Expression and Function of the POU4F2/Brn3b Transcription Factor in the Heart during Development and in Responses to Stress

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Declaration

I, Lauren Maskell, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: Date of signature:
Abstract

Heart morphology, structure and function in the developing and adult heart is tightly controlled by cellular processes including proliferation, differentiation and apoptosis which determine cell fate. Defects in these cellular processes and hence heart development or complications in the adult heart in response to stress can lead to congenital heart disease or cardiovascular disease respectively which cause high rates of mortality globally. The fate of cardiomyocytes during development and in response to stress are highly dependent on changes in gene expression. Hence, factors that control such changes will be important for facilitating cardiac development and hypertrophic responses to stress in the heart. The molecular mechanisms however are not fully understood. The transcription factor (TF) POU4F2/ Brn3b is expressed in cardiomyocytes and regulates several target genes involved in cardiac development. It is highly expressed in the foetal heart and reduced in adult cardiomyocytes, but is re-expressed in response to injury. As such, the role of Brn3b was investigated in the developing heart in more detail by undertaking morphological and molecular analysis of Brn3a KO mouse embryonic hearts. In this study, Brn3b KO mutant mice and wild type (WT) controls were also used to assess the role of this TF in hypertrophic responses induced by Angiotensin II (AngII) infusion (pathological stress) or exercise (physiological stress), by analysing for changes in cardiac morphology, function and genetic changes (parallel studies were also undertaken in cardiomyocyte cultures, e.g. NRVM and H9C2 cells). Functional parameters such as cardiac output, fractional shortening, and ventricular mass, etc. were measured using echocardiography, and histological changes were assessed (e.g. Masson’s trichrome staining) at baseline and 4 weeks post AngII treatment/ exercise. This data showed that cardiac hypertrophy was attenuated in Brn3b KO mice following AngII treatment/ exercise. This suggests that Brn3b may be a novel foetal gene that is required for heart development and for mediating hypertrophic responses following pathological and physiological stress; and may have distinct roles in males and females.
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<td>ANF</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Apic 4</td>
<td>Apical 4 chamber view</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B2M</td>
<td>Beta 2 Microglobulin</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma/leukemia 2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>Bcl extra large</td>
</tr>
<tr>
<td>ß-MHC</td>
<td>Beta myosin heavy chain</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyl transferase 1</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ECG</td>
<td>Echocardiography</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>Oestrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Oestrogen receptor beta</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal Related Kinase</td>
</tr>
<tr>
<td>F</td>
<td>Forward</td>
</tr>
<tr>
<td>FAC</td>
<td>Fractional area change</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA binding protein 4</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Hsp27</td>
<td>Heat shock protein 27</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>H1</td>
<td>Histone H1</td>
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<tr>
<td>H2A</td>
<td>Histone H2A</td>
</tr>
<tr>
<td>H2AX</td>
<td>Histone H2AX</td>
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<tr>
<td>H2B</td>
<td>Histone H2B</td>
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<tr>
<td>H3</td>
<td>Histone H3</td>
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<tr>
<td>H4</td>
<td>Histone H4</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin like growth factor 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IVS</td>
<td>Interventricular septum</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LCCA</td>
<td>Left common carotid artery</td>
</tr>
<tr>
<td>LV mass</td>
<td>Left ventricular mass</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MV</td>
<td>Mitral valve</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NRVM</td>
<td>Neonatal rat ventricular myocyte</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS/T</td>
<td>Phosphate Buffered saline/Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>POU</td>
<td>Pit-1 Oct-1/2 and Unc-86</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PSLAX</td>
<td>Parasternal long axis view</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Reverse</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immunoprecipitation assay</td>
</tr>
<tr>
<td>RCCA</td>
<td>Right common carotid artery</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>Ribonucleic acid polymerase two</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SAX</td>
<td>Short axis mode</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS/T</td>
<td>Tris buffered saline/Tween-20</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Description</td>
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</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TRPC1</td>
<td>Transient receptor potential canonical channel</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter One

Introduction
1.0 Introduction

Cardiovascular disease is the most common cause of mortality globally and caused 31% of all global deaths (17.7 million) in 2015. Examples of cardiovascular disease include: congenital heart disease, cardiomyopathy, and myocardial infarction which is responsible for over 80% of deaths caused by cardiovascular disease (WHO, 2017). The high rate of mortality from such diseases is due to progression to heart failure, whereby the heart muscle fails to effectively pump blood around the body. At least 26 million people worldwide suffer from heart failure which has a poor prognosis for patients; 30-40% of patients diagnosed with heart failure die within one year (Cowie et al., 2000, Savarese and Lund, 2017). In this regard, it is essential to understand the cellular and molecular mechanisms that lead to the development of heart failure in order to reduce patient mortality.

