown has been found to produce altered calcium handling in cell model lines, including reduced calcium transient amplitudes during CICR, coupled with prolonged transient decay times (Landstrom et al., 2011). Additionally, variability in the amplitudes of calcium transients between JPH2 knockout embryos is observed, with some exhibiting almost complete absence of transients (Takeshima et al., 2000). Transients that did occur in this model were asynchronous with the heartbeats, with the presence of spontaneously occurring transients even in the absence of calcium in the extracellular solution (Takeshima et al., 2000). These findings indicated that the SR may be overloaded with calcium in JPH2 knockout, or potentially that release mechanisms of the RyR are altered, such as they exhibit increased 'leakiness'.

Further evidence for altered RyR calcium release mechanisms is provided by the inducible cardiac JPH2 knockdown adult mouse model. It was found that the relative release of SR calcium via the RyR as a fraction of total SR content was increased in these model mice, despite having reduced SR calcium stores (Van Oort et al., 2011). This was also associated with an observed decrease in DHPR-triggered RyR calcium transients, although normal DHPR currents ($I_{Ca}$) were maintained, indicating a reduction in EC coupling gain (Van Oort et al., 2011, Wu et al., 2012). This idea is strengthened by the observation that there is less co-localisation of DHPR and RyR in JPH2 knockdown cardiomyocytes without changes in their expression levels. Spontaneous calcium release via the RyRs is also increased following JPH2 knockdown, with increased calcium waves and a higher frequency of calcium sparks observed, supporting the idea of potentially leaky RyR (Van Oort et al., 2011). It has also been shown that in a myocardial infarction model there is a reduction in co-localisation between JPH2 and RyR (due to JPH2 down-regulation), with subsequent dysynchrony of the calcium transient (Wagner et al., 2012). Interestingly, by introducing a small peptide fragment of JPH2 containing amino acid E169 into JPH2 knockdown mice, there was improvement in
the calcium handling properties providing further evidence that this region of the protein may play a key role in stabilising RyR calcium release properties (Beavers et al., 2013).

Despite significant changes in calcium signalling, the expression levels of key calcium handling proteins are not altered in JPH2 knockdown (Landstrom et al., 2011, Van Oort et al., 2011), providing further support of the hypothesis that JPH2 plays a vital role in maintaining the organisation of the junction, which is essential for the regulation of EC coupling gain.

1.4.3.4 Potential mechanisms

Several of these studies implicate JPH2 as playing a role in regulating the resting level of cytosolic calcium, as well as influencing the calcium transients (Takeshima et al., 2000, Landstrom et al., 2007, Woo et al., 2010). This is seen by the higher than normal level of resting calcium in JPH2-modified cardiomyocytes as well as reduced amplitude and increased decay time of the calcium transients (Landstrom et al., 2011, Van Oort et al., 2011). It has previously been suggested that an overloaded SR was contributing to the changes observed in intracellular calcium properties due to reduced SR calcium release (Takeshima et al., 2000), however more recent studies have shown that the SR is not overloaded following reduced JPH2 levels (Van Oort et al., 2011, Hirata et al., 2006). Based on the findings of previous studies, there are three main mechanisms proposed by which JPH2 may influence calcium handling in cardiomyocytes, all of which could potentially contribute to the development of heart failure. These include changes to: 1) junctional morphology and protein organisation, 2) t-tubule organisation and 3) RyR stabilisation.

Junction Organisation

The first of these mechanisms is an alteration of junctional space morphology in response to decreased levels of full-length wild-type JPH2. Abnormal organisation, such as an increase in the distance across the junctional space, could result from altered JPH2 interactions with the sarcolemmal membrane caused by mutations in the protein, or a reduced number of full length JPH2 molecules. The reported cleavage of JPH2 (Murphy et al., 2013) would reduce its ability to anchor the SR and t-tubule in close proximity, potentially allowing for an increase in the distance across the junction. Increasing the junctional space would consequently increase the distance between the DHPRs and RyRs which are usually closely juxtaposed across the dyadic cleft (Franzini-Armstrong et al., 1999), and is supported by the observed reduction in DHPR-RyR co-localisation following JPH2 knockdown (Van Oort et al., 2011). This uncoupling of key calcium handling proteins would lead to a reduced efficiency of DHPR-mediated calcium entry to activate the RyRs, and subsequently a reduction of RyR-mediated
calcium release from the SR. The consequence of this would be the observed reduction in the amplitude of the calcium transient, despite normal DHPR currents being maintained (Van Oort et al., 2011, Wu et al., 2012). Therefore, modification of the junctional space mediated by changes in JPH2 could lead to reduced EC coupling gain, as observed in heart failure.

T-tubule Organisation

Alteration of t-tubule organisation is another potential mechanism by which JPH2 could influence calcium handling in cardiomyocytes. Evidence for this comes from the observed disorganisation of the t-tubule system following JPH2 knockdown (Van Oort et al., 2011), which could result in a reduced synchronicity of calcium fluxes throughout the cardiomyocyte and subsequently produce abnormal calcium transients (Brette and Orchard, 2003). Furthermore, as previously mentioned (section 1.4.2), a potential secondary consequence of t-tubule disorganisation is the reduced proximity of the t-tubule and SRJ membranes, leading to increased junctional cleft distances and reduced association of DHPR and RyR. This impairs the efficiency of CICR, further contributing to abnormal calcium handling due to t-tubule disorganisation. The mechanism by which JPH2 is involved in t-tubule organisation remains unclear, however, the cleavage of JPH2, as previously described, has also been found to be associated with the development of t-tubule disorganisation (Wu et al., 2014). This suggests that JPH2 may play a role in anchoring the t-tubule membrane in close proximity to the SRJ, and maintaining the z-disk alignment of these structures. While the exact processes underlying t-tubule growth and maturation are not fully established (see Chapter 6 for further details), several studies have identified JPH2 as a key protein in regulating this development (Chen et al., 2013, Reynolds et al., 2013, Han et al., 2013), and it is possible that identified mutations or reduced expression of JPH2 could influence t-tubule organisation from early in development. Interestingly, in a heart failure model study, it was identified that decreased BIN1 but not JPH2 was associated with t-tubule loss, with JPH2 maintaining sarcomeric distribution (Caldwell et al., 2014). This suggests that BIN1 is important for maintenance of t-tubule abundance while JPH2 is more involved in overall organisation.

RyR Stability

A third mechanism by which the observed calcium-handling abnormalities could arise is through the development of leaky RyRs and it has been suggested that this occurs due to the loss of functional JPH2, which associates with, and potentially stabilises RyRs (Van Oort et al., 2011, Beavers et al., 2013). This includes an identified JPH2 mutation being linked to atrial fibrillation, which is thought to be caused by spontaneous RyR release, triggering
irregular contractions (Beavers et al., 2013). Consistent with this role of JPH2, impaired calcium transients are observed in a model of myocardial infarction, associated with a reduction of co-localisation between JPH2 and RyR (Wagner et al., 2012). The increased leak of calcium via destabilised RyRs would give rise to the observed increased in spark frequency (Van Oort et al., 2011, Beavers et al., 2013) and may contribute to the high resting cytosolic calcium levels seen with reduced wild-type JPH2 levels (Landstrom et al., 2011). RyR leakiness could also potentially cause a decrease in SR store load, which would be a mechanism behind the reduced amplitudes of calcium transients, with the prolonged release of calcium via leaky RyR producing the increased decay times observed for these transients (Van Oort et al., 2011, Landstrom et al., 2011).

It is likely that all three potential mechanisms contribute to the influence of JPH2 in alterations to cardiomyocyte function. Overall, research to date has shown the importance of JPH2 in maintaining the organisation of the junctional cleft, in cardiomyocytes as well as the t-tubules. In addition, a reduction in wild-type JPH2 protein levels has detrimental effects on the calcium signalling within cells. The combination of reduced EC coupling gain and increased leakiness of RyRs suggest that abnormal JPH2 protein expression could play a role in the development or progression of heart disease and failure.

1.5 Project Aims

The aims of this thesis were to investigate aspects of the cardiac structure-function relationship, and the role of JPH2 in this relationship. It is clear that JPH2 plays an important role in many facets of cardiac physiology, including development and modulation of EC coupling, as well as disease progression. While many aspects of the role of JPH2 in cardiomyocytes have been increasingly supported in recent literature, the majority of these studies have focus on functional techniques, often in culture models, with limited evidence on the distribution and organisation of JPH2 and EC coupling proteins, particularly in mammalian cardiac samples. This includes lack of information regarding the sub-cellular organisation of these proteins, particularly at the nanoscale level. Therefore, this study has been designed to utilise super resolution imaging techniques for determining the nanoscale arrangement of key proteins implicated in EC coupling, including JPH2. This has been used in combination with confocal imaging to provide multi-scale information on the structural basis behind many of the proposed functional roles of JPH2, and the implication in overall cardiac function. This has
been done across a range of mammalian species, using a combination of experimental models.

Specific aims of this project were to examine three main aspects of the relationship between cardiomyocyte organisation and function:

1) To determine the role of JPH2 in the maintenance of junctional protein and t-tubule organisation in response altered JPH2 expression, at the confocal and super resolution level using, JPH2 transgenic mice. These changes have been correlated with altered calcium handling properties observed in these mice.

2) To examine changes in the organisation of structural and junctional proteins (including JPH2) in human heart failure and to correlate this with functional data acquired, by examining of cardiac trabeculae from explanted human hearts in end-stage heart failure.

3) To investigate the relationship between JPH2 distribution and EC coupling maturation, including the t-tubules, in the developing mammalian heart. Maturation of structural components have been associated with known developmental changes in EC coupling properties, using the fetal sheep as a model of cardiac development in large mammals.

The methods used throughout this study are described in Chapter 2. Following this, Chapter 3 focuses on changes in the organisation of the RyR clusters (CRUs) due to altered JPH2 expression in transgenic mice. Due to the nanoscale nature of the junctional space and CRUs, this has been examined using established super resolution microscopy techniques (Baddeley et al., 2009b, Jayasinghe et al., 2012a). Novel findings regarding the size and density of RyR clusters were revealed as a result of altered JPH2 protein levels. These changes identified in the nanoscale organisation of the RyR were related to observed functional changes in these transgenic mice, including altered calcium spark properties (X.H.T. Wehrens, unpublished), revealing the role of JPH2 in maintaining the organisation of EC coupling proteins localised to the junctional space.

As a logical progression from the RyR organisation, Chapter 4 uses the same transgenic mice to examine the influence of JPH2 expression on additional structures and proteins implicated in EC coupling. This includes the t-tubules, which have been studied at both the confocal and super resolution level to determine both the cell-wide changes as well as nanoscale changes to co-localisation with JPH2. Additionally, the association between the RyR and myofibrils was examined using super resolution imaging, as this relationship is important in the efficiency of RyR-mediated calcium release to trigger mechanical contraction.
of the cardiomyocyte. Furthermore, super resolution microscopy identified nanoscale changes in the junctional localisation of NCX in the JPH2 transgenic mice. Together, findings from these experiments revealed that altered JPH2 expression further influences the organisation of EC coupling proteins and structures beyond RyR.

In Chapter 5, human cardiac trabeculae were used to directly determine how changes in cardiomyocyte organisation, at both the tissue and cellular level, correlate with changes in force development in the failing heart. Changes in both myocyte content and protein organisation at the confocal level were identified as correlating with variation in active stress production. The force-frequency response was also examined, along with determination of the biological variability observed in trabeculae versus the ventricular wall structure to validate the suitability of cardiac trabeculae as a model for ventricular function.

The maturation of t-tubule and EC coupling protein organisation has been studied in Chapter 6, using fetal sheep hearts as a model of large mammalian cardiac development. While it has been well established that t-tubules develop and mature postnatally in rodents (Chen et al., 2013, Sedarat et al., 2000, Haddock et al., 1999), early EM work indicated this might not be the case for larger mammals. Findings presented in this chapter confirm this, with t-tubule structure identified in the fetal sheep heart. The relationship between this t-tubule development and JPH2 distribution was examined. Furthermore, the development of EC coupling protein organisation (RyR, NCX) was assessed in relation to the temporal changes in EC coupling dynamics previously described.
Chapter 2. General methods

Experiments performed for the data series in this thesis involved the use of a combination of adult mammalian cardiac tissue and isolated myocytes, together with fetal mammalian samples. This included JPH2 transgenic adult mouse cardiomyocytes and myocardium (Chapters 3 & 4), adult human myocardium (Chapter 5) and fetal sheep myocardium samples (Chapter 6). In addition, adult rat myocardium and cardiomyocytes were used for the preliminary establishment of techniques. Immunofluorescent histochemical and cytochemical techniques on fixed samples were predominantly used to visualise proteins involved in calcium handling in ventricular myocytes, as well as to determine the organisation and alteration of these proteins, together with supporting live trabeculae experiments (human samples). A combination of confocal laser scanning microscopy (LSM; Chapters 4-6) and super resolution imaging techniques (Chapters 3 & 4) were used for visualising the fluorescent labelling of these proteins in the specimens, with sample preparation and image analysis adjusted accordingly to suit the imaging modality used, with dSTORM super resolution imaging providing ~10-fold improvement in resolution such that the nanoscale organisation of proteins could be determined.

A combination of isolated cardiomyocytes and sections of myocardium were used for experimental procedures. Transgenic mouse isolated cardiomyocytes were used to investigate the organisation of key structures and EC coupling proteins in response to altered JPH2 expression. The mounting of myocytes between the slide and coverslip typically lets the myocytes fall flat on their side (long axis of cell perpendicular to imaging optical path), which allows us to examine structure organisation in the longitudinal orientation, including displacement of proteins from the z-disk. However, information regarding the transverse arrangement of structures within the cardiomyocyte was also of interest, and so sections of myocardium were used in which the cells were organised in transverse arrangement (long axis parallel to the optical path). In both the human and fetal sheep studies, the organisation of cardiomyocytes at both the tissue and cellular level was investigated. This required the sole use of myocardium sections, with cells aligned both longitudinally and transversely used in imaging experiments.
2. General methods

2.1 Sample Collection and Preparation

While the experimental results presented in this thesis were predominantly from mouse, sheep or human samples, rat cardiac ventricular tissue and isolated cardiomyocytes were primarily used for testing of antibody labelling and analysis techniques prior to use on other species samples. This was to enable establishment and optimisation of methods prior to obtaining the experimental samples which were limited in supply. General sample preparations based on those established in rat specimens are detailed below, with other species specific protocols for sample preparation and imaging outlined in the appropriate chapters.

2.1.1 Rat ventricular myocytes and myocardium

Ethical approval was obtained from the University of Auckland Animal Ethics Committee for all experimental procedures performed. 200-300 g male Wistar rats were used for these experiments, in which an intraperitoneal injection of lethal dose pentobarbital (100 mg/kg) was administered. After successful anaesthetisation (assessed by loss of toe pinch and corneal reflexes), cervical dislocation was performed immediately before quick excision and briefly rinsing of the heart in either phosphate buffered saline (PBS, pH 7.4) or calcium-free Tyrode’s solution (140 mM NaCl, 10 mM Hepes, 4 mM KCl, 1 mM MgCl₂, 10 mM glucose, pH 7.4). The heart was then cannulated via the aorta onto a retrograde Langendorff perfusion system for either enzymatic myocyte isolation or whole heart perfusion fixation. The perfusion systems used for these experiments were custom made and included the use of solution chambers at a specified height from the cannula (63 cm) to provide a constant gravitational force for perfusion of the hearts. The cannulae used for perfusion were custom made from a 16 gauge needle with a 14 gauge needle collar for securing the aorta to the cannula with 2/0 size braided silk suture thread (George Tiemann & Co., New York).

2.1.1.1 Ventricular myocyte enzymatic isolation

For isolation of ventricular myocytes, after cannulation the heart was perfused with oxygenated calcium-free Tyrode’s solution for 5 min to clear the vasculature before being perfused with oxygenated Tyrode’s solution with 0.2 mM CaCl₂, 1 mg/mL Type 2 Collagenase (Worthington Biochemicals, NJ) and 0.1 mg/mL Type I Protease (Sigma-Aldrich, NZ) for 10-20 min. The ventricles were then dissected into Tyrode’s solution containing 0.15 mM CaCl₂ and quickly diced into small pieces (~1 mm blocks). The tissue was then progressively triturated with a wide-bore Pasteur pipette to release single cells, with the resulting
supernatant being decanted to collect isolated myocytes. All solutions were at 37°C for the isolation of the myocytes. Fixation of the isolated myocytes involved adding equal volume of room temperature 4% paraformaldehyde in PBS, pH 7.4 (PFA) to the isolated myocyte solution. The myocyte suspension was then gently stirred (to prevent sedimentation) and fixed with a final concentration of 2% PFA at room temperature for 10 min. Following fixation, the myocytes were centrifuged at ~1100 rpm for 2 min and the supernatant was discarded. This was followed by re-suspension and a 10 min wash with PBS before further centrifugation with supernatant removal. The fixed myocytes were then re-suspended in storage buffer: 0.5% bovine serum albumin (BSA; Life Technologies, NZ) + 0.1% sodium azide in PBS and stored at 4°C until required for immunocytochemistry.

2.1.1.2 Myocardium perfusion fixation

For perfusion fixation, following cannulation, the whole heart was perfused with Tyrode’s solution with 0.1 mM CaCl₂ for 5 min to clear the vasculature before perfusion fixing with 2% PFA for 10 min. The ventricles were then selectively dissected and cryoprotected for freezing by sequential immersion in 10%, 20% and 30% sucrose solution in PBS with 0.1% sodium azide. Immersion in 10% and 20% sucrose was for 1 hour each (or until the tissue blocks had sunk), with 30% sucrose solution immersion overnight, all at 4°C. The tissue was then frozen in methyl butane cooled with liquid nitrogen, and stored at -80°C until use. For immunohistochemistry, the frozen tissue was sectioned using a Leica 3050 cryostat such that it contained transverse or longitudinal sections through the ventricle walls. Either 8 µm (super resolution imaging) or 16 µm (confocal LSM) sections were collected onto size 1.5 coverslips. All coverslips were pre-cleaned by soaking in methanol saturated with NaOH for 10 min and then double rinsed in ddH₂O. This was particularly important for reducing background signal in super resolution imaging. Following air drying, the coverslips were coated with 0.05% (w/v) L-poly-lysine (Sigma-Aldrich) for 5 min, then rinsed with ddH₂O for 5 min and allowed to air dry before use. The collected cryosections were then immediately hydrated with PBS for immunohistochemical labelling.

2.1.2 Immunohistochemistry and immunocytochemistry

For immunolabelling procedures, tissue sections and isolated myocytes were selected based on visual cell structure, particularly the presence of clear, well-spaced (~1.8-2 µm) sarcomere striations, as well as isolated myocytes being rod-shaped with sharp outlines. The protocols used for immunohistochemical and immunocytochemical labelling were adapted from those
2. General methods

previously described for cardiac myocytes and tissue (Jayasinghe et al., 2012a, Jayasinghe et al., 2012b), with appropriate modification dependent on imaging technique to be used.

2.1.2.1 Immunohistochemistry

For immunolabelling, 8 µm or 16 µm tissue cryosections were selected for super resolution or confocal imaging respectively, and rehydrated with PBS immediately prior to immunolabelling. The sections were permeabilised with 1% triton-X100 for 10 min at room temperature followed by blocking with Image-iT signal enhancer (Life Technologies), for 1 hour at room temperature. Prior to applying blocking or incubation solutions, excess existing solution was blotted from the sections using filter paper, while being careful not to dehydrate the samples. The sections were then briefly washed with PBS before incubating with primary antibody diluted in antibody incubation solution (see ‘Antibodies and stains’) overnight at 4°C. The final antibody dilutions which were used are summarised in Table 2.1. The sections were washed three times with PBS for 15 min per wash before being incubated with the secondary antibody diluted 1:200 in incubation solution for 2 hours at room temperature. The sections were then washed three times for 15 min with PBS prior to mounting for imaging. The mounting medium that was selected depended on the imaging modality to be used (see below for details). The coverslips with the sections on them were mounted onto pre-cleaned slides and allowed to settle, such that there was even distribution of the mountant with displacement of any air bubbles, before sealing with nail varnish.

2.1.2.2 Immunocytochemistry

Isolated rat cardiomyocytes were centrifuged to form a loose pellet so that the storage solution supernatant could be removed. Changing between all solutions required centrifugation at 3000 rpm for 2 min with supernatant removal. The myocytes were permeabilised in 1% Triton-X100 for 10 min at room temperature before blocking treatment with 10% normal goat serum (NGS; Life Technologies) + 0.05% NaN₃ in PBS for 1 hour at room temperature. They were then re-suspended with the primary antibody diluted in incubation solution and incubated overnight at 4°C. The myocytes were then washed three times with PBS for 30 min each before application of the secondary antibody diluted in incubation solution for 2 hours at room temperature. The isolated myocytes then underwent another wash cycle of three 30 min washes with PBS. Following the final wash, the cells were allowed to sediment under gravity before the supernatant was removed. The myocytes were then suspended in mounting medium (see below for details), with a small aliquot (7 µL)
of the cell suspension mounted onto pre-cleaned size 1.5 coverslips and slides. This was allowed to settle before sealing the coverslip with nail varnish. The isolated mouse cardiomyocytes (used for Chapters 3 & 4) were processed using these same techniques, with the difference of allowing gravity settling of the myocytes between solution changes instead of centrifugation (with the exception of following permeabilisation). This was due to formation of excessively robust pellets that the mouse myocytes compacted into during centrifuging, making re-suspension less effective for keeping cells separated.

2.1.2.3 Confocal microscopy

Samples immunolabelled for LSM confocal microscopy were mounted in ProLong Gold mounting medium containing anti-fade reagent (Life Technologies). The coverslips were sealed only in the corners to hold the coverslip in place while allowing the mounting medium to set prior to imaging to increase the refractive index of the medium. ProLong Gold sets to a refractive index (>1.44) which is well-matched for oil-immersion imaging, however with the disadvantage of taking approximately 48 hours to achieve the desired refractive index. ProLong contains an anti-fade reagent which reduces deterioration over the time allowing for an increased potential imaging lifetime of the sample (Fisher, 2014). This feature justified the long curing time between the labelling and imaging steps for these samples.

2.1.2.4 Super resolution imaging

The sections and myocytes labelled for super resolution imaging were mounting using a fluorophore ‘switching’ buffer to promote the generation of single molecule events. The switching buffer was glycerol based with 10% glucose (w/v), 5 mM cysteamine (Sigma-Aldrich), 250 µg/mL glucose oxidase (Sigma-Aldrich), 25 µg/mL catalase (Sigma-Aldrich) and 10% PBS, pH ~7.4. This combination of components produces an oxygen scavenging system which helps to reduce the oxygen content of the mounting medium as well as containing a thiol-reducing agent (cysteamine). This promotes the fluorophores to produce single molecule events by facilitating their entry into a stable and long-lasting ‘dark state’ when illuminated with the far-red laser, while also protecting them from irreversible photobleaching, which prolongs the potential imaging life of the sample (Van De Linde et al., 2009). In addition, the high glycerol content provides an improved refractive index compared to a PBS-based mounting medium, while also reducing the diffusion of oxygen through the mounting medium. The coverslips were completely sealed with nail varnish to prevent
additional atmospheric oxygen from penetrating the mounting medium, while also holding the coverslip secure.

### 2.1.3 Antibodies and stains

Combinations of primary antibodies were selected to enable the visualisation of the distribution of several structural proteins as well as those involved in EC coupling. The primary antibodies used for the immunolabelling are summarised in Table 2.1 including the source of the antibody and the final dilution used. The antibodies that were used are all of commercial origin which have been well characterised, and have been previously used successfully in cardiac immunolabelling experiments. To ensure minimal cross-reactivity during immunolabelling, double labelling experiments involved combining primary antibodies raised in mice with those raised in rabbits, rather than single-species dual labelling. The antibodies were diluted in a PBS-based solution containing either 2% NGS, 2% BSA, 0.05% Triton-X100 + 0.05% NaN₃ (for immunocytochemistry), or 1% BSA, 0.05% Triton-X100 + 0.05% NaN₃ (for immunohistochemistry). Negative controls for the primary antibodies were prepared identically to experimental samples with the exception of omitting the primary antibody(ies) from the incubation solution.

<table>
<thead>
<tr>
<th>Target</th>
<th>Host; Clonality</th>
<th>Final Dilution</th>
<th>Company</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actinin</td>
<td>Mouse; monoclonal</td>
<td>1:200</td>
<td>Sigma</td>
<td>A7811</td>
</tr>
<tr>
<td>Caveolin-3</td>
<td>Mouse; monoclonal</td>
<td>1:200</td>
<td>BD Biosciences</td>
<td>610420</td>
</tr>
<tr>
<td>Caveolin-3</td>
<td>Rabbit; polyclonal</td>
<td>1:500</td>
<td>Abcam</td>
<td>ab2912</td>
</tr>
<tr>
<td>JPH2 (c-term)</td>
<td>Rabbit; polyclonal</td>
<td>1:50</td>
<td>Invitrogen</td>
<td>40-5300</td>
</tr>
<tr>
<td>JPH2 (c-term)</td>
<td>Rabbit; polyclonal</td>
<td>1:100</td>
<td>Yenzym (custom)</td>
<td>YZ2635(1)</td>
</tr>
<tr>
<td>NCX1</td>
<td>Mouse; polyclonal</td>
<td>1:200</td>
<td>Swant</td>
<td>R3F1</td>
</tr>
<tr>
<td>RyR2</td>
<td>Mouse; monoclonal</td>
<td>1:100</td>
<td>Thermo</td>
<td>ma3-916</td>
</tr>
<tr>
<td>RyR2</td>
<td>Rabbit; polyclonal</td>
<td>1:50</td>
<td>Sigma</td>
<td>HPA016697</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Mouse; monoclonal</td>
<td>1:200</td>
<td>Abcam</td>
<td>Ab184613</td>
</tr>
</tbody>
</table>

Table 2.1 Table of the primary antibodies used for immuno-histochemistry and cytochemistry with final dilutions used and source of the antibodies.

T-tubule labelling was achieved by using a combination of primary antibodies from a single species which targeted proteins localised to the t-tubules, specifically caveolin-3 (Scriven et al., 2005) (BD Biosciences) and NCX1 (Frank et al., 1992, Kieval et al., 1992) (Swant)
following previously established methods (Hou et al., 2015). For the JPH2 immunolabelling experiments, the Invitrogen commercial antibody was used in mouse samples, whereas the Yenzym custom antibody was used in human, rat and sheep tissue labelling due to observed differences in optimal labelling achieved. The secondary antibodies used for LSM confocal microscopy immunolabelling were highly cross-adsorbed goat-anti-mouse or goat-anti-rabbit IgG (H+L) antibodies conjugated to Alexa Fluor 488, 568, 594 or 647 (488 + 594 for dual labelled; 488 + 568 + 647 for triple labelled samples). The antibodies that were used for super resolution immunolabelling were also goat-anti-mouse or goat-anti-rabbit IgG (H+L) antibodies; however these were conjugated to Alexa Fluor 680 or 750, with the Alexa 680 being highly cross-adsorbed. All secondary antibodies were from Life Technologies.

Along with the immunolabelling of cardiomyocytes and tissue, additional stains were used to label organelle structures to complement the antibody-targeted protein labelling. These stains were wheat-germ agglutinin (WGA) and phalloidin (both from Life Technologies) which were conjugated to either Alexa Fluor 488, 594 or 647. WGA is a lectin which binds to the sialic acid and N-acetylglucosamine residues, which are sugar-based modifications found on the cell surface (Bhavanandan et al., 1977), making it a convenient stain for visualising the plasma membrane, including the t-tubule system in some species (Jayasinghe et al., 2015). It is also useful for identifying changes in connective tissue content as it binds to glycosylated residues in the connective tissue (Kostrominova, 2011). Phalloidin is a high-affinity toxin derived from the Amanita phalloides mushroom which selectively binds to f-actin (Lengsfeld et al., 1974), making it an excellent marker of the thin filaments in cardiac myocytes, thereby allowing visualisation of the contractile apparatus. Samples were incubated with these stains concomitantly to the secondary antibody incubation at a dilution of 1:200 for WGA or 1:50 for phalloidin.

Detailed protocols for the different biological specimens used are outlined in the relevant chapters. Fixed transgenic mouse samples were used for experiments in Chapters 3 and 4, which were kindly supplied by Prof Xander Wehrens’ laboratory, Baylor College of Medicine, Houston, TX for dSTORM and confocal imaging analysis. Experiments performed in Chapter 5 used human tissue which was acquired in accordance with approval from the New Zealand Health and Disabilities Ethics committee (NTY/05/08/050) and informed consent of the donor and/or their family. Fetal sheep hearts used for experiments in Chapter 6 were kindly provided by the Fetal Physiology and Neuroscience Laboratory, University of Auckland. Human and fetal sheep tissue samples were processed according to confocal immuno-histochemical procedures described above.
2. General methods

2.2 Image Acquisition and Analysis

Myocytes were selected for imaging based on the presence of clear sarcomeric striations in transmitted light as well as an adequate contrast of immunolabelling with epifluorescence. Isolated myocytes were used to obtain information on the longitudinal distributions of the labelled proteins both on the surface and within the cells, while the myocardium tissue sections provided information about intracellular labelling, particularly in the transverse orientation. In instances where isolated cardiomyocytes were not available, myocardium samples were used to visualise both transverse and longitudinal protein organisation. Specialised imaging analysis procedures are outlined in each relevant results chapter.

2.2.1 Confocal microscopy

2.2.1.1 Image acquisition

The isolated cardiomyocytes and cardiac tissue sections processed for confocal microscopy were imaged using either a Zeiss Axiovert LSM 410 confocal microscope with a 63x NA 1.25 oil-immersion objective (Chapter 4 confocal images) or a Zeiss LSM 710 confocal microscope with a 63 x NA 1.4 oil-immersion objective (Chapter 5 & 6 results). The fluorophores were excited using a combination of lasers, with Alexa Fluor 488 and 594 being excited using Ar+ 488 nm and HeNe 543 nm lasers respectively, on the LSM 410 system. The Zeiss LSM 710 system also used an Ar+ 488 nm laser to excite Alexa Fluor 488 in conjunction with HeNe 561 nm and HeNe 633 nm lasers to excite the 568/594 and 647 fluorophores respectively.

To ensure adequate Nyquist sampling was achieved, images were acquired such that the pixel scale was finer than 90 nm per pixel based on the typical ~200-300 nm lateral resolution which can be obtained by the confocal microscope (Huang et al., 2008a). Due to the reduced maximal resolution of the confocal microscope in the z-dimension (~500 nm), image stacks were acquired with a focal stepping distance of 200 nm. In addition to the imaging of the labelled samples, confocal stacks were also acquired of 200 nm yellow-green, orange-red or dark red fluorescent microspheres (Life Technologies) mounted in the same mounting medium as the sample in order to obtain information on the point-spread function (PSF) of the confocal system. These PSF image stacks were acquired using the same lateral pixel scale as the sample imaging, but with a focal stepping distance of 100 nm.
2.2.1.2 Image processing

Deconvolution was applied to the images using the PSF data acquired from the appropriate colour-matched beads, with Alexa Fluor 488 using yellow-green beads, Alexa Fluor 568 or 594 using red-orange beads and 647 using dark red beads. The PSF was estimated by averaging images of 10-12 of these beads using PYME software written by Dr David Baddeley which was then applied to deconvolve the image stacks. Maximal-likelihood deconvolution was performed on the data using the same software by applying a Richardson-Lucy algorithm at 20-50 iterations, with data padding (removed upon completion) to avoid edge artefacts, with this methods previously established for use on confocal image stacks (Soeller and Cannell, 1999). This process greatly improved the signal-to-noise ratio of the images. Following the application of deconvolution, customised image analysis was performed, with details provided in the appropriate chapters. In some instances, intracellular regions of the images were selected to exclude labelling at the surface sarcolemma, which provided measurements related to intracellular protein distributions only. Co-localisation of dual labelled images was also performed, which is detailed below in section 2.2.3.

2.2.2 Super resolution imaging

Single molecule localisation microscopy (super resolution) was implemented to visualise the nanoscale organisation of junctional proteins in the transgenic mouse samples. The system that was used for super resolution imaging was custom built by Dr David Baddeley and Prof Christian Soeller, and is based on the direct stochastic optical reconstruction microscopy (dSTORM) approach. All software for acquisition and processing of dSTORM data series was developed by Dr David Baddeley using the Python platform. Previous optimisation of sample preparation protocol was performed by Dr Isuru Jayasinghe. The implementation of dSTORM imaging techniques provides for a ~10-fold improvement of resolution compared to that achieved in confocal imaging, enabling the visualisation of nanoscale organisation of proteins of interest that are known to localise to the junction. Lateral resolution of <30 nm could routinely be achieved (Baddeley et al., 2009a).

The underlying principles behind single molecule localisation microscopy techniques such as dSTORM have been well characterised and previously described (see (Hell, 2007) for review). Standard illumination of fluorescent samples results in simultaneous excitation of all fluorochromes present, leading to the creation of the resolution or diffraction limit. In dSTORM imaging, the sample is initially illuminated such that it generates a diffraction-limited epifluorescent image (Figure 2.1A); however the combination mounting medium and the
intensity of the laser results in the rapid decay of emission intensity. This is due to both permanent and ‘reversible’ photobleaching of the fluorophores, with the latter being a reversible transition from the ground state to an excited state, followed by a meta-stable dark state (as described in detail (Van De Linde et al., 2009)). The result is that the majority of fluorophores in the sample are temporarily ‘switched off’ such that the field of view is predominantly dark, enabling the detection of the stochastic reversal of individual fluorophores back into the excitation (or bright) state. These single molecule stochastic transitions are typically sparse in comparison to the overall density of dye present in the sample, such that they appear as spontaneous diffraction-limited ‘blinks’, called localisation events (Figure 2.1B). The efficiency of pushing fluorophores into the reversible dark state and the rate of their spontaneous blinking are dependent on the mounting medium properties and the illumination intensity (Baddeley et al., 2009a).

Figure 2.1 Detection of single molecule localisation events
Sample labelled with mouse anti-RyR antibody and Alexa Fluor 680 secondary in which A) initial illumination produces simultaneous fluorescence of all fluorophores in the sample in an epifluorescent manner. Continual exposure to high intensity illumination results in B) a decrease of overall sample intensity due to ‘reversible photobleaching’ with the occurrence of spontaneous single molecule localisation ‘events’ (shown in red boxes). Note that intensities of each frame have been individually scaled to allow visualisation of localisation events.

2.2.2.1 Image acquisition
The custom setup used to achieve super resolution microscopy for experiments presented in Chapters 3 & 4 of this thesis have been previously described (Baddeley et al., 2010, Baddeley et al., 2011). The isolated myocytes and cardiac tissue which were processed for
super resolution imaging were imaged using a Nikon Te2000 inverted microscope with a
Nikon 60x NA 1.49 oil-immersion objective. The sample was illuminated using a solid state
671 nm laser (Viasho, China), at a power output of 300-330 mW and a high beam angle such
that an inclined beam of light was generated, providing an improvement of signal contrast
when imaging within optically thick sections compared to standard illumination methods
(Tokunaga et al., 2008). The focal plane was controlled by a piezo focussing unit (Physik
Instrumente), with the objective holder fixed onto the underside of the microscope stage. This
aided in the reduction of thermal and mechanical drift contributing to imaging artefacts. As
previously described, upon excitation by the laser, the fluorophores (Alexa Fluor 680 and 750)
in the switching buffer get pushed into the ‘dark state’ through reversible photobleaching and
can stochastically fluoresce, producing a brief 2D Gaussian shape in the image (single
molecule event) which is captured by the EM-CCD camera. Each frame was captured over a
50 ms integrated exposure with each image acquired over ~24,000 frames in which several
thousand single molecule localisation events were potentially detected. A schematic of the
optical pathway is provided in Figure 2.2A.