The heart is the first organ to develop within the embryo and is essential for life. As such, defects in heart development are detrimental to life and can cause miscarriages or congenital heart disease in babies. For instance, in the UK, approximately every 9 in 1000 babies are born with congenital heart disease such as ventricular and atrial septal defect, which can be life limiting and require significant medical care (NHS, 2015). This in turn can lead to a poor quality of life in infants. Consequently, it is also essential to understand the mechanisms that cause congenital heart defects in order to develop treatments to provide a better quality of life for children suffering from this condition.

The heart is composed of a variety of cell types, including contractile cardiomyocytes, endothelial cells, smooth muscle cells, epicardial cells and fibroblasts which all contribute to the normal function, structural and mechanical properties of the heart (see Figure 1.0).
Figure 1.0: Diagram showing the different cell types that contribute to the morphology, structure and function of the heart, including cardiomyocytes, epicardial cells, endothelial cells, smooth muscle cells, fibroblasts, Purkinje fibres and pacemaker cells. Cardiomyocytes are responsible for contraction of the heart muscle, whereas cardiac fibroblasts surround and support the cardiomyocytes and assist cardiomyocyte shortening (Bernardo et al., 2010). Endothelial cells are essential for forming the lining of the blood vessels which supply oxygen and nutrients to the heart, and smooth muscle cells are able to facilitate efficient contraction of the major blood vessels of the heart. Furthermore, pacemaker cells and Purkinje fibres are essential for providing and transmitting electrical impulses to the ventricles of the heart which allow the cardiomyocytes of the heart to contract (Xin, Olson and Bassel-Duby., 2013).

SAN = Sino Atrial Node AVN = Atrial Ventricular Node
The normal structure and hence function of the heart is tightly controlled by a number of different cellular processes such as proliferation, differentiation and apoptosis. For instance, in the developing heart proliferation of pre-cardiac mesodermal cells eventually give rise to the left ventricle of the heart (Black, B. L., 2007). Proliferation also plays an important role in responses to stress in the heart, e.g. proliferation of cardiac fibroblasts contributes to increased cardiac fibrosis following prolonged hypertension. In addition, cell differentiation is essential for generation of the multiple specialised cell types which have different morphologies and functions that contribute to normal heart function. For instance, cardiomyocytes have contractile properties and as such are responsible for contraction of the heart muscle, whereas cardiac fibroblasts generate a network of extracellular matrix proteins which surround the cardiomyocytes and assists cardiomyocyte shortening (McMullen and Jennings, 2007).

Apoptosis, or programmed cell death is another process that has important roles during heart development. For instance, apoptosis plays an important role in forming the outflow tract (OFT) of the heart (Poelmann et al., 1998). However, excess apoptosis of cardiomyocytes following chronic stress can be detrimental to heart function. This is because cardiomyocytes lose their proliferative capacity shortly after birth, therefore cardiomyocytes that are lost do not get replaced which leads to poor cardiac function and eventually heart failure (Cowie et al., 2000).

Importantly, these cellular processes must be tightly controlled and balanced in order for the heart to develop and function efficiently to provide the body with sufficient oxygen and nutrients to sustain life. Thus, important cellular processes such as proliferation, differentiation and apoptosis of the multiple cell types of the heart are regulated by cell signalling pathways which converge on gene expression changes that drive cell fate determination in the heart. Such changes in gene expression are controlled by transcription factors (TFs), which are DNA binding proteins that either activate or repress expression of target genes. Hence, transcription factors mediate cell fate determination and can be considered master regulators of gene expression which are essential for normal heart development and function. Therefore understanding their regulation and mechanism of action in different cell types is essential for understanding how cardiac homeostasis is maintained during development and in response to stress.
1.1 Gene regulation

Cell fate determination is controlled by changes in gene expression which in turn is regulated at a number of different levels (see Figure 1.1). For instance, chromatin modification (epigenetic control) enables genes to become accessible to the basal transcriptional machinery. Gene transcription, or synthesis of messenger RNA (mRNA), is facilitated by RNA polymerase II (RNA Pol II), an enzyme that synthesises single stranded mRNA from the DNA template. This process is tightly regulated and controlled by many proteins (transcription factors which help RNA Pol II bind to the DNA template) that work together to either enhance or inhibit gene expression (Harvey Lodish, 2007). Regulation of gene expression can also occur following the synthesis of mRNA; for example, alternative splicing to generate multiple transcripts from the same mRNA by rearrangement or removal of exons (Latchman and Latchman, 2007). Furthermore, translation of the mRNA by ribosomes to produce a polypeptide chain (required for protein synthesis) and post-translational modification of proteins is necessary for producing functional protein.

**Levels of gene regulation**

![Diagram showing levels of gene regulation](image)

**Figure 1.1**: Diagram showing levels of gene regulation, including chromatin modification, gene transcription, post transcriptional regulation (e.g. mRNA splicing), translation and post translational regulation, e.g. protein acetylation (Ac) and phosphorylation (P) (Nature, 2017b, Nature, 2017c, Socratic, 2017)
1.1.1 Chromatin modifications

The human and mouse genome consists of $3 \times 10^9$ base pairs (approximately 2 metres in length), however the average cell diameter is between 5µm-10µm (Harvey Lodish, 2007). As such, DNA is wrapped around histone proteins to form a nucleosome (10nm fibre), which consists of 146 base pairs of DNA wrapped around a histone octamer composed of two H2A, H2B, H3 and H4 histone proteins (see Figure 1.2 (A)) (Lowndes and Toh, 2005). Folding of multiple nucleosomes forms a 30nm chromatin fibre which allows further condensing of DNA so that it fits into the cell nucleus (see Figure 1.2 (B)) (Cooper, G. M and Sunderland, M. A., 2000). However, when DNA is packaged in this manner genes are not accessible to the transcriptional machinery that facilitates activation of gene expression. Although every cell within an organism has the same DNA content, different tissues within an organism must express different sets of target genes at specific times; such as in response to an external signal. Consequently, regions of chromatin are either more or less condensed depending on whether genes within the chromatin need to be silenced or activated, which plays an important role in gene regulation.

Therefore histone modifications mediated by histone modifier enzymes play an important role in either making genes more accessible to transcriptional machinery when their expression is required, or making genes less accessible to transcriptional machinery when they need to be silenced (Cooper, G. M and Sunderland, M. A., 2000). Several types of post translational modifications on histone proteins can alter the structure of chromatin, which are mediated by a number of different enzymes (see Figure 1.3). Importantly, these modifications make gene promoter and enhancer elements either more or less accessible to basal transcriptional machinery. For instance, histone acetyltransferase (HAT) enzymes facilitate histone acetylation which opens up the chromatin, and mediates transcriptional activation. Histone methylation, ubiquitination and phosphorylation on specific amino acid residues are carried out by methyl transferase, histone ubiquitin ligase and kinase enzymes, and can cause either activation or silencing of genes depending on the site of methylation, ubiquitination or phosphorylation (Cao and Yan, 2012, Greer and Shi, 2012). Activation or repression of
gene expression can also be achieved by removal of histone modifications which may be carried out by demethylase, deacetylase, deubiquitinase and phosphatase enzymes.

Figure 1.2: A: DNA wraps around an octamer of two H2A, H2B, H3 and H4 histone proteins to form a nucleosome, stabilised by the H1 histone proteins. B: Nucleosome folding forms a 30nm chromatin fibre, allowing packaging of DNA into the cell nucleus (Cooper, G. M and Sunderland, M. A., 2000).

Figure 1.3: Examples of histone modifications that act to either activate or repress gene transcription by making chromatin more or less easily accessible to transcriptional machinery (Kouzarides, 2017).
1.1.2 Cis-regulatory elements associated with gene regulation

Core Promoter

The core promoter is essential for basal gene transcription and is often located just upstream of the transcription start site (see Figure 1.4). It contains several short consensus DNA sequences (cis elements) which are recognised by trans-factors such as general transcription factors through their DNA binding domains (will be discussed later). As such, cis elements are necessary for activating transcription since they facilitate binding of trans-factors which recruit RNA polymerase II to the gene promoter. Thus, the core promoter represents the minimal region of DNA sequence that is able to direct transcription initiation (Latchman and Latchman, 2007).