A dichroic mirror (Q680LP; Chroma Technology) was used to separate the collected emission
light from the excitation wavelengths, before it then passed through an emission filter
(XF3104-690ALP; Omega Optical). A custom-built splitter (as previously detailed; (Baddeley
et al., 2011)) was then used to separate the Alexa Fluor 680 and 750 emission spectra using
a FF741-Di01 dichroic mirror (Semrock), which allows for wavelengths longer than 741 nm to
be transmitted. The resulting split beams were projected onto two halves on the EM-CCD
chip (each half being 512 x 256 pixels) by the use of adjustable mirrors which could be
aligned to correct for shifts between the two images. The resulting images on each half of the
EM-CCD therefore approximately mirrored each other. The apparent misalignment of the two
images was determined by detecting fluorescent microsphere images on each half of the EM-
CCD, and calculating the vectors which represented the shift between the same structures on
the corresponding images to sub-pixel accuracy, as previously described (Baddeley et al.,
2011). A map of these vectors across the imaging field was determined (see Figure 2.2B)
and applied post-hoc to the acquired images to correct for the residual shifts between the two
EM-CCD halves. Single molecule localisation events were then detected independently in
each of these two halves.
2. General methods

Figure 2.2 dSTORM acquisition properties
A) Schematic of the optical light path for excitation and emission of the microscope for dual labelled samples and B) and example of a map of the calculated shift vectors for alignment of the two EM-CCD halves [panels A and B reproduced from Baddeley et al 2011; copyright]. C) Resulting data point clouds from pixel intensities in the two imaged channels to assign events to the correct fluorophore by determining the linear fit (red and blue lines for channel 1 and 2 respectively).
2.2.2.2 Separation of spectral channels

Although the emission of Alexa Fluor 680 and 750 were ‘split’ by the final dichroic mirror, there is an overlap of the emission spectra of these two dyes resulting in the ‘events’ being observed in each of the two image halves. However, these fluorophores have distinctive spectral symmetry characteristics from the splitter, such that the corresponding pixel intensities on each EM-CCD half can be used to determine which fluorophore they belong to. By plotting the intensities from each image half, two distinct clouds of points are formed from the two dyes, as shown in Figure 2.2C. As seen, there is a high degree of separation between these two clouds, such that linear fits can be applied to determine the predicted theoretical intensities of the two channels. This can then be used to select the regions of the scatterplot clouds which give a high probability of the events within that region being correctly assigned to the appropriate fluorophore. This results in a high degree of accuracy for designing localisation events to the correct channel without cross-talk.

2.2.2.3 Image rendering and analysis

Given that each super resolution image is captured over ~20-25 minutes, slight movement of the sample over time can occasionally occur due to thermal changes. This leads to ‘drift’ in the resulting image which needs to be corrected. This correction is achieved by selecting a region that contains visible temporal drift, which is identified by pseudo-colouring the events in the image based on temporal acquisition (Figure 2.3A). The Python-based software then uses an algorithm to automatically detect the best 5-piece linear fit for the drift correction, which can then be applied to the image (Figure 2.3B-D).

Visualisation of the localisation events involved reconstructing a fluorophore density map based on the position of the detected events in each channel (as described above; shown in Figure 2.2C). This was generated by applying Delaunay triangularisation to the fluorophore positions with the resulting triangle intensities assigned in proportion to the area they covered (Baddeley et al., 2010). Jittering was randomly applied to the data points at a distance which was half of that to the nearest neighbouring point, and repeated independently for ten reconstructions. These were averaged and used to produce a smooth triangularisation visualisation to reduce the sharpened appearance of the visualisation due to limited accuracy of localisation. The Delaunay rendering algorithm was used rather than the commonly applied Gaussian rendering approach as it results in improved resolution preservation (Baddeley et al., 2010). The visualisation points were mapped onto 5 x 5 nm pixel grid to produce a grayscale resultant image, with the intensity of each pixel being proportional to the
point density of local fluorophores such that images were able to be analysed using a similar approach to ‘standard’ grayscale fluorescent images.

![Figure 2.3 Drift correction](image)

**Figure 2.3 Drift correction**

Processes used to correct for spatial drift of the sample involved A) pseudo-colouring the detected localisation events according to relative time of acquisition (red earliest through to magenta as the last) with a small region of interest selected (white box). A 5-piece linear drift correction was then applied, with the corresponding ROI shown B) before and C) after drift correction. This resulted in removal of the drift effect across the entire image (shown in panel D).

### 2.2.3 Co-localisation analysis

Dual labelled samples were processed for co-localisation analysis according to previously described techniques (Jayasinghe et al., 2014, Hou et al., 2015). In most instances, the whole image was assessed, while in some cases the intracellular labelling only was of interest and so was selectively cropped for analysis. Co-localisation analysis was performed using Python-based software which was custom written by Dr David Baddeley, based on analysis outlined in Jayasinghe et al. (2009). The region of interest (Figure 2.4A) was then subjected to thresholding, with isodata or mode plus standard deviation applied to confocal images, while dSTORM images were thresholded according to the fraction of total labelling intensity such that a set signal fraction was captured with the threshold (0.7 or 0.8 signal fraction). A mask for each protein label was then generated from the result (Figure 2.4B). Euclidean distance maps were created such that the distance between the mask of ‘protein A’ was then determined from the nearest region of labelling for ‘protein B’ to generate a distance distribution plot (Figure 2.4C). Distances were processed into bins of 10 nm for dSTORM or
100 nm for confocal images. The fraction of protein labelling in each of the bins was calculated, with overlapping (co-localised) regions of the two masks represented as a negative distance. The sum of the fraction of labelling with distances up to and including zero was used as the total fraction of co-localisation of the two labels (dark bars Figure 2.4C,D).

![Figure 2.4 Co-localisation analysis process](image)

Figure 2.4 Co-localisation analysis process
Python based software was used for co-localisation analysis in which A) the original image (confocal or super resolution) dual labelled for ‘protein 1’ (red) and ‘protein 2’ (green) was applied with the appropriate threshold to B) generate protein labelling masks. From this, the Euclidean distance was determined to generate plots for the fraction of one protein label against the distance to the nearest labelling of the second protein label to produce distance distribution plots of C) protein 1 labelling from protein 2, and D) protein 2 labelling from protein 1. The dark bars in each distribution plot represent the distances below and equal to zero which are summed to give the final fraction of protein co-localisation.
Chapter 3. RyR cluster organisation in adult JPH2 transgenic mice

3.1 Background

The ability for cardiomyocytes to function by producing synchronous contractions relies on the efficiency of processes involved in EC coupling. This is influenced by many factors, including the close proximity of the DHPRs and RyRs, such that they functionally couple for sufficient CICR to occur. As outlined in Chapter 1, JPH2 has been suggested as playing a vital role in both the formation and maintenance of junctional organisation (Takeshima et al., 2000, Chen et al., 2013, Han et al., 2013, Reynolds et al., 2013), with many of the key proteins involved in EC coupling localising to the junction, including RyR – the SR calcium release channel (Franzini-Armstrong et al., 1999). Therefore, the organisation of the junctions (and subsequently the junctional proteins) is critically linked to the efficiency of EC coupling, potentially making JPH2 a key protein in the regulation of EC coupling gain and playing a vital role in maintaining cardiac function.

JPH2 has been implicated in forms of human cardiomyopathy as well as in several animal models of heart failure. This includes evidence that in human heart failure, JPH2 protein expression levels can be significantly decreased (Landstrom et al., 2011), or that the protein may be mutated (Landstrom et al., 2007, Matsushita et al., 2007, Beavers et al., 2013). The loss of JPH2 has been shown to result in the development of acute heart failure in adult mice, as well as leading to calcium handling abnormalities and structural disorganisation (Van Oort et al., 2011, Takeshima et al., 2000, Landstrom et al., 2011, Wu et al., 2012). JPH2 knockdown mice also show a reduction in the co-localisation between DHPR and RyR, indicating an increase in the distance across the junctional cleft and junctional protein re-organisation, with reduced EC coupling efficiency (Van Oort et al., 2011). In contrast, the over-expression of JPH2 has been found to leave EC coupling dynamics unchanged in mice (Guo et al., 2014).

While functional studies have been performed on these animal models which provide key insights as to calcium handling alterations, to date the underlying structural changes and EC
coupling protein organisation have only been touched on using confocal microscopy or EM. Given the nanoscale nature of the junction, many subtleties in changes to the junctional protein organisation may be lost using confocal techniques, while the interpretation of results of EM studies are heavily influenced by sample orientation (Franzini-Armstrong, 2010), and specific proteins are often difficult to identify, with the interactions between many proteins of interest difficult to determine. Therefore, the experiments presented here have been designed to investigate the influence of JPH2 expression on the nanoscale organisation of key EC coupling proteins using recent developments in super resolution imaging. This not only provides a high level of detail, but it can be combined with immunolabelling techniques, allowing for specific protein(s) to be identified. The aim of these experiments was to determine the relationship between structural changes associated with the alteration of JPH2 expression levels in adult mammalian cardiomyocytes, and how these may contribute to the functional changes that have been previously observed, as described in detail below.

3.1.1 Calcium release properties of JPH2 transgenic mice

While JPH2 knockout has been shown to be embryonically lethal (Takeshima et al., 2000), the ability to induce cardiac-specific knockdown in the adult mouse has enabled the examination of changes in cardiac function as a result of reduced JPH2 protein levels. Research by Prof Wehrens’ laboratory revealed that following JPH2 knockdown, adult mice develop acute heart failure within 1-2 weeks of induction, with gross dilation of the heart chambers without hypertrophy (Van Oort et al., 2011). This was associated with observed alteration in calcium handling properties. Despite maintaining a normal DHPR-mediated calcium influx ($I_{Ca}$) and exhibiting an increased fractional release from the SR compared to controls, the amplitude of the calcium transient was reduced in these mice, indicating reduced EC coupling gain, which is at least in part, due to reduced SR content (Van Oort et al., 2011). Furthermore, there was an observed increase in both the frequency and size of calcium sparks, indicating increased ‘leakiness’ of the RyR. The findings of this study suggest that JPH2 plays a role in stabilising the RyR channels, and that loss of JPH2 results in abnormal calcium handling which is detrimental to the cardiomyocytes, and therefore the whole heart.

Following on from this work have been studies investigating the influence of over-expressing JPH2 in mice. While initial work by Guo et al (2014) indicated that there were no changes to calcium handling properties of these mice at either the spark or transient level, more recent work has revealed that there may be subtle changes in the release properties from the RyR, but overall EC coupling function is not affected (Wehrens’ laboratory, unpublished).
Figure 3.1 Calcium handling properties in control and JPH2-OE mice

Functional studies revealed A) calcium sparks in JPH2-OE mice compared to controls showed B) decreased frequency and C) decreased size (FWHM). D) The calcium transients showed heterogeneity across the wavefront (arrows) with no overall change in E) amplitude of the transient, despite F) increased SR content. Note that temporal and spatial scale bars apply to both images. X.H.T. Wehrens' laboratory (unpublished data).

Differences in these findings may be a result of the specific transgenic mouse strain used. The work presented in this chapter utilises cardiac samples from the same transgenic mouse strain as in the more recent study, and so these functional changes will be considered in detail. Functional work carried out in Prof Wehrens’ lab revealed that the calcium sparks are
altered as a result of JPH2 over-expression, with a reduction in both the size and frequency of sparks observed compared to controls (unpublished; Figure 3.1A-C). It was also observed that despite the changes to spark properties, there was no difference in the overall amplitude of the calcium transient (Figure 3.1D,E), which may, in part, be attributed to the increased SR calcium load (Figure 3.1F). Interestingly, when discrete points along the calcium wave are examined, it is seen that there is heterogeneity in the profile of the waveform (Figure 3.1D), indicating that there may be subcellular variation in the organisation of the CRUs, or RyR clusters. These potential changes in RyR organisation in JPH2 transgenic mice are investigated in this chapter.

3.2 Experimental Techniques and Analysis

3.2.1 JPH2 transgenic mouse samples

The isolated mouse cardiomyocytes and myocardium used for the experiments outlined in this section were kindly provided by Prof Xander Wehrens (Baylor College of Medicine, Houston, TX). All procedures were approved and performed in accordance with Baylor College of Medicine Animal Care and Use Committee regulations. Fixed cardiomyocytes and whole hearts were supplied from established transgenic mouse strains in which JPH2 expression levels had been altered, and functional studies previously performed by Prof Wehrens’ laboratory. The generation of adult cardiac specific inducible JPH2 knockdown (JPH2-KD) and corresponding control mice were as previously described (Van Oort et al., 2011), while the JPH2 over-expressing mice (JPH2-OE) have been described elsewhere (Wehrens’ laboratory, unpublished), and are briefly covered below. Compared to controls, the JPH2-KD mice showed a ~60% reduction in JPH2 protein levels (Van Oort et al., 2011), while the JPH2-OE mice showed a 2.2-fold increase in JPH2 protein expression levels compared to control mice (Wehrens’ laboratory, unpublished).

Due to the embryonic lethality of JPH2 knockdown, an inducible system had to be used to determine the effect of reduced JPH2 protein in the heart. In addition, since JPH2 is also expressed in skeletal muscle, a cardiac-specific knockdown system was required. The mouse strains used to achieve this have been previously detailed (Van Oort et al., 2011). In brief, to generate the JPH2 knockdown strain (JPH2-KD), mice were generated which contained a U6 promoter inactivated by insertion of a loxPneo cassette mid-sequence, all upstream of a JPH2-targeting short-hairpin RNA (shJPH2) sequence. An additional strain was
generated in which a MerCreMer (MCM) sequence was inserted downstream of a cardiac specific promoter – the α-myosin heavy chain (αMHC). This MCM strain was then crossed with the shJPH2 mouse strain to produce double transgenic offspring (MCM-shJPH2). In order to induce knockdown of JPH2, the MCM-shJPH2 transgenic mice were administered tamoxifen, which is a ligand for the modified estrogen receptor (Mer), allowing the expression and activation of the Cre recombinase protein. This recombinase is then able to cause site-specific recombination of lox-containing sequences, which in this mouse model results in the excision of the loxPneo cassette from the U6 promoter sequence. This in turn enables the activation of the promoter, and transcription of the downstream shJPH2 sequence. Expression of the shJPH2 RNA results in the silencing of the endogenous JPH2 RNA, and therefore produces acute JPH2 protein expression knockdown (JPH2-KD). Tamoxifen injections (100 μL) were administered daily over five consecutive days, with MCM mice (controls) also administered tamoxifen at the same dosage as the JPH2-KD mice, with mice aged 3-5 months. This was to ensure that any differences found were not as a result of the insertion of the transgenic sequences or tamoxifen treatment, but due to the knockdown of JPH2. Isolated cardiomyocytes from the tamoxifen treated control mice were also supplied for analysis.

Along with the JPH2 knockdown and controls, JPH2 over-expressing (JPH2-OE) mouse samples were provided for analysis. This mouse strain was generated as previously described (Wehrens’ laboratory, unpublished), by insertion of an additional JPH2 sequence downstream of a αMHC promoter, making it cardiac-specific over-expression. This involved insertion of JPH2 mouse cDNA into a αMHC vector between HindIII and NotI restriction enzyme sites. Following linearization of the vector, it was injected into fertilised C57/BL6 oocyte pronuclei, which were then implanted into pseudopregnant mice. Confirmation of the level of JPH2 expression was performed on the different mouse strains using Western blot analysis (Van Oort et al., 2011) (Wehrens’, unpublished data). The preparation of samples followed protocols similar to those detailed in the General Methods for rat cardiomyocyte and myocardium samples. Upon receiving the whole hearts, cryoprotection and cryosectioning were performed according to methods detailed in section 2.1.1.2 (General Methods), with the additional step of embedding the tissue blocks in Tissue-Tek® O.C.T. Compound (ProSciTech) prior to freezing. The immunocytochemical, histochemical and imaging techniques described in General Methods were used to determine the influence of various JPH2 modifications on the organisation of junctional proteins. For results presented in this chapter, the primary antibodies used were rabbit anti-JPH2 (Invitrogen) and mouse anti-RyR2 (Thermo), which were used in combination, followed by goat-raised secondary antibodies,
which were conjugated to Alexa Fluor 680 or Alexa Fluor 750 for dual colour super resolution (dSTORM) imaging. In order to avoid photobleaching of the samples, only one image per cell was acquired for both the isolated cardiomyocytes (longitudinal view) and myocardium sections (transverse view). Super resolution images were processed and rendered as previously described in General Methods. Image processing and analyses were performed using custom-written programming in ImageJ, IDL (Exelis Inc.) and Python-based software, as detailed below.

### 3.2.2 RyR cluster detection and analysis

In order to quantify the distribution of RyR labelling in the super resolution images from JPH2-KD, control and JPH2-OE genotypes, RyR cluster analysis was performed with the steps shown in Figure 3.2. This involved performing thresholding on the rendered images using

![Figure 3.2 RyR macro-cluster analysis steps](image)

**Figure 3.2 RyR macro-cluster analysis steps**

Steps used for detection and analysis of RyR macro-clusters in super resolution images. A) Original rendered super resolution image, B) 0.8 signal fraction image, C) resulting binary mask with filled holes and D) particle detection analysis of RyR super-clusters output from ImageJ. Scale bar: 2.5 µm.
Python software, whereby the threshold value was set 0.8 signal fraction, such that 80% of the total labelling signal was captured (Figure 3.2B). This was then converted into a binary mask with holes filled (Figure 3.2C) and particle detection was used on the image masks in ImageJ. For total cluster analysis, ‘particles’ which were equal to or greater than 1 RyR channel in size (~1000 nm²; based on dimensions of individual channels, (Franzini-Armstrong and Protasi, 1997, Yin and Lai, 2000)) were detected and counted, using an approach derived from those previously described (Hou et al., 2015). The mean number and size of all detected RyR clusters were then determined for each genotype. For RyR ‘macro-cluster’ analysis, clusters ≥200,000 nm² (≥200 RyR channels) were detected and counted (Figure 3.2D). Due to variations in the size of imaged regions of interest, and that in many cases the cardiomyocyte imaged did not take up the entire frame, the area of each image accounting for cardiomyocyte labelling was determined. Based on the rendered image pixels scaled to 5 nm each, the number of total macro-clusters detected was then converted into the density per 100 μm² of cardiomyocyte. This was performed in longitudinal and transverse images.

3.2.2.1 Longitudinal dimension analysis

The longitudinal properties of RyR clusters were also analysed, using the already generated cluster masks from longitudinal images (Figure 3.3A). These masks were used to fit an ellipse to the cluster (Figure 3.3B), with the largest 25% of clusters from each cardiomyocyte used for the analysis due to increased accuracy of fitting an ellipse to larger clusters (Figure 3.3C), compared to those of relatively small sizes. The parameters from the resulting ellipse were used to calculate both the longitudinal diameter (2r) and the longitudinal extension (w) of the cluster, relative to the longitudinal axis of the cardiomyocyte (Figure 3.3D).

Using trigonometrical functions, the longitudinal diameter (2r) of the cluster was calculated as:

\[
r\theta = \frac{ab}{\sqrt{(b \cos\theta)^2 + (a \sin\theta)^2}}
\]

While the longitudinal extension (w = 2x) was calculated by:

\[
x = a \cos(t) \times \cos(\theta) - b \sin(t) \times \sin(\theta)
\]

Where:

a = semi-major axis of ellipse
b = semi-minor axis of ellipse
\(\theta\) = angle between long axis of ellipse and longitudinal axis of cardiomyocyte
t = angle from longitudinal axis of cardiomyocyte to widest point of ellipse, where:

\[
\tan(t) = -b \cos(\theta) / a
\]
3. RyR cluster organisation in adult JPH2 transgenic mice

Figure 3.3 RyR cluster longitudinal analysis
Analysis of longitudinal diameter and extension used A) the RyR cluster masks to B) fit ellipses, with C) an overlaid example shown. D) Parameters from the fitted ellipse, including major (a) and minor (b) axes, longitudinal radius (r), the ellipse's rotational angle from longitudinal (θ), widest point of ellipse (x) and angle to widest point (t) of the ellipse were used to calculate longitudinal measurements. Arrow indicates the direction of the longitudinal axis of the cardiomyocyte.

3.2.2.2 Labelling density analysis
The densities of protein labelling detected in the super resolution images were determined, with various aspects assessed using ImageJ. Initially, the total labelling density across the image was analysed by measuring the mean 32-bit grayscale pixel value for the entire image. This provided a measure of cell-wide protein labelling intensity. For JPH2 labelling, this analysis was performed on longitudinal images, while for the RyR, transverse images were used. In addition, the protein density within individual clusters was of interest. To analyse this, the previously acquired RyR cluster masks were used to determine the regions of interest. The density of either RyR or JPH labelling within these clusters was quantified based on the 32-bit grayscale intensity, with the raw integrated density value divided by the

54
3. RyR cluster organisation in adult JPH2 transgenic mice

The area of the cluster. For cluster density analysis, transverse images were used for both proteins.

Further to the labelling density of the rendered images, the density of localisation events detected within the cluster regions in the raw super resolution image data was determined, with event detection performed according to algorithms detailed in General Methods. This analysis was performed by Dr Isuru Jayasinghe. For both the labelling and event density analyses, to circumvent variation in labelling density being due to changes in image acquisition properties of the super resolution imaging (such as the activity of the mounting medium, which can influence the event detection), analysis was performed on images from the three genotypes which were acquired on the same selected imaging days.

3.2.2.3 Distance analysis

The distribution of the RyR clusters within the cardiomyocytes was determined by measuring the nearest neighbour edge-to-edge distances. This involved analysing the previously obtained RyR cluster masks, such that the Euclidean distance was determined between each cluster and the edge of its nearest neighbour. In addition, the analysis of RyR 'super-clusters' was performed using previously described methods (Hou et al., 2015). This was based on the previously reported ability of RyR clusters being within 100 nm apart to potentially co-activate each other at calcium concentrations >10 μM, as can be achieved in the junctional cleft (Sobie et al., 2006). Therefore, using the Euclidean distance analysis, the RyR clusters separated by ≤100 nm of each other were identified and the area of labelling of these 'super-clusters' was determined. Both the nearest neighbour and super-cluster analyses were performed by Dr Isuru Jayasinghe.

3.2.3 Co-localisation analysis

The co-localisation of RyR and JPH2 labelling was determined from the dual labelled dSTORM images, and was performed according to protocols described in General Methods. In brief, using Python software, binary masks of the labelling from each protein were created using a threshold value which captured 80% of the total signal intensity (0.8 signal fraction). From these masks, the fraction of labelling of the first protein (e.g. RyR) was determined as a function of the Euclidean distance to the nearest edge of the labelling of the second protein (e.g. JPH2), and used to generate a distance distribution histogram. From this histogram, the percentage of protein one that was co-localised with protein two could be determined, which is represented by the sum of nearest edge distances of below or equal to zero (as illustrated...
3. RyR cluster organisation in adult JPH2 transgenic mice

in General Methods Figure 2.4). The software for this analysis was written by Dr David Baddeley, using algorithms previously described (Baddeley et al., 2010, Jayasinghe et al., 2012a).

3.2.4 Statistical analysis

Unless stated otherwise, the results are reported as mean with the standard error of the mean (SEM) as uncertainty. Statistical tests were performed using IBM SPSS Statistics v22 software. Mann-Whitney U- or Kruskal-Wallis k-testing with post-hoc analysis was performed on the data, determined by the number of groups analysed.

3.3 Changes in Response to JPH2 Expression Levels

Before determining the influence of JPH2 expression on the organisation of cardiomyocytes, the changes in JPH2 distribution itself was examined. This also assessed the ability to detect varying levels of JPH2 labelling with the imaging technique utilised in order to validate the detection of protein expression levels corresponding to those previously reported in these transgenic mice by Western blots (Van Oort et al., 2011)(Wehrens' laboratory, unpublished). Super resolution imaging of JPH2 immunolabelled cardiomyocytes from each of the three genotypes revealed that different expression levels of the protein appeared to alter how it is organised throughout the cells (Figure 3.4A-C). While the control mice showed small JPH2 clusters arranged in regular transverse rows across the cardiomyocytes (Figure 3.4B), the clusters in JPH2-KD mice typically appeared smaller and more widely dispersed throughout the cell with reduced organisation, and a reduced presence of labelling (Figure 3.4A). The JPH2-OE mice showed a similar pattern of distribution to the controls with the additional appearance of a subset of enlarged JPH2 clusters (see Figure 3.4C, arrows) and an apparent increase in protein labelling present. To quantify the observations of the amount of labelling present, density of JPH2 labelling was assessed across the images, with distribution plots generated. Compared to controls, these showed a shift towards lower labelling densities in JPH2-KD mice; while the JPH2-OE distribution was shifted towards higher densities (Figure 3.4D). These observations were further confirmed with the mean JPH2 labelling density significantly reduced by ~38% in the JPH2-KD (n= 3 animals, 38 cells; p <0.001) and significantly increased by ~1.6-fold in the JPH2-OE (n= 3 animals, 40 cells; p <0.01) when compared to control mice (n= 3 animals, 29 cells), as shown in Figure 3.3E. These findings
3. RyR cluster organisation in adult JPH2 transgenic mice

confirm that the JPH2 labelling observed as a result of super resolution imaging aligns with protein expression values reported for these genotypes.

**Figure 3.4 JPH2 expression levels influence the pattern of localisation**
Super resolution images rendered to show JPH2 immunolabelling in A) JPH2-KD, B) control and C) JPH2-OE isolated adult mouse cardiomyocytes. Arrowheads indicate large JPH2 clusters; scale bars: 2 μm. D) Distribution plots of JPH2 density for JPH2-KD (red), control (blue) and JPH2-OE (green) cells with E) mean JPH2 labelling density analysis. Data displayed as mean ± SEM; **p<0.01, ***p<0.001.

3.3.1 RyR organisation is influenced by JPH2 expression

As there have been many previous reports of a link between JPH2 and the organisation of EC coupling proteins (Takeshima et al., 2000, Van Oort et al., 2011, Wu et al., 2012), the organisation of the SR calcium release channel, the RyR, was investigated in these JPH2...
transgenic mice. While there has been some work previously done to examine this, it has primarily been performed using confocal microscopy (Van Oort et al., 2011), and due to the nanoscale nature of the junctional space, many of the details in organisation may have gone unidentified, which can now be observed with the application of the super resolution imaging techniques used throughout this chapter.

3.3.1.1 RyR cluster size

dSTORM imaging revealed the overall appearance of RyR immunolabelling across the three different genotypes, with the pattern of cluster distribution throughout the longitudinally orientated cardiomyocytes visibly different (Figure 3.4A-C), particularly in the JPH2-KD mice. While the control cells showed RyR clusters arranged linearly in regular, tight transverse rows across the cardiomyocyte (Figure 3.4B), following knockdown of JPH2, the RyR clusters appeared disorganised with apparent dispersion from a tight linear arrangement (Figure 3.4A). Over-expression of JPH2 does not appear to alter the regularity of RyR cluster localisation throughout the cell, with a similar pattern of distribution to control mice observed in JPH2-OE. However, it was observed that there is an additional appearance of apparently enlarged clusters (Figure 3.4C), similar to those observed with JPH2 labelling in these mice. Furthermore, these clusters often appeared elongated, making longitudinal extensions within the cardiomyocyte.

The size of the RyR clusters, or calcium release units (CRUs), contributes to the properties of the calcium spark (and subsequent transient) following calcium release from the SR (Franzini-Armstrong et al., 1999). Analysis of RyR cluster size has therefore been determined in these JPH2 transgenic mice. This revealed that while there was no change in mean cluster size in the JPH2-KD mice (22.61 ± 0.45 x 10^3 nm^2) compared to controls (23.44 ± 0.54 x 10^3 nm^2), the mean cluster size was significantly increased (29.95 ± 0.78 x 10^3 nm^2, p<0.001) as a result of JPH2 over-expression by ~28% compared to control mice (Figure 3.4D). In addition, the prevalence of RyR 'macro-clusters' was assessed, with a macro-cluster defined as being larger than 200,000 nm^2, such that each cluster could contain approximately >200 RyR channels (based on reported RyR dimensions (Lai et al., 1988, Franzini-Armstrong and Protasi, 1997)). This revealed a significantly higher density of RyR macro-clusters in the JPH2-OE cells of 7.52 ± 0.64 per 100 µm^2 (n= 2 animals; 15 cells; p<0.001 for both), compared to both the control (1.84 ± 0.68 per 100 µm^2; n= 2 animals; 12 cells), and JPH2-KD cells (1.91 ± 0.41 per 100 µm^2; n= 2 animals; 11 cells), with these results, along with mean cluster size findings, confirming visual observations of an increased RyR cluster size in JPH2-OE mice.
Figure 3.5 JPH2 expression causes longitudinal reorganisation of the RyR clusters
Super resolution images showing RyR immunolabelling in isolated cardiomyocytes from A) JPH2-KD, B) control and C) JPH2-OE mice, with RyR macro-cluster examples shown with arrowheads and longitudinal connections indicated by arrows. Scale bars: 2.5 µm. Bar graphs showing D) mean cluster sizes and density analysis of RyR macro-clusters as number of macro-clusters per 100 µm² in JPH2-KD cells, control and JPH2-OE cells. Data displayed as mean ± SEM; ***p<0.001.

Based on the observation of increased longitudinal extensions of many of the larger clusters in the JPH2-OE mice, the extent of longitudinal projection was determined in these mice. Due to the majority of smaller clusters not containing sufficient RyR channels to assess directionality, the largest 25% of clusters were analysed. By applying an elliptical fit to these clusters (as described in section 3.2.2), both the longitudinal diameter and the extent of longitudinal projection relative to the longitudinal axis of the cardiomyocyte could be determined. The findings of this analysis confirmed the observation that large RyR clusters in JPH2-OE cardiomyocytes have an increased longitudinal appearance, with both the longitudinal diameter (Figure 3.6A) and extension (Figure 3.6B) significantly greater than control mice, with values of 429.1 ± 8.0 nm and 473.1 ± 8.9 nm in JPH2-OE mice,
3. RyR cluster organisation in adult JPH2 transgenic mice

respectively, compared to 294.5 ± 4.7 nm and 327.3 ± 5.1 nm in controls (p<0.001 for both). Furthermore, these parameters were both found to be significantly smaller in the JPH2-KD mice, decreasing to 222.8 ± 2.9 nm and 248.9 ± 3.2 nm, respectively (p<0.001 for both), indicating a reduction in longitudinal orientation of these RyR clusters.

![Figure 3.6 Changes to longitudinal component of RyR clusters due to JPH2 expression](image)

**Figure 3.6 Changes to longitudinal component of RyR clusters due to JPH2 expression**

RyR cluster analysis from elliptical fitting showing A) longitudinal diameter and B) longitudinal extension measurements for the three genotypes. Data displayed as mean ± SEM; ***p<0.001.

Due to the complex, three-dimensional structure of the junctional space, RyR cluster organisation was also examined in transversely orientated cardiomyocytes using myocardium sections. In regions of the cardiomyocyte where the z-disks were in focus, super resolution imaging revealed that overall the appearance of RyR clusters was of a dense network throughout the cells, with areas devoid of labelling, which were presumably due to the presence of other cellular structures, such as mitochondria and myofibrils. Furthermore, while there was variability in the sizes of clusters observed, it was seen that there appeared to be larger clusters present in the JPH2-OE cardiomyocytes, matching the observation from longitudinal imaging. Confirming this, cluster analysis revealed that transversely orientated cardiomyocytes also showed a significant increase in the mean RyR cluster size of ~27% in JPH2-OE mice (p<0.001) compared to control mice (Figure 3.7D). Furthermore, there was still no significant change in transverse cluster size between controls and JPH2-KD mice observed. In transverse orientation, the mean cluster size in controls was found to be 35.6 ± 1.0 x 10^3 nm² (n= 2 animals, 11 cells), 35.9 ± 0.8 x 10^3 nm² in JPH2-KD mice (n= 2 animals,
RyR cluster organisation in adult JPH2 transgenic mice

12 cells), and $45.3 \pm 1.8 \times 10^3$ nm$^2$ in the JPH2-OE mice (n= 2 animals, 9 cells). Macro-cluster analysis in the transverse sections also agreed with the findings from the isolated myocytes, with JPH2-OE mice showing a significantly higher density of RyR macro-clusters compared to both control and JPH2-KD mice (Figure 3.7E).

**Figure 3.7 JPH2 expression causes transverse reorganisation of the RyR clusters**
Super resolution images of RyR immunolabelling in transversely orientated cardiomyocytes from A) JPH2-KD, B) control and C) JPH2-OE myocardium. Scale bars: 2.5 µm. Bar graphs showing D) mean cluster sizes and E) density analysis of RyR macro-clusters as number of macro-clusters per 100 µm$^2$ in JPH2-KD cells, control and JPH2-OE cells. Data displayed as mean ± SEM; **p<0.01, ***p<0.001.

3.3.1.2 Clusters distances and density of RyR
The synchronous release of calcium throughout the cardiomyocyte is important for contraction of the cell. The distribution of CRUs throughout the cell therefore plays a role in ensuring the myofibrils receive this triggering calcium, with the distance between RyR clusters
determining the distance required for calcium diffusion to adequately activate all myofibrils. For this reason, the nearest neighbour (edge-to-edge) distances of the three genotypes were examined. It was found that there was a shift towards larger distances in both the JPH2-OE and JPH2-KD mice, compared to the distribution observed in controls (Figure 3.8A). The mean nearest neighbour distances revealed to be significantly increased in JPH2-KD (48.1 ± 2.6 nm; n= 2 animals, 6 cells; p<0.05) and JPH2-OE mice (59.2 ± 2.8 nm; n= 2 animals, 7 cells; p<0.001), compared to control (39.8 ± 2.3 nm; n= 2 animals, 5 cells), as shown in Figure 3.8B.

**Figure 3.8 RyR cluster distance analysis**

Analysis of RyR inter-cluster distances showing A) the frequency distribution of nearest neighbour distances for JPH2-KD (red), control (blue) and JPH2-OE (green) mice, with B) the mean nearest neighbour distance determined. C) The area of RyR labelling contained within ‘super-clusters’ of the three genotypes. Data displayed as mean ± SEM; *p<0.05; ***p<0.001.