Examples of cis elements include the TATA box, an AT rich sequence which is recognised by TATA box binding protein (TBP) and located within the core promoter. However, not all gene promoters contain a TATA box sequence, and instead may contain other cis elements such as an Initiator element (Inr) or the TFIIB-Recognition Element (BRE) which is recognised by TFIIB (described later) (Latchman, 2005, Maston et al., 2006). The Downstream Promoter Element (DPE), Downstream Core Element (DCE), and Motif Ten Element (MTE) are other examples of cis elements (see Figure 1.4). Interestingly, many gene promoters do not contain elements such as the TATA box or BRE and contain different cis elements within their promoters that are required for transcription initiation (Lee et al., 2005, Maston et al., 2006). For example, the cyclin D1 gene promoter contains consensus sequences such as the cyclic AMP response element (CRE) and binding sites for specific transcription factors such as E2F and Brn3b (Budhram-Mahadeo et al., 2008, Guo et al., 2011, Herber et al., 1994).
Enhancers are short DNA sequences that are located either upstream, downstream or within the transcriptional unit of the core promoter of a target gene and act at a distance from its target gene (Latchman, D. S., 2005). Enhancers were first identified within the Histone H2A transcriptional start site, and are necessary for increasing promoter activity (up to 100 fold) in a tissue specific manner (Busslinger et al., 1979). For example, the GLUT4 promoter contains an enhancer element that facilitates activation of GLUT4 expression specifically in skeletal muscle (Moreno et al., 2003). Enhancer elements are particularly important for activating gene expression since the transcription initiation complex is only able to stimulate a low level of transcription. Enhancers can act to increase expression of a target gene when a transcriptional activator recognises and binds to a DNA sequence within an enhancer, which in turn can interact with a protein bound to the promoter region of a target gene (e.g. a general transcription factor). Since enhancers are located at a distance from a target gene, this interaction causes DNA looping and facilitates recruitment of additional general transcription factors and RNA
polymerase II to the gene promoter (see Figure 1.5) (Latchman, 2005, Maston et al., 2006).

Alternatively, enhancer elements may also activate gene transcription indirectly. For instance, histone modification enzymes (e.g. histone acetyltransferase enzymes) may recognise and bind to a consensus sequence within an enhancer of one gene and modify histone proteins (e.g. histone acetylation) on a nearby nucleosome. As such, these histone modifications make a different gene promoter become more accessible to the basal transcription machinery, which may also be facilitated through chromatin remodelling (facilitated by the SWI/SNF complex) (Latchman and Latchman, 2007).

*Figure 1.5:* Diagram showing one mechanism by which enhancers activate gene transcription. On binding of a transcriptional activator (A) to an enhancer element (E), DNA looping occurs which enables interaction with a protein (B) bound to the promoter region of a target gene (e.g. a general transcription factor). This in turn facilitates recruitment of additional general transcription factors and RNA polymerase II to the gene promoter (adapted from (Latchman and Latchman, 2007)).
Silencers

Similar to enhancers, silencer elements are also short DNA sequences that are distally located to the core promoter of a target gene (upstream or downstream) and work in either orientation relative to the promoter (see Figure 1.6). Silencer elements have been identified in many genes, including c-myc, and collagen type II. Silencers inhibit promoter activity and act to repress gene transcription. This may occur by altering the structure of chromatin, which in turn makes the promoter region of different target genes less accessible to the basal transcription machinery. Transcriptional repressor proteins may also bind to a silencer element which prevents general transcriptional factors and RNA polymerase II from being recruited to the gene promoter, repressing gene expression (Latchman, 2005, Maston et al., 2006).

![Silencer](image)

**Figure 1.6**: Silencers upstream, downstream or within the transcriptional unit of a target gene promoter inhibit promoter activity and repress gene expression (Maston et al., 2006, Latchman, 2005).

Insulators and the Locus Control Region (LCR)

As previously described, enhancers and silencers can activate and repress gene expression from a distance as these elements are usually a distance away from their target gene. Therefore they may also be able to alter the expression of nearby target genes. In this regard, insulator elements ensure that the effects of enhancers/silencers are confined to their target genes only. This is achieved by preventing the interaction between the enhancer and promoter of a neighbouring gene, which prevents the recruitment of RNA polymerase II to the promoter region of that neighbouring gene (see Figure 1.7). For example, insulator elements flank the β-globin gene cluster located on human chromosome 11 which prevent the enhancer sequences that regulate this gene...
cluster from activating the expression of the neighbouring Folate receptor and Odorant receptor gene (Latchman, 2005).

Locus control regions (LCRs) also play a role in regulating gene expression, and consist of multiple short DNA sequences that are closely grouped together which act to regulate expression of a group of genes in a tissue specific manner (see Figure 1.7). For example, the β-globin gene cluster also contains a locus control region that regulates the expression of the β-globin genes in erythrocytes during development (Chakalova et al., 2005). Locus control regions regulate gene transcription by recruiting a number of different transcriptional regulators including transcription factors, co-activators and co-repressors, which either activate or repress gene expression (Ahuja et al., 2007). Insulator and locus control region elements also make genes more accessible to the basal transcription machinery through chromatin modification, which has also been demonstrated with the β-globin gene cluster (Latchman, 2005).