In addition to the previous macro-cluster analysis, analysis of RyR ‘super-clusters’ was performed to determine the area of labelling with each super-cluster. A super-cluster was defined as being the combination of individual single clusters within a 100 nm distance of
3. RyR cluster organisation in adult JPH2 transgenic mice

each other such that they are within a close enough distance to form a functional super-cluster based on the diffusion of calcium concentrations released in a calcium spark (Sobie et al., 2006). Based on distance analyses, it was found that in control mice, 91.2% of RyR clusters were within ≤100 nm distance, which was unchanged at 91.4% in JPH2-KD, but reduced to 83.3% in JPH2-OE mice (Figure 3.8A). Interestingly, further analysis revealed that the mean area of labelling within these super-clusters was significantly increased in the JPH2-OE mice compared to control and JPH2-KD mice, with no difference between JPH2-KD and control (Figure 3.8C). Mean labelling area was found to be 59.2 ± 5.0 x 10^3 nm^2 in control mice (n= 2 animals, 16 cells), 70.0 ± 5.2 x 10^3 nm^2 in JPH2-KD (n= 2 animals, 14 cells), and increased by ~57% to an average of 93.1 ± 12.0 x 10^3 nm^2 following JPH2 over-expression (n= 2 animals, 15 cells; p<0.05). This indicates that despite having a greater average separation distance between clusters, there is more labelling within the super-cluster regions in JPH2-OE mice, showing that JPH2 expression is influencing the subcellular organisation of RyR clusters.

As there have been reports of JPH2 expression also influencing the expression of other junctional proteins (Guo et al., 2014), the total image RyR labelling density was assessed. Interestingly, it was observed that the overall RyR labelling density across the entire image was not significantly changed between the three genotypes (Figure 3.9A), indicating that there is a comparable amount of RyR protein present (control: n= 2 animals, 28 cells; JPH2-KD: n= 2 animals, 20 cells; JPH2-OE: n= 2 animals, 27 cells). Furthermore, while the size of clusters provides valuable information regarding the area covered by RyRs for calcium release from an individual CRU, potential changes in size do not necessarily correlate with the content of RyR channels within the cluster. Therefore, the density of RyR labelling within the individual clusters was determined.

This revealed that there is a significant reduction in the density of RyR labelling within both JPH2-OE and JPH2-KD RyR clusters (p<0.001 for both) compared to control clusters (Figure 3.9B; control: n= 2 animals, 11 cells; JPH2-KD: n= 2 animals, 12 cells; JPH2-OE: n= 2 animals, 9 cells), with these same findings reflected in the RyR localisation event densities (Figure 3.9C; n= 1 animal each, 3 images [JPH2-KD, control] or 4 images [JPH2-OE]; p<0.05 JPH2-KD vs control, p<0.01 JPH2-OE vs control). These findings indicate that, although JPH2-OE mice exhibit an increased cluster size, this reduction in RyR labelling density within clusters results in the overall RyR labelling density across the entire image being unchanged compared to controls. However, for the JPH2-KD mice, there is a reduced density within clusters of the same size as controls, but with the same density of overall RyR labelling present. This indicates that there is an additional change occurring to result in reduced intra-
RyR cluster organisation in adult JPH2 transgenic mice

Cluster density while maintaining cluster size and overall labelling density. Therefore, the number of RyR clusters present was assessed as a density per area of cardiomyocyte. While there was no difference between controls and JPH2-OE mice, it was found that there was ~19% more RyR clusters present in the JPH2-KD mice compared to controls, although this was not statistically significant (Figure 3.9D; control: n= 2 animals, 11 cells; JPH2-KD: n= 2 animals, 12 cells; JPH2-OE: n= 2 animals, 9 cells).

Figure 3.9 RyR labelling and cluster densities following JPH2 expression changes

Density analyses results showing A) the total RyR labelling density across the super resolution images, B) the RyR labelling density within the RyR clusters with C) the corresponding localisation event density, and D) the density of RyR clusters within the cardiomyocyte. Data displayed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.
3.3.2 Altered co-localisation between RyR and JPH2

Due to previous reports of the interaction of JPH2 with RyR to potentially aid in channel stabilisation (Van Oort et al., 2011), the co-localisation between these two proteins was examined at the super resolution level. This dual labelling revealed an apparent decrease in RyR-JPH2 association in JPH2-KD mice (Figure 3.10A) compared to controls (Figure 3.10B), while there appeared to be an increase in the JPH2-OE mice (Figure 3.10C). Analysis of these images confirmed that there was very little co-localisation apparent between the remaining JPH2 and RyR in the JPH2-KD cells (n= 3 animals, 35 cells), with co-localisation analysis revealing that only 20.1% of RyR was co-localised with JPH2 and 27.6% of JPH2 co-localised with RyR (Figure 3.10D,E). Statistical analysis confirmed that these values were significantly reduced compared to values from control cells (n= 3 animals, 15 cells), with RyR-

![Super resolution images showing dual immunolabelling of RyR (red) with JPH2 (green) in A) JPH2-KD, B) control, and C) JPH2-OE mouse cardiomyocytes. Scale bars: 1.5 µm. Co-localisation analysis for D) RyR with JPH2 and E) JPH2 with RyR immunolabelling in super resolution images from all three genotypes. Data displayed as mean ± SEM; **p<0.01, ***p<0.001.](image-url)
3. RyR cluster organisation in adult JPH2 transgenic mice

JPH2 32.8% co-localised and 45.1% of JPH2 co-localised with RyR (p<0.001 for both). In addition, experiments showed that the large RyR clusters are associated with the previously observed large JPH2 clusters in the JPH2-OE cells (Figure 3.10C). Co-localisation analysis confirmed this observation, with 43.4% of RyR co-localised with JPH2, and 43.1% of JPH2 co-localised with RyR (n= 3 animals, 38 cells). Compared to controls, there was a significant increase in the level of RyR-JPH2 co-localisation (p<0.01), however there was no change in JPH2-RyR co-localisation level compared to control cells. Together, these findings provide evidence that altering JPH2 levels can influence the extent of co-localisation with RyR, particularly following knockdown.

3.3.2.1 Relative density of RyR and JPH2 within junctions

While the co-localisation analysis assesses the relative interactions between these two proteins based on total labelling present, the relative densities of each protein within individual RyR clusters was also assessed to determine how they contribute to the composition of the junction. Considering that a junction, or dyad, is a functional region where the DHPR and RyR channels interface to enable CICR (Scriven et al., 2000), the RyR cluster masks were used to determine the location of potential junctions. Further to the density of RyR, which has already been analysed, the density of JPH2 labelling within these junctions was assessed in the control and JPH2-OE mice. This allowed it to be determined whether the increased levels of JPH2 in the over-expressing mice resulted in higher levels of the protein localised specifically to the junction, or whether the increased JPH2 was distributed throughout the cardiomyocytes. Due to the reduced density of JPH2 in the knockdown mice, and the already observed reduced co-localisation of RyR labelling with JPH2, and vice versa, these mice were not included in the analysis. Visual assessment of labelling within the junctions confirmed the reduced density of RyR within the clusters of JPH2-OE mice, and that compared to controls, there appeared to be an increased density of corresponding JPH2 labelling (Figure 3.11A). Analysis revealed that, compared to controls, JPH2-OE cardiomyocytes had a significantly higher density of JPH2 labelling within the RyR clusters (Figure 3.11B), at ~2.1-fold higher than controls, and furthermore, that the ratio of RyR:JPH2 labelling within the clusters was reduced in JPH2-OE mice (Figure 3.11C), or conversely, that the JPH2:RyR labelling ratio is increased compared to control.
3. RyR cluster organisation in adult JPH2 transgenic mice

Figure 3.11 JPH2 over-expression alters junctional JPH2 and RyR densities
A) Super resolution images showing corresponding immunolabelling of RyR (red) and JPH2 (green) within the same clusters from control and JPH2-OE mouse cardiomyocytes. Scale bars: 0.25 µm. B) The labelling density of JPH2 within the junction and C) the corresponding ratio plots of the densities of junctional RyR and JPH2 in control and JPH2-OE mice. Data displayed as mean ± SEM; ***p<0.001.
3.4 Discussion

This chapter has presented super resolution images which have been used in determining the nanoscale organisation of the junctional proteins JPH2 and RyR. The findings regarding JPH2 organisation indicate that the pattern of protein expression is altered in response to the level of JPH2 expressed. The observed regular, linear distribution of JPH2 throughout the control and JPH2-OE cardiomyocytes is as expected based on previous findings (Takeshima et al., 2000, Wagner et al., 2012), which is consistent with the role of JPH2 as an anchoring protein between the SR J and t-tubule (Takeshima et al., 2000), both of which align at the z-disks in cardiomyocytes (Fawcett and McNutt, 1969). The apparent loss of this pattern following JPH2-KD is likely to be due to there being insufficient protein remaining to show a clear distribution pattern. The overall trends of labelling density changes detected across the whole image from the three genotypes were consistent with reported altered protein expression levels (Van Oort et al., 2011)(Wehrens’ laboratory, unpublished), however, the extent of this detected change in both JPH2 knockdown and over-expressing mice compared to control was lower than that reported in Western blots. This could potentially be due to only isolated ventricular myocytes being imaged, while whole heart lysates were used in protein expression analyses (Van Oort et al., 2011), which may include atrial cardiomyocytes which also express αMHC (Schiaffino and Reggiani, 1996). It may have been of interest to also examine additional cell types individually, such as epithelial or smooth muscle cells as well as isolated atrial cardiomyocytes; however the αMHC promoter used to drive cardiac-specific genetic manipulation is reported to be restricted to primarily cardiac myocytes (Gupta et al., 1998).

Variability in the accuracy of super resolution localisation event detection can occur between imaging days (due to changes in activity of the thiol-based reducing agent in the mounting medium; (Van De Linde et al., 2011)) which could also contribute to differences in reported protein labelling, however the effects of this have been minimised by analysing images from the different genotypes that were acquired on the same days. Overall, these results have shown a clear difference in JPH2 protein labelling in the three mouse strains, validating the ability of this super resolution imaging technique to detect changes in protein expression levels. Therefore, this imaging technique can be suitably applied for the assessment of additional protein changes in JPH2 transgenic mice. The rest of the findings discussed below provide structural information on the organisation of the RyRs, which support the potential mechanisms by which the calcium handling function has been altered in these mice, including organisation of additional junctional proteins (RyR) and altered RyR channel stability.
3.4.1 Altered organisation of RyR clusters

The main focus of results presented in this chapter was to identify changes in the organisation of the RyR clusters, or CRUs, as a result of altered JPH2 expression. The identification of RyR clusters in control and JPH2-OE mice predominantly localising in regular transverse arrangement throughout the isolated cardiomyocytes is in agreement with previous reports of the protein localising to the z-disks, where they can form close associations with DHPR in the t-tubule membrane (Scriven et al., 2000). The apparent dispersion of RyR clusters in the JPH2-KD mice away from a tight z-disk alignment indicates that loss of JPH2 has resulted in reduced localisation of the SR J (where RyR are localised; (Chen-Izu et al., 2006, Soeller et al., 2007) to the z-disk. As JPH2 has been shown to link the SR J and t-tubule membranes, via membrane-spanning domains and MORN motifs respectively (Takeshima et al., 2000), these findings could be due to aberrations in t-tubule organisation away from z-disk alignment (examined in Chapter 4). The network-like organisation of clusters throughout the transversely orientated control cardiomyocytes is consistent with previous reports, indicating that the clusters follow the pattern of t-tubule organisation wrapping around the myofibrils (Hou et al., 2015). Both JPH2-KD and JPH2-OE mice showed the same overall transverse pattern of cluster distribution, suggesting the subcellular localisation of RyR was not altered in response to changes in JPH2 expression when viewed in this orientation. In longitudinal views of cardiomyocytes, t-tubules and SR J typically tightly align with the z-disk in parallel to the optic path, making it easier to identify deviations from this arrangement (due to dislocation from the z-disk occurring laterally in the image). In comparison, in transverse cardiomyocytes, these structures align parallel to the focal plane, and so shifts in longitudinal organisation would appear as out-of-focus labelling. Due to the helicoid nature of the z-disks (Jayasinghe et al., 2010), and the axial resolution limit of this super resolution imaging technique in thick samples (Hou et al., 2015), it is not possible to distinguish the difference between out-of-focus RyR clusters being due to z-disk orientation or impaired alignment of the RyR to the z-disk. This emphasises the importance of imaging cardiomyocytes in the two orientations to obtain a more complete picture of the nanoscale changes occurring.

3.4.1.1 RyR cluster size

RyR cluster sizes were determined for the three genotypes, with control cardiomyocytes showing an average cluster size of \( \sim 23 \times 10^3 \text{ nm}^2 \) longitudinally, and \( \sim 36 \times 10^3 \text{ nm}^2 \) in transverse orientation, which is equivalent to maximum of \( \sim 23 \) and \( \sim 36 \) RyR channels, respectively, depending on the density of RyR packing (see below for further discussion).
The discrepancy between these two reported sizes highlights the importance of determining the orientation of the cardiomyocyte, as these clusters are clearly more extended in the transverse orientation than longitudinally. The complex 3D structure of the t-tubule network wrapping around the myofibrils means that although the t-tubules align at the level of the z-disk, when viewed at a longitudinal orientation they are not always in the focal plane, or they may be running at an oblique angle into the cell. In these latter instances, the corresponding RyR clusters observed would also be at an angle to the focal plane and so their size may be under-reported. In contrast, when viewing cardiomyocytes in transverse and the z-disk is in focus, the majority of t-tubules will be in the same focal plane and so the corresponding RyR clusters will be aligned 90° to the optical path, making more of the cluster visible and therefore reported sizes will be larger compared to longitudinal orientation. The reported cluster sizes for both orientations are lower than those recently reported in transverse rat cardiomyocytes (~63 RyR) using the same super resolution techniques (Hou et al., 2015), but the transverse measurements are not dissimilar to reported sizes in dyadic couplings in wild-type mice (~43 RyR), utilising a combined 3D EM and super resolution approach (Das and Hoshijima, 2013), indicating potential inter-species variation.

The observation of increased RyR cluster size in the JPH2-OE mice was consistently reported in both longitudinal and transverse samples, with an increased (~27-28%) mean size and prevalence of RyR macro-clusters (clusters ≥200 x 10^3 nm²). These findings indicate that JPH2 also plays a role in the organisation of junctional proteins other than itself, in particular the RyR. While this is the first study to directly investigate the organisation of the RyR in this mouse model, the results are in general agreement with previous work by Guo et al (2014) who used EM to examine the contact points between the t-tubules and SR in a similar mouse strain, which revealed a ~2-fold increase in the length of these contact points following JPH2 over-expression. Potential explanations for the discrepancy in the extent of this increase between studies are discussed later on. One of the likely explanations for the detected increased mean cluster size is the observation of a higher prevalence of RyR ‘macro-clusters’, with these particularly large RyR clusters also recently reported in the Wistar rat (Hou et al., 2015). While the exact mechanism underling the observed increased cluster size is unclear, it has previously been reported that JPH2 directly interacts with RyR (Van Oort et al., 2011) and that there are often RyR channels ‘orphaned’ from the t-tubules present in healthy cardiomyocytes (Jayasinghe et al., 2009). It is therefore possible that increased levels of JPH2 are able to ‘hold’ more RyR channels within the junctional cleft, potentially sequestering the otherwise non-junctional RyR. However, further density analysis findings from this study (discussed below) suggest that this is not the case, and that the increased RyR cluster size is not due to increased RyR content within the junction. What the results
from this study, along with previous work, suggests to be more likely, is that the enlarged RyR cluster size resulting from JPH2 over-expression is due to the reported increase in contact area between the SR_j and t-tubule membranes (Guo et al., 2014), such that there is an increased area for RyR channels to localise within in these regions, leading to the observed enlargement of clusters.

Further results revealed that the largest 25% of RyR clusters in JPH2-OE mice showed exaggerated longitudinal projections into the cardiomyocyte, as seen by an increase in both the longitudinal diameter and extension measurements compared to control. One potential explanation for this observation would be an increased presence of longitudinally orientated t-tubules, allowing for junctions to extend in this direction, which is investigated in Chapter 4. This is in concurrence with the observation of apparently longitudinally orientated clusters in the super resolution images, which appear in JPH2-OE mice, and to a lesser extent in controls but are virtually absent in JPH2-KD cardiomyocytes. This absence in JPH2-KD cells indicates a potential explanation as to why there are reduced longitudinal measurements in these mice.

The findings regarding RyR cluster size indicate that there are larger CRUs present as a result of increased JPH2 expression, and therefore we would potentially expect to see larger calcium sparks in these mice compared to controls. However, as detailed in section 3.1.1, a reduction of spark size is actually observed in these JPH2-OE mice. Furthermore, it has previously been reported that knockdown of JPH2 results in increased calcium spark size (Van Oort et al., 2011), and yet findings from this study indicate that there is no difference in the average RyR cluster size compared to control. Taken together, these findings suggest that there are additional changes occurring within the junction that are contributing to the observed functional changes which cannot be explained by differences in CRU size, and so labelling density analyses were also performed (discussed in section 3.4.1.3).

3.4.1.2 Cluster distances

The distance separating RyR clusters determines the likelihood of inter-cluster activation due to calcium spark activity. It was found that in control cardiomyocytes, the average nearest neighbour distance was ~40 nm, which is smaller than previously reported values from dSTORM imaging in the rat (Hou et al., 2015), yet about twice that reported in mice using EM (Hayashi et al., 2009). This inconsistency in reported values is likely to be due to a combination of species differences, along with methodological variation. The distance between RyR clusters was, on average, found to be significantly increased in both the JPH2-
3. RyR cluster organisation in adult JPH2 transgenic mice

KD and JPH2-OE mice to ~48 nm and ~59 nm, respectively, indicating greater calcium diffusion would be required to activate neighbouring CRUs as a result of RyR calcium release. Based on work by Sobie et al. (2006), clusters with ≤100 nm separation have the potential ability to trigger CICR in each other. It was found that the majority of control clusters were inside this range, with ~91.6% of clusters within ≤100 nm of their nearest neighbour. A similar proportion of clusters were observed within this distance in JPH2-KD mice (~91.4%), but only ~83.3% from JPH2-OE mice. Further analysis of these distances revealed the extent of super-clustering in the JPH2 transgenic mice, in which the amount of RyR labelling within ≤100 nm of each other was assessed. Interestingly, the findings revealed an opposing relationship when compared to nearest neighbour distances, with JPH2-OE mice showing significantly more labelling with super-clusters compared to controls and JPH2-KD. This suggests that while the number of clusters at ≤100 nm separation distance is reduced in these mice, the clusters which are involved in super-clustering are larger and therefore overall result in greater area of RyR labelling present. This is consistent with the findings regarding RyR cluster sizes previously discussed.

Structural information provided from these analyses may provide insights as to possible mechanisms contributing to the heterogeneity of the calcium transient observed in JPH2-OE mice (Wehrens’ laboratory, unpublished). The observation of high prevalence of macro-clusters in these mice means that the release of calcium from the RyR as a result of $I_{Ca}$ would be spread over a larger area. However, the increased distance between neighbouring RyR clusters suggests that the synchronicity of activation could be impacted following the action potential. These two findings coupled together indicate that there could be heterogeneity observed across the calcium wave, but since the total cell density of RyR is unchanged, globally, there would be a similar amount of calcium released during the transient, resulting in the overall amplitude remaining unchanged compared to controls, which is exactly what is observed in these mice (Wehrens’ laboratory, unpublished).

3.4.1.3 RyR channel density

Density analysis of RyR labelling was performed in this study, which revealed interesting results in the JPH2 transgenic mice. There was no difference found in the total labelling of RyR between any of the genotypes, which is consistent with Western blot reports from previous studies, indicating that RyR protein expression levels are unchanged following knockdown or over-expression of JPH2 (Van Oort et al., 2011, Guo et al., 2014)(Wehrens’ laboratory, unpublished). This suggests that the previously observed enlargement of RyR
3. RyR cluster organisation in adult JPH2 transgenic mice

clusters in JPH2-OE mice is due to redistribution of the protein, rather than an increase in the amount of RyR protein present in the cardiomyocytes.

Previous studies have investigated the density of RyR channels within the junction and have suggested the idea that there may be incomplete filling of the junction (Das and Hoshijima, 2013). This means that while measurements based on RyR channels size provide an upper limit on the maximum number of RyR within the junction, it is likely that the actual number of channels present is lower than this value, dependent on their packing density (Das and Hoshijima, 2013). While the total density of RyR labelling was unchanged between genotypes, there was a reduction of labelling within clusters of both the JPH2-KD and JPH2-OE mice, which further suggests sub-cellular protein redistribution as a result of altered JPH2 expression. RyR density within clusters was found to be reduced in JPH2-KD mice, which was coupled with both total RyR density and cluster size remaining unchanged. However, while not statistically significant, there was a trend towards an increase in the density of RyR clusters per area of cardiomyocyte in JPH2-KD mice compared to control. A higher density of clusters would indicate that the same number of RyR channels is dispersed over a larger number of clusters, such that there are fewer channels within each cluster, resulting in the observed reduced RyR density within clusters. Reduction of RyR density was also observed within JPH2-OE clusters, which fits in with the previous findings of enlarged clusters but unchanged total labelling density. Furthermore, the number of RyR clusters present per area of cardiomyocyte was unchanged compared to control. Together, these findings suggest that the same number of RyR channels is now spread across a similar number of clusters, but these clusters are larger in size, such that each larger cluster still contains the same number of channels as control clusters. This indicates that, as observed, we would expect to see a reduced density of RyR channels within each cluster for the JPH2-OE mice.

Interestingly, previous reports using EM found that there was an increase in the number of contact points between the t-tubules and SR following JPH2 over-expression, indicating the presence of more junctions (Guo et al., 2014), and that there is a reduction in the number of junctional membrane complexes in JPH2-KD mice (Van Oort et al., 2011); however these findings were not supported in the present study. Possible reasons for these contrasting observations may be due to subtle differences in the generation of the over-expressing mouse strains (which have slightly different JPH2 over-expression levels), or potentially because of the difference in methods used for detection. Furthermore, the previous study normalised SR-t-tubule ‘contact point’ counts to the number of inter-myofibrillary spaces (or per sarcomere), as opposed to cardiomyocyte area. This present study used dSTORM to examine RyR clusters using indirect fluorescent labelling, compared to the detection of
3. RyR cluster organisation in adult JPH2 transgenic mice

‘dyadic feet’ in EM (Guo et al., 2014), in which reported sizes can be influenced by the orientation of the specimen. To minimise the possibility of orientation effect in this work, both longitudinal and transverse orientated cardiomyocytes were separately analysed.

Given the overall density of RyR labelling, combined with the density of clusters, it would be expected that these transgenic mice would present with similar calcium spark frequencies, potentially with a subtle increased following JPH2 knockdown. However, the findings regarding RyR density within clusters would indicate that JPH2-KD mice would show reduced calcium spark sizes compared to controls, and that JPH2-OE mice would be comparable, or larger (given the balance of cluster size and RyR density). However, this is not the case, with JPH2-KD mice showing more, larger sparks (Van Oort et al., 2011) and JPH2-OE mice calcium sparks being reduced in size and frequency (Wehrens’ laboratory, unpublished). While the findings presented so far provide clear evidence of changes in RyR cluster organisation as a result of altered JPH2 expression, it is unclear how these relate to the observed functional changes in these mice. Further potential mechanisms relating to calcium spark data are explored below.

3.4.2 Changes to RyR stability

The mechanism underlying the link between JPH2 and RyR has not been fully revealed in research to date; however it has been found that these two proteins co-immunoprecipitate, indicating that there is a physical link between them, and that research suggests that JPH2 potentially acts to stabilise RyR channels (Van Oort et al., 2011, Beavers et al., 2013). Therefore, the extent of association between these two proteins was investigated.

3.4.2.1 Co-localisation with JPH2

The co-localisation of JPH2 and RyR was investigated at the super resolution level, with dual dSTORM imaging revealing that, in control mice, ~33% of RyR was co-localised with JPH2, while ~45% of the JPH2 labelling was co-localised with RyR. These findings suggest a reduced level of association compared to previous reports from both peripheral couplings (Jayasinghe et al., 2012a) and intracellular junctions in rats (Hou et al., 2015), with both of these studies utilising dSTORM imaging. It is possible that there are species differences in these measurements, as well as in method used (such as transverse versus longitudinal image analysis).
In addition, compared to control, there was a reduction in the co-localisation of these two proteins following JPH2 knockdown, and an increase in the fraction of RyR associated with JPH2 labelling in JPH2-OE mice. Given the altered expression levels of JPH2 in these mice, it is expected that the amount of RyR co-localising with JPH2 would be reduced in JPH2-KD and increased in JPH2-OE mice, as there is relatively less or more JPH2, respectively, present in the cardiomyocyte for RyR to associate with. However, what these findings also show is that following knockdown of JPH2, there is a reduced proportion of the remaining JPH2 co-localised with RyR. Given the previous evidence of JPH2 as a stabiliser of the RyR channels (Beavers et al., 2013, Van Oort et al., 2011), this reduced association of RyR-JPH2 would be expected to result in an increased frequency of calcium sparks in JPH2-KD mice, and is in fact observed in these mice (Van Oort et al., 2011). It is also possible that due to the reduced presence of JPH2 to stabilise the RyR channels within each cluster, the spontaneous leak of calcium from a single channel would be more likely to trigger release from a neighbouring cluster within the CRU, leading to the observed increase in calcium spark size following JPH2 knockdown (Van Oort et al., 2011).

Furthermore, the large JPH2 clusters identified were observed to co-localise with the RyR macro-clusters. The increased co-localisation of RyR labelling with JPH2 in the over-expression mice indicates that there is increased association of these two proteins, and therefore suggests that there are potentially more RyR channels being stabilised. This is supported in the functional data of these mice, in which there is a reduction in both the frequency and size of the calcium sparks (Wehrens’ laboratory, unpublished). However, it was found that the percentage of JPH2 labelling associated with RyR was unchanged compared to control, indicating that the increase in JPH2 protein levels is maintaining the relative proportion of junctional versus non-junctional distribution. Therefore, the relative density of JPH2 and RyR were also assessed within the junctional regions of these mice.

3.4.2.2 Relative density with JPH2

While the co-localisation analysis provided information on the relative interactions between RyR and JPH2 based on total labelling present, the relative densities of these two proteins within individual RyR clusters was also assessed to determine how they contribute to the composition of the junction. It was found, that compared to controls, JPH2-OE mice showed a ~2.1-fold increase in ‘junctional’ JPH2 labelling, indicating that the increased protein present is indeed localising to the junction. In JPH2-OE mice, the combination of reduced RyR density with an increase in JPH2 density within the junctions means that each RyR channel has relatively more JPH2 present, compared to in control mice. This means that the potential
ability for JPH2 to stabilise the RyR in the JPH2-OE mice is increased, further contributing to the reduction in calcium spark frequency and size observed in these cells. Based on these density findings, in combination with previous reports of ‘convoluted dyads’ in JPH2-OE mice (Guo et al., 2014), a proposed model of the change in junctional organisation following over-expression of JPH2 is presented in Figure 3.12. This highlights that junctions from each genotype contain a similar number of RyR channels, but that they are distributed over a larger cluster size in JPH2-OE mice, leading to a reduction in density. This is coupled with the increased junctional density of JPH2, such that there is more JPH2 protein per RyR channel in these mice.

![Figure 3.12 Junctional RyR stabilisation following JPH2 over-expression](image)

Schematic representing the organisation of the junction with the relative densities of RyR (red squares) and JPH2 (green dots) in control and JPH2-OE mice. Generated by Dr Isuru Jayasinghe.

### 3.5 Concluding Remarks

The use of dSTORM super resolution imaging techniques has enabled the characterisation of the nanoscale organisation of the CRUs in JPH2 transgenic mice. It was identified that this
approach is able to detect novel changes in protein labelling density as a result of altered JPH2 expression levels, and that this was associated with changes in the properties of the RyR clusters. Knockdown of JPH2 did not influence average RyR cluster size, but did result in reduced RyR density within clusters and decreased association between RyR and JPH2. Over-expression of JPH2 resulted in enlargement of the RyR clusters with a reduced intra-cluster density but an increase in both inter-cluster distance and super-cluster labelling. Changes in junctional protein densities and co-localisation provide potential explanations for the changes in calcium handling properties observed in these transgenic mice. This information is important for understanding the link between JPH2 and the organisation of the junctional proteins, in particular RyR, which plays a vital role in cardiac EC coupling.
Chapter 4. **T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice**

4.1 **Background**

The synchronous contraction of the cardiomyocytes relies not only on the organisation of the junctions, but also on the organisation of regular t-tubules for the rapid propagation of the action potential into the cell interior (Brette and Orchard, 2003) and having close apposition to the SR, for the formation of junctions (Franzini-Armstrong et al., 1999). These properties are highly important in ensuring EC coupling processes can occur efficiently and synchronously throughout the cardiomyocyte. As outlined in Chapter 1, JPH2 has been implicated in promoting both the formation and maintenance of the t-tubule network organisation (Van Oort et al., 2011, Wei et al., 2010, Chen et al., 2013, Reynolds et al., 2013), suggesting that JPH2 is an important protein involved in supporting the efficiency of EC coupling, and therefore potentially playing a role in regulating cardiac function. Furthermore, as previously described in this thesis, JPH2 has been implicated in human and animal models of cardiomyopathy and heart failure, including either mutated or decreased expression of the protein (Landstrom et al., 2011, Landstrom et al., 2007, Matsushita et al., 2007, Beavers et al., 2013). Heart failure develops in adult mice following the acute knockdown of JPH2, with loss of JPH2 found to be associated calcium handling abnormalities and disruption of the t-tubule organisation (Van Oort et al., 2011, Takeshima et al., 2000, Landstrom et al., 2011, Wu et al., 2012), which is a well characterised structural change in heart failure (Crossman et al., 2015, Wei et al., 2010, Lyon et al., 2009). In contrast, a JPH2 over-expression mouse model has been found to have EC coupling dynamics unchanged, while being protective of pressure over-load induced disruption of the t-tubule system (Guo et al., 2014). In contradistinction to previous reports, as described in Chapter 3, the specific strain of JPH2 over-expressing mice used in the current series of experiments do exhibit alterations in calcium handling properties.
In addition to previously observed alterations in RyR calcium handling properties (in the form of calcium sparks, detailed in Chapter 3 (Van Oort et al., 2011)), it has been shown that NCX activity is also altered in response to decreased JPH2 expression levels (Wang et al., 2014). Following induced knockdown of JPH2, adult mice exhibited a 50% reduction in NCX activity, despite no changes in the protein expression level. Furthermore, it has been shown that pharmacological inhibition of NCX to a similar extent (using cadmium) was able to induce an increased calcium spark size (Wang et al., 2014). These findings implicate JPH2 as playing a role in both the direct and indirect regulation of EC coupling/junctional proteins, and that these changes can produce detrimental alterations in calcium handling dynamics.

The end result of EC coupling is the mechanical contraction of the cardiomyocyte, which is mediated by the myofilaments, or myofibrils. This involves the interaction of the thick and thin filaments (as described in section 1.1.1) to produce cross-bridge cycling and the generation of force (Bers, 2002). The thin filaments are anchored at the z-disks by the protein α-actinin, which helps maintain their organisation within the sarcomere (Frank et al., 2006). The mechanism of cardiomyocyte contraction is partially determined by the ability of activating calcium released from the SR to bind to the myofibrils. Therefore, the organisation of the SR calcium release channels, the RyRs, in relation to the myofibrils is a contributing factor to the efficiency of EC coupling. Consequently, in addition to disruption of t-tubule organisation, it has been previously found that in human heart failure there is a reduction in the density of RyR clusters in relation to the volume of myofibrils (Crossman et al., 2011).

The underlying structural changes relating to impaired EC coupling have been widely investigated, however, the majority of these studies have utilised confocal or EM imaging. This either limits the ability to resolve fine structural changes (confocal), or the ability to examine the interactions of specific proteins due to many smaller proteins being difficult to accurately identify in sufficient contrast without use of antibody-targeted fluorescent labelling, along with being dependent on tissue orientation (EM) (Franzini-Armstrong, 2010). Therefore, these experiments have been conducted using a combination of confocal and super resolution imaging (dSTORM) techniques to investigate both the cell-wide and nanoscale changes to t-tubule and EC coupling protein organisation in response to altered JPH2 expression levels. These structural changes have then been correlated with previously reported functional changes in these mice. The aim of these experiments was to determine the influence of JPH2 expression level on the organisation of key structures and EC coupling proteins, including the t-tubules, myofibrils and NCX, in adult mammalian cardiomyocytes, and how these may provide novel insights as to mechanisms underlying the associated functional changes.
4.2 Experimental Techniques and Analysis

The transgenic mouse isolated cardiomyocytes and myocardium used for the experiments performed in this chapter were kindly provided by Prof Xander Wehrens, with JPH2 over-expressing (JPH2-OE) and knockdown (JPH2-KD) samples, as well as controls provided, as previously detailed in Chapter 3. Techniques described in General Methods were used for immunofluorescent labelling and imaging experiments to determine the influence of JPH2 expression levels on the organisation of EC coupling proteins and t-tubules. For results presented in this chapter, the primary antibodies used were: polyclonal rabbit anti-JPH2 (Invitrogen) or anti-RyR (Sigma), together with mouse monoclonal anti-NCX1 (Swant), anti-caveolin-3 (Abcam) or anti- α-actinin (Sigma), which were used in combination followed by secondary antibodies of goat origin. The mouse anti-NCX1 and anti-cav-3 were combined together in an ‘antibody cocktail’ for t-tubule labelling, as in previously established protocols (Hou et al., 2015). For confocal imaging, the combination of secondary antibodies used were conjugated to Alexa Fluor 488 and 594, while for dual colour super resolution (dSTORM) imaging Alexa Fluor 680 and 750 conjugated secondary antibodies were used. Photobleaching of the samples was reduced by acquiring only one image per cell for both the isolated cardiomyocytes and myocardium sections. For improvement of signal to noise ratio, the image stacks that were acquired using confocal imaging techniques were deconvolved and super resolution images were processed and rendered, all as described in General Methods. Customised image processing and analyses were performed using custom-written programming in ImageJ, IDL (Exelis Inc.) and Python-based software, as per details provided below.

4.2.1 Fast Fourier transform and t-tubule angle analysis

Confocal imaging was performed to visualise the t-tubule labelling in the three genotypes, using isolated cardiomyocytes. Following deconvolution of the confocal image stacks, Fast Fourier transform (FFT) analysis was performed on a single slice from the deconvolved images to quantify the extent of regularity of the t-tubule labelling. This technique transforms information from the spatial domain to the frequency domain (Wei et al., 2010) and has previously been established as a measure of t-tubule regularity (Crossman et al., 2015, Wei et al., 2010). FFT was performed on the t-tubule images using custom-written scripts in IDL software, written by Dr David Crossman. A single representative confocal image was selected and rotated such that the longitudinal axis of the cardiomyocyte was in the vertical direction (Figure 4.1A). From this, the surface sarcolemma was excluded by cropping the
region of the image containing intracellular labelling only (Figure 4.1B). FFT analysis was then performed on this image selection, to produce a power spectrum (Figure 4.1C). A smooth Gaussian curve was then computationally fitted to the peak in the resulting power spectrum, with the sigma of the fit adjusted manually if required. The amplitude of the resulting Gaussian peak was used as a relative measure of strength of regularity (TT-power; see Figure 4.1D). These power spectrum amplitudes were measured for all deconvolved confocal t-tubule images, with one-way ANOVA performed on the resulting data, with Tukey post-hoc analysis (SPSS, IBM software).