![Image showing Insulator and Locus Control Region elements](image)

**Figure 1.7:** A: Insulator elements prevent expression of specific genes from being influenced by transcriptional machinery recruited to neighbouring genes. B: Locus control regions are clusters of short DNA sequences that regulate the expression of a group of genes in a temporal and spatial manner (Maston, G, A, et al., 2006)
1.1.3 Trans-factors and regulation of gene expression

Eukaryotic gene transcription is highly complex, and requires many cis-elements and trans-factors which all work together to generate a tightly regulated network of gene expression to produce proteins that are important for driving changes in cell fate. Trans-factors (e.g. transcription factors) are modular proteins that contain different domains which are able to mediate different effects which facilitate activation or repression of gene expression. Trans-factors achieve this by binding directly to cis-elements (i.e. DNA sequences within a gene promoter). Once bound to a cis element, trans-factors are then able to recruit RNA polymerase II to a target gene. Trans-factors can also indirectly regulate gene transcription by interacting with other trans-factors. For instance, the general transcription factors TFIID, TFIIA and TFIIB are examples of trans-factors since they bind to consensus DNA sequences within the gene promoter (e.g. TATA box and BREs). These general transcription factors enable recruitment of RNA polymerase II to the gene promoter (via TFIIB) (Latchman and Latchman, 2007).

In addition, transcriptional activator proteins are important for activating gene expression by binding to DNA sequences within promoter or enhancer elements. Upon binding to such cis elements transcriptional activators are then able to interact with and recruit RNA polymerase II and the basal transcriptional machinery to the promoter region. Co-activator proteins which can also bind to the basal transcriptional machinery (RNA polymerase II, etc.) or recruit chromatin modifiers to nearby nucleosomes can also interact with transcriptional activators to activate gene expression (Latchman and Latchman, 2007). Similarly, transcriptional repressor proteins are also important for gene regulation, and repress gene expression. This is achieved when transcriptional repressors bind to silencer elements which either prevents the basal transcriptional machinery from being recruited to the gene promoter, or by causing chromatin condensation which makes gene promoters/ enhancers less accessible to the basal transcriptional machinery (Latchman and Latchman, 2007).

However, transcriptional activators, repressors, co-activators and co-repressors, etc. are not the only factors involved in gene regulation. The Mediator complex also activates gene expression and was first identified in yeast and mammals (Kornberg, 2007, Poss et
al., 2013). The Mediator complex consists of at least 30 different subunits, and also plays an important role in regulating gene expression since mutations in each of the subunits have been shown to cause embryonic lethality in mammals (Ito et al., 2002, Poss et al., 2013, Stevens et al., 2002, Tudor et al., 1999, Westerling et al., 2007). The Mediator complex does not have DNA binding activity, however it is able to interact with other transcription factors such as a transcriptional activators and the C-terminal domain of the RPB1 subunit of RNA polymerase II (see Figure 1.8). As such, interaction of the Mediator complex with a transcriptional activator bound to a DNA sequence within an enhancer or promoter region can help recruit RNA polymerase II to the transcriptional start site and stimulate its activity and stabilise its binding. Transcriptional repressors have also been shown to interact with the Mediator to reduce expression of target genes (James D. Watson, 2008, Latchman, 2005, Poss et al., 2013).

![Diagram showing how the Mediator complex can activate expression of target genes, by interacting with both a transcriptional activator and RNA polymerase II, facilitating recruitment of the basal transcriptional machinery to the gene promoter. CTD: C-terminal domain; TATA: TATA box (James D. Watson, 2008)](image-url)

**Figure 1.8:** Diagram showing how the Mediator complex can activate expression of target genes, by interacting with both a transcriptional activator and RNA polymerase II, facilitating recruitment of the basal transcriptional machinery to the gene promoter. CTD: C-terminal domain; TATA: TATA box (James D. Watson, 2008)
1.2 Gene transcription

Gene transcription describes synthesis of messenger RNA (mRNA) that encode proteins which are involved in determining cell fate in different tissues. In eukaryotes, gene transcription is carried out by three RNA polymerase enzymes – RNA polymerase I, II and III which are large multi-subunit enzymes, several of which are conserved between them. Interestingly, the structure of RNA polymerase I, II and III is also similar, which indicates that the mechanism by which they synthesise RNA is also conserved (James D. Watson, 2008). RNA polymerase I transcribes ribosomal RNA genes (e.g. 28S, 18S, and 5.8S) and RNA polymerase III is responsible for transcribing 5S ribosomal RNA, transfer RNA, and small nuclear RNA U6 (Latchman and Latchman, 2007).