Figure 4.1 FFT analysis process of t-tubule immunolabelling
Steps used to determine TT power from FFT analysis using IDL, in which A) the t-tubule immunolabelled image is aligned in the vertical direction and B) the surface sarcolemma labelling is excluded. Scale bars: 5 µm. C) The FFT power spectrum output from FFT analysis of the t-tubule image is the determined with D) a Gaussian fit to resulting peak (red), and the representative height for TT-power is measured (vertical red bar).
Following deconvolution of the confocal t-tubule image stacks, maximal projection was applied to a small sub-stack (3 consecutive slices) using ImageJ. Images were orientated such that the longitudinal axis of the cardiomyocyte was in the vertical direction (0°), and the image was converted to a binary mask after application of manual thresholding. Manual thresholding was used to ensure adequate labelling signal was captured to obtain a true representation of the original image. The surface sarcolemma was then cropped from the image, and the resulting intracellular t-tubule labelling was skeletonised using ImageJ (Figure 4.2A). These skeletonised images were then used for t-tubule angle analysis.

Figure 4.2 Steps for angle analysis of t-tubule labelling
Steps used to analyse angles present in skeletonised images of t-tubule labelling. A) Skeletonised image of t-tubule labelling with B) the conversion to colour-coded angle bins using Python software. The resulting angles are then converted into C) a frequency distribution graph, with t-tubule angles ranging from 0-180°, and then used to produce D) a normalised frequency distribution graph of t-tubule angles ranging from 0-90°.
Angle analysis of the skeletonised t-tubule images was performed to determine the angles of the t-tubule labelling in relation to the longitudinal axis, where longitudinal is represented by 0° and 180°, while transverse is 90° (see Figure 4.2B). To do this, t-tubules were assigned local angle orientation (Figure 4.2B) and were grouped accordingly into 20 bins of 9° each (panel C) using custom-written scripts in Python-based software written by Prof Christian Soeller. These bins were then normalised to give values with a range of 0-90°, for example 0-9° was grouped with 171-180° to form one bin (Figure 4.2D). The distribution of the fraction of tubules occurring at these angles in JPH2-KD and JPH2-OE cells was compared to the control distribution using $\chi^2$ analysis. In addition to determining the overall distribution of t-tubule angles in the isolated cardiomyocytes from the different genotypes, the proportion of transversely orientated t-tubules was directly compared to the proportion of longitudinally orientated t-tubules. To do this, for each genotype, the normalised fraction of t-tubules occurring in each of the two most ‘transverse’ bins (72-81° + 81-90°) were summed, as was the normalised fraction of t-tubules in the two ‘longitudinal’ bins (0-9° + 9-18°).

4.2.2 Co-localisation analysis

The co-localisation of dual labelled dSTORM images was performed according to procedures described in General Methods, using Python software written by Dr David Baddeley, with the algorithms involved previously described (Baddeley et al., 2010, Jayasinghe et al., 2012a, Jayasinghe et al., 2009). In brief, binary masks of each protein label were created using a threshold value which captured a determined percentage of the total label signal intensity. For JPH2-t-tubule co-localisation analysis, a threshold value which captured 0.8 of the total signal fraction (80% total signal intensity) was used, while for the NCX-RyR analysis 0.7 signal fraction thresholding was used to generate masks. From these masks, the fraction of labelling of ‘protein A’ a function of the Euclidean distance to the nearest edge of the labelling of the ‘protein B’ was determined and used to generate a distance distribution histogram. From this distribution, the percentage of ‘protein A’ co-localising with ‘protein B’ could be determined, which was found by the summing the nearest edge distances that were below or equal to zero (as illustrated in General Methods Figure 2.4).

4.2.3 Analysis of RyR-myofibril organisation

In order to characterise the organisation of the RyR clusters around the myofibrils, the perimeter of each myofibril was manually outlined based on α-actinin labelling at the level of the z-disk using transverse sections of the mouse ventricle tissue (Figure 4.3, magenta lines).
The contact region between the RyR clusters and the myofibril was also outlined (Figure 4.3, yellow regions), with half the perimeter of this region used as the measure of RyR-myofibril contact distance. The sum of these contact regions was calculated for each myofibril, which was divided by the total myofibril perimeter to obtain the percentage of RyR-myofibril contact to total myofibril perimeter determined, based on previous methods (Hou et al., 2015).

Figure 4.3 Analysis of perimeter of myofibril contacting RyR
Super resolution dual immunolabelling for RyR (red) and α-actinin (cyan) with the corresponding analysis of the fraction of the myofibril perimeter (magenta) in contact with RyR clusters (yellow). Scale bar: 1 µm.

4.2.4 Statistical analysis
Unless stated otherwise, the results are reported as mean with the standard error of the mean (SEM) as uncertainty, with the statistical tests performed using IBM SPSS Statistics v22 software. Mann-Whitney U- or Kruskal-Wallis k-testing with post-hoc analysis was performed on the data, determined by the number of groups analysed, unless otherwise stated.
4. T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice

4.3 T-tubule and EC Coupling Protein Changes in Response to Altered JPH2 Expression

4.3.1 T-tubule organisation

Previous studies have investigated the influence of JPH2 expression on t-tubule organisation (Van Oort et al., 2011, Guo et al., 2014), and reported a disrupted organisation following JPH2 knockdown. While these studies were performed in live isolated cardiomyocytes at the confocal level, this study has used a different approach of examining fixed cardiomyocytes with immunocytochemical techniques, combined with both confocal and super resolution imaging techniques. Therefore, the organisation of the t-tubules was assessed in JPH2-KD mice along with controls and JPH2-OE to determine the reproducibility of previous findings with the present techniques.

4.3.1.1 T-tubule regularity and orientation is influenced by JPH2 expression

Following deconvolution, the confocal images revealed clear changes in the organisation of the t-tubules as a result of altered JPH2 expression. Isolated cardiomyocytes from control mice showed a predominantly regular arrangement throughout the cells, with the majority of t-tubules orientated in a transverse direction (Figure 4.4A). In comparison, it was observed that there was disorganisation of the t-tubule system in the JPH2-KD cardiomyocytes, with an apparent loss in regularity throughout the cell, and t-tubules appearing randomly orientated without a clear dominant angle (Figure 4.4B). Following over-expression of JPH2, the overall t-tubule system organisation appeared to remain intact when compared to the control cells (Figure 4.4C). JPH2-OE cardiomyocytes showed a predominantly regular, transverse orientation of t-tubules present, with the additional appearance of an apparent increase in longitudinal branching observed. To quantify these observed changes in t-tubule regularity in JPH2-KD mice, FFT analysis was performed to determine the TT-power as a measure of regularity of t-tubule labelling. From this analysis, it was found that the TT-power amplitude was significantly reduced in JPH2-KD (n= 2 animals; 15 cells) compared to control (n= 2 animals; 11 cells; p<0.001), confirming that the regularity of t-tubule organisation throughout the cardiomyocyte was decreased following JPH2 knockdown (Figure 4.4D). In contrast, there was no statistically significant difference in TT-power between the JPH2-OE (n= 2 animals; 12 cells) and control cells, suggesting that the regularity of transverse t-tubule labelling is maintained in these mice.
4. T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice

While FFT analysis measures of the overall t-tubule labelling regularity, it does not provide an indication as to how the t-tubule organisation has changed within the cardiomyocyte. Therefore, to determine how the t-tubule network had been re-organised following altered JPH2 expression, angle analysis was also performed on the deconvolved confocal images. This confirmed that in control cardiomyocytes, there is a clear, strong prevalence of transversely orientated t-tubules (72-90°), with very low proportions occurring at longitudinal or oblique angles (Figure 4.5A). In contrast, the JPH2-KD cells showed a reduction in the proportion of transverse tubules present compared to controls, with all angles occurring at relatively similar proportions (Figure 4.5B). This indicates that there is severe disorganisation...
of the t-tubule network and confirms the visual observation of randomly orientated t-tubules following JPH2 knockdown. The overall change in the angle distribution of t-tubules was confirmed to be significantly different between the control and JPH2 knock-down cells ($\chi^2$ analysis; p<0.001). It was found that the JPH2-OE cells showed an overall distribution similar to that of controls, with the highest proportion of t-tubules occurring at transverse angles (Figure 4.5C). However, it was also seen that compared to controls there was an increased proportion of t-tubules occurring at longitudinal angles, correlating with the observed longitudinal branching in these cardiomyocytes, with $\chi^2$ analysis of the angle distributions revealing a significant difference between control and JPH2-OE cells (p<0.001).

Figure 4.5 JPH2 knockdown leads to t-tubule disorganisation

Angle analysis showing normalised frequency distributions of t-tubule angles, for A) control, B) JPH2-KD, and C) JPH2-OE mouse cardiomyocytes. D) Analysis of the proportion of longitudinal (white; 0-18°) versus transverse (black; 72-90°) orientated t-tubules in JPH2-KD, control and JPH2-OE cardiomyocytes. Data displayed as mean ± SEM; *p<0.05, ***p<0.001 longitudinal between genotypes; #p<0.05, ###p<0.001 transverse between genotypes; +++p<0.001 angles within genotype.
4. T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice

To further investigate the re-organisation of t-tubules, the proportion of t-tubules occurring at longitudinal angles (0-18°) was compared to the transverse (72-90°) proportion of t-tubules in all three genotypes (Figure 4.5D). This confirmed that in control cardiomyocytes, the majority of t-tubules are running at transverse angles (55.4 ± 2.8%), with longitudinal t-tubules occurring at a significantly lower frequency (10.3 ± 1.1%; p<0.001). This same trend was observed in the JPH2-OE mouse cardiomyocytes, with a significantly higher proportion of transverse orientated t-tubules compared to longitudinal (p<0.001); however, when compared to control, the proportion of transverse tubules was reduced (32.2 ± 2.1%; p<0.05), with an increased frequency of those at longitudinal angles (18.7 ± 1.1%; p<0.05). Following JPH2 knockdown, it was observed that there was no difference in the proportions of t-tubules occurring at longitudinal and transverse angles, confirming that there is significant disruption to t-tubule organisation. Furthermore, compared to controls, there was a significant reduction in the frequency of transversely orientated tubules in the JPH2-KD cardiomyocytes (23.4 ± 2.2%; p<0.001), which was accompanied by a significantly increased proportion of longitudinal t-tubules (23.9 ± 1.7%; p<0.001). Together, these findings reveal that there is significant remodelling of the t-tubule network due to altered JPH2 expression.

4.3.1.2 JPH2 expression influences co-localisation between t-tubules and JPH2

Due to the observed finding that JPH2 expression influences t-tubule organisation, dual dSTORM imaging was performed to examine the nanoscale interaction between JPH2 and the t-tubules. This super resolution imaging revealed that in control cardiomyocytes, both the t-tubules and JPH2 showed clear striations in the transverse direction, with the two labels aligned throughout the cell, and a moderate percentage of co-localisation apparent (Figure 4.6B). T-tubule disorganisation was also seen in super resolution images of ventricular myocytes from JPH2-KD mice, with a limited presence of JPH2 apparent and reduced levels of co-localisation between the two labels compared to controls (Figure 4.6A). Over-expression of JPH2 was found to produce regularly aligned t-tubules throughout the cardiomyocyte, with additional longitudinal connections present (Figure 4.6C), confirming the observations from confocal imaging. In addition, dual labelling revealed that the large JPH2 clusters previously observed (see Chapter 3) are localised along the t-tubules and are often seen at the branch points, with a similar degree of co-localisation compared to controls.

Co-localisation analysis was performed on these dual labelled dSTORM images, which confirmed the above visual observations. A reduction in the percentage of co-localisation in JPH2-KD mice was observed, with 9.9 ± 1.3% of the t-tubule labelling co-localised with JPH2, while only 12.8 ± 1.3% of JPH2 was co-localised with t-tubule labelling (n= 2 animals; 17
cells), which is significantly reduced compared to 20.5 ± 1.5% and 22.2 ± 1.4%, respectively, in the control cells (n= 2 animals; 17 cells; p<0.001 both). Furthermore, this analysis revealed that following JPH2 over-expression, 23.9 ± 1.2% of JPH2 was co-localised with t-tubule labelling, while 20.6 ± 0.9% of t-tubule labelling was co-localised with JPH2 (n= 2 animals; 16 cells) in JHP2-OE cells. These values were not statistically different from those in control cells. These findings reveal that loss of JPH2 has an impact on association of JPH2 clusters with the t-tubules, and further confirms the influence of JPH2 expression on the organisation of the t-tubules at the nanoscale level.

Figure 4.6 Reduced co-localisation of JPH2 with t-tubules after knockdown

Super resolution images showing dual immunolabelling of t-tubules (grey) with JPH2 (green) in A) JPH2-KD, B) control and C) JPH2-OE mouse cardiomyocytes. Scale bars: 1.5 µm. Analysis showing the percentage of D) t-tubule co-localised with JPH2 labelling and E) JPH2 co-localised with t-tubule labelling in super resolution images from the three genotypes. Data displayed as mean ± SEM; ***p<0.001.
4.3.2 NCX organisation is influenced by JPH2 expression

While it has been established that NCX localises to the t-tubules (Frank et al., 1992), previous research provided inconsistent reports of whether it is also localised to the junctional region in cardiomyocytes, and the extent of this junctional NCX, with values of ~6-42% reported (Scriven et al., 2000, Jayasinghe et al., 2009). Furthermore, recent findings indicate that NCX activity is altered in response to loss of JPH2, which also influences EC coupling processes (Wang et al., 2014). In order to determine the distribution of NCX, and if this is influenced by the expression of JPH2, dual labelling with RyR (as a junctional marker) was performed in the transgenic mouse isolated cardiomyocytes. Some of these findings have previously been accepted for publication (Wang et al., 2014).

dSTORM imaging of the dual-labelled cardiomyocytes revealed that the NCX distribution is altered following the knockdown of JPH2 compared to controls. The control cells show a regular, linear organisation of NCX occurring transversely across the cardiomyocyte, with occasional longitudinal connections present, and a modest degree of association with RyR (Figure 4.7B). In the JPH2-KD cardiomyocytes NCX appeared disorganised compared to controls, with a reduction in the regular, transverse labelling present and an increase in the longitudinally orientated labelling (Figure 4.7A). In addition, there appeared to be a loss of association between NCX and RyR following knockdown of JPH2. Over-expression of JPH2 showed an overall pattern of NCX labelling similar to that observed in controls, with a regular, transverse arrangement and an apparent increased association with RyR (Figure 4.7C). In all genotypes the same overall pattern of RyR labelling observed was the same as that previously detailed in Chapter 3.

These dual labelled dSTORM images were processed for co-localisation analysis, which confirmed the observation of a reduction in NCX-RyR association following JPH2 knockdown compared to control. It was found that 11.7 ± 0.8% of NCX was co-localised with RyR in JPH2-KD cells (n= 2 animals; 25 cells), which was significantly reduced from 17.7 ± 0.8% in control cardiomyocytes (n= 2 animals; 23 cells; p<0.001). The fraction of RyR co-localised with NCX was also reduced to 17.1 ± 0.9% in JPH2-KD mice, compared to 25.8 ± 1.6% in control (p<0.01; Figure 4.7D). Additionally, this analysis confirmed the observation of JPH2-OE cardiomyocytes exhibit an increased the level of association between these two proteins compared to controls, with 31.6 ± 0.8% of NCX co-localised with RyR, and 31.5 ± 0.9% of RyR co-localised with NCX (n= 2 animals; 23 cells; p<0.001 and p<0.05 respectively; Figure 4.7D). These findings indicate that JPH2 expression plays a role in the localisation of NCX to the junctional region.
4.3.3 Altered RyR-myofibril organisation

In order to achieve contraction of the cardiomyocyte, the calcium released from the SR by the RyR must reach the contractile proteins, the myofilaments (Bers, 2002). Therefore the organisation of the RyR channels in relation to the myofilaments is important for determining the efficiency of EC coupling. Transverse sections of transgenic mouse ventricular myocardium were used so that the organisation of the RyR clusters around the myofibrils at the level of the z-disk, as indicated by α-actinin labelling, could be determined. It was observed that the α-actinin labelling was very dense within the z-disk for all three genotypes, with inter-myofibrillar spaces which were occupied by RyR clusters, and presumably additional organelles, such as mitochondria. In control cardiomyocytes, the RyR clusters
were predominantly found localised around α-actinin, with the RyR clusters appearing to wrap around the myofibrils (Figure 4.8A). This same overall pattern was observed in the JPH2-KD mice, however, there was an apparent reduction in the size of RyR clusters contacting the myofibrils (Figure 4.8B). The JPH2-OE mice showed the presence of RyR clusters larger than those found in control mice, as previously reported (Chapter 3), with RyR clusters often extending more towards the inter-myofibrillar spaces rather than extending along the perimeter of the myofibril, leading to reduced contact with the myofibril (Figure 4.8C).

**Figure 4.8 Changes in contact between RyR and myofibrils in JPH2 transgenic mice**

Dual labelled super resolution images showing RyR (red) and α-actinin (cyan) in transverse myocardium sections from A) control, B) JPH2-KD and C) JPH2-OE mice. Scale bar: 1 μm. D) Graph of the resulting contact analysis showing the percentage of the myofibril perimeter in contact in RyR. Data displayed as mean ± SEM; *p<0.05.
Analysis of the contact between RyR clusters and the myofibrils revealed that in all three genotypes there is a linear correlation between increasing myofibril size (perimeter) and the length of RyR label making contact with the myofibril, with this same relationship previously reported in rat cardiomyocytes (Hou et al., 2014). Following further analysis, it was observed that the percentage of the myofibril perimeter that was in direct contact with RyR clusters was significantly reduced in both JPH2-KD (n= 2 animals, 5 cells) and JPH2-OE (n= 2 animals, 7 cells) cardiomyocytes, to 31.7 ± 1.1% and 31.6 ± 1.0%, respectively, compared to 35.1 ± 1.2% in control (n= 2 animals, 7 cells; Figure 4.8D; p<0.05 for both). These results indicate that JPH2 plays a role in the organisation of the CRUs in relations to key EC coupling structures in the cardiomyocyte.

4.4 Discussion

A combination of confocal and super resolution imaging have been used in this chapter to present findings on the changes in the organisation of the t-tubules and EC coupling proteins in response to altered JPH2 expression levels. These results complement not only those findings presented in chapter 3, but also the previously detailed functional experiments from these transgenic mice.

4.4.1 JPH2 influence on t-tubule organisation

One of the primary structures examined in this chapter was the t-tubule network, looking at both the cellular (confocal) and nanoscale (super resolution) organisation. At the cellular level, there is an overall regular, transverse pattern of organisation observed in the isolated cardiomyocytes from control mice. This is in agreement with the widely observed localisation of the t-tubules to the z-disk in cardiac myocytes, where they play a role in action potential propagation and are involved in the formation of junctions with the SRJ (Brette and Orchard, 2003). The visual observation of aberrant t-tubules running throughout the cardiomyocytes following knockdown of JPH2 provided evidence of disruption to t-tubule organisation. This was confirmed by the significant decrease in TT-power amplitude in the JPH2-KD mice compared to controls, with this reduction of TT-power in agreement with previous work on live cardiomyocytes from the same mouse strain (Van Oort et al., 2011), indicating that loss of JPH2 results in t-tubule disorganisation. The consistency in these findings provides support for the claim that these results are not an artefact of cellular fixation or immunolabelling techniques used, enabling confidence in these and further immunolabelling results achieved.
Furthermore, it has repeatedly been shown in previous studies that disorganisation of t-tubules is one of the hallmark structural changes in heart failure (Song et al., 2006, Wei et al., 2010, Crossman et al., 2015, Wagner et al., 2012), with our results further supporting this, as acute knockdown of JPH2 results in heart failure development in these mice. In contrast, confocal imaging of cardiomyocytes over-expressing JPH2 revealed that the regular, transverse organisation of t-tubules is maintained, which was confirmed by the FFT results revealing no significant change in TT-power in the JPH2-OE mice compared to control. These results are in agreement with recent findings by Guo et al 2014, which used a similar JPH2-OE mouse strain and also identified no changes to t-tubule regularity using in situ MM-4-64 labelling in perfused hearts.

Additional analysis performed in this study was able to reveal a greater level of detail into the organisation of t-tubules, and how this is altered in these JPH2 transgenic mice, which has previously not been reported. In control mice, this revealed a strong preference toward t-tubules orientated transverse to the longitudinal axis of the cardiomyocyte, with the majority (~55%) of t-tubules occurring at transverse angles, which strongly supports both the visual observations made and the functional role of the t-tubules. Additionally, the observation of t-tubules occurring at longitudinal or oblique angles is in agreement with previous reports of ~40% of t-tubules occurring between z-disks, forming longitudinal extensions (Soeller and Cannell, 1999). The angle analysis also revealed insights as to how the disorganisation of the transverse component of the t-tubule network was occurring following loss of JPH2. The observation of ‘randomly’ orientated t-tubules is supported by the finding that the t-tubules are occurring at similar proportions for the different angles throughout the cardiomyocyte, with no significant difference in the proportions of transverse and longitudinal t-tubules in JPH2-KD mice. The increase in the longitudinal component, with the reduction in the transverse t-tubules compared to control has also been previously reported in the SHR heart failure rat model (Song et al., 2006). These findings indicate that there has been a loss of anchoring of the t-tubules to a z-disk localisation, such that they have become aberrantly organised. Given the evidence of JPH2 playing a role in the anchoring of the t-tubule to the SR, (and therefore z-disk) (Takeshima et al., 2000), the reduction of JPH2 protein levels in these mice provides a clear mechanism for this t-tubule disorganisation. Furthermore, this loss of t-tubule organisation has been suggested as one of the potential mechanisms by which JPH2 influences EC coupling processes (Van Oort et al., 2011). The loss or reduced regularity of the transverse t-tubules leads to a reduction in synchronous activation of the CRUs (Øyehaug et al., 2013), and results in abnormalities of the calcium transient, including a reduction in amplitude (Song et al., 2006, Brette et al., 2002). This is one of the observed functional changes in these mice, with significantly reduced calcium transient amplitudes (Van Oort et
In addition, the displacement of the t-tubules from the z-disk could result in an increased distance across the junctional cleft. Based on the theory of local control, this would result in a reduced ability to control local ion concentrations within this space, which results in a higher $I_{Ca}$ being required to trigger calcium release from the RyR through CICR to achieve release equivalent to that of a healthy dyad (Stern, 1992, Wier et al., 1994). This is observed as a loss of EC coupling gain, which has also previously been reported in these JPH2 deficient mice (Van Oort et al., 2011).

The observation of increased longitudinal branching in JPH2-OE mice was confirmed with the detailed t-tubule angle analysis. It was found that while the majority of t-tubules were occurring at transverse angles, this proportion was reduced in comparison to control mice, with a complementing increase in the proportion of longitudinal t-tubules. Unlike the JPH2-KD mice, in which the altered angle distribution reflected t-tubule disorganisation, in the JPH2-OE mice, the increased longitudinal fraction is due to increased branching, which is additional to the regular transverse organisation, rather than their re-organisation. This is supported by the lack of change in the TT-power compared to controls, indicating that the underlying transverse regularity is indeed maintained. The exact mechanism by which increased JPH2 expression results in enhanced t-tubule branching is unclear, however one potential factor is that, unlike the JPH2-KD mice in which expression changes were induced in adult mice (to circumvent embryonic lethality), the JPH2-OE mice are over-expressing the protein prior to birth. It has been shown that JPH2 plays a role in t-tubule development in the post-natal mouse (Chen et al., 2013, Han et al., 2013), and that over-expression of JPH2 results in an enhanced rate of maturation (Reynolds et al., 2013). Coupled with this, is the observation that in the early stages of t-tubule development in striated myocytes, there is initially a higher density of longitudinal elements, becoming more transverse with further development (Takekura et al., 2001, Han et al., 2013). This means that it is possible that the observed increased longitudinal branching in JPH2-OE mice is the result of reduced ‘pruning’ or re-organisation of these branches during t-tubule maturation. Regardless of whether these changes occur in utero or postnatally, it is clear that the increased JPH2 protein levels influence the organisation of the t-tubule network. Functionally, over-expression of JPH2 does not appear to have detrimental effects in these mice, with global calcium handling maintained in the form of calcium transient amplitude (Wehrens’ laboratory, unpublished), which was also seen in a similar mouse strain (Guo et al., 2014). These findings indicate that the increased branching of the t-tubule network observed in these mice is not producing abnormal electrical signalling within the cardiomyocytes, and furthermore, JPH2 over-expression was found to be protective against t-tubule remodelling in a pressure-overload model of heart failure (Guo et al., 2014).
4. T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice

4.4.1.1 Co-localisation with JPH2

dSTORM imaging of the t-tubule network/JPH2 dual labelled isolated cardiomyocytes revealed that, in control mice, ~20% of the t-tubule labelling is associated with JPH2. This value is lower than previous reports of the fraction of t-tubules associated with junctional SR regions (~40%) in mouse myocardium (Page and Surdyk-Droske, 1979); however, this may be due to previous methods relying on approximations for whole cell measurements based on small regions of imaging, along with potential angle orientation artefacts from EM imaging. Co-localisation analysis also indicated that, in these control mice, ~22% of JPH2 was associated with the t-tubules. Despite this seeming to be a low association given the reported role of JPH2 in anchoring the t-tubules and SRJ together (Takeshima et al., 2000), it can be seen that the majority of the protein still follows the organisation of the t-tubules across the cardiomyocytes. Given the complex three-dimensional network of the mouse t-tubule system (Hayashi et al., 2009, Jayasinghe et al., 2015), it is possible that these junctional regions are associated with t-tubules occurring out of the focal plane, particularly as it has been seen that the formation of junctions can involve the SRJ wrapping around the t-tubule from multiple sides (Hayashi et al., 2009). It may also represent incomplete visualisation of the t-tubule labelling in the dSTORM images due to the reported inability of many conventional dyes and antibodies to fully label the t-tubule network in fixed samples (Jayasinghe et al., 2015), however, the effects of this have been reduced by using a previously established combination of antibodies targeting NCX and cav-3, both of which are known to localise to the t-tubules (Thomas et al., 2003, Frank et al., 1992, Scriven et al., 2005), and summate to provide the most complete impression of the t-tubules available to date (Jayasinghe et al., 2012b).

Following the knockdown of JPH2, it was found that of the remaining JPH2, a reduced proportion was co-localised with t-tubules, and furthermore, there was significantly less t-tubule labelling associated with JPH2. These findings are in agreement with the previously reported reduction in the number of functional junctional regions in JPH2-KD mice based on EM measurements (Van Oort et al., 2011). As JPH2 contains an SR membrane spanning domain (Takeshima et al., 2000), it would be expected that this protein would therefore follow the organisation of the SR, which is typically localised to the z-disk (Fawcett and McNutt, 1969). Therefore, the observed disorganisation of the t-tubules away from the z-disk in these mice would reduce the ability for potential interaction with JPH2. In addition to the t-tubules, it has been reported that the SR network undergoes remodelling in a paced sheep heart model of heart failure (Pinali et al., 2013). Considering that the remaining JPH2 is not found predominantly localised to the z-disk, it is therefore likely that a combination of t-tubule disorganisation and SR remodelling are contributing to the reduced association between
4. T-tubules and EC coupling protein organisation in adult JPH2 transgenic mice

these labels in the JPH-KD mice. While the number of CRUs was found to be unchanged in these mice, the above findings along with previous EM work (Van Oort et al., 2011) indicate that there is a reduction in the functional coupling of the t-tubules with the SR$_J$. As previously discussed, this would result in reduced EC coupling gain and an inability to synchronously activate CRUs, both of which are observed abnormalities in these mice.

Interestingly, it was observed that over-expression of JPH2 did not alter the extent of association between JPH2 and t-tubules. This indicates that despite the increased level of JPH2 protein present in these mice, it is distributed in the same overall pattern as in control mice, such that the same proportion is associated with the t-tubules. It would be expected that with more JPH2 present in the cardiomyocyte, more of the t-tubule network would be associated with the protein. However, the fact that the fraction of t-tubule labelling co-localised was unchanged in these mice suggests that the increased branching of the network is also forming associations with JPH2. This can be seen by the presence of large JPH2 clusters often occurring at the branch points of the t-tubules, meaning that they are co-localised with more of the t-tubule network at these points compared to a non-branched region. The similarity in co-localisation between the t-tubules and JPH2 suggests that the docking of the SR$_J$ and t-tubules is maintained and therefore, functionally, these mice over-expressing JPH2 are comparable to controls in terms of global EC coupling function. This is indeed what is observed in these mice (Wehrens’ laboratory, unpublished)(Guo et al., 2015).

However, it was observed that in the mouse strain used in this study, there was heterogeneity in the wavefront of the calcium transient, which when summated over the cardiomyocyte resulted in the overall calcium transient amplitude being unchanged compared to controls (Wehrens’ laboratory, unpublished). As detailed in Chapter 3, JPH2 over-expression was associated with an observed increase in the size of the RyR clusters, with longitudinal extensions into the cardiomyocytes often present. It was further observed that these larger RyR clusters showed high co-localisation with large JPH2 clusters. This coupled with the identification of these JPH2 clusters often localising to t-tubule branch points suggests that the large RyR clusters may often be occurring at longitudinal branch points of the t-tubules. This would not only form a basis for the extension of these RyR clusters longitudinally into the cell along the t-tubules, but could also influence the appearance of the calcium transient at these points. The longitudinal branching of the t-tubule would allow for the propagation of the action potential in this direction, activating the CRUs localised along this t-tubule connection. This activation of large, longitudinally extending RyR clusters would result in large calcium release along the cardiomyocyte, contributing to the ‘high’ points observed in the calcium transient.
transient. However, when combined with the observed increased nearest neighbour distance the overall result is of a calcium transient of similar amplitude to controls.

4.4.2 Alterations in junctional organisation of NCX

The co-localisation of NCX with RyR was determined using dual dSTORM labelling to investigate the extent of junctional localisation of the exchanger. It was observed that the NCX labelling primarily followed a regular, transverse organisation throughout the control cardiomyocytes, with the occasional longitudinal extension. This is similar to the pattern observed for t-tubule organisation in these mice, and given the high localisation of NCX to the t-tubules (Frank et al., 1992, Kieval et al., 1992) it is not unexpected that NCX would show the same general distribution. In control cardiomyocytes, it was also revealed that there was a modest proportion of NCX labelling co-localised with RyR, suggesting ~18% of NCX is directly localised to the junctional regions. Further confirming this, ~26% of RyR labelling was associated with NCX, clearly indicating that a sub-population of NCX has a junctional localisation. NCX is the main calcium extrusion mechanism in the cardiomyocyte (Bers, 2001), with previous studies providing inconclusive evidence as to the extent of its localisation to the junctional space (Jayasinghe et al., 2009, Scriven et al., 2000), where it has been suggested to play a role in CICR via the reverse mode of the exchanger (Leblanc and Hume, 1990). Findings from this study clearly support the nanoscale localisation of NCX to the junction, where it would have the ability to contribute to EC coupling mechanisms.

Knockdown of JPH2 resulted in visible re-organisation of NCX throughout the cardiomyocyte, with a reduction in transverse labelling coupled with increased longitudinal distribution. These changes correspond with the overall pattern of t-tubule re-organisation observed in the JPH2-KD mice. In addition to the visible alteration in the pattern of protein distribution, JPH2 expression levels were also found to influence the degree of co-localisation between NCX and RyR, indicating changes in the junctional localisation of NCX. In JPH2-KD cardiomyocytes, there was a reduction in the junctional sub-population of NCX, with only ~12% of NCX co-localised with RyR, and ~17% of RyR co-localised with NCX. Interestingly, previous studies of human and animal heart failure have reported up-regulation of NCX as a potential compensatory mechanism (Hasenfuss et al., 1999, Pogwizd et al., 1999, Sipido et al., 2000), however there was no change in NCX expression in this JPH2-KD mouse model of heart failure (Wang et al., 2014). As there is also no change in RyR expression levels in these mice (Van Oort et al., 2011), the changes observed in NCX co-localisation with RyR are therefore due to protein redistribution. Recent work has suggested that in addition to ‘leaky’
RyR channels via reduced stabilisation by JPH2, JPH2-KD mice also show a reduction in NCX activity which may contribute to the observed increase calcium spark rate by increasing junctional calcium levels (Wang et al., 2014). The super resolution co-localisation data supports these findings (and has been included in the recent publication), whereby the reduced junctional localisation of NCX impairs the ability to extrude calcium following the calcium transient. As there is no compensatory change in SERCA expression (Van Oort et al., 2011), this leads to a build-up of calcium in the junctional cleft which is able to trigger spontaneous calcium release from the RyR (Wang et al., 2014), resulting in increased RyR channel leak. These findings provide a second potential mechanism by which loss of JPH2 results in abnormal calcium handling of the RyR (as seen by increased calcium spark frequency) in a model of heart failure development.

In JPH2-OE cardiomyocytes, the distribution of NCX was again found to follow the overall observed organisation of the t-tubules. In addition, it was found that the junctional sub-population of NCX was increased compared to control, with ~32% of NCX associated with RyR labelling, and ~32% of RyR co-localised with NCX. Interestingly, while there is no evidence of RyR expression changes as a result of JPH2 over-expression, studies have found that over-expression of JPH2 leads to a subsequent increase in expression levels of NCX (Guo et al., 2014)(Wehrens’ laboratory, unpublished). In the present transgenic mouse strain, a ~1.4-fold increase in NCX protein expression was found compared to control mice (Wehrens’ laboratory, unpublished), which is a modest up-regulation compared to previous studies (Guo et al., 2014). This suggests that some of the observed changes in NCX localisation are a result of altered protein expression in these mice. The increased co-localisation of NCX and RyR following JPH2 over-expression is most likely to be due to a combination of increased NCX expression together with the previous observation of increased RyR cluster size (see Chapter 3). Therefore, not only is there more NCX present to localise to the junctional region, but the RyR clusters within the junction cover a larger area to potentially associate with NCX. JPH2-OE mice appear to have no detrimental effect on overall function due to increased NCX expression or junctional localisation, which is interesting based on previous findings from NCX over-expressing mice, which show impaired EC coupling properties, including altered EC coupling gain and depressed calcium transients (Pott et al., 2004, Goldhaber et al., 2005, Reuter et al., 2004). It is possible that in this JPH2 transgenic mouse strain that the increased NCX levels result in a decreased junctional calcium concentration at rest, further contributing to the reduced frequency and size of calcium sparks observed in these mice. However, it has not as yet been determined if the increased NCX protein levels in these mice correspond to an increase in NCX activity.
4.4.3 Association between myofibrils and RyR

The association of the RyR clusters with the myofilaments is important for the ability of calcium released from the CRUs to trigger mechanical contraction of the cardiomyocyte (Bers, 2001). In control mice, it was observed that ~35% of the myofibril perimeter (as determined by α-actinin labelling) was in contact with RyR labelling. While this value is lower than previous reports from similar measurements in rat of ~57% (Hou et al., 2015), one possibility for this is subtle differences in dSTORM image acquisition techniques. The present study used single plane imaging, while previous work acquired sequential z-steps such that the final image used was a projection of a small range of focal planes. Given the non-planar alignments of the z-disk (Jayasinghe et al., 2010), this latter approach would potentially detect more of the RyR clusters associated with myofibrils at a slightly shifted focal planes, resulting in a greater incidence of identified RyR-myofibril association. Despite this, the present findings still indicate a clear relationship between myofibril perimeter and the extent of associated RyR clusters in these mice, with similar reported findings of the proportionality of RyR cluster contact to the myofibril perimeter (Hou et al., 2015).

Following loss of JPH2, there is a small, yet statistically significant reduction in the myofibril perimeter associated with RyR labelling (~32%) compared to controls (35%). While it is possible that this small degree of change would not be physiologically significant to the overall function of the cardiomyocyte, when coupled with the decrease in EC coupling gain observed in these JPH2-KD mice (Van Oort et al., 2011), it is likely that the contractility of the myocyte is even further impaired as result of reduced RyR-myofibril contact. Furthermore, these findings are consistent with previous observations that in human heart failure there is a reduction in the density of RyR clusters per volume of myofibril (Crossman et al., 2011). While this induced knockdown of JPH2 in adult mice does result in acute heart failure development with reduced ejection fraction (Van Oort et al., 2011), it is difficult to distinguish the contribution of impaired mechanisms determining EC coupling gain and the possibility of reduced myofilament activation based on currently available evidence.

The percentage of the myofibril perimeter in contact with RyR clusters was also found to be reduced in JPH2-OE mice to ~32%. Based on the previous finding of increased RyR cluster size in these mice, it may have been expected to see an increase in this parameter following JPH2 over-expression. However, it was observed that while the particularly large RyR macro-clusters previously identified were typically in contact with α-actinin labelling, they often extended towards the cytosol, rather than following the contour of the myofibril. Based on the large area covered by these clusters, it is possible that upon calcium release, the ion diffusion...
would still be sufficient to activate myofilament contraction. However, again, the physiological significance of this reduction in RyR-myofibril contact cannot be determined based on current functional data for these mice.

4.4.4 Significance of findings

The findings presented in this chapter, along with those presented in chapter 3, reveal a clear relationship between the expression of JPH2 and the organisation of key cardiomyocyte components, including the RyR clusters, t-tubules and junctional protein organisation. The use of dSTORM imaging has enabled the identification of novel information regarding these components and has provided new insights into the potential mechanisms underlying the functional role of JPH2. These include maintenance of junctional protein organisation, including RyR and NCX, stabilisation of the RyR, and maintaining t-tubule organisation. Not only has the structural basis of many aspects of altered EC coupling as a result of reduced JPH2 levels been identified, but it has been shown that changes in the nanoscale organisation of junctional proteins do not always result in detrimental cardiac functional changes, as observed following JPH2 over-expression. Previous studies have shown that reduction of JPH2 is associated with both human and animal models of heart failure (Landstrom et al., 2011, Woo et al., 2010, Xu et al., 2007, Minamisawa et al., 2004). Proposed mechanisms contributing to this protein down-regulation include µ-calpain mediated cleavage of full-length JPH2 protein (Murphy et al., 2013, Wu et al., 2014), as well as interference at the mRNA level by miRNA-24 (Li et al., 2013, Xu et al., 2012). While the exact mechanism is not fully understood, there has been increasing interest in determining the link between this JPH2 reduction and the associated EC coupling alterations. This study has been able to provide strong support for potential mechanisms contributing to this link. It has been proposed that JPH2 could be targeted for therapeutic treatment of heart disease and failure based on previous findings (Van Oort et al., 2011). The identification of non-harmful effects of JPH2 over-expression to the nanoscale organisation of key EC coupling structures (and previously reported functional changes (Wehrens’ laboratory, unpublished)) suggest that if JPH2 gene therapy was implemented, the potential for moderate over-compensation of protein expression levels would not necessarily be detrimental to the patient.

4.4.5 Limitations and future direction of JPH2 transgenic mouse study

One of the key differences in development of the JPH2 transgenic mouse strains is that JPH2 knockdown is induced in the adult mouse (to circumvent embryonic lethality), while the over-
expression of JPH2 is present from embryogenesis. It is therefore possible that a different pattern of alterations in t-tubule and RyR cluster organisation would be observed if over-expression was induced in adult mice. This would also more closely mimic the potential use of JPH2 for gene therapy treatment in human heart disease or failure. Further studies examining these or similar mouse strains could potentially investigate this line of transgenic expression. Furthermore, future work examining the role of JPH2 on cardiac function could potentially examine its role in the establishment of adult EC coupling in the cardiac muscle at the tissue or cellular level (such as trabeculae or isolated myocytes). This would help determine the differences between CICR impairment in these mice, and other EC coupling mechanisms, such as the ability of the calcium released from the RyR to trigger contraction of the myofilaments.

4.5 Concluding Remarks

Based on previous studies, three main potential mechanisms were proposed as to how JPH2 contributes to EC coupling in the cardiomyocyte by maintaining: 1) junctional protein organisation, 2) RyR channel stability, and 3) t-tubule organisation. The use of dSTORM super resolution imaging in these experiments has allowed the identification of changes in the nanoscale organisation of key EC coupling and structural proteins which are associated with altered JPH2 expression levels. These changes in organisation provided evidence to support all three of the proposed mechanisms of the functional role of JPH2 in the cardiomyocyte. Both loss and over-expression of JPH2 altered junctional protein organisation in the form of either RyR cluster properties or its co-localisation with other junctional proteins (JPH2, NCX). The spatial association between JPH2 and RyR was also altered in response to JPH2 expression levels, and perhaps more interestingly, so was the relative densities of these proteins within the junction, which have the potential to alter the stabilisation effect of JPH2 on the RyR channel. Findings presented in this chapter clearly showed a link between JPH2 levels and the organisation of the t-tubules at both the cellular and nanoscale level. Together, all these results provide novel findings regarding the mechanistic role of JPH2 in the organisation of the cardiomyocyte, supporting the proposed role of this protein in influencing key functional EC coupling properties.
Chapter 5. **Structure-function relationship in human heart failure**

5.1 Background

Cardiovascular disease, including cardiomyopathy and heart failure, is the leading cause of morbidity and mortality in the developed world, and is associated with significant healthcare costs (WHO, 2014). Dilated cardiomyopathy (DCM) is the most common form of heart disease, and is characterised by the dilation of one or more chambers of the heart, while ventricle wall thickness typically remains unchanged (Mestroni et al., 2014). While there is a high prevalence of familial cases of DCM caused by genetic mutations, idiopathic DCM (IDCM) is the most prevalent from of this pathology. DCM commonly progresses into heart failure, with DCM being the most prevalent underlying cause of heart transplant procedures (Hong et al., 2012). Loss of cardiac function is the hallmark of heart failure in which there is an inadequate ability to pump blood to meet metabolic demands of the body (Forbes and Sperelakis, 1984). Heart failure can develop from a number of causes including the progression of cardiomyopathy. There is a poor prognosis of ~50% mortality within 5 years of diagnosis associated with heart failure (Bers, 2001), and it affects ~4/1000 people in the general population, with an increasing prevalence in the elderly (McMurray and Stewart, 2000). While animal models are invaluable for identifying and understanding potential mechanisms involved in development and progression of heart disease and failure, it is essential that these processes are also examined in human heart failure samples to validate the translation of these identified mechanisms.

5.1.1 Structural and EC coupling changes in human heart failure

The inability of the failing heart to meet metabolic demands of the body has been attributed to several changes at both the tissue and cellular level which result in loss of cardiac function.
However, it has been shown that within failing human hearts there is heterogeneity in the severity of impaired function (Crossman et al., 2015). At the tissue level, it has been well characterised that there is increased interstitial fibrosis present throughout the diseased or failing myocardium (Maron et al., 1975, Vahl et al., 1993, Weber et al., 1989). This causes stiffening of the ventricle wall such that the reduced compliance makes it harder to generate force (Vahl et al., 1993). Due to the increased collagen content of the myocardium, there is a decrease in the fractional myocyte content of the tissue, which can occur in the absence of necrosis (Weber et al., 1989). This makes it difficult to distinguish whether this composition change is due to an actual loss of cardiomyocytes or an increase in the extracellular matrix (ECM), or both. Within the cardiomyocytes there are several well-characterised changes to structure, including the disruption of the t-tubule system (Lyon et al., 2009, Crossman et al., 2015, Zhang et al., 2013). It has also been shown that the severity of this disorganisation is strongly correlated with extent of cardiac dysfunction, as identified using tagged MRI in heart failure patients to measure circumferential shortening of the ventricle wall (Crossman et al., 2015). The dislocation of the t-tubules from the z-disk results in reduced regularity of the network, which impairs the calcium transient and the synchronicity of contraction (Louch et al., 2004), which are characteristic of heart failure (Lyon et al., 2009, Guo et al., 2013). T-tubule disruption has been identified as impairing action potential propagation (Sacconi et al., 2012), which directly negatively impacts the calcium transient (Crocini et al., 2014). In addition, due to the displacement of the t-tubules, there is a loss of their association with the SR, which subsequently results in ‘orphaned’ RyR (Song et al., 2006). There is also increased RyR cluster distances from the sarcolemma (Sachse et al., 2012) and reduced RyR co-localisation with DHPR (Crossman et al., 2011). This leads to reduced gain of EC coupling due to the increased distance \( I_{Ca} \) must diffuse to activate the RyR (via CICR), as well as a reduced ability of RyR-mediated calcium release to inactivate \( I_{Ca} \) (Song et al., 2006).

In the healthy human heart, the force-frequency response (FFR) is positive, with increasing contractile strength as heart rate is increased; however, in addition to the overall impairment of cardiac function, one of the commonly reported alterations in human heart failure is the development of a blunted, or even negative, FFR (Brixius et al., 2001, Mulieri et al., 1992, Schwinger et al., 1993). It has been suggested that this is attributed to a combination of cellular mechanisms resulting in abnormal calcium homeostasis, including the loss of SERCA expression/activity (Pieske et al., 1995, Gwathmey et al., 1987, Takahashi et al., 1992) which can also be coupled with increased activity of NCX (Hasenfuss et al., 1999). This shifts the balance of calcium removal from the cytosol towards extrusion from the myocyte, as opposed to the usually favoured SR re-uptake (via SERCA) (Pieske et al., 1999). Overtime, this
results in the progressive decline of SR calcium content, leading to the decreased amplitude of the calcium transient, and subsequently reduced active force generation (Pieske et al., 1999). It has also been proposed that altered sensitivity and responsiveness of the myofilaments plays a role in the development of the attenuated FFR in the diseased human heart (Gwathmey et al., 1990).

5.1.2 JPH2 in human cardiomyopathy

The role of JPH2 in heart disease and failure is not yet fully understood, although both down-regulation (Landstrom et al., 2011, Zhang et al., 2013) and mutations of the protein (Landstrom et al., 2007, Matsushita et al., 2007) have been observed in human heart failure. JPH2 is thought to promote the formation and maintenance of the cardiac junctions, as well as maintaining the organisation of the t-tubules (Takeshima et al., 2000, Van Oort et al., 2011, Zhang et al., 2013). As both these structures have been identified as being altered in heart failure, it has been suggested that JPH2 may play a role in the development or progression of human heart disease. One of the proposed mechanisms underlying the alteration of JPH2 function in heart disease is through impaired trafficking of the protein due to microtubule densification (Zhang et al., 2014). The observed redistribution of JPH2 was also associated with t-tubule remodelling, providing further evidence of a mechanistic link between JPH2 and potential alterations in cardiomyocyte organisation in heart disease and failure (Zhang et al., 2014). Furthermore, it has been shown that increased microtubule densification was associated with reduced fractional shortening of the ventricle wall following aortic stenosis (Wang et al., 1999), suggesting a potential relationship between these structural alterations and cardiac function.

The aim of the experiments presented in this chapter was to examine the cardiac structure-function relationship in the failing human heart. In particular, changes in cardiomyocyte organisation and the role of JPH2 in this relationship were of interest. Cardiac trabeculae from failing human hearts were used for both functional and structural experiments, which enabled direct correlation of structural alterations and the resulting functional performance. They were also used to compare with structural results from the ventricular wall of the same failing hearts, and non-failing controls, to determine the validity of cardiac trabeculae as a model for the ventricular myocardium. Changes to the organisation of key EC coupling structures and proteins were investigated using confocal microscopy, including the t-tubules, ECM, JPH2, the SR calcium release channel, RyR, and the cytoskeletal protein α-tubulin.
5.2 Experimental Techniques and Analysis

Ethical approval was obtained from the New Zealand Health and Disabilities Ethics Committee (NTY/05/08/050) for the collection of human samples, along with written, informed consent of the heart transplant recipients and the families of organ donors (in the case of control tissue). A combination of myocardium and cardiac trabeculae from non-ischemic IDCM hearts in end-stage heart failure were used for these experiments, together with pathologically normal hearts as ‘non-failing’ controls. Cardiac trabeculae were not able to be obtained from non-failing hearts as none became available during the tissue collection period for this study, however previously collected non-failing free wall tissue samples were available for immunohistochemical analysis. These non-failing samples were obtained from previous organ donors that could not be matched to transplant recipients, and had a mean age of 57.7 years. Non-failing hearts were found to have parameters of cardiac function within the normal range, including ejection fraction and LV diameters based on echocardiogram assessment. Failing samples were obtained from explanted hearts during heart transplant procedures from patients diagnosed with IDCM, with a mean age of 51.2 years and cardiac ejection fractions of 9-27%, all of which were below the range for normal cardiac function (55-70%; (AHA, 2015)). A summary of the patient details are provided below in Table 5.1.

<table>
<thead>
<tr>
<th>Heart ID</th>
<th>Age</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>EF (%)</th>
<th>LVEDD</th>
<th>LVESD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH32</td>
<td>58</td>
<td>M</td>
<td>IDCM, NYHA class III-IV</td>
<td>9</td>
<td>8.2</td>
<td>N/A</td>
</tr>
<tr>
<td>DH36</td>
<td>56</td>
<td>M</td>
<td>IDCM, NYHA class III</td>
<td>25</td>
<td>7.5</td>
<td>6.6</td>
</tr>
<tr>
<td>DH37</td>
<td>53</td>
<td>M</td>
<td>IDCM, NYHA class III</td>
<td>27</td>
<td>5.2</td>
<td>4.3</td>
</tr>
<tr>
<td>DH38</td>
<td>23</td>
<td>M</td>
<td>IDCM, NYHA class III</td>
<td>19</td>
<td>8</td>
<td>N/A</td>
</tr>
<tr>
<td>DH40</td>
<td>66</td>
<td>M</td>
<td>IDCM, NYHA class III</td>
<td>21</td>
<td>7.6</td>
<td>6.9</td>
</tr>
<tr>
<td>N1</td>
<td>54</td>
<td>F</td>
<td>Normal</td>
<td>74</td>
<td>5.1</td>
<td>2.9</td>
</tr>
<tr>
<td>N2</td>
<td>62</td>
<td>F</td>
<td>Normal</td>
<td>N/A</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>N3</td>
<td>57</td>
<td>F</td>
<td>Normal</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.1 Details of patients for human heart samples

Details for the heart samples used in these experiments, including the identification number of the heart, patient age (in years), gender (M = male, F = female), pathological diagnosis (IDCM = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association functional classification of failure). Parameters as indicators of cardiac function, including ejection fraction (EF), left ventricle end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) in centimetres are also included. N/A = details not available.
5. Structure-function relationship in human heart failure

5.2.1 Preparation of failing human heart samples

Upon availability, samples were collected from explanted human hearts following transplant procedures at Auckland Hospital. This involved acquiring tissue block samples from the left and right ventricles, as well as the septum, from the failing hearts (Figure 5.1 left panel), which were collected into chilled, pre-oxygenated Tyrode’s solution (detailed in General Methods) with 0.25 mM CaCl₂ + 20 mM 2,3-butanedione monoxime (BDM, Sigma-Aldrich) to prevent cross-bridge cycling and injury to the dissected tissue (Mulieri et al., 1989). These samples were then kept oxygenated (bubbled with 100% O₂) and transported on ice back to the laboratory as soon as possible, where potentially suitable trabeculae were identified for functional experiments (Figure 5.1 right panel). Tissue block sample collection and fixation was performed by Dr David Crossman and the author, while trabeculae dissection and functional experiments were performed by Dr Xin Shen. After dissection of suitable trabeculae, the remaining wall tissue samples were dissected into small blocks and immersion fixed in 2% PFA solution (diluted in the BDM-Tyrode's solution) for 1 h at 4°C. Following completion of functional experiments (detailed in section 5.2.2), the trabeculae were pinned at a length similar to that used for functional experiments and also fixed in 2% PFA solution for 1 h at 4°C, after which they were cut in half transversely.

Figure 5.1 Human cardiac trabecula
Explanted human heart with IDCM (left panel) after the removal of left ventricular wall sample shown. Right panel shows an underlying trabecula on the septal wall prior to dissection (indicated with arrows).
All samples were then cryoprotected and processed for immunohistochemistry following the same procedure as described in General methods. The trabeculae were embedded in OCT prior to freezing and orientated such that one half could be sectioned in the transverse direction, and the other in the longitudinal direction. Immunohistochemistry was performed according to previously detailed protocols (see General methods). For these samples, the primary antibodies used were rabbit anti-JPH2 (Yenzym) together with either mouse anti-RyR (Thermo) or mouse anti-α-tubulin (Abcam). This was then followed by goat raised species-specific secondary antibodies conjugated to Alexa Fluor 488 and 568. In addition, wheat germ agglutinin (WGA) conjugated to Alexa Fluor 647 was included in the secondary incubation solution to label the t-tubule network (Jayasinghe et al., 2015) and ECM (Kostrominova, 2011). Cryosections of cardiac trabeculae and myocardium were processed together, along with the corresponding no primary antibody controls. Samples were imaged using an inverted Zeiss LSM710 confocal microscope, as described in General methods. The use of trabeculae enabled the determination of the correlation between structure and function, as well as comparison of the biological variability with the ventricular wall samples in diseased and non-failing human hearts, to assess the use of cardiac trabeculae as models for the ventricular wall.

5.2.2 Trabeculae functional experiments

Potential experimental trabeculae were identified according to appropriateness of length and diameter to fit in the force transducer rig, with small to medium diameters preferred (mean diameter 296 ± 26 µm) to ensure adequate oxygen diffusion of the tissue (Goo et al., 2009). Experimental setup and procedures were adapted from those previously described (Ward et al., 2003, Shen et al., 2013). Cardiac trabeculae were dissected from the endocardial wall of the tissue block samples (LV, RV or septum) with small blocks of ventricular tissue at each end, and transferred to a tissue bath positioned on an inverted Nikon Eclipse TE2000 microscope stage. The trabeculae were held at fixed length ($L_0$) by placing the tissue block at one end in a wire cradle which extended from a force transducer (KX801 Micro Force Sensor, Kronex Technologies). The opposite tissue block was secured in a nylon loop attached to a manipulator to enable control of trabecula length. Trabeculae were continuously superfused with oxygenated Tyrode’s solution containing 1.5 mM extracellular calcium, maintained at 37°C, unless otherwise stated. Trabeculae were field stimulated with electrical pulses at a supramaximal voltage 10% above threshold and 5 ms in duration (model D100, Digitimer™, UK).
5. Structure-function relationship in human heart failure

Force-frequency data were acquired from the trabeculae by varying the frequency of field stimulation. Stimulation was varied in random order for frequencies of 0.1, 0.2, 0.5, 1 and 2 Hz to obtain force data on the FFR in these failing muscle preparations. In some instances, ectopic beats were observed at lower frequencies of stimulation and so these frequencies were excluded from the FFR analysis.

5.2.2.1 Justification of cardiac trabeculae use

Isolated cardiomyocytes have been useful in the assessment of protein distributions through indirect immunocytochemistry; however the ability to determine functional performance has previously been restricted mainly to calcium imaging, with information on force generation difficult to acquire, with many studies relying on unloaded fractional shortening as an alternative (Ljubojevic et al., 2014). Myocardial strips connected to a force transducer can be used to perform functional experiments to directly determine force measurements, with cardiac trabeculae a popular choice. Cardiac trabeculae are multi-cellular strips of myocardium lining the endocardial wall of the ventricles (and atria) of the mammalian heart. While the exact role of trabeculae in the adult mammalian heart has not fully been determined, it has been suggested that during embryonic maturation they are important for mediating nutrient and gas exchange for the myocardium prior to the development of the coronary arteries (Sedmera et al., 1997), with hypo-trabeculation potentially being embryonically lethal (Captur et al., 2015). The trabeculation of the mammalian ventricle becomes less extensive with development, with a shift towards a more ‘compact’ myocardium (Captur et al., 2015). However, it has been identified that hyper-trabeculation is present in an animal model of DCM (Shou et al., 1998), and that excessive numbers of abnormal trabeculae can result in ‘non-compacted’ cardiomyopathy (Captur et al., 2015).

In the rat ventricle, trabeculae are typically 150-500 µm in diameter and ≤3 mm long (Backx and Ter Keurs, 1993, Goo et al., 2009); while in the human ventricle there is great variation in size and length (see Vahl et al (1997) Fig. 1). They can be attached along their length to the wall of the ventricle, or in some cases, attached only at either end and ‘free-running’ in the middle (Captur et al., 2015), with the latter preferable for functional studies. Like the wall of the ventricle, trabeculae contain multiple cell types, including cardiomyocytes aligned along the length of the trabecula, endothelium and ECM containing collagen – which would be lost during the enzymatic isolation of cardiomyocytes (Hanley et al., 1999). The similarity in composition of the trabeculae with the rest of the myocardium has led to the use of trabeculae as representative of the ventricle wall. In addition, the process of isolating cardiomyocytes does not produce a yield of 100% rod-shaped, clearly striated cardiomyocytes, leading to
potential selection bias for the cells with a ‘healthy’ appearance. When examining normal cardiac physiology of healthy animals, this is generally not a problem; however in the case of human heart failure samples, it is difficult to determine the difference between abnormal-looking cardiomyocytes due to pathological changes or those damaged in the isolation process, leading to potential for selection bias towards the less extreme phenotype of damage (Crossman et al., 2011). For this reason, cardiac trabeculae and whole myocardium samples can be advantageous for studying cardiomyopathies, in particular when examining the development of force.

5.2.3 Functional data processing and analysis

Several parameters were measured to assess mechanical performance in the trabeculae samples. The peak twitch force was measured as the difference between the diastolic force and the peak active force developed. Additional mechanical performance parameters measured included time to peak stress development (TPS) and time to 50% relaxation of force ($T_{50\%}$). TPS was measured as the time taken from onset of contraction to the development of peak force. $T_{50\%}$ was found by determining when active force was reduced to half the peak value, and measuring the time taken to reach this point from the beginning of muscle relaxation. These parameters were measured and averaged across 5-6 sequential contractions from each trabecula at 1 Hz stimulation. These procedures were carried out manually using Excel (Microsoft Corp.).

It has been long recognised that developed force is proportional to muscle size, in particular cross-sectional area (CSA) (Bruce et al., 1997). Force measurements were therefore normalised to area and expressed as stress (mN/mm$^2$) to account for differences in CSA between the trabeculae. Confocal imaging revealed a large variation in the proportion of myocytes between trabeculae, which was found to be positively correlated with developed force. Therefore, force was also normalised to myocyte CSA (MCSA) of the trabecula, with both stress per CSA and stress per MCSA (at 1 Hz stimulation, 37°C, 1.5 mM [Ca$^{2+}$]$_o$) determined for all trabeculae. In addition, the force-frequency relationship (FFR) was investigated for each trabecula. The FFR was calculated by fitting a line of best fit to a scatterplot of peak active stress (normalised to CSA) against frequency of stimulation. From this, the gradient of the fit was determined which indicated directionality (positive or negative) as well as the strength of the relationship. For the FFR, TPS and $T_{50\%}$, those trabeculae with negligible active force were excluded from the analysis.
5.2.4 Confocal image processing and analysis

Imaging of the human samples was carried out using a Zeiss 710 LSM inverted confocal microscope, with 40x magnification tissue overviews acquired, followed by image stacks at 63x magnification (see General Methods for details). Deconvolution of the 63x magnification image stacks was performed according to procedures described in General Methods.

5.2.4.1 Tissue cardiomyocyte content analysis

It has been well established that fibrosis is a pathological change observed in diseased and failing heart tissue, leading to a relative increase in collagen and extent of the ECM (Maron et al., 1975). Therefore the relative fraction of the sample occupied by cardiomyocytes was determined in both the trabeculae and ventricular wall samples (failing and non-failing), using ImageJ. This was performed on images obtained with a 40x magnification objective in transversely orientated samples (Figure 5.2A). The total CSA of the trabecula was determined based on the WGA labelling in which the labelling was manually thresholded and subsequently used to generate a mask. The outline of this mask was manually traced to determine the region of interest and the area of labelling was established (see Figure 5.2B). Cardiomyocyte area was determined based on the JPH2 labelling within the transverse trabecula, with manual thresholding and a mask generated. From the JPH2 mask, the area of each region of labelling was found, along with the area fraction of labelling by measuring the ‘particles’ >20 µm² in ImageJ. From this, the total area of JPH2 labelling (MCSA) was calculated to give (Figure 5.2C). This was then converted into the percentage of total CSA.

![Figure 5.2 Cardiomyocyte content analysis](image)

A) Transverse sections of tissue dual labelled for WGA (blue) and JPH2 (green) were used to determine the cross-sectional area attributed to cardiomyocyte presence by generating masks of B) total area based on WGA labelling and C) area of cardiomyocyte labelling as determined by JPH2 labelling. Scale bar = 50 µm.
5.2.4.2 T-tubule-RyR association

As the functional coupling of the t-tubule and SR$_i$ membranes is important for the formation of junctions and efficient EC coupling (Bers, 2001), the degree of association between the SR calcium channels, the RyR, with the t-tubules was determined. To do this, a threshold value calculated as the mode pixel grayscale value plus the standard deviation (Sachse et al., 2012, Li et al., 2015) was applied to the RyR labelling and a mask created from this using ImageJ. Particle analysis was performed to identify RyR clusters and saved as regions of interest (ROIs). The same thresholding method was then applied to the corresponding WGA labelled image and overlaid with the RyR ROIs. This allowed identification of RyR clusters associated with t-tubule labelling (ROIs with mean pixel grayscale value >0). This number was then converted into a percentage of total RyR clusters present. The reasons for using this approach for determining the association of the two labels rather than the methods of co-localisation analysis performed on the dual immunolabelled samples (outlined below in 5.2.4.3) are discussed in a following section (section 5.4.2).

5.2.4.3 Co-localisation analysis

The association of paired proteins of interest was assessed with co-localisation analysis using Python based software written by Dr David Baddeley, as detailed in General methods. This involved examining RyR-JPH2 co-localisation as well as JPH2-α-tubulin co-localisation in the longitudinally orientated 63x magnification objective acquired deconvolved images. For RyR-JPH2 dual labelled images, masks were generated in ImageJ based on mode plus standard deviation thresholding which were then opened in Python software, while for α-tubulin-JPH2 this involved applying isodata thresholding in Python software to generate the protein labelling masks. From each protein label, the distance to the nearest labelling of the mask of the other protein was determined using Euclidean distance maps to generate a distance distribution plot. The distances up to and including zero nm were summed as a measure of the fraction of protein co-localisation.

5.2.4.4 Protein density analysis

Previous work by Zhang et al (2014) found that there is increased densification of the microtubules in the failing heart, and that this is associated with impaired trafficking and function of JPH2. The density of microtubules was assessed in the failing (trabeculae and ventricular wall) and non-failing cardiac samples by examining the labelling density of α-tubulin, one of the monomers involved in microtubule formation (Phung et al., 2006).
Because the cardiac trabeculae did not fill the entire image frame, a ROI was manually drawn around the region corresponding to cardiac tissue, with labelling density determined within this region. To do this, isodata thresholding was applied to the images, and converted into a mask. From this, the fractional area covered by the mask was determined and used as a measure of relative density. This analysis was performed on the longitudinally orientated images (taken with 40x magnification objective). In addition, as it has been shown that α-tubulin expression is high in the vascular smooth muscle cells of capillary blood vessels (Phung et al., 2006), the same analysis was also performed on the longitudinal images (63x magnification objective), with the intra-cardiomyocyte labelling specifically selected. This enabled comparison of tissue-wide densification of the microtubules with that observed within cardiomyocytes. Given the previous findings that in some cases of human heart failure there is reduced expression of JPH2 (Landstrom et al., 2011, Zhang et al., 2013), density analysis was also performed on confocal images labelled for JPH2 and RyR (taken with a 40x objective) and to determine the relative protein labelling densities within the tissue.

5.2.5 Statistical analysis

Correlation analysis between variables was performed using Pearson’s correlation in SPSS, with differences between diseased trabeculae and ventricular wall samples determined with student’s t-test, as was comparison of means between diseased and control wall samples. 2-tailed t-tests were performed between failing samples, with 1-tailed performed between failing and non-failing ventricle wall samples, as it was hypothesised that non-failing samples would exhibit improved cardiomyocyte organisation. Unless stated otherwise, data are presented as mean ± standard error of the mean (SEM).

5.3 Structure-Function Relationship in Heart Failure

In order to investigate the cardiac structure-function relationship in the samples from failing human hearts, information on both aspects was collected from the same trabeculae. Data regarding function was provided from the mechanical experiments performed on the trabeculae, while structural information was obtained from confocal imaging following immunohistochemical staining. In addition, to determine the appropriateness of trabeculae as a model for ventricular myocardium, free wall tissue samples from the failing hearts were also processed and imaged using confocal microscopy. Samples from the myocardium of non-
failing donor hearts were used for comparison of the structural changes observed in heart failure.

### 5.3.1 Variability of functional performance in failing trabeculae

One of the primary findings regarding the contractile performance of trabeculae isolated from the failing human heart was the high degree of variability between samples (see Table 5.2). This was found to exist both between and within individual explanted hearts. It was therefore decided to utilise this inherent variability to explore the relationship between function and structural changes in myocytes organisation at the tissue and cellular level.

<table>
<thead>
<tr>
<th>Heart</th>
<th>Trab</th>
<th>Force (mN)</th>
<th>CSA (mm²)</th>
<th>Stress (mN/mm²)</th>
<th>TPS (ms)</th>
<th>T50% (ms)</th>
<th>FFR gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH32</td>
<td>RV3*</td>
<td>N/A</td>
<td>0.126</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A</td>
</tr>
<tr>
<td>DH32</td>
<td>Sept3</td>
<td>1.29</td>
<td>0.166</td>
<td>7.76</td>
<td>353</td>
<td>270</td>
<td>-3.33</td>
</tr>
<tr>
<td>DH36</td>
<td>RV3</td>
<td>0.15</td>
<td>0.073</td>
<td>2.05</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DH36</td>
<td>RV4</td>
<td>3.26</td>
<td>0.108</td>
<td>30.30</td>
<td>365</td>
<td>263</td>
<td>-2.25</td>
</tr>
<tr>
<td>DH37</td>
<td>LV3</td>
<td>0</td>
<td>0.137</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DH37</td>
<td>RV2</td>
<td>0.32</td>
<td>0.035</td>
<td>9.38</td>
<td>333</td>
<td>193</td>
<td>-6.05</td>
</tr>
<tr>
<td>DH37</td>
<td>Sept2</td>
<td>0</td>
<td>0.044</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DH37</td>
<td>Sept3</td>
<td>0</td>
<td>0.077</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DH38</td>
<td>RV1</td>
<td>1.35</td>
<td>0.031</td>
<td>42.99</td>
<td>243</td>
<td>173</td>
<td>+26.47</td>
</tr>
<tr>
<td>DH38</td>
<td>RV2</td>
<td>0.10</td>
<td>0.018</td>
<td>11.12</td>
<td>248</td>
<td>156</td>
<td>+3.97</td>
</tr>
<tr>
<td>DH38</td>
<td>Sept1</td>
<td>0.16</td>
<td>0.011</td>
<td>13.93</td>
<td>195</td>
<td>93</td>
<td>+1.85</td>
</tr>
<tr>
<td>DH38</td>
<td>Sept2</td>
<td>0.95</td>
<td>0.111</td>
<td>8.54</td>
<td>240</td>
<td>160</td>
<td>+4.06</td>
</tr>
<tr>
<td>DH40</td>
<td>RV1</td>
<td>1.90</td>
<td>0.083</td>
<td>22.83</td>
<td>390</td>
<td>315</td>
<td>+3.56</td>
</tr>
<tr>
<td>DH40</td>
<td>RV2</td>
<td>0.01</td>
<td>0.051</td>
<td>0.189</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DH40</td>
<td>Sept1</td>
<td>2.20</td>
<td>0.074</td>
<td>29.51</td>
<td>263</td>
<td>343</td>
<td>+4.15</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.835±0.28</td>
<td>0.076±0.012</td>
<td>12.76±3.65</td>
<td>292±23</td>
<td>218±28</td>
<td>+3.60±3.12</td>
</tr>
</tbody>
</table>

**Table 5.2 Variability of contractile performance in failing cardiac trabeculae**

Summary of contractile performance parameters measured in 15 trabeculae from failing human hearts, including patient heart ID number, trabecula number from heart (RV = right ventricle, LV = left ventricle, sept = septum). Parameters measured include peak twitch force production at 1 Hz stimulation (at 37°C, 1.5 mM [Ca²⁺]o), cross-sectional area (CSA) of trabecula, normalised stress development, time to peak stress (TPS) and time to 50% relaxation (T50%). The gradient from FFR analysis is also included based on peak stress measurements. Bottom row shows averaged data from trabeculae displayed as mean ± SEM. N/A = peak twitch force was too low to accurately measure parameter. *Trabecula exhibited ectopic contractions so data was not collected at 1 Hz.
5.3.1.1 Force-frequency relationship

One of the well-described changes in contractile function in the failing human heart is the shift from a positive force-frequency response (FFR) toward a blunted or negative FFR (Brixius et al., 2001, Schwinger et al., 1993). Therefore, the FFR was examined over a range of frequencies that encompassed the \textit{in vivo} heart rate to determine the variability of this response in these failing cardiac trabeculae. It was found that the gradient of the FFR was highly varied between trabeculae, with some showing a strong positive relationship (e.g. DH40 Sept1), while others showed a blunted positive relationship (e.g. DH38 Sept1), or even negative (DH36 RV4; see Table 5.2). These FFR gradients have been compared with the structural parameters assessed to determine potential cardiomyocyte organisational changes contributing to the high variability observed.