In contrast, RNA polymerase II transcribes all protein encoding genes and is composed of 12 subunits – a 10 subunit core and a heterodimer of RPB4 and RPB7 which form a structure that resembles a ‘crab claw’ (see Figure 1.9). RPB1 and RPB2 are the largest subunits of RNA polymerase II and form the active site of the enzyme, and RPB1 contains a flexible linker to the C-terminal domain (CTD) which contains a phosphorylation site which is essential for transcriptional activation (Armache et al., 2003, James D. Watson, 2008). Gene transcription by RNA polymerase II is complex since it does not directly bind to the gene promoter. Therefore RNA polymerase II gets recruited to the promoter by general transcription factors - proteins that help to recruit RNA polymerase II to the transcription start site on the DNA template. Furthermore, gene transcription occurs in three stages – initiation, elongation and termination which will now be described in more detail.
For gene transcription to take place, a number of factors are required, including trans-factors, proteins which help to recruit RNA polymerase II to the DNA template (such as the general transcription factors) and cis-elements – DNA sequences within the target gene being transcribed which are recognised by trans-factors and are hence required for recruitment of RNA polymerase II to the target genes by trans-factors.

The start of transcription occurs within the promoter region of a target gene, which is composed of a number of consensus DNA sequences (cis-elements) that are bound by proteins that recognise them, which upon binding help to recruit RNA polymerase II to the start site of transcription. One of the most common consensus sequences that is found in the majority (but not all) of gene promoters is the TATA box, which is an A-T rich DNA sequence located just upstream of the start site of transcription (Latchman and Latchman, 2007). This short DNA sequence is recognised by the general transcription factors.
factor TFIID by the TATA box Binding Protein (TBP), located within TFIID (see Figure 1.10). As such, TFIID binds to the TATA box in the first step of transcription initiation and causes DNA bending which allows its interaction with other general transcription factors (e.g. TFIIB) and RNA polymerase II (Latchman and Latchman, 2007). This is facilitated by another general transcription factor TFIIA which also has DNA binding activity and also gets recruited to the promoter region of the target gene. Next, TFIIB (another general transcription factor) gets recruited to the promoter region through recognition of the TFIID/DNA complex, and recognises TFIIB Recognition Elements (BRE) consensus sequences within the gene promoter which facilitate its binding (see Figure 1.10). TFIIB is particularly important since it is able to directly recruit RNA polymerase II to the promoter itself and ensures that they are correctly orientated for interaction with one another (Latchman and Latchman, 2007).

Following recruitment of TFIIB and RNA polymerase II to the gene promoter in association with the TFIIF transcription factor, the TFIIE and TFIIH transcription factors get recruited to form the pre-initiation complex (see Figure 1.10). This allows promoter melting and initiation of gene transcription (Latchman, 2005, Maston et al., 2006). TFIIH is a multi-subunit complex consisting of a core complex and a cyclin-dependent kinase (CDK) - activating kinase (CAK) complex. The kinase activity of TFIIH phosphorylates the C-terminal domain of the RBP1 subunit of RNA polymerase II which is important for its activation and transcription initiation. In addition, the helicase activity of the core complex facilitates DNA unwinding and promoter melting to enable mRNA synthesis from the coding strand of DNA (Mydlikova et al., 2010).

Interestingly, as the DNA strand moves through the RNA polymerase enzyme, it becomes obstructed by a wall of protein which causes it to bend at a 90 degree angle, allowing ribonucleotides to be added to the new mRNA strand. Separation of the mRNA strand from the template DNA also occurs as the DNA-RNA hybrid encounters another wall of protein, called the ‘rudder’ (Latchman and Latchman, 2007). Once RNA polymerase II has transcribed the first few nucleotides of the mRNA strand and moves downstream of the DNA template to complete transcription, TFIIA and TFIID remain bound to the gene promoter. This allows a basal level of transcription (of certain genes) to occur since they are able to recruit a holoenzyme complex – whereby some RNA
polymerase II within a cell is associated with TFIIB, TFIIE, TFIIF and TFIIH (see Figure 1.11) (Latchman and Latchman, 2007).

**Figure 1.10:** Diagram showing the stepwise recruitment of the general transcription factors TFIID (via the TATA box Binding Protein – TBP), TFIIA, TFIIB, TFIIF, TFIIE and TFIIH to form the pre-initiation complex (Latchman and Latchman, 2007).

**Figure 1.11:** Diagram showing the recruitment of the holoenzyme (RNA polymerase II, TFIIB, TFIIF, TFIIE and TFIIH – how some RNA polymerase II is found in the cell) to form the pre-initiation complex at the gene promoter (Latchman and Latchman, 2007).
1.2.2 Transcriptional elongation

Following activation of RNA polymerase II by phosphorylation of serine 5 within the C-terminal domain of the RPB1 subunit, transcription initiation begins by adding complementary nucleotides to the coding DNA strand to synthesise mRNA in a 5’ to 3’ direction. However, once 20-30 nucleotides have been transcribed, RNA polymerase II pauses and does not continue transcribing. This plays an important role in gene regulation since it allows gene expression to be activated only upon receipt of an extracellular signal. For example, many genes involved in the MAP Kinase signalling pathway (see later) are regulated by polymerase pausing and as such are only activated when RNA polymerase II is activated (Latchman and Latchman, 2007). In this regard, the C-terminal domain of the RPB1 subunit of RNA polymerase II is phosphorylated by the kinase enzyme pTEF-b which activates RNA polymerase II, enabling it to continue adding ribonucleotides to the mRNA strand. Importantly, the DNA of the gene being transcribed located downstream of the transcription start site is not accessible to the transcriptional machinery since it is wrapped around histone proteins. Consequently, histone acetylase enzymes are recruited by RNA polymerase II which acetylate these histones, which opens up the chromatin structure and makes the DNA encoding the rest of the gene accessible to RNA polymerase II. Finally, addition of a modified guanine nucleotide to the 5’ end of the nascent mRNA is known as capping, which facilitates mRNA translation by the ribosome and prevents mRNA degradation by exonucleases (Latchman and Latchman, 2007).
1.2.3 Transcriptional termination

The last stage of transcription is transcriptional termination which occurs when RNA polymerase II reaches the end of the template DNA sequence. At the 3’ end of the nascent mRNA strand, a polyadenylation signal is transcribed which is recognised by the cleavage/ polyadenylation complex (see Figure 1.12). Binding of the cleavage/ polyadenylation complex to RNA polymerase II is then able to recruit Rat1. Rat1 is an exonuclease enzyme which cleaves the mRNA strand downstream of the polyadenylation signal. Next, poly (A) polymerase adds up to 200 adenosine nucleotides to the free 3’ end of the cleaved mRNA, which facilitates its translation by the ribosome and prevents its degradation. Finally, transcription is terminated when Rat1 exonuclease binds to (‘torpedoes’) RNA polymerase II and causes a conformational change in the enzyme which in turn causes it to fall off of the DNA template (see Figure 1.12) (Latchman and Latchman, 2007).

Figure 1.12: Diagram showing polyadenylation of the nascent mRNA strand which mediates its translation by the ribosome and prevents its degradation by exonuclease enzymes - adapted from (Jeremy M Berg, 2010).
1.3 Tissue specific Transcription Factors (TFs)

General transcription factors are essential for basal transcription of protein coding genes because they form a transcription initiation complex with RNA polymerase II. In addition, tissue specific transcription factors are important for regulating the rate of transcription of genes within particular tissues. As such, these transcription factors play a key role in regulating expression of genes that code for proteins which drive cellular processes such as growth, differentiation and apoptosis within specific subsets of cells.

Tissue specific transcription factors make up approximately 2% of the total genome and contain several domains which facilitate their function. For instance, transcription factors contain an activation domain that facilitates transcriptional activation. This may occur through protein-protein interaction with other transcription factors by increasing their binding to a target gene promoter or by stimulating their activity if they are already bound. Three main classes of activation domain have been identified, including the acidic region, the glutamine rich region and the proline rich region (Latchman, 2005) (Latchman and Latchman, 2007). Tissue specific transcription factors also contain an interface for binding to other proteins which may or may not be an activation domain. Finally, these transcription factors also have a DNA binding domain that is able to recognise and bind to a specific DNA sequence within a promoter or enhancer element of a target gene; however it is not always sufficient for activating gene expression alone.

On binding of a transcription factor to a gene promoter via its DNA binding domain, transcriptional activation may occur either by directly recruiting the basal transcriptional machinery to the target gene promoter, or indirectly by recruiting additional transcription factors.