5.3.2 Altered tissue cardiomyocyte content

In order to determine the extent of fibrosis and the potential contribution to alterations in contractile performance, the CSA corresponding to cardiomyocyte area was determined in the experimental trabeculae. On visual assessment of confocal images, it was found that the cardiomyocyte content greatly varied between samples (Figure 5.3A-C), with some trabeculae exhibiting very little, or complete absence of cardiomyocytes, with these latter samples also typically producing ‘poor’ or no contractile function (Figure 5.3A). Interestingly, cardiac trabeculae with ‘intermediate’ and ‘strong’ peak stress production both appeared to show increased cardiomyocyte content compared to weaker trabeculae, although with similar proportions of cardiomyocytes present in these two samples (Figure 5.3B,C). Due to the known observation of total muscle CSA correlating with force production, this relationship was examined in these trabeculae (Figure 5.3D) along with the relationship between myocyte CSA and force (Figure 5.3E). It was found that in these failing cardiac trabeculae there was a weak correlation between total CSA and force production, but no statistically significant relationship found between these parameters (Pearson’s $R^2 = 0.2429$; $p>0.05$). In contrast, however, there was a much stronger, significant correlation observed between absolute cardiomyocyte CSA (MCSA) and peak force generation in the failing trabeculae ($R^2 = 0.6317$; $p<0.001$). Furthermore, it was observed that there was a significant correlation between peak stress production and cardiomyocyte content as a proportion of total CSA (% myocytes), regardless of whether peak stress was normalised to total CSA or MCSA (both $p<0.01$; Figure 5.3F). Together, these results show that in failing cardiac trabeculae, cardiomyocyte
Figure 5.3 Cardiomyocyte content correlates with mechanical performance

Dual labelling of WGA (blue) and JPH2 (green) in cardiac trabeculae with A) weak, B) intermediate and C) strong contractile performance (upper panels), and the corresponding peak stress developed at 1 Hz (lower panels). Scale bars: 50 µm. Correlation between force and either D) total trabecula CSA or E) cardiomyocyte CSA. F) Relationship between fractional cardiomyocyte area and stress normalised to total CSA (orange) or cardiomyocyte CSA (green). G) Relationship between total CSA and the content fraction of cardiomyocyte.
content is a stronger predictor of contractile function than total CSA of the muscle strip. Furthermore, these findings indicate that in the failing human heart, the organisation of cardiomyocytes at the tissue level is a factor contributing to the ability to generate contractile force. Despite these significant findings, when stress is normalised to cardiomyocyte content, there is still variability observed across samples (as seen by the scatter of data points in Figure 5.3F). Therefore, additional organisational properties of the tissue and cardiomyocytes were examined. Functional results presented from here onwards have been normalised to MCSA rather than total CSA when normalising force to stress, in an attempt to identify additional significant contributors to the structure-function relationship in these samples. It was also identified that there was no clear relationship between the total CSA of the trabeculae and the fractional content of cardiomyocytes (Figure 5.3G), with mid-range sized trabeculae exhibiting both very high and very low myocyte content.

5.3.3 Changes to EC coupling structural organisation

While the variability observed in cardiomyocyte content was found to correlate with contractile function, it does not explain all the variability in stress observed in these failing trabeculae. It was therefore also of interest to examine if there was altered organisation within the myocytes themselves, as it has been shown that EC coupling structures and processes can be altered in human heart failure, in particular disruption to the t-tubule organisation (Lyon et al., 2009, Zhang et al., 2013) and loss of CICR gain (Guo et al., 2013, Song et al., 2006).

5.3.3.1 RyR-t-tubule association relationship with function

Given the importance of the formation and maintenance of junctions between the t-tubule and SR membranes for EC coupling (Bers, 2001), the extent of association between the SR calcium release channels, RyR, with the t-tubules was assessed in these samples. There appeared to be loss or disruption of t-tubule labelling in some of the samples, with cardiomyocytes from some samples exhibiting an absence of t-tubule labelling, which was typically observed in trabeculae with low active stress production (Figure 5.4A). In many cases, t-tubules were observed at oblique angles, while a few appeared to maintain a transverse organisation. In comparison, RyR clusters were typically observed in a striated arrangement throughout the majority of cardiomyocytes (Figure 5.4B,C), with an occasional few exhibiting a disrupted distribution, with no clear organisation present (Figure 5.4A).
Confocal imaging of RyR and t-tubule dual labelled samples revealed that there was variability in the degree of association between the RyR clusters and the t-tubules (labelled with WGA) across the different trabeculae samples, with some showing a moderate number of RyR clusters associated with t-tubules (Figure 5.4B,C), while others showed very little or virtually no association, partially due to loss of t-tubule labelling (Figure 5.4A). The trabeculae which were identified as producing moderate or intermediate active stress measurements appeared to show higher RyR-t-tubule association (Figure 5.4C). However, this image analysis could not be performed on some samples (2 trabeculae; 13%) due to the complete absence of cardiomyocytes. Analysis of the extent of RyR-t-tubule association revealed that there was no significant correlation found between this parameter and stress production in the diseased trabeculae, even once cardiomyocyte content had been corrected for, as shown in Figure 5.4D ($R^2 = 0.1777$; p>0.05). Interestingly, however, it was observed that there was a
strong, positive correlation between the myocyte content of the trabeculae and the proportion of RyR clusters associated with t-tubule labelling ($R^2 = 0.6284$, $p<0.001$; Figure 5.4E). This indicates that there is a relationship between trabeculae tissue composition and the structural organisation of the cardiomyocytes.

### 5.3.3.2 Densities and co-localisation of JPH and RyR

It has previously been reported that JPH2 protein expression levels are reduced in some cases of human heart disease and failure (Landstrom et al., 2011, Zhang et al., 2013), with some studies also suggesting loss of RyR as well (Go et al., 1995, Vatner et al., 1994). To determine if changes in the expression of either of these proteins was associated with cardiac

![Figure 5.5 Density of JPH2 and RyR protein labelling in failing trabeculae](image)

Figure 5.5 Density of JPH2 and RyR protein labelling in failing trabeculae

Tissue density was plotted against active stress development (normalised to cardiomyocyte CSA) for A) RyR and B) JPH2 labelling. C) Further analysis revealed a significant correlation between JPH2 labelling density and the percentage of RyR clusters associated with t-tubule labelling.
function, their labelling densities across the trabeculae sections were determined. Analysis of confocal images revealed that there was variability in the apparent labelling densities of both JPH2 and RyR in these failing samples. It was further identified that while there was a weak, positive relationship between RyR density and active stress development which was not significant (Figure 5.5A), there was a stronger, significant relationship observed between JPH2 density and stress production in the failing trabeculae ($R^2= 0.297$, $p<0.05$; Figure 5.5B). Furthermore, it was found that there was a significant, positive relationship between the density of JPH2 labelling within the tissue and the extent of RyR clusters associated with t-tubule labelling ($R^2= 0.6316$, $p<0.01$; Figure 5.5C). This suggests that JPH2 does indeed play a role in maintaining EC coupling organisation. There were also significant, positive relationships observed between both JPH2 and RyR density with the fractional cardiomyocyte content of the trabeculae (data not shown, $R^2= 0.7790$ and $0.6837$, respectively; both $p<0.001$).

JPH2 has not only been implicated in promoting the formation and maintenance of junctions (which are important in EC coupling) (Takeshima et al., 2000), but has also been identified as potentially stabilising RyR channels, reducing calcium ‘leak’ from the SR (Van Oort et al., 2011). Therefore, the co-localisation of JPH2 and RyR was assessed in dual labelled samples to determine if this is a contributing factor to the functional variability observed. Again, due to lack of cardiomyocyte content (and therefore absence of RyR and JPH2 labelling), two samples were excluded from this analysis. It was observed that there was an overall striated appearance of RyR clusters in the majority of cardiomyocytes analysed. JPH2 was found to be distributed throughout the cardiomyocyte with varying degrees of organisation, whereby in the majority of samples there was an intracellular distribution, with varying extents of surface labelling. In some instances where RyR labelling was largely absent from cardiomyocytes, JPH2 labelling was still observed, although with a reduced organisation (Figure 5.6A). Additional to the intracellular transverse distribution, in many cases there was further labelling observed at the surface sarcolemma (Figure 5.6B,C). Of the analysed dual labelled samples, confocal imaging revealed that there was great variability in the extent of co-localisation of these two proteins, with some trabeculae exhibiting an apparent reduction in the degree of protein association (Figure 5.6A) compared to others (Figure 5.6B,C). While there appeared to be greater organisation in trabeculae with increasing contractile function, analysis revealed that there was no significant correlation or trending relationship observed between either the fraction of RyR co-localised with JPH2, or the fraction of JPH2 co-localised with RyR, and the development of active stress in the failing trabeculae (Figure 5.6D,E respectively).
5. Structure-function relationship in human heart failure

Figure 5.6 Co-localisation of RyR and JPH2 in failing trabeculae
Deconvolved confocal images showing dual labelling of RyR (red) with JPH2 (green) in failing cardiac trabeculae with A) weak, B) intermediate, and C) strong contractile function. Scale bars: 10 µm. Analysis reveals no significant relationship between either D) the fraction of RyR co-localised with JPH2 or E) the fraction of JPH2 co-localised with RyR and active stress production (normalised to cardiomyocyte CSA) in failing trabeculae.

5.3.4 Microtubule and JPH2 alterations

Previous studies have indicated that there is densification of microtubules as a result of heart failure in both animal and human studies (Heling et al., 2000, Schaper et al., 1991), and that this densification is inversely correlated with cardiac function (Wang et al., 1999, Tagawa et al., 1998). Therefore, the density of α-tubulin was assessed at both the tissue and cardiomyocyte level in the cardiac trabeculae. Throughout the trabeculae tissue sections it was observed that there was α-tubulin labelling identified within the cardiomyocytes, and also in the surrounding ECM regions of the tissue, often with high intensity (Figure 5.7A-C). At the cellular level, α-tubulin was found to be distributed in apparent thin strands (microtubules)
forming a network throughout the length of the cardiomyocyte (Figure 5.7D-F). Interestingly, it was also often observed that the α-tubulin appeared to wrap around an intracellular region, assumed to be the nucleus (see Figure 5.7F). Both tissue and cellular densities of α-tubulin appeared to be highly variable across samples. When plotted against peak stress development (normalised to cardiomyocyte CSA), it was revealed that neither tissue (Figure 5.7G) nor cellular (Figure 5.7H) α-tubulin labelling density were significantly correlated.

Figure 5.7 Density of α-tubulin in failing cardiac trabecula tissue and myocytes
Confocal images showing α-tubulin labelling (magenta) in A-C) cardiac trabeculae tissue and D-F) cardiomyocytes with occasional apparent nuclear envelopment (arrow). WGA (grey) labelling indicates cell boundaries. Scale bars A-C = 50 µm; D-F = 10 µm. Analysis of α-tubulin labelling density for G) whole tissue and H) cardiomyocytes from failing trabeculae correlated with active stress production (normalised to cardiomyocyte CSA).
Following from the previously identified densification of α-tubulin in heart failure, it has been suggested that this cytoskeletal protein alteration results in disorganisation of JPH2 and subsequent disruption of the t-tubules through impaired JPH2 trafficking (Zhang et al., 2014). Therefore, the co-localisation of α-tubulin and JPH2 was analysed to determine if changes in association of these two proteins with the cardiomyocytes correlate with cardiac function in the failing trabeculae. Confocal imaging revealed that dual labelled samples showed high variation of protein co-localisation (Figure 5.8A-C). Analysis of these images revealed that there was no relationship observed between the peak stress production with either α-tubulin co-localised with JPH2, or with JPH2-tubulin co-localisation (Figure 5.8D,E respectively).

**Figure 5.8 Co-localisation of α-tubulin and JPH2**

Confocal images of dual labelling of α-tubulin (magenta) with JPH2 (green) in cardiomyocytes from trabeculae with A) weak, B) intermediate and C) strong contractile function. Scale bars: 10 µm. Analysis reveals the extent of both D) α-tubulin co-localisation with JPH and E) JPH2 with α-tubulin and the respective correlations with active stress development in failing trabeculae, normalised to cardiomyocyte CSA.
5.3.5 Structural relationship with FFR and time course of contraction

One of the commonly reported alterations in failing heart function is the loss of the strong positive FFR which is observed in the healthy human heart (Mulieri et al., 1992, Schwinger et al., 1993, Brixius et al., 2001), as well as being frequently shown that there are alterations to the temporal aspects of mechanical performance in heart failure, including prolongation of the time to peak stress (TPS) and time to 50% relaxation ($T_{50\%}$) (Gwathmey et al., 1990, Mulieri et al., 1992). In this study, it was identified that there was substantial variability in these parameters in the failing trabeculae (see Table 5.2). Therefore, these parameters were examined in relation to the assessed structural changes in the failing trabeculae. While none of the structural parameters were found to form a significant relationship with FFR, there were a small number of significant correlations identified with the temporal aspects of contraction. Despite a significant relationship observed between TPS and $T_{50\%}$ (Figure 5.9A), the structural correlations with each of these measures differed. It was observed that TPS showed a modest, positive correlation the fraction of JPH2 co-localised with $\alpha$-tubulin (Figure 5.9B), such that increased co-localisation is associated with an increased, or prolonged, TPS. This same parameter was not found to correlate with $T_{50\%}$, and while a weak, non-significant positive relationship was observed with tissue labelling density of $\alpha$-tubulin (not shown), there were no significant structural correlations identified with $T_{50\%}$.

![Figure 5.9](image.png)

**Figure 5.9 Structural correlates with temporal mechanics**

Correlation analyses reveal a positive relationship between A) time to peak stress (TPS) and time to 50% relaxation ($T_{50\%}$). B) TPS was observed to form a significant, positive relationship with the fraction of JPH2 co-localised with $\alpha$-tubulin.
5.3.6 Structural comparisons between failing and non-failing wall

While cardiac trabeculae are valuable preparations for investigating cardiac function, the ability to extrapolate findings to the ventricle wall as a whole has not previously been clearly determined, particularly from failing hearts. Therefore, in addition to correlating structural changes in trabeculae organisation with contractile function, the overall organisation of these samples was compared to the free wall of the failing human heart. Furthermore, to determine if the changes observed were attributed to biological variability or the progression of heart failure, the failing ventricular wall samples were also compared to non-failing human hearts.

**Figure 5.10 Altered tissue composition in the failing heart**
Dual labelled confocal images showing ECM (WGA; blue) and JPH2 (green) in myocardium sections from the A) failing and B) non-failing human ventricle. Scale bars: 50 µm. Content analysis reveals C) mean cardiomyocyte content from all failing trabeculae, failing ventricle and non-failing ventricle; as well as D) mean cardiomyocyte content from trabeculae with weak or strong contractile force and failing ventricle. Data displayed as mean ± SEM.
The first aspect of organisation examined in the three different samples was the relative content of cardiomyocytes throughout the cardiac tissue. While the high variability in the trabeculae has previously been shown (Section 5.3.2), there was an apparent increase in the extent of ECM labelling in the failing ventricle myocardium (Figure 5.10A) compared to that observed in the non-failing heart (Figure 5.10B). Content analysis revealed that the mean cardiomyocyte fraction of $41.52 \pm 7.19\%$ in failing trabeculae is significantly lower than that in samples from the ventricular wall of the same hearts ($64.17 \pm 4.28\%$; Figure 5.10C). In addition, it was observed that there was a higher degree of variability present in the trabeculae samples compared to the wall. Therefore, if the trabeculae were split into two groups according to stress production ($n= 7$ in each group), it can be seen that those which develop lower peak stress have greater variability and significantly lower cardiomyocyte content than those that develop high stresses ($26.43 \pm 11.04\%$ vs $56.61 \pm 5.15\%$; Figure 5.10D). Furthermore, the trabeculae with stronger contractile function do not show a significant difference in content in comparison to the failing ventricle wall. It was also revealed that there is a significant reduction in the proportion of cardiomyocytes in the myocardium from failing ventricles compared to non-failing (Figure 5.10C), with mean cardiomyocyte content in the non-failing ventricle of $79.01 \pm 3.04\%$.

The remodelling of the t-tubule network in heart failure has been well established (Wei et al., 2010, Zhang et al., 2013), and has been implicated in influencing the association of RyR clusters with the sarcolemma (Sachse et al., 2012). Confocal imaging revealed disrupted t-tubule organisation in failing myocardium sections, with oblique angles and longitudinal extensions present (Figure 5.11A) compared to the predominantly transverse arrangement observed in the non-failing ventricle (Figure 5.11B). Analysis revealed that the percentage of RyR clusters associated with t-tubule labelling was not significantly different between the failing trabeculae and wall samples, with $22.95 \pm 2.62\%$ and $25.43 \pm 1.51\%$, respectively. However, in comparison to the non-failing ventricle wall ($32.28 \pm 2.01\%$), there was a modest, yet significantly reduced RyR-t-tubule association observed in the failing free wall samples ($p<0.05$; Figure 5.11C). Dual labelling of JPH2 and RyR in the human heart revealed that there appeared to be similar overall distributions in the failing (Figure 5.11D) and non-failing hearts, with an increased transverse organisation of JPH2, associated with increased RyR co-localisation in the non-failing heart (Figure 5.11E). The tissue labelling densities of JPH2 and RyR were also assessed in these samples, revealing that there were no differences between the failing trabeculae and ventricle, or between failing and non-failing ventricular myocardium (Figure 5.11F). Furthermore, it was observed that there was a significant reduction in the fraction of RyR co-localised with JPH2 in the failing ventricle wall ($46.86 \pm$
2.57%) compared to in the non-failing (62.30 ± 6.62%; Figure 5.11G; p<0.05) but no significant difference between the failing trabeculae and ventricle. Additionally, there was no difference between failing samples, or between failing and non-failing ventricle myocardium in the extent of JPH2 co-localised with RyR (Figure 5.11H).

**Figure 5.11 RyR and JPH2 organisation in heart failure**

Distribution of RyR (red) and t-tubule (WGA; grey) labelling in A) failing and B) non-failing ventricle myocardium. Analysis revealed C) the percentage of RyR clusters associated with t-tubule labelling in these samples, along with the mean from the failing trabeculae. Confocal images of JPH2 (green) and RyR (red) distribution in D) failing and E) non-failing ventricle myocardium, with F) corresponding tissue labelling density analysis in the three sample types. Scale bars: 10 µm. Co-localisation analysis reveals the extent of G) RyR co-localised with JPH2 and H) JPH2 co-localised with RyR in the three samples. Data displayed as mean ± SEM; *p<0.05.
To investigate the reported densification of microtubules in human heart failure, the labelling density of α-tubulin was assessed at the total tissue and myocyte levels in the failing and non-failing samples. Confocal imaging revealed that in both the failing (Figure 5.12A) and non-failing ventricle (Figure 5.12B) there was apparent labelling present both within and between...

Figure 5.12 Organisation of α-tubulin in human heart failure
Confocal imaging revealing the distribution of α-tubulin (magenta) throughout the myocardium from A) failing and B) non-failing human ventricle; cell boundaries indicated by WGA (grey) and C) tissue labelling density in all three samples. Scale bars: 50 µm. Dual labelling of α-tubulin (magenta) and JPH2 (green) in D) failing and E) non-failing ventricle. Scale bars = 10 µm. Analysis reveals F) cellular labelling density of α-tubulin with the fraction of co-localisation of G) α-tubulin with JPH2 and H) JPH2 with α-tubulin. Data displayed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.
the cardiomyocytes, with inter-myocyte labelling appearing more intense than within the myocytes in the non-failing myocardium. Interestingly, it was found that the total α-tubulin density was unchanged between the failing and non-failing ventricle wall; however there was a significantly higher labelling density observed in failing trabeculae compared to the wall samples from the same hearts, increased by ~1.7-fold (Figure 5.12C). Dual labelling of α-tubulin and JPH2 showed the presence of strands of α-tubulin labelling running throughout the cardiomyocytes, with an apparent increase in the density of microtubules in the failing myocytes, and increased association with JPH2 (Figure 5.12D) compared to the non-failing heart (Figure 5.12E), which typically showed only high α-tubulin labelling between neighbouring myocytes. This observation was confirmed by myocyte density analysis, in which the mean α-tubulin labelling density was significantly increased in the heart failure ventricle by ~3.4-fold compared to the non-failing ventricle wall (Figure 5.12F). There was no difference in cellular tubulin density between the failing trabeculae and ventricle wall samples.

Co-localisation analysis of α-tubulin and JPH2 in the three sample sets revealed significant differences in the association of these two proteins. There was significantly higher co-localisation of both α-tubulin with JPH2 (32.61 ± 2.79% versus 7.61 ± 0.86%), and JPH2 with α-tubulin (22.24 ± 2.15% versus 12.70 ± 1.93%) in the failing cardiac trabeculae compared to the failing ventricle wall (p<0.001 and p<0.01, respectively; Figure 5.12G,H). Furthermore, the extent of both protein co-localisation measures was increased in the failing ventricle compared to the non-failing ventricle (α-tubulin with JPH2 of 3.96 ± 1.17%, and JPH2 with α-tubulin of 6.75 ± 1.94%; both p<0.05).

5.4 Discussion

The results presented in this chapter have focused on the analysis of confocal images to identify correlations between cardiomyocyte organisation and the contractile function of trabeculae from the failing human heart. Analysis indicates that there is a high degree of variability between trabeculae in the three measures of mechanical performance – peak active stress, time to peak stress (TPS) and time to 50% relaxation (T50%). Previous research has shown that compared to the non-failing human heart, in heart failure there is an increased duration of both the TPS and T50% (Gwathmey et al., 1990). While the present study was not able to compare these temporal parameters to non-failing hearts under the same experimental conditions, it was found that despite the inter-sample variability, the overall mean for both TPS and T50% were in agreement with previous reports of these parameters in
human heart failure in some instances (Gwathmey et al., 1990), but not in other studies (Mulieri et al., 1992, Schwinger et al., 1993). Furthermore, in previous experiments performed at the same temperature as the present study (37°C), it has been shown that in IDCM there is a reduction in active stress development compared to the non-failing heart, which develops ~23 mN/m² peak stress (Mulieri et al., 1992). The mean stress development in this study is comparable to measurements obtained in IDCM from one previous study (Mulieri et al., 1992), but not comparable to others performed at room temperature (Schwinger et al., 1993). Overall, this indicates that due to differences in experimental conditions, including the use of larger myocardium strips rather than trabeculae, frequencies of stimulation and different extracellular calcium concentrations, it is difficult to directly compare measurements of mechanical performance between the present and previous studies. Therefore, given the unavailability of non-failing trabeculae, and the high variability observed in the experimental failing trabeculae in this study, it was decided that this inherent functional variability would be utilised to identify potential structural correlates in the failing human heart, which may be implicated in the cardiac structure-function relationship. The following discussion covers how aspects of tissue and cardiomyocyte organisation fit into this relationship, particularly the relationship with peak stress development.

5.4.1 Cardiomyocyte content and peak stress development

The standard practice of normalising active force development by muscle between samples has been to account for the cross-sectional area (CSA) of the muscle to produce a measurement of stress (Krivickas et al., 2011). However, findings presented in this thesis indicate that, in the failing human heart for cardiac trabeculae the total CSA does not form a strong relationship with force production, and that instead, the CSA accounted for by cardiomyocytes is a stronger predictor in determining contractile function. It has been shown that in the SHR rat model of heart failure there is increased collagen content of the cardiac trabeculae, which is associated with a reduction in active stress development (Ward et al., 2003). The findings from cardiomyocyte content analysis in this study are in agreement with these reports, whereby an increased proportion of myocytes (and therefore a corresponding decrease in ECM/collagen content) was positively correlated with the development of higher active stress. Furthermore, it was identified that both within and between failing hearts there was a high degree of variability in the myocyte content of trabeculae.

While the observation of variability in the cardiomyocyte content of trabeculae is not new, with Sands et al. (2011) showing that collagen content of ‘normal’ rat trabeculae can range from
1.1-100%, this is the first study to directly correlate this change in trabecula tissue composition with active force/stress production from the same samples. Furthermore, findings from healthy hearts indicate that only the smaller trabeculae show high proportions of collagen content, while trabeculae with larger CSA show relatively low collagen staining (Sands et al., 2011). This same trend was not observed in the failing human trabeculae, with many of the ‘mid-range’ sized trabeculae exhibiting the lowest percentage of cardiomyocytes. This difference may be due to a combination of the increased range of trabeculae sizes present in the human ventricle, as well as potential fibrosis through the progression of DCM and heart failure. While in the myocardium of the failing heart it has also been shown that there is extensive fibrosis (Maron et al., 1975, Weber et al., 1989), it is difficult to determine if the reduction in cardiomyocyte content is due to cell death or increased ECM (or a combination of both). In the trabeculae samples it was evident that there was loss of cardiomyocytes from many of the samples, with the remaining cells often appearing irregular. However, the trabeculae samples in which there were virtually no cardiomyocytes present, the lack of non-failing trabeculae for comparison makes it difficult to determine if this is due to cell loss associated with disease progression or whether these trabeculae are naturally occurring collagenous strips. While the exact cause of the variation in trabeculae composition is unclear at present, these findings indicate that in the failing myocardium the relative cardiomyocyte content is a stronger predictor of contractile force than total CSA of the muscle. Therefore, normalisation of force to stress should perhaps be performed based on cardiomyocyte CSA as opposed to the standard total CSA, particularly in failing preparations.

5.4.2 EC coupling protein organisation in failing trabeculae

The close association of the SR and t-tubule membranes leads to the formation of junctions (Franzini-Armstrong et al., 1999) which are functional domains important for regulating the local ion concentrations and the efficiency of CICR in the cardiomyocyte (Stern, 1992). Remodelling of the t-tubule system is now well recognised as one of the pathological changes to the cardiomyocyte in heart disease and failure (Cannell et al., 2006, Zhang et al., 2013, Wei et al., 2010), with the severity of remodelling inversely correlated with fractional shortening of the ventricle wall (Crossman et al., 2015). The loss of t-tubule regularity in heart failure has been associated with a reduction in synchronicity of the calcium transient (Louch et al., 2004), as well as decreased association of RyR clusters with the membrane due to t-tubule displacement from the z-disk (Sachse et al., 2012). Interestingly, after normalisation to cardiomyocyte content, the peak stress production of the failing trabeculae was not found to be significantly related to the proportion of RyR clusters associated with the
5. Structure-function relationship in human heart failure

t-tubule network, indicating that increased coupling of the sarcolemmal and SRJ membrane was not associated with improved contractility. This said, however, there was a positive trend observed between these parameters, but due to high variability and moderate sample numbers, this was not significant. It is possible that a more extensive investigation could potentially confirm a positive link between these parameters, as it would be expected that increased association of the RyR with t-tubules would lead to enhanced EC coupling, and therefore improved contractile performance (Bers, 2001). The method used for determining RyR-t-tubule association differed from that used for measuring dual protein co-localisation (using Python software). The main reason for this difference was the use of WGA staining to label the t-tubules. WGA also labels the ECM (Kostrominova, 2011), making the WGA staining not t-tubule specific. Therefore, using a signal fraction mask of the WGA labelling would be based inappropriate as it would contain ECM and t-tubules, rather than solely t-tubule labelling.

On closer examination it was discovered that the RyR-t-tubule association is closely linked to the composition of the trabeculae, showing increasing contact between these structures when there is a higher cardiomyocyte presence. This reveals that in trabeculae with increased myocyte content, the cardiomyocytes present also exhibit improved cellular organisation, suggesting that having more myocytes is potentially protective of pathological ultrastructural changes. Increased fibrosis in cardiac muscle results in stiffening of the tissue which leads to higher passive tension and increased resistance on the myocytes when attempting to contract (Vahl et al., 1993). This results in higher forces (stress) being necessary to result in myocyte shortening (Hooke’s Law). The development of strain is proportional to the stresses applied in an elastic material (Atanackovic and Guran, 2000), and it has subsequently been shown that exposure to enhanced strain leads to the remodelling of the t-tubules in cardiomyocytes (McNary et al., 2011). Together, these findings support the hypothesis that decreased cardiomyocyte content in trabeculae could potentially influence the organisation of key cellular structures, in particular, the t-tubules.

One of the commonly observed EC coupling alterations in heart failure is the depressed amplitude of the calcium transient, contributing to the reduction in force development (Bers, 2001). There are several potential mechanisms contributing to this observation, including evidence for reduced expression of the SR calcium release channel, the RyR (Go et al., 1995, Vatner et al., 1994, Yano et al., 2000). Not surprisingly, a significant positive relationship was observed between increased cardiomyocyte content and RyR labelling density, indicating increased amounts of RyR within the tissue as more cardiomyocytes are present.
However, analysis from this study did not identify RyR labelling density as a significant contributing factor to active stress production in the failing trabeculae. This suggests that other mechanisms are responsible, such as altered RyR activity, SR calcium content or organisation of the junctional proteins (Bers, 2001). JPH2 has been implicated as playing a key role in the formation and maintenance of key EC coupling structure organisation in the cardiomyocyte, including the t-tubules and junctions (Takeshima et al., 2000, Chen et al., 2013, Van Oort et al., 2011, Wu et al., 2012). Interestingly, there was a modest positive relationship found between JPH2 labelling density and active stress development in the failing trabeculae. While the increased JPH2 density could be indicative of increased cardiomyocyte content, (which was also correlated with both JPH2 density and stress production), it is also possible that increased JPH2 levels leads to improved formation of junctions and therefore enhanced efficiency of EC coupling. Supporting this proposed mechanism is the observation that there was a strong correlation between increased JPH2 labelling density and the proportion of RyR clusters associated with the t-tubules. This indicates that there is potentially enhanced coupling of the SR, to the t-tubules with increased JPH2 labelling density, supporting the role of JPH2 as a mediator of junctional formation and maintenance (Takeshima et al., 2000). Furthermore, the co-localisation between RyR and JPH2 is thought to be important for maintaining EC coupling gain, as well as stabilisation of RyR (Van Oort et al., 2011). Despite these previously reported findings, there was no association found between either RyR co-localising with JPH2 or JPH2 co-localised with RyR and the development of active force in the failing trabeculae.

5.4.3 Microtubules and JPH2

In addition to the development of fibrosis, previous studies have identified the densification of the cytoskeletal protein, tubulin (both α and β subunits), as occurring in the diseased and failing myocardium (Aquila-Pastir et al., 2002, Schaper et al., 1991, Heling et al., 2000). Throughout the myocardium it was observed, that in addition to positive labelling within the cardiomyocytes, there was α-tubulin labelling present in the non-cardiomyocyte regions. These may be attributed to either smooth muscle cells of the capillaries (Phung et al., 2006) or, activated myofibroblasts (Rudolph and Woodward, 1978) which are involved in the proliferation of collagen in the ECM (Weber et al., 1989). The observed distribution of α-tubulin within the cardiomyocytes themselves was in agreement with previous reports of longitudinal strands as apparent microtubules, as well as a network around the perinuclear region (Schaper et al., 1991, Heling et al., 2000). It has been suggested that in the failing heart, the microtubules play a role in contractile dysfunction through the inhibition of
sarcotome shortening due to increased resistive stresses as densification occurs (Tsutsui et al., 1993). In addition, it has been proposed that microtubules are not only potentially associated with the junctions (Iribe et al., 2009), but that they may play a role in modulation of calcium handling including $I_{Ca}$ and release via the RyR (Gómez et al., 2000, Kerfant et al., 2001, Prosser et al., 2011) as well as maintaining the integrity of the SR structure (Vega et al., 2011). Interestingly, despite these suggested mechanisms, there was no relationship observed between peak stress development and $\alpha$-tubulin density, at either the tissue or cellular level in these trabeculae.

Further to densification, Zhang et al (2014) also suggested that this altered microtubule organisation is responsible for impaired trafficking and function of JPH2, with redistribution of JPH2 towards the cell periphery and altered cardiac function. Despite these reports, in the present study it was determined that there was no significant correlation between the extent of co-localisation between JPH2 and $\alpha$-tubulin with the development of active stress in the failing trabeculae. This indicates that the increased association of JPH2 with the microtubules does not result in impaired cardiac contractility; however, it is possible that it is not the intermediary interaction of JPH2 with the microtubules that results in functional impairment, but the end result of altered trafficking in heart failure. This could lead to the altered distribution of JPH2 throughout the cardiomyocytes, such as the increased localisation at the surface sarcolemma previously been reported, where there is a reduced ability for JPH2 to maintain the junctional cleft, resulting in loss of EC coupling gain (Zhang et al., 2014). One further possibility of the lack of a clear relationship between the properties of $\alpha$-tubulin and contractile function in this study is the etiology of the disease. While tubulin up-regulation has previously been observed in DCM (Schaper et al., 1991, Heling et al., 2000), the majority of reports regarding microtubule densification and altered function in human and animals have primarily involved hypertrophic models (such as aortic stenosis), in which there are changes in response to pressure overload of the heart (Aquila-Pastir et al., 2002, Tsutsui et al., 1993, Wang et al., 1999, Zile et al., 2001). In comparison, the present study solely used failing samples from patients with IDCM, which may result from differential mechanisms.

5.4.4 The FFR and temporal mechanics

Along with reduced peak stress development, the loss of a strong, positive FFR is one of the commonly reported functional changes in human heart failure, with either a blunted or reversed inotropic response with increasing frequency (Brixius et al., 2001, Mulieri et al., 1992, Schwinger et al., 1993). One of the aims of this study was to identify structural
changes in failing trabeculae which correlate with the FFR observed. Despite the numerous structural parameters examined, none were identified as forming a clear relationship with the gradient of the FFR in these samples. While this was not the anticipated outcome, it is possible that further structural components which were not able to be investigated within the scope of this project may prove to play an important role in the FFR in the failing heart. One potential candidate, which has previously been implicated in this relationship, would be the amount of SERCA present (Hasenfuss et al., 1994), and even its proximity to the junctional complex. Perhaps of even greater interest would be the ratio of SERCA and NCX expression and activity, which would determine the balance of calcium removal mechanisms from the cytosol. It has previously been suggested that the shift towards increased calcium extrusion versus SR re-uptake is implicated in the decline of the positive FFR in heart failure (Pieske et al., 1995, Hasenfuss et al., 1999). Unfortunately, due to tissue limitations, this was not able to be examined within the constraints of this project, but would warrant further investigation.

The observation of ectopic beats in a subset of failing trabeculae at low frequencies of stimulation meant their exclusion from FFR analysis. However, the occurrence of these abnormalities provides further insights as to the potential impairment of homeostasis in the failing human heart. Given the suggested shift towards systolic calcium extrusion from the cell by NCX in heart failure (Pieske et al., 1995), this would result in a corresponding influx of Na⁺ ions, potentially depolarising the membrane and triggering an action potential (delayed after-depolarisation), resulting in an ectopic contraction (Bers, 2001). In the rat, cardiomyocytes show a relatively high resting Na⁺ level in the cytosol, which through NCX activity, is responsible for maintaining a high SR calcium load (Bers, 2001). There is a naturally occurring overall negative FFR at low frequencies in rat, although some isolated myocytes exhibit a positive ionotropic response (Capogrossi et al., 1986, Frampton et al., 1991). During contraction in the rat, however, NCX favours calcium extrusion (Shattock and Bers, 1989), such that with an increasing stimulation frequency, there is a progressive extrusion of calcium from the cell leading to the dominant negative FFR observed (Frampton et al., 1991). Given that a similar change in resting Na⁺ occurs in the failing human heart (Despa and Bers, 2013), it is possible that the altered intracellular Na⁺ homeostasis contributes not only to the occurrence of ectopic beats, but also the decline of the FFR.

The prolongation of both the TPS and T₅₀% are commonly reported in heart failure (Ljubojevic et al., 2014, Gwathmey et al., 1990), which further indicates impairment of EC coupling mechanisms. In the present study it was revealed that there was a significant positive correlation between TPS and T₅₀%, indicating that as these failing trabeculae exhibit
impairment of speed of contraction, they are more likely to show impaired relaxation dynamics as well. In terms of structural influences, it was found that TPS was associated with changes in the co-localisation of JPH2 with α-tubulin, whereby increased association of JPH2 with microtubules was related to an increased TPS. It has recently been suggested that altered JPH2 trafficking in response to microtubule densification is involved in the remodelling of the t-tubules (Zhang et al., 2014). The increased association of JPH2 with α-tubulin may indicate more extensive trafficking occurring, removing it from the typical junctional location where it helps to maintain the close coupling of the SRJ and t-tubule membranes (Takeshima et al., 2000). While a significant relationship was not identified between JPH2-α-tubulin co-localisation and the extent of RyR cluster association with t-tubule labelling in this study (data not shown), it is possible that there is an additional element of t-tubule remodelling present in these samples. For example, in addition to the observed occurrence of oblique angles, the loss of t-tubule connection to the surface sarcolemma has been reported to occur in failing cardiomyocytes (Lyon et al., 2009), and is linked to the loss of EC coupling through desynchronisation of the calcium transient (Brette et al., 2002). While the exact mechanisms remain unclear at this point, the findings from the present study suggest that the association of JPH2 with microtubules plays a role in mechanical dysfunction in the failing myocardium. No significant structural correlates were identified with T_{50%}, which is likely to be due to the inability to include examination of SERCA and NCX organisation in the present project.

In addition to this suggested structural component, the observed variability in both the FFR and temporal aspects of mechanical performance may in part be explained by alterations to the calcium handling properties of the cardiomyocytes. It has been shown that there is a reduction in calcium transient amplitude (Sipido et al., 1998, Pieske et al., 1995), as well as a delay in the kinetics of the transient in heart failure (Gwathmey et al., 1990). Due to limitations on both the experimental protocol and number of samples available, only three trabeculae were able to be loaded with calcium indicator in this project (data not shown). However, due to this very low sample number, the resulting transients were not considered to be sufficient for investigating potential correlative relationships with the mechanical data. Another potential factor contributing to both the FFR and temporal characteristics of stress would be impaired energetics and mitochondrial function. It has been previously suggested that mitochondrial dysfunction results in an inability to meet metabolic demands of the cardiomyocyte, leading to ATP-dependent processes to become impaired (see (Vogt and Kubler, 1998) for review). This includes the cross-bridge cycling of the myosin ATPase which is important for the development of force and therefore TPS, as well as cellular relaxation which could be impaired through insufficient SERCA ATPase activity, lengthening the T_{50%}
5. Structure-function relationship in human heart failure

(Bers, 2001). Unfortunately, investigation of this as a potential contributing component to the FFR and temporal dynamics of contraction was outside the scope of the present study.

5.4.5 Alterations in organisation in the failing human heart and trabeculae as a model for failing myocardium

As discussed, fibrosis of the myocardium is commonly observed in cardiomyopathy and heart failure (Vahl et al., 1993, Maron et al., 1975). This is further supported by findings presented in this chapter which identified myocardium samples from the failing ventricle as showing a reduced cardiomyocyte content compared to the non-failing heart. Initially, the remodelling of the ECM is thought to be an adaptive process in response to pressure overload through increased collagen deposition to generate increased systolic stiffness (Weber et al., 1989). However, with the further progression of cardiomyopathy, this increase in ECM volume becomes decompensatory, impairing the mechanical performance of the myocardium. This cardiac fibrosis leads to stiffening of the ventricle wall, increasing the resistance which cardiomyocytes must overcome to generate shortening, contributing to the reduced ability of the failing heart to generate sufficient active force (Weber et al., 1989). While increased collagen deposition is also associated with the response to cell loss as a result of myocardial infarction or pressure overload (Weber et al., 1988), it is unclear exactly what triggers this ECM proliferation in other forms of heart disease, as it has also been observed to occur in the absence of necrotic cell death (Doering et al., 1988). Interestingly, it was observed that when all failing trabeculae samples were grouped together there was significantly lower cardiomyocyte content compared to the failing ventricle wall, but when the trabeculae were grouped according to contractile performance, those with improved function showed no difference compared to the wall myocardium. These findings highlight the higher degree of variability in tissue composition observed in cardiac trabeculae, which has also been previously reported in the healthy rat heart (Sands et al., 2011). While the function of cardiac trabeculae in the adult heart is not fully understood, the findings in the present study suggest that the trabeculae are potentially more susceptible than the ventricular myocardium to ECM changes as a result of altered mechanical performance in the failing heart. Clearly shown through these experiments is that the relative cardiomyocyte content of the myocardium is highly important for determining the ability to develop active stress, and that this is a high predictor of contractile function in the failing human heart.

The coupling of the t-tubule and SR$_J$ membranes is essential for the formation of junctions, which are necessary for the efficiency of CICR and EC coupling processes through the close
association of DHPR and RyR (Bers, 2001), which are localised to the respective membranes (Scriven et al., 2000, Soeller et al., 2007). It has been previously shown that in a canine model of heart failure, there is a reduction of RyR association with the sarcolemma, despite maintaining cell-wide organisation of the clusters, indicating that it is primarily due to the remodelling of the t-tubule network (Sachse et al., 2012). The observation of reduced RyR-t-tubule association in the failing myocardium compared to non-failing is in agreement with these previous reports, suggesting improved cellular organisation in the non-failing samples. Based on the additional finding of a strong correlation between cardiomyocyte content and RyR-t-tubule association, it is not surprising to observe both of these parameters to be reduced in the failing heart, as previously discussed. The loss of association of the RyR clusters with t-tubules in these samples indicates that there is likely to be impaired EC coupling dynamics through the decreased formation of junctions, in agreement with previous studies in heart failure models (Song et al., 2006, Zhang et al., 2013).

It was found that there was no change to the labelling density of RyR in the failing myocardium in this study. While there have been reported discrepancies regarding the presence of reduced RyR levels in human heart failure (Go et al., 1995, Yano et al., 2000, Sainte Beuve et al., 1997, Gomez et al., 1997), the reduction of JPH2 expression has been commonly found in human cardiomyopathy (Landstrom et al., 2011, Zhang et al., 2013). However, this was not supported in analysis of labelling density in the present study, which indicated no change in JPH2 in the failing myocardium compared to non-failing. Interestingly, this is in agreement with recent Western blot findings from our laboratory, which did not identify altered JPH2 expression in human heart failure samples (Crossman et al, unpublished data). Together, these findings suggest that structural changes associated with altered JPH2 function are likely to be the result of protein reorganisation, rather than loss. Although there was no difference observed in the extent of JPH2 co-localised with RyR between failing and non-failing samples, the finding of reduced RyR co-localised with JPH2 in the failing myocardium is in agreement with the previous observation of decreased RyR-JPH2 association in a myocardial infarction model of heart failure (Wagner et al., 2012). While there was no relationship observed between this structural parameter and active stress development in the failing trabeculae, this finding indicates a reduced degree of junctional organisation in the failing myocardium. The decreased association of RyR clusters with JPH2 may lead to increased leak of the channels (Van Oort et al., 2011), contributing to higher diastolic calcium concentrations and reduced SR content, both of which are observed in heart failure (Gwathmey et al., 1990, Beuckelmann et al., 1992, Lindner et al., 1998). The organisation of EC coupling structures in the cardiomyocytes of failing trabeculae were not
found to be different from that observed in the ventricle wall, with the above parameters unchanged between samples. This indicates that in terms of RyR cluster organisation in respect to both t-tubules and JPH2, the cardiac trabeculae are a good overall model for cardiomyocyte organisation in the ventricular myocardium.

While the densification of microtubules was first described over 20 years ago (Tsutsui et al., 1993), this cytoskeletal alteration has recently become an area of interest in the failing myocardium, particularly in relation to JPH2 (Zhang et al., 2014). Findings from this study further confirm the presence of increased tubulin density in the failing myocardium at the cellular level. Increased microtubule density has been suggested as physically impairing contractile function through increased stiffness within the myocytes (Tagawa et al., 1998). It has also been shown that disruption of microtubule polymerisation reduces the impairment of t-tubule organisation and contractile dysfunction in heart failure (Tsutsui et al., 1993, Tagawa et al., 1998, Koide et al., 2000, Zhang et al., 2014). There is additional evidence that microtubule dynamics are able to modulate EC coupling properties, including $I_{Ca}$ and the calcium transient, both of which are enhanced by depolymerising microtubules (Gómez et al., 2000). Together, there have been multiple plausible links between increased microtubule density and impaired function in the diseased heart. The observed 'wrapping' of the microtubules around the nucleus in many samples is in agreement with previously reported distributions in both failing and non-failing cardiomyocytes (Heling et al., 2000, Schaper et al., 1991), where they may play a role in nuclear envelope protein trafficking (Park et al., 1984). It has been shown that perinuclear protein expression and organisation is altered in both human and rabbit heart failure, which was also associated with changes in calcium handling (Ljubojevic et al., 2014). While it is unclear if there is a link between these two observations, given the role of microtubules in trafficking, it is possible that they play a role in perinuclear organisation and an additional role in myocyte function.

Interestingly, it was observed that there was increased tubulin density within the tissue of failing trabeculae compared to the ventricle wall. However, there was no difference observed in myocyte α-tubulin density, suggesting that the tissue-wide increase is likely to be due to non-cardiomyocyte cells. This could include activated myofibroblasts which are involved in the deposition of increased collagen during fibrosis (Weber et al., 1989), which have been shown to express a high density of microtubules (Rudolph and Woodward, 1978). This is in agreement with the observation of the high prevalence of α-tubulin positive staining outside of the cardiomyocytes in the trabeculae. It was further observed that there was increased co-localisation between α-tubulin and JPH2 in the trabeculae compared to ventricle myocardium.
Although not related to stress development, this co-localisation is considered an adverse alteration as it was correlated with prolongation of TPS. This is also supported by the observation of increased JPH2-α-tubulin co-localisation in the failing myocardium compared to non-failing. While this latter observation may be in part due to the increased density of α-tubulin in the myocytes, the exact mechanism underlying this apparent pathological change remains unclear. Previous research has indicated that a signalling complex exists between the ECM and the cytoskeleton (Carmignac and Durbeej, 2012); it is therefore possible that, coupled with the increased ECM content, the failing myocardium (in particular the trabeculae) are also more prone to ECM-mediated alterations to the cytoskeleton, including the microtubules. The increased association of JPH2 and α-tubulin in the failing myocardium indicates the potential for enhanced intracellular trafficking of JPH2, which is in agreement with previous findings from Zhang et al (2014) in human and mouse failing hearts.

5.4.6 Significance, limitations and future directions

Despite the prevalence of cardiac trabeculae studies to investigate cardiac function, this is one of the first studies to directly investigate the link between cardiac structure and the corresponding function within the same samples. This has provided valuable novel information regarding the cardiac structure-function relationship in the failing human heart, particularly regarding the importance of cardiomyocyte content for contributing to peak stress development. From these findings, it would be recommended that future studies examining cardiac trabeculae need to determine the composition of the tissue, as this would be a significant contributor to functional results, especially in the failing heart. Furthermore, it was discovered that there is a strong correlation between the organisation of cardiomyocytes at both the tissue and cellular level, with increased myocyte content appearing to be associated with improved protein organisation.

The main limitation throughout this study was the inability to obtain trabeculae from non-failing hearts due to unavailability. This means that functional experiments performed on failing samples could not be directly compared with results from non-failing samples to determine the contribution of pathological changes as opposed to biological variation. However, this was largely overcome by the ability to use this variation in the failing samples to our advantage, whereby structural correlates were directly linked to functional performance. While there was high variability in stress production observed between trabeculae collected from different hearts, care was taken to ensure this was not the underlying cause of overall variation identified in parameters measured. This was achieved by selecting samples from
failing hearts in which multiple suitable trabeculae were able to be investigated, and confirming that at least one of the trabeculae was functionally responsive. By confirming the responsiveness of one (or more) trabeculae from a single heart, reassurance was obtained that the inability of some failing trabeculae to generate force was not due to delays in collection of the samples, or in the processing of the tissue. Based on these experimental measures, it was determined that there was substantial variation between trabeculae from a single heart, which was comparable to that observed overall between failing hearts. This indicated that inter-heart variation was not a significant contributing factor to the overall findings in this study.

One further limitation was the difficulty in obtaining a large number of suitable tissue samples for histological analysis, particularly from non-failing human hearts. This is largely due to the fact that the majority of non-failing hearts that became available were designated for the transplant recipients, and so research samples could not be obtained from these hearts. While this did limit the number of non-failing hearts able to be used in this study, a high degree of structural similarity was observed between those that were used. This indicates that the structural parameters analysed in this study showed low variability between non-failing hearts, providing confidence in the reported findings, despite low sample numbers. While it may have been possible to source additional samples from other tissue banks, those available had not been processed following the same fixation protocol, and subsequently could lead to further confounding factors in the interpretation of results. Unfortunately, further to this was the limitation that obtaining gender-matched non-failing ‘controls’ was not possible, so there may be unidentified sex-based confounding factors when comparing results to the non-failing myocardium.

An additional limitation was the physical size of the trabeculae, as there was a restriction on the number of proteins which could be examined within the confines of this study due to tissue sectioning. It would therefore be of interest in future studies of this nature to investigate additional proteins for which the structural organisation may play a role in the functional performance of the myocardium. This would potentially include SERCA and NCX, as well as attempting to obtain substantial numbers of calcium recordings for examining the calcium transients and SR content of the myocytes.
Chapter 6. Development of t-tubule and EC coupling organisation in the fetal sheep

6.1 Background

The reliance on a heartbeat for the delivery of nutrients to meet metabolic demand throughout the body begins long before we are born. The development of the cardiovascular system begins in the early stages of embryonic life, with a rudimentary, beating heart present from approximately 21 days in utero in humans (Brand, 2003). At birth, the heart undergoes remodelling during the fetal-neonatal transition to adapt to the change in supply of oxygen from the placenta to the lungs. However, prior to this transitional remodelling, structural and functional changes occur within the cardiomyocytes of the heart to adapt to the increasing metabolic demand as the fetus increases in size. Morphologically, this includes both the proliferation and enlargement of existing myocytes through hyperplasia and hypertrophy, respectively (Burrell et al., 2003). Associated with this cardiomyocyte enlargement is a shift in the mechanisms underlying EC coupling processes (Louch et al., 2015), requiring a corresponding change in the proteins and structures involved in these mechanisms. This includes progressive alterations to the organisation of key calcium handling proteins including NCX, SERCA, RyR as well as the t-tubules.

6.1.1 Development of EC coupling organisation in rodents

The ability of the heart to contract efficiently in adult mammals is reliant on the development and maintenance of the organisation of key proteins and structures involved in EC coupling, as outlined in Chapter 1. This involves the specific localisation of key proteins to the appropriate regions within the cardiomyocyte, including the t-tubules (for DHPR and NCX), the SR (for RyR and SERCA), as well as the functional coupling of these two membranes for the formation of junctions. In the fetal and neonatal heart, the requirement for protein
organisation is no less important, however the smaller cell size (Jonker et al., 2007) and the absence of t-tubules in early developing cardiomyocytes means that a different pattern of protein localisation is required (Louch et al., 2015). The majority of studies to date examining functional and structural changes occurring throughout the developmental transition have been performed in neonatal rodents and rabbits. While there is contractility of the embryonic rodent heart, with expression of many of the proteins involved in adult EC coupling (Louch et al., 2015), the organisation of these into internal functional calcium release units (CRUs) typically does not occur until after birth. This includes clear evidence that t-tubules, and therefore intracellular junctions, develop only after birth in rodents (Haddock et al., 1999, Reynolds et al., 2013), however, peripheral couplings are found at the surface membrane during embryogenesis (Takeshima et al., 2000). This suggests that fetal and early developmental EC coupling relies on a different mechanism from the traditional CICR localised along t-tubules that is observed in adult rodent hearts.

Proteins previously identified as being located to the t-tubules in adult cardiomyocytes show a differential pattern of localisation throughout progression from newborn to adult cells in rodents and rabbits. In particular, the NCX is predominantly localised to the t-tubules and on the surface sarcolemma in adult cardiomyocytes (Frank et al., 1992, Kieval et al., 1992) including within dyadic regions (Thomas et al., 2003). Several studies have shown that at birth and in early rodent postnatal development, NCX is initially solely localised to the surface sarcolemma, and it becomes increasingly internally localised from the age at which t-tubule development starts, being around P11 in rabbits (Haddock et al., 1999, Chen et al., 1995). As cardiomyocyte development progresses, NCX distribution becomes more striated in appearance and co-localises with the developing t-tubular system in the cell interior (Chen et al., 1995). Furthermore, it has been shown that compared to the adult and juvenile, there is higher expression of NCX in the fetal and early neonatal heart (Artman, 1992). This is coupled with evidence to suggest that early developmental EC coupling involves the reverse mode of NCX, which not only triggers SR calcium release, but can also directly provide the triggering calcium for contraction (Huang et al., 2008c), as opposed to primarily DHPR-mediated CICR in adult cardiomyocytes (Bers, 2002). Embryonic cells also rely on T-type calcium channels for $I_{Ca}$, which declines with development (Tohse et al., 2004, Leuranguer et al., 2000). There is progressive decline of expression (Artman, 1992, Liu et al., 2002) and surface sarcolemmal localisation of NCX (Chen et al., 1995), which is associated with a decreasing reliance on NCX for triggering contraction (Sipido et al., 1997). A corresponding increase in the amount of SERCA expressed is observed as NCX expression levels decline (Vetter et al., 1995). This is associated with the shift from reliance on NCX for cytosolic
calcium removal towards an increasing extent of SR calcium re-uptake (Vetter et al., 1995) and is also coupled with NCX activity shifting to increasingly favour the forward direction for calcium extrusion (Artman et al., 2000).

The calcium transient in embryonic and early neonatal rodent hearts is typically 'U-shaped' due to the absence of t-tubules, and therefore there is reduced activity and synchronisation of intracellular RyR clusters (Perez et al., 2005, Haddock et al., 1999, Seki et al., 2003). Studies examining the early distribution of RyR have shown that there are clusters present intracellularly prior to t-tubule formation, localised in corbular SR (Franzini-Armstrong et al., 2005), with Sedarat et al showing that in rabbits even as young as P3 there is already clear, striated intracellular organisation of the calcium channel (Sedarat et al., 2000, Haddock et al., 1999, Dan et al., 2007, Ziman et al., 2010). Embryonic distribution of RyR is predominantly peripheral, with a gradual increase in the content of calcium channels in these clusters (Franzini-Armstrong et al., 2005). Early neonatal RyR distribution shows a combination of surface and intracellular localisation, with a shift towards more prominent intracellular localisation that increases with postnatal development (Dan et al., 2007). As t-tubules, and therefore intracellular junctions, develop, the density of RyR and DHPR within these junctions progressively increases (Franzini-Armstrong et al., 2005). The protein expression level of DHPR also increases as the cardiomyocytes mature (Liu et al., 2002). DHPR has been found to be initially localised to the surface sarcolemma, and progressively change from P10 to a striated distribution in parallel with the formation of the t-tubules, to gradually co-localise with the RyR (Sedarat et al., 2000, Seki et al., 2003, Wibo et al., 1991). This increased co-localisation is also associated with the timing of calcium spark appearance within the myocytes (Seki et al., 2003).

Together with the organisation of RyR and DHPR into junctions, the ability of cardiomyocytes to produce calcium sparks is dependent on the maturation and content of the SR (Louch et al., 2015). Functional SR has been found to be present in young neonatal hearts (Perez et al., 2005) and it has been shown that both fetal and neonatal rodent cardiomyocytes rely on SR release for contributing calcium for initiating contractions (Haddock et al., 1999), with this contribution increasing with postnatal development (Bassani and Bassani, 2002, Escobar et al., 2004). To maintain calcium homeostasis, the amount of calcium released from the SR needs to balance re-uptake via SERCA (Bers, 2002). It has been shown that SERCA expression and activity gradually increases with maturation after birth in rodents (Liu et al., 2002), with z-disk alignment of SERCA present from early developmental ages in rats (Perez et al., 2005). Increased protein levels are associated with an increased capacity of the SR to
store (and release) calcium (Korhonen et al., 2009, Ziman et al., 2010), due to both increased up-take and a shift towards increased calsequestrin presence for buffering (Ioshii et al., 1994, Franzini-Armstrong et al., 2005). There is also a parallel increase in phospholamban expression with the increased SERCA levels (Liu et al., 2002). At early stages of development, calcium provided for SR loading is primarily via NCX entry, shifting towards DHPR-mediated influx in later stages (Huang et al., 2007).

6.1.2 T-tubule formation in rodents

To date, the exact mechanisms and processes underlying the development of both t-tubules and junctions for EC coupling in mammalian cardiomyocytes remains unclear. There are two main proposed mechanisms for the formation of t-tubule invaginations from the plasma membrane. The first involves the inward invagination of the plasma membrane whereby DHPR containing vesicles merge with the surface membrane, increasing the lipid content allowing for inward extension of the membrane (Di Maio et al., 2007). The DHPR expressed in these budding t-tubules then secondarily associate with the intracellular RyRs (localised in SR vesicles) to form junctions along the developing t-tubule membrane. The second proposed mechanism suggests that the DHPR-containing intracellular vesicles associate with RyR-containing SR vesicles within the cell interior prior to the fusion with the plasma membrane. These dual vesicles then progressively merge with each other to gradually build the t-tubule network from inside-out (Di Maio et al., 2007). A hybrid model of these two mechanisms has been suggested as the most plausible (Di Maio et al., 2007). Whichever the mechanism of formation, previous work has identified that the final pattern of t-tubule network organisation in healthy adult animals is species-dependent (Jayasinghe et al., 2012b).

The majority of recent studies examining the development of the cardiac t-tubule system in mammals also primarily focus on rodents. The findings of these studies are all in agreement that in rats, mice and rabbits the t-tubule system develops in the postnatal hearts of these animals, with it virtually absent prenatally or at birth. In mice, it is between ages P10-P19 that this development begins and matures until the t-tubule system resembles a phenotype identical to that in adult mice, with the earliest beginnings of sarcolemmal membrane invagination suggested at P5 (Chen et al., 2013, Reynolds et al., 2013). In newborn rabbits (P1-5) there is an absence of t-system in ventricular cardiomyocytes, with development also identified as beginning around the P10 age (Haddock et al., 1999, Sedarat et al., 2000). A similar, however delayed, pattern is also observed in rats, with identifiable t-tubules becoming apparent at age of P15 (Chen et al., 2013, Han et al., 2013, Ziman et al., 2010). Together, all
these studies to date on the developing rodent heart provide evidence of the development of t-tubules as occurring after birth.

6.1.3 The role of JPH2 in cardiac development

The formation of junctions has long been implicated as an important process for the generation of normal EC coupling in adult cardiomyocytes (Bers, 2002). Junctions are formed by the close association of the plasma membrane (surface and t-tubule) and the SR membrane to produce a ~12-15 nm cleft (Franzini-Armstrong et al., 1999). A key protein implicated in forming and maintaining junctions in cardiomyocytes is junctophilin-2 (JPH2) (Takeshima et al., 2000). In embryonic mice, JPH2 first becomes detectable at E9.5, which coincides with the age at which JPH2-knockout becomes embryonically lethal (Takeshima et al., 2000). JPH2 expression levels are found to increase in mouse cardiac tissue preceding the initiation of t-tubule development, with expression levels also increasing during the early stages of t-tubule maturation, at which point protein expression plateaus (Chen et al., 2013). While early localisation is predominantly on the surface, it has been shown that JPH2 starts to become intracellularly localised in developing rat and mouse cardiomyocytes prior to t-tubule formation (Ziman et al., 2010, Reynolds et al., 2013). This suggests that JPH2 localises to the SR membrane first where it plays a role in guiding the developing t-tubules and promoting the formation of junctions. As increasing levels of JPH2 are found intracellularly (and surface JPH2 decreases), the fraction co-localised with RyR also increases, indicating an improved level of junctional organisation, which is synchronised with the formation of t-tubules (Ziman et al., 2010).

In addition, recent studies have shown that in JPH2-knockdown mice, t-tubule development is delayed, while over-expression of JPH2 leads to faster maturation towards a fully developed t-tubule network in postnatal animals (Reynolds et al., 2013, Chen et al., 2013). T-tubules that do form in these JPH2-KD mice are often disorganised, and display an inability to form a mature t-tubule network with a sufficient number of junctions, resulting in postnatal mortality prior to the age at which t-tubule maturation is complete in control mice (Reynolds et al., 2013). This indicates that JPH2 plays an essential role in normal t-tubule development, although the exact mechanism remains unclear.
6.1.4 Development of EC coupling organisation in large mammals

While there is a lack of studies in larger mammals which utilise more recent advancements in fluorescent techniques, a handful of previous developmental studies have been based on EM work. These include the use of fetal sheep (Brook et al., 1983, Sheldon et al., 1976), guinea pig (Forbes and Sperelakis, 1976), or human tissue (Kim et al., 1992). Although guinea pigs are classified as rodents, they have a longer gestational period (68 days; (National Research Council, 1996)) compared to mice, rats and rabbits (19, 22 and 32 days respectively; (Sissman, 1970)), and so will be considered as ‘larger mammals’ for developmental purposes. These studies on larger mammals suggest an initial prenatal t-tubule development, with preliminary invaginations of the surface sarcolemma and caveolae extensions observed in line with the z-disk. Rudimentary t-system has been identified as early as 32 weeks in the fetal human (term: 40 weeks; (Kim et al., 1992)), 8 weeks gestation in guinea pig (term: 9 weeks; (Forbes and Sperelakis, 1976)) and 90 days gestation in the sheep (term: 145 days; (Sheldon et al., 1976)). While the t-tubule organisation in these fetal tissues is far from adult phenotype, it does provide evidence that t-tubule development may begin prenatally in some larger mammals, with further development continuing after birth to resemble the adult organisation. However, due to the nature of EM work, it can be difficult to extrapolate findings from such small tissue sections to accurately determine the overall cellular or tissue-wide occurrence of these structures. Therefore, confocal imaging was utilised in the present study to further examine and confirm the pattern of t-tubule development in a large mammal fetus.

In fetal sheep, the ability to take up calcium into the SR increases with age (Spencer et al., 2006), with expression and activity levels of SERCA reduced compared to the adult sheep, along with reduced phospholamban expression levels (Mahony and Jones, 1986). A human study found that SERCA expression levels do not change with postnatal cardiac development (Wiegerinck et al., 2009). However, this same pattern was not observed in an additional study, which identified progressively increasing SERCA protein levels from 15 weeks gestation through to adulthood in human hearts (Qu and Boutjdir, 2001). A similar trend was observed with DHPR expression, which was coupled with a progressive decline in T-type calcium channel levels in the immature human heart (Qu and Boutjdir, 2001). It has also been shown that in postnatal human development there is decreasing NCX expression which shows an increasing intracellular distribution with maturation (Wiegerinck et al., 2009). While these expression studies provide valuable information, there is a lack of data in regards to the distribution and organisation of these EC coupling proteins in large mammal development.
While findings suggest that unlike rodents, larger mammals have rudimentary t-tubule structures developing prenatally, to date the majority of studies examining the development of EC coupling protein organisation have focussed on rodents. This leaves a potential gap in current knowledge as to how the organisation of key proteins involved in EC coupling develops in larger mammals, which are suggested to show a similar temporal pattern to human cardiac t-tubule development. The aim of this chapter was to conduct a preliminary study to investigate the changes in EC coupling protein organisation during the development of the large mammalian heart. This involved confocal imaging of myocardium sections from the left ventricle (LV) of fetal sheep of increasing gestational ages, to examine the distribution of the t-tubules, RyR, JPH2 and NCX. The identified EC coupling organisation in these fetal hearts was compared to that observed in adult sheep hearts.

6.2 Experimental Techniques and Analysis

6.2.1 Fetal sheep myocardium samples

Romney/Suffolk fetal sheep hearts were kindly provided by the Fetal Physiology and Neuroscience Laboratory (University of Auckland), from which tissue samples from the LV were collected and immersion fixed in 1% PFA on ice for 2 hours. In order to investigate the development of cardiac structure, samples were obtained from fetuses of varying gestational ages, with full-term gestation being 145 days (Davidson et al., 2015). Gestational ages of 108, 114, 125-126 and 135-136 days were used (n= 3 animals per age), covering preterm through to near-term fetal development. In addition, adult maternal LV samples (n= 3 animals) were also collected and processed to assess and compare protein organisation in the adult sheep heart. Tissue samples were processed and immunolabelled according to the protocol detailed in General methods.

Several proteins and structures of interest were examined to investigate the development of EC coupling organisation in the sheep heart. This involved dual labelling with primary antibodies, including mouse anti-RyR2 (Thermo) or anti-NCX1 (Swant), with the RyR antibodies coupled with rabbit anti-JPH2 (Yenzym). This was followed by species-specific secondary antibodies conjugated to Alexa Fluor 488 or 568, together with incubation of Alexa Fluor 647 conjugated WGA to label the surface sarcolemma and t-tubule network (Jayasinghe et al., 2015). Additional samples were prepared which were stained with WGA.
(Alexa Fluor 594 conjugated) in combination with phalloidin conjugated to Alexa Fluor 488 to label the contractile machinery by binding to f-actin (Lengsfeld et al., 1974). These samples were imaged using an inverted Zeiss LSM 710 confocal microscope with a 40x objective.

6.2.2 Image processing and analysis

Confocal stacks were acquired of the EC coupling protein labelled samples, which were subjected to deconvolution, as detailed in General methods, followed by t-tubule analysis using ImageJ. Longitudinal images were used to assess the density of transverse elements (TE) of the t-tubule system. Maximal projections of three consecutive deconvolved image slices of WGA labelling were generated, followed by manual thresholding to ensure the resulting mask was representative of the original labelling. Intracellular regions were selected, with the percentage of WGA positive pixels showing clear transverse organisation determined to provide a measure of TE density across the different aged animals. Single focal plane images of t-tubule labelling in transverse orientated cells were also analysed. Percentile thresholding was applied to images acquired of WGA labelling in the transversely orientated cardiomyocytes. This threshold assumes that 0.5 signal fraction is attributed to foreground labelling. The intracellular region was then selectively cropped for five cells per image, with the percentage of WGA positively labelled pixels within each cell determined and the mean compared across the different age groups to measure the t-tubule prevalence.

6.2.2.1 Co-localisation analysis

In order to quantify the organisation of EC coupling proteins in relation to each other and the developing t-tubules, co-localisation analysis was performed in ImageJ on the double labelled samples in longitudinal orientation following deconvolution. In brief, this involved applying isodata thresholding to the confocal images of each of the protein labels of interest to generate a binary mask. The regions corresponding to labelling within the mask of one protein were then determined and overlaid onto the mask of the second protein. The number of pixels overlapping with the second protein were then determined and converted into a percentage of the total number of pixels in the mask. This analysis was performed following selectively segmenting the internal regions of the cardiomyocytes to determine intracellular co-localisation. This method was used in favour of the PYME based analysis detailed elsewhere due to the apparently highly variable expression and distribution of proteins between the different aged animals.
6. Development of t-tubule and EC coupling organisation in the fetal sheep

6.2.3 Statistical analysis

Statistical tests were performed using IBM SPSS Statistics v22 software, with either Student's t-test or ANOVA applied where appropriate. Unless stated otherwise, data are presented as mean ± standard error of the mean (SEM).

6.3 Development of EC Coupling Organisation in Fetal Sheep

6.3.1 Development of t-tubule network organisation

To further investigate previous reports of t-tubule system development as beginning in fetal sheep (Sheldon et al., 1976, Brook et al., 1983), confocal imaging was used to examine WGA staining in the LV of increasing gestational ages of fetal sheep and adult myocardium. Phalloidin staining was used to determine the organisation of the myofibrils into organised sarcomeres. Visible striations of the myofibrils (f-actin stained with phalloidin) were clearly present and organised into sarcomeres in the youngest fetal sheep (108 days; Figure 6.1A) and maintained through all ages of development. It was also observed that myocyte diameter appeared to increase with developmental age. In the youngest animals it was seen that there was very weak evidence of t-tubule presence in both the longitudinal (Figure 6.1B) and transverse orientation (Figure 6.1C), with only a few small budding invaginations of the surface membrane identified in a sparse number of myocytes. With advancing gestational age, there was increasing evidence of small plasma membrane invaginations into the cell interior to form the potential beginnings of t-tubules in the fetal sheep. This was seen as an overall increase in striated appearance of WGA labelling within the cardiomyocytes in longitudinal view (Figure 6.1, middle panels), as well as a greater number of observed plasma membrane ‘buddings’ in the transverse view (indicated by arrows in Figure 6.1, right panels). By 135-136 days gestation, there were clearly immature t-tubules present within the majority of cardiomyocytes, with moderate lengths of plasma membrane extension into the cell interior (Figure 6.1K,L). These membrane extensions were typically present as multiple apparent invaginations, side-by-side within the cell, aligning at neighbouring z-disks of the cardiomyocyte (as determined by phalloidin labelling). Although these t-tubules were not as extensive as those observed in the adult heart, they do provide clear evidence of t-tubule development beginning in the fetal sheep heart. In the adult myocardium it was observed that...
6. Development of t-tubule and EC coupling organisation in the fetal sheep
Figure 6.1 T-tubule organisation in fetal sheep (previous page)

Confocal micrographs showing dual staining of phalloidin (cyan) and WGA (red) in fetal sheep of increasing gestational ages. Left-hand column shows longitudinal f-actin staining with phalloidin and the corresponding t-tubule labelling with WGA is presented in the middle column. Right-hand column shows representative transverse sections of t-tubule labelling. Gestational ages: A-C) 108 days, D-F) 114 days, G-I) 125-126 days and J-L) 135-136 days. Arrows indicate immature t-tubule budding of the plasma membrane. M-O) Phalloidin and WGA labelling in adult sheep myocardium. Scale bars: 10 µm left and middle panels, 5 µm right panels.

WGA staining clearly labelled the surface sarcolemma as well as revealing the presence of regular t-tubules throughout the length of the cardiomyocyte with ~1.8 µm spacing (Figure 6.1N). These were seen to typically align with the z-disk in longitudinal orientation, and when viewed in transverse, a clear radial spoke-like arrangement was seen (Figure 6.1O). These were identified as being present in all cardiomyocytes imaged from the adult heart.

Assessment of the prevalence of intracellular WGA labelling as a marker for t-tubules was performed to quantify these findings. This confirmed the visual observation that there was low presence of t-tubules in the younger fetal sheep hearts, and that with increasing gestational age, this was enhanced (Figure 6.2). The density of TE of the t-tubule system in longitudinal myocytes was found to progressively increase in the developing fetal sheep heart, reaching a maximum in the adult (Figure 6.2A). In addition, when assessed in transverse orientation at the level of the z-disk, there was a dramatically higher prevalence of t-tubule labelling in the adult ventricle (26.9%) compared to the most mature fetal hearts (7.4% at 135-136 days), further supporting visual observations (Figure 6.2B). Statistical analyses on these data showed that, compared to the youngest fetal samples (108 days; n= 5 long cells, 2 animals; 10 trans cells, 2 animals), there was a significant increase in the prevalence of t-tubule labelling in transverse orientation from 114 days (p<0.05; n= 6 long cells, 3 animals; 20 trans cells, 3 animals), with both prevalence and TE density significantly increased from 126 days (n= 6 long cells, 3 animals; 20 trans cells, 3 animals; p<0.01 TE density versus 108 days, p<0.001 prevalence versus 108 days) onwards (Figure 6.2A,B). Furthermore, there was also a significant increase in both parameters of t-tubule presence identified between the oldest fetus of 136 days gestation (n= 8 long cells, 3 animals; 20 trans cells, 3 animals) and the adult sheep hearts (n= 5 long cells, 2 animals; 20 trans cells, 2 animals; p<0.01 TE density, p<0.001 transverse prevalence). Together, these findings demonstrate that t-tubule development begins in utero, with maturation continuing after birth in the sheep heart.
6. Development of t-tubule and EC coupling organisation in the fetal sheep

Figure 6.2 Prevalence of t-tubule labelling in developing fetal sheep hearts

Analysis of t-tubule (WGA) labelling in cardiomyocytes from fetal sheep hearts of increasing gestational age, as well as adult sheep hearts examining the A) TE density in longitudinal cardiomyocytes, and B) extent of t-tubule presence at the level of the z-disk in transverse orientation, both as percentages of cell area. Data displayed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 all ages versus 108 days; ##p<0.01, ###p<0.001 136 day fetus versus adult.

6.3.2 RyR and JPH2 development

The formation of intracellular CRUs is an important step in the maturation of EC coupling processes in the mammalian heart. For these to further develop into functional junctions, they must associate with the t-tubule membrane, which has been shown to involve JPH2 (Takeshima et al., 2000). Therefore, multi-labelled samples were used to assess changes in the extent of RyR cluster association with the t-tubules (WGA) and JPH2, as well as JPH2 co-localisation with the t-tubules in the developing sheep heart. Confocal imaging revealed that in all ages examined there were clear intracellular RyR clusters observed. In the youngest fetal samples there was also surface labelling observed (Figure 6.3A), with an apparent increasing number of intracellular clusters observed with gestational maturation as the myocytes underwent developmental hypertrophy. Furthermore, these intracellular clusters showed an enhancement in the striated arrangement with advancing gestational age (Figure 6.3B-D). In contrast, JPH2 was identified as being primarily distributed along the surface plasma membrane in the fetal sheep, with little evidence of clear organisation in intracellular regions at gestational age of 108 days. Beginning at gestational age 114 days, JPH2 could be identified within the cardiomyocytes, appearing as diffuse clusters (Figure 6.3B). In the 114-136 day fetal sheep, the RyR and JPH2 clusters were often observed associating with the developing t-tubule system. In the adult sheep heart, both RyR and JPH2 were observed to form clear, regular intracellular clusters aligned with the mature t-tubules with reduced
Development of t-tubule and EC coupling organisation in the fetal sheep

surface labelling in comparison to within the cells (Figure 6.3E). Visual assessment of the JPH2-RyR dual labelled samples suggested that there is an increasing extent of association between these two proteins within the cardiomyocytes. Analysis of intracellular labelling revealed that there was a progressive increase in the percentage of pixels within the cardio-

Figure 6.3 Development of RyR and JPH2 distributions
Deconvolved confocal images showing RyR (red) and JPH2 (green) labelling, with corresponding WGA (grey) staining in heart samples from A) 108 day, B) 114 day, C) 126 day, D) 136 day fetus and E) adult sheep. Scale bars: 5 µm. Co-localisation analysis of intracellular labelling reveals the extent of F) RyR and JPH2 association, as well as both G) RyR and H) JPH2 with t-tubule labelling. Data displayed as mean ± SEM; **p<0.01.
myocytes showing co-localisation between RyR and JPH2 with gestational development, and further increasing in the adult (Figure 6.3F). It was confirmed that compared to the 108 day fetus, there was a significant increase in the percentage of RyR-JPH2 co-localised pixels from 126 days onwards (p<0.01 for 126, 136 and adult versus 108 days; 108 days: n= 6 cells, 2 animals; 114 days: n= 7 cells, 3 animals; 126 days: n= 4 cells, 2 animals; 136 days: n= 8 cells, 3 animals; adult: n= 5 cells, 2 animals). There was an apparent further increase in the extent of RyR-JPH2 co-localisation in the adult sheep heart compared to the 136 day fetus; however this was not statistically significant. Unfortunately, due to the variability in t-tubule prevalence within the developing cardiomyocytes, quantitative co-localisation analysis involving the t-tubule labelling was very difficult; therefore we were not able to perform meaningful comparisons due to the confounding changes in prevalence, which made the use of a consistent thresholding procedure unfeasible.

6.3.3 NCX development

The changes in NCX distribution have been well studied in rodent cardiac development and to a lesser extent in the postnatal human (Wiegerinck et al., 2009), however no such studies to date have been performed in the large mammalian fetal heart. It has been shown that the functional role of this protein in EC coupling processes changes dramatically with cardiac maturation as internal structure develop (Artman et al., 2000). Therefore, this protein was also investigated in the fetal sheep heart in relation to the development of the t-tubules. It was identified that all fetal sheep examined, NCX was primarily localised to the surface plasma membrane (Figure 6.4A-D). In the older fetal sheep (in which t-tubule development was more pronounced) there was also identification of an additional intracellular NCX distribution. While this labelling was relatively sparse, it showed a pattern of alignment with the early t-tubules present in these cells (Figure 6.4C,D). This was clearly observed in the 126-136 day gestation fetal sheep, with evidence of early inward ‘budding’ of NCX labelling along the plasma membrane in 114 day animals (Figure 6.4B). In comparison, there was a more prominent degree of intracellular NCX labelling observed in the adult sheep, which clearly showed an overall striated appearance and was associated with the location of t-tubules (Figure 6.4E).
6. Development of t-tubule and EC coupling organisation in the fetal sheep

![Figure 6.4 Development of NCX organisation in the fetal sheep](image)

Confocal images of NCX (magenta) and WGA (grey) dual labelling in fetal and adult sheep. A) 108 days, B) 114 days, C) 126 days and D) 136 days gestation fetal with E) adult sheep myocardium. Scale bar: 5 µm.

6.4 Discussion

The results presented in this chapter form the basis of a pilot study investigating the development of EC coupling structure and protein organisation in the large mammal heart. This involved confocal imaging of hearts from increasing gestational age fetal sheep to examine the maturation of the t-tubules, as well as RyR, JPH2 and NCX organisation in comparison to the adult sheep heart.

6.4.1 T-tubule development in fetal sheep

The primary structure of interest was the t-tubule network and the potential prenatal development of this key cardiomyocyte structure in large mammals. There was evidence to suggest that there is rudimentary t-tubule budding from the surface plasma membrane...
identified in fetal sheep as young as 108 days gestation. As gestational age increased there was greater prevalence of this membrane budding in the maturing cardiomyocytes, with a significant increase in observed t-tubules in ovine fetal hearts from 114 days and older. The most mature fetal sheep hearts (135-136 days gestation) consistently showed multiple internal membrane extensions occurring within individual cells, as observed in both longitudinal and transverse orientation. This was observed in multiple cells within a single myocardium tissue section. These findings are in agreement with previous reports from EM studies in which the beginnings of t-tubules are identified from 90 days gestation in the fetal sheep, and clearly present from 110 days onwards with increasing prevalence and extent through to term (Sheldon et al., 1976, Brook et al., 1983). The importance of identifying these structures with the methods used in this study is that, unlike EM, the use of WGA staining and immunofluorescence allows specific labelling of several proteins of interest, making the identification of these structures a lot less subjective. By specifically targeting t-tubules with WGA throughout entire cardiomyocytes, the ability to identify their presence and pattern of distribution is greatly improved compared to previous EM approaches which rely on a small section of tissue to interpret ultrastructural changes with low contrast non-specific labelling. The transverse t-tubule organisation observed in the adult ovine hearts was substantially more developed than that in the oldest fetuses, with radial spoke-like projections observed in transverse orientation, with high prevalence in all cardiomyocytes. This mature phenotype is similar to that previously reported in the ventricle of adult sheep hearts (Richards et al., 2011). The further significant increase of identifiable t-tubule labelling in the adult heart compared to the 136 day fetus indicates that there is further development and maturation of the t-tubule network after this prenatal age, potentially occurring in the newborn or juvenile lamb to achieve an adult phenotype.

The presence of t-tubules is essential in mammalian ventricular myocytes to allow for efficient EC coupling to occur. This includes their essential role in propagating actions potentials into the cell interior to aid in the synchronisation of DHPR activation throughout the myocyte (Brette and Orchard, 2003). The dependence on t-tubules for synchronous EC coupling becomes more important with thicker myocytes, in which there would be a substantial temporal delay if they were to solely rely on calcium diffusion from the surface membrane (Brette et al., 2005). Therefore, as developing cardiomyocytes undergo hypertrophy, there is an increasing requirement for t-tubule formation to play a role in maintaining cardiac function. This is supported by findings from this work, as well as previous studies on postnatal rodent cardiac development. It is seen that there is very little evidence of t-tubules at young ages of cardiac development, being largely absent from the young (108 day) pre-term fetal sheep and
reported as absent from the newborn and young postnatal rodent (Chen et al., 2013, Reynolds et al., 2013, Haddock et al., 1999), both of which have relatively narrower cardiomyocytes than their older counterparts (Burrell et al., 2003, Dan et al., 2007). As mammalian cardiomyocytes mature, they enlarge through hypertrophy (Jonker et al., 2007, Louch et al., 2015) and subsequently require the development of t-tubules in order to maintain adequate EC coupling.

6.4.2 EC coupling protein maturation in fetal sheep

While early t-tubule formation has been previously identified with EM in large mammals, including sheep and humans (Sheldon et al., 1976, Kim et al., 1992), there have been no such studies examining the organisation of EC coupling proteins prior to birth in these large mammals. Immunofluorescent labelling revealed that there was an intracellular, striated appearance of RyR clusters from the youngest fetal sheep examined, which further increased in striated intracellular organisation with maturation, similar to that observed in early postnatal rodent cardiomyocytes (Sedarat et al., 2000, Haddock et al., 1999, Ziman et al., 2010). This suggests that the SR in these animals is at least partially developed with the capacity to support early intracellular CRUs, despite the absence of a mature t-tubule network. Indeed, it has been shown in rodents that these RyR clusters are localised along the corbular SR prior to t-tubule formation (Franzini-Armstrong et al., 2005). Furthermore, these intracellular CRUs are functional in the immature rodent heart but show a reduced synchronicity of activity (Perez et al., 2005). As the cardiomyocyte matures and develops t-tubules, there is an increasing reliance on traditional CICR mechanisms for cardiac function (Louch et al., 2015), which requires the organisation of RyR into clusters localised along the t-tubules. The pattern of RyR distribution observed with ovine cardiac maturation suggests that this is beginning to occur at a prenatal age in large mammals, with intracellular RyR clusters typically associating with any observed t-tubules that develop, potentially forming early junctions.

There have been several studies suggesting a key role of JPH2 in the formation of both the junctions and t-tubules in the rodent heart (Takeshima et al., 2000, Chen et al., 2013), with intracellular protein localisation observed prior to t-tubule development (Reynolds et al., 2013, Ziman et al., 2010). The findings presented in this study are in agreement with this, whereby it was observed that JPH2 formed clear intracellular clusters in the 114 day fetal sheep – an age at which t-tubule formation was only beginning to become apparent. With further development, there was an increased prevalence of clusters, which were also observed to be associated with immature t-tubules. This association with the t-tubules appeared to coincide
with the timing for the observed increase in co-localisation between JPH2 and RyR. In the postnatal rat, this co-localisation has also been shown to synchronise with the formation of t-tubules, with the co-occurrence of these events indicating that intracellular junctions are forming at this time (Ziman et al., 2010). Together, the findings from this study support the hypothesis that a similar pattern of junction maturation is occurring in the large mammal heart; however, this occurs in the prenatal period in the sheep compared to the postnatal period in rodents.

The distribution of NCX throughout the fetal myocardium was observed to be predominantly surface membrane labelling for all ages examined, while a strong intracellular distribution was observed in the adult sheep heart. In the 126-136 day fetal sheep, there was a small proportion of NCX observed within the cardiomyocyte which appeared to be associated with developing t-tubules. While the expression or activity of NCX has not been previously examined in the ovine heart, it has been previously shown in the young postnatal human heart that there is a progressive decline in expression along with a shift towards an intracellular distribution (Wiegerinck et al., 2009). A similar trend is observed in the developing postnatal rodent and rabbit heart (Haddock et al., 1999, Chen et al., 1995). Despite a lack of young postnatal samples, the findings from the present study are in general agreement with these previous reports with clear intracellular NCX only observed in those animals which also show evidence of t-tubule development. Given that NCX is known to localise to the plasma membrane (Frank et al., 1992, Kieval et al., 1992), it would be expected that intracellular distribution would only be possible in parallel, or following, the appearance of t-tubules.

6.4.3 Significance, limitations and future directions

While there is a plethora of research investigating rodents, this is the first immunofluorescent study to examine the development of t-tubules and EC coupling proteins in the large mammal heart prior to birth. Findings from this study show that, unlike rodents, there is early development of the t-tubules in the fetal sheep, and that this is associated with progressive maturation of EC coupling organisation, including RyR clusters forming intracellular junctions with an increasing association with both the t-tubules and JPH2. This included a significant increase in the co-localisation of intracellular JPH2 and RyR clusters with fetal maturation. The primary limitation of this study was the inability to include additional samples from infant or juvenile sheep, providing an intermediary stage of development between the fetal and adult samples. This was due to unavailability of such samples at the time that the study was
6. Development of t-tubule and EC coupling organisation in the fetal sheep

Conducted. Continuation of this study into newborn and infant lambs to further follow the progressive maturation of the t-tubules and EC coupling proteins would be of interest. Furthermore, obtaining isolated cardiomyocytes from fetal, newborn and adult sheep in future studies would allow for functional experiments to be performed. This would enable assessment of calcium transients and sparks for comparison between the ages to correlate directly with EC coupling protein organisation. One of the observations in this study was the variability of protein labelling across the different aged animals, which limited the ability to perform co-localisation analysis between the WGA labelling and the EC coupling proteins. Accordingly, Western blot analysis of protein expression levels would be a logical progression of these findings, particularly focussing on the EC coupling proteins. In addition, it would be of interest to examine these cardiomyocytes (isolated or tissue sections) using super resolution imaging to determine the changes to the nanoscale organisation of the peripheral couplings and intracellular junctions with development. Utilising this imaging modality would also allow for the possibility of improving on co-localisation analysis performed between the EC coupling proteins and the t-tubules. Furthermore, determining the implications of these findings in human fetal cardiac disease warrants further investigation.
Chapter 7. The relationship between cardiomyocyte organisation and cardiac function

This thesis presents a multi-scale study into the relationship between cardiomyocyte organisation and cardiac function. Not only does this cover examination of structural properties ranging from the tissue composition at the macroscopic scale, down to the organisation of protein clusters at the nanoscale, but it also covers the temporal scale of changes to cardiac function, from fetal development through to end-stage failure in the adult heart. Several structural components were investigated over the different facets of this study, including the calcium release units – the RyR clusters, and the t-tubules. Of particular interest was the role of JPH2 in the cardiac structure-function relationship. While this protein was considered in all aspects of cardiac function examined, it was primarily investigated by examination of JPH2 transgenic mice. Although recent studies have also begun to investigate JPH2 expression changes, they have been limited to the use of standard fluorescent techniques (e.g. confocal microscopy) and EM imaging modalities (Van Oort et al., 2011, Guo et al., 2014). This left a substantial gap in current knowledge as to how EC coupling proteins interact at the nanoscale level, which is difficult to determine with previous techniques. The use of super resolution imaging (Chapters 3 & 4) has helped to fill this gap by combining antibody-targeted protein labelling with sub-diffraction resolution. This allowed for the identification of nanoscale organisation of vital cellular structures – the junctions and t-tubules, and determination of how these were influenced by altered expression of JPH2. Subtle changes were revealed in the organisation of RyR clusters within these mice, which have not been previously reported. Linking these and additional structural changes with the calcium handling data supplied from these animals provided novel insights as to the role of JPH2 in the organisation and function of cardio-myocytes, including those from JPH2-knockdown mice which develop acute heart failure.

The examination of samples from the failing human heart made it clear that there is a high degree of variability in cardiac organisation and function, both between and within hearts
The relationship between cardiomyocyte organisation and cardiac function (Chapter 5). This emphasised the need to directly correlate structural alterations with the resulting contractile function, particularly in the absence of non-failing counterpart samples. The ability to determine both the function and structure within a single sample provided a novel approach for investigating the structure-function relationship in human heart failure. This revealed that the predominant determinant of cardiac function was the cardiomyocyte content of the trabeculae, and that this in turn was correlated with the microscopic organisation of EC coupling proteins within the individual cells.

While there is a vast availability of rodent studies, there is a surprising absence of studies investigating cardiac development in large mammals, particularly utilising advances in fluorescent imaging techniques to examine EC coupling protein distributions. This is the first study to apply these techniques systematically in the investigation of the prenatal development of cardiomyocyte organisation in a large mammal – the fetal sheep (Chapter 6). Compared to early EM studies (Brook et al., 1983, Sheldon et al., 1976), which are sensitive to tissue orientation and typically do not present specific protein distributions, this immuno-fluorescent work has shown target-specific evidence of the early development of the t-tubules, as well as revealing EC coupling protein organisation prior to birth in large mammals.

Cardiomyocyte organisation examined in this thesis ranges from the macroscopic tissue content down to the organisation of protein clusters at the nanoscale. The organisation of cardiomyocytes plays a vital role in the efficiency of EC coupling and subsequent cardiac function, which differs in both development and heart failure. The major findings of this thesis will be discussed in the following sections in relation to how these novel reports integrate into current knowledge of EC coupling mechanisms.

7.1 JPH2 in the Cardiac Structure-Function Relationship

The primary findings from the investigation of the JPH2 transgenic mice were the significant changes to the organisation of cardiomyocytes as a result of altered JPH2 expression. While altered calcium handling has been accepted as occurring in JPH2-knockdown mouse hearts (Van Oort et al., 2011, Wang et al., 2014), evidence from JPH2 over-expressing mouse strain showing changes to calcium handling (Wehrens’ lab; unpublished data) contradicts findings previously reported from a similar mouse model, despite the presence of structural modifications (Guo et al., 2014). Since the cardiomyocytes utilised in these experiments were provided from the former mouse strain, the structural results from this thesis will be discussed
in relation to functional reports from these same mice. The findings from these studies provide evidence of three mechanisms through which JPH2 is able to modulate EC coupling, and therefore cardiac function: 1) organisation of the junction and junctional proteins, 2) modulation of t-tubule organisation, and 3) stabilisation of the RyR channels.

### 7.1.1 Junctional organisation

The properties of the CRUs are critical in determining the EC coupling response of cardiomyocytes and subsequent cardiac function (Bers, 2001). One of the key findings from the super resolution imaging of the JPH2 transgenic cardiomyocytes in Chapter 3 was the identification of changes in the nanoscale organisation of the RyR clusters as a result of JPH2 expression levels. Detailed analysis of dSTORM images revealed that while there was no change in RyR cluster size in JPH2-KD mice, they showed a reduction in RyR labelling density within the junctions compared to control mice. This was not due to changes in protein expression level as both Western blot (Van Oort et al., 2011) and image labelling density analysis revealed that this was not affected in these mice. Interestingly, these transgenic mice have been found to have an increase in calcium sparks size (Van Oort et al., 2011), which could suggest a potentially increased CRU size, although this was clearly not the case. This indicates that there are additional factors contributing to the alteration of EC coupling processes in these mice, and subsequently, additional junctional properties were examined. Further findings from Chapter 4 revealed alterations to the composition of the junction as a result of reduced JPH2 expression, whereby there was an apparent reduction in the proportion of junctional NCX in these mice. This was associated with a loss of NCX activity in JPH2-KD mice with no change in protein expression levels (Wang et al., 2014), which while contributing to the observed increased frequency of calcium sparks (Van Oort et al., 2011), still does not fully explain the increase in spark size. Other mechanisms through which JPH2 is implicated in modulating EC coupling were therefore investigated.

Over-expression of JPH2 led to an average enlargement of RyR clusters by ~27% in comparison to control mice, together with an increase in the prevalence of ‘macro-clusters’ containing >200 RyR channels. Coupled with the observations that the RyR protein expression levels (Wehrens’ lab, unpublished data) and total labelling density were not changed between these animals, this indicates that the increase in CRU size is not due to an increased RyR channel content, but that a similar number of channels are distributed over a larger junctional area compared to controls. Indeed, these enlarged JPH2-OE clusters were associated with a reduction in the density of RyR labelling within the junction. Findings from
EM work by Guo et al (2014) revealed an increased contact area between the SR and t-tubule membranes in a similar strain of JPH2-OE mice, which supports the hypothesis of an enlarged potential area in which the RyR can be contained. This suggests that there is incomplete packing of the junctional space, such that with an increased RyR cluster size there is a loss of density due to the channels being more ‘spread out’. This is supported by previous findings which suggest that in wild-type rodent cardiomyocytes, there is incomplete filling of RyR channels within the junction (Jayasinghe et al., 2012a, Hayashi et al., 2009, Das and Hoshijima, 2013). While the mean inter-cluster nearest neighbour distance was increased in these mice, so too was the labelling area of RyR super-clusters, in which a ‘super-cluster’ was defined as the combination of individual clusters within a 100 nm distance, such that there is substantial potential for inter-cluster activation (Sobie et al., 2006). Interestingly, these results would suggest an increase in the size of calcium sparks released from the RyR clusters; however, functional data shows that there is a reduction in both size and frequency of calcium sparks (Wehrens’ lab, unpublished data). These findings do, however, provide a potential structural basis for the observed heterogeneity in the calcium wavefront in these mice, whereby the larger clusters with increased inter-cluster spacing could produce the observed peaks and troughs of the calcium transient (Wehrens’ lab, unpublished data). It has also been shown that in JPH2-OE mice there is an associated up-regulation of NCX (Guo et al., 2014)(Wehrens’ lab, unpublished data). Super resolution imaging of these mice revealed that there is a corresponding increase in the proportion of NCX co-localised with RyR, indicating that the proportion of NCX localised within the junction is enhanced. While the functional implications of this are still uncertain, it further supports of the role of JPH2 as influencing the EC coupling protein composition, and therefore organisation, of the junction.

The findings from investigation of the nanoscale structure of the junction in these transgenic mice clearly demonstrate that there is alteration of junctional organisation as a consequence of JPH2 expression levels. While these changes are likely to impact EC coupling mechanisms, they may not explain all calcium handling functional changes observed in these mice (including the perhaps surprising absence of major changes in calcium handling following JPH2 over-expression), leading to further investigation of additional contributing factors.

7.1.2 Maintenance of t-tubule structure

One of the primary findings of Chapter 4 was the complex pattern of t-tubule re-organisation due to altered JPH2 expression. While the role of JPH2 in the formation and maintenance of
the t-tubule system has been investigated in recent studies (Chen et al., 2013, Reynolds et al., 2013, Van Oort et al., 2011, Wei et al., 2010), this was the first in-depth examination of the effect of JPH2 expression on the distribution of tubule angles throughout the cardiomyocyte. In support of previous reports from the same mouse model (Van Oort et al., 2011), key findings from this study revealed that in response to induced knockdown of JPH2 there is a reduction of the regularity of the t-tubule network, with the additional detailed identification of a loss of ‘transverse’ tubules with an associated increased proportion of longitudinal and oblique orientated tubules. This pattern of t-tubule remodelling is consistent with that observed in human and animal heart failure (Crossman et al., 2015, Wei et al., 2010, Lyon et al., 2009), and indeed, these JPH2-KD mice develop acute heart failure (Van Oort et al., 2011). Aberrant t-tubule reorganisation such as this, results in the displacement of t-tubules from the z-disk, reducing the synchronicity of CRU activation as well as leading to dissociation of the opposing membranes of the junctional cleft (Song et al., 2006, Øyehaug et al., 2013, Zhang et al., 2013). Consequently, this is associated with a reduction in the amplitude of the calcium transient and a corresponding loss of EC coupling gain (Louch et al., 2004, Song et al., 2006, Brette et al., 2002), with these functional abnormalities observed in the JPH2-KD mice (Van Oort et al., 2011).

In contrast, over-expression of JPH2 does not alter the overall regularity of the t-tubules with regular transverse tubules maintained, which is in agreement with live cell work from Guo et al (2014) using a similar mouse strain. The ability to assess the angle profile of the t-tubules in the present study resulted in the additional novel finding that, while there was no loss of transverse tubules, there was an increased prevalence of longitudinal tubules following JPH2 over-expression. While the exact mechanism underlying this increased t-tubule branching is unclear, the overall function of these cardiomyocytes appeared to be unaffected by this additional component of the t-tubule network, with no difference in the amplitude of the calcium transient (Wehrens’ lab, unpublished data). Furthermore, it has even been suggested that over-expression of JPH2 is protective of t-tubule disorganisation in a model of pressure-overload heart failure (Guo et al., 2014).

Together, findings from these transgenic mice provide clear evidence that JPH2 plays a key role in maintaining the organisation of the t-tubules in adult cardiomyocytes, as well as being implicated in proliferation of the network. These results also confirm that t-tubule remodelling can be detrimental to cardiac function; however, novel findings also indicate that there are instances where increased longitudinal extensions of the t-tubule system appear to have no negative impact on overall cardiomyocyte function (Wehrens’ laboratory, unpublished).
7.3 Evidence for stabilisation of RyR

Detailed analysis performed in Chapter 3 revealed that altered expression of JPH2 influences the extent of co-localisation between JPH2 and RyR. Not surprisingly, as a result of JPH2 knockdown, there was a reduction in the extent of RyR labelling co-localised with JPH2, and conversely, this was increased in JPH2-OE mice. However, there was also a reduced extent of JPH2 co-localising with RyR in the JPH2-KD mice, indicating that there was not only a decrease in JPH2 protein levels, but that of the remaining protein there was a smaller fraction associated with RyR. One of the more recently proposed roles of JPH2 is that it acts to help stabilise the RyR channels to reduce calcium ‘leak’ from the SR, effectively by reducing RyR open probability at a given intracellular calcium concentration (Van Oort et al., 2011, Beavers et al., 2013). JPH2-KD mice have been shown to exhibit increased calcium spark frequency and size despite no changes to RyR expression levels, suggesting an increased leak of the RyR channels (Van Oort et al., 2011). Given the combination of reduced JPH2 protein levels, and a further reduction in co-localisation with RyR, these findings support the role of JPH2 as stabilising RyR channels, with this functional ability impaired in the JPH2-KD mice. This co-localisation result provides a structural explanation for the changes identified in calcium handling in the absence of alteration to the size of the RyR clusters in these knockdown mice.

There was no change in the proportion of JPH2 co-localised with RyR in the JPH2-OE mice across the entire cardiomyocyte; however as there is an increased amount of protein present, this same proportion now represents more JPH2 molecules associated with RyR compared to controls. Further analysis revealed that there was a significant increase in the labelling density of JPH2 within the junction in the JPH2-OE mice, indicating that the additional protein is localising to the CRUs. Furthermore, when combined with the previously discussed decrease in RyR junctional density, it was found that there was a significantly higher ratio of JPH2:RyR within the junction compared to controls, such that each RyR channel has relatively more JPH2 available. This gives a greater potential for JPH2 to stabilise these calcium release channels in the JPH2-OE, providing a molecular basis for the observed decrease in calcium spark size and frequency (see Figure 3.1; Wehrens’ lab, unpublished data). A proposed model of what these enlarged junctions with altered protein densities would look like is presented in Section 3.4.2.2.

Together with findings from the JPH2-KD mice, results from both the co-localisation and junctional density analyses not only provide further evidence that JPH2 expression alters the composition of the junction, but that this leads to the potential for a change in the degree of interaction between RyR and JPH2. Subsequently, the organisational changes identified
support the hypothesis of JPH2 acting to stabilise the RyR channels, providing a clear mechanism contributing to the functional changes observed in these transgenic mice.

7.2 Implications in the Failing Heart

The primary aim of Chapter 5 was to determine contributing structural factors that result in the loss of cardiac function in the failing human heart. It was revealed that there are multi-scale components of cardiomyocyte organisation which play a role in this relationship. One of the clear findings from examination of cardiac trabeculae from human hearts in end-stage failure was the high degree of variability in active force production between samples, with this variability present both between and within individual hearts. The ability to generate force is dependent on the number of myofibrils present in the muscle, and the number of myofibrils is typically proportional to the size (cross-sectional area; CSA) of the muscle, making active force proportional to total CSA (Bruce et al., 1997). Consequently, force is often normalised to CSA to provide a comparative measure of stress between samples (Krivickas et al., 2011). However, in the failing human cardiac trabeculae, it was found that there was no significant relationship between force and total CSA, and that there was high variability in the observed proportional content of cardiomyocytes (and therefore myofibrils) in the trabeculae. The CSA attributed to cardiomyocyte content within the trabeculae (MCSA) was positively correlated with force, with this macro-scale organisation identified as the largest contributing factor to the variability observed in active force production. Unlike findings from non-failing rat hearts (Sands et al., 2011), there was no relationship identified between total trabeculae CSA and the percentage attributed to cardiomyocytes. It has therefore been proposed that in studies utilising cardiac trabeculae, rather than normalising force to the total CSA (which is current practice), force should be normalised to MCSA, particularly for samples from failing hearts (although we have to qualify this as we do not currently have data on the variability of cardiomyocyte content in trabeculae from non-failing human hearts). It seems reasonable to speculate that the high variability in the failing human heart is related to the fibrosis generally observed in failure (Maron et al., 1975, Weber et al., 1989).

Microscopic changes to structural organisation were also examined, and although not significantly correlated with stress, it was identified that a significant relationship existed between the percentage of RyR clusters associated with the t-tubules and the fraction of CSA accounted for by cardiomyocytes. This indicates that protein organisation of the cardio-
The relationship between cardiomyocyte organisation and cardiac function

myocytes is improved when there is a higher prevalence of these cells in the myocardium, suggesting that increased cardiomyocyte tissue content may be protective of microscopic re-organisation in heart failure. Additionally, both stress and RyR-t-tubule association were correlated with the density of JPH2 labelling in the human cardiac trabeculae. As previously discussed, JPH2 has been implicated in regulating both t-tubule and junctional organisation. These findings support this role of JPH2 in maintaining these aspects of cardiomyocyte organisation, both of which are important for regulating EC coupling function, explaining the relationship between JPH2 density and stress. The loss of t-tubule regularity is widely accepted as a hallmark structural change in the failing heart, and as this has previously been identified as correlating with impaired cardiac function in the human heart (Crossman et al., 2015), this relationship was not re-examined in this study.

Findings from the cardiac trabeculae revealed the complexity of changes in cardiomyocyte organisation in failing human hearts with a high extent of variability possible at both the tissue and cellular level. For the majority of structural parameters investigated, these cardiac trabeculae were found to be a good approximation of results from the myocardium of the ventricular free wall from the same failing hearts, supporting the use of these samples as an appropriate experimental model for the whole ventricular myocardium from failing hearts. In addition, there was significant alteration to many of the parameters examined when comparing the failing myocardium to that of a non-failing heart. This indicates that there are a large number of multi-scale structural changes occurring in the failing human myocardium to potentially contribute to the loss of cardiac function.

7.3 Implications in the Developing Heart

Following the examination of cardiac development in the fetal sheep (Chapter 6), the main finding was the identification of the presence of early t-tubules in fetuses as young as 108 days gestation, with increasing prevalence and clear development through to near-term age of 136 days (with full-term being 145 days (Davidson et al., 2015)). While early EM work has also provided evidence of this finding (Brook et al., 1983, Sheldon et al., 1976), the interpretation of structures within EM sections can be influenced by tissue orientation (Franzini-Armstrong, 2010) and non-specific targeting can make structures difficult to identify. This study is the first to examine the development of large mammal cardiac structure in the fetus using fluorescent imaging techniques, resulting in the definitive identification of t-tubules
in the fetal sheep heart with increasing prevalence with gestational development. This is clearly different from the temporal pattern of t-tubule development in the rodent and rabbit, which only begin to show t-tubule formation after birth (Haddock et al., 1999, Chen et al., 2013, Reynolds et al., 2013). Furthermore, findings from both this thesis and previous studies reveal that the pattern of t-tubule network organisation in the adult human heart shows a closer resemblance to that in the adult sheep heart and other large mammals compared to rodents, including rats and mice (Richards et al., 2011, Jayasinghe et al., 2015). This suggests a greater need to investigate these developmental processes in large mammals, such as sheep, particularly when translation to human physiology is of interest.

The pattern of several EC coupling proteins was also determined throughout fetal sheep cardiac development, in which it was found that there is progressive internalisation of several key proteins including RyR, NCX and JPH2. This was coupled with increasing co-localisation between JPH2 and RyR within the cardiomyocytes, as well as an observed increased association of each of these proteins with the developing t-tubules. Although there is a discrepancy in the timing of these events, the same pattern of protein distribution changes are also observed in the rodent heart after birth (Haddock et al., 1999, Chen et al., 1995, Sedarat et al., 2000, Ziman et al., 2010). While there is a distinct lack of functional data from studies on the fetal sheep and other large mammals, this pattern of EC coupling protein organisation is in agreement with the temporal changes observed in EC coupling mechanisms in rodents. This includes a shift away from dependence on surface NCX for triggering CICR, as well as the development of internal junctions as the SR and t-tubules mature for greater reliance on DHPR for RyR activation (Huang et al., 2007). These changes mark an increasing need for t-tubules and internal junctions to allow for synchronised EC coupling as the cardiomyocytes enlarge with cardiac maturation (Louch et al., 2015).

7.4 Multi-scale Cardiomyocyte Organisation as a Determinant of Cardiac Function

This thesis has presented a series of experiments which demonstrate that there are many aspects of cardiomyocyte organisation across a range of spatial scales which can be altered to produce significant consequences for cardiac function. The ability to assess potential alterations in different samples required careful utilisation of appropriate imaging techniques and data analysis. The findings from assessment of transgenic mouse strains provide novel
insights as to how JPH2 contributes to cardiac function by acting through three potential mechanisms to influence EC coupling processes. The ability to detect the subtle nanoscale changes in junctional organisation in the transgenic mouse strains was dependent on the use of super resolution imaging. This allowed antibody targeted protein labelling to be combined with sub-diffraction resolution to provide a ~10-fold improvement in structural information to be determined compared to confocal imaging (Huang et al., 2008b, Baddeley et al., 2009a).

The novel approach of performing functional and structural experiments in a single sample meant that, following the identification of functional variability in trabeculae from failing human hearts, direct correlation with structural parameters was possible. This resulted in several aspects of cardiomyocyte organisation at both the tissue and sub-cellular level being implicated in cardiac function in the failing heart, many of which were further supported by identified impairment compared to the non-failing myocardium. At the opposite end of the temporal scale, examination of fetal sheep samples revealed significant maturation of EC coupling structures prior to birth in the hearts of large mammals. This included progressive development of immature t-tubules and the organisation of junctional proteins to associate with each other and the t-tubules within the cardiomyocytes. These findings have significant implications as they suggest that there is a dramatic shift in the timing of the maturation of EC coupling mechanisms between mammalian species, in particular differences between rodents and large mammals – potentially including humans.

The formation of junctions, or dyads, in cardiomyocytes involves the close association of the DHPR and RyR on the sarcolemma and SR membranes respectively, such that they are functionally coupled (Bers, 2001). Thus, in order to determine the prevalence and organisation of functional dyads, the examination of both RyR and DHPR would be required. Unfortunately, access to suitable antibodies specifically targeted to DHPR was not possible for this study, and so data regarding the organisation of this protein was not able to be obtained with confidence. This remains a substantial problem for the progress of EC coupling related research; our own finding of issues related to the lack of reliability of commercial anti-DHPR antibodies has also been observed in the laboratories of many colleagues we have contacted. Subsequently, the location of junctions was determined based on the distribution of RyR. This may have included ‘orphaned’ or non-junctional clusters which were not associated with the t-tubules or DHPR, and so resulted in a slight over-estimation of the prevalence of functional dyads. It would be of great interest in future studies to obtain a suitable DHPR antibody to provide further information regarding the composition of dyads and potential alterations in the different models examined in this thesis.
References


References


References


References


FABiATO, A. 1983. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology - Cell Physiology,* 14, C1-C14.


References