Transcription factors can be grouped into different classes depending on the amino acid structure of the DNA binding domain within the transcription factor, which mediate binding to DNA within the gene promoter in different ways. For example, this may occur through insertion of the DNA binding domain into the major groove of the DNA sequence within the promoter/ enhancer region of the target gene (Latchman and Latchman, 2007). These classes include the helix turn helix, Zinc Finger, Helix Loop Helix, Steroid receptor and Leucine zipper transcription factors (see Figure 1.13).
<table>
<thead>
<tr>
<th>Transcription Factor (TF) class</th>
<th>Schematic Diagram</th>
<th>Examples of TFs in class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix turn helix/ Homeodomain</td>
<td><img src="image" alt="Helix turn helix/ Homeodomain" /></td>
<td>Brn3b, Brn3a, Pit1, Oct1, Oct2, Unc86</td>
</tr>
<tr>
<td>Zinc Finger</td>
<td><img src="image" alt="Zinc Finger" /></td>
<td>KLF5, GATA4, GATA5, GATA6</td>
</tr>
<tr>
<td>Leucine Zipper</td>
<td><img src="image" alt="Leucine Zipper" /></td>
<td>c-fos, c-jun,</td>
</tr>
<tr>
<td>Helix Loop HELIX</td>
<td><img src="image" alt="Helix Loop HELIX" /></td>
<td>c-myc, Hand1, Hand2</td>
</tr>
<tr>
<td>Steroid receptor</td>
<td><img src="image" alt="Steroid receptor" /></td>
<td>Glucocorticoid receptor, Oestrogen receptor</td>
</tr>
</tbody>
</table>

**Figure 1.13:** Classes of DNA binding transcription factors and examples of transcription factors which belong to each class
Transcription factors that contain a helix turn helix motif (two alpha helices approximately 20 amino acids long connected by a beta turn – see Figure 1.14) are of particular interest since many of them have been identified to play an important role in regulating the expression of target genes during development. For instance, homeotic genes were identified in Drosophila (fly) (e.g. Ubx and Exd) to play an important role in regulating development, since mutations in such genes caused developmental defects (Gehring, 1987, Latchman, 2005, Pabo and Sauer, 1992). Gene cloning identified that these homeotic genes contained a sequence specific DNA binding domain, suggesting that the homeotic genes were able to facilitate development by regulating the expression of target genes. Gene cloning and X-ray crystallographic analysis of the DNA binding domain in the homeotic genes confirmed that this domain contained an 180bp DNA sequence that encoded a helix turn helix motif (60 amino acids in length) consisting of three alpha helices. The second alpha helix of this motif contacts the major groove of the DNA, known as the recognition helix; whereas the third alpha helix is necessary for correctly positioning the recognition helix into the major groove (Latchman and Latchman, 2007). The first alpha helix also facilitates DNA binding by contacting base pairs within the minor groove of the DNA (see Figure 1.14) (James D. Watson, 2008).

**Figure 1.14:** The homeodomain consists of three alpha helices and contains a helix turn helix motif (helices 2 and 3) which facilitates DNA binding. The recognition helix (3) contacts the base pairs within the major groove of the DNA molecule which is stabilised and correctly positioned by the second alpha helix (2). The N-terminal domain of the first alpha helix (1) also facilitates DNA binding by contacting base pairs within the minor groove of the DNA molecule (Alberts, B, et al., 2002).
1.3.1 POU family of transcription factors

The POU family of transcription factors are examples of helix turn helix transcription factors. The POU domain is a short region of protein that was first identified in the original members of this subclass of TFs Pit-1, Oct-1, Oct-2, and Unc-86 which all have important roles in growth and development (Herr et al., 1988). For instance, Pit-1 facilitates gene transcription of growth hormone genes and prolactin via this domain. Pit-1 has been shown to play an important role in growth and development since mice lacking this transcription factor suffer from dwarfism (Nelson et al., 1988). Oct-1 and Oct-2 both recognise an octameric DNA sequence in the promoter region of target genes via their POU domain; Oct-1 is universally expressed in tissues and Oct2 is expressed in B cells (Clerc et al., 1988, Scheidereit et al., 1987). Unc-86 is another POU protein identified in C. elegans which is required for neuronal differentiation (Chalfie et al. 1981), which has been demonstrated by (Olsson-Carter and Slack, 2011) who showed that loss of Unc-86 in C. elegans caused defects in axon growth. Based on the primary sequence of the POU domain, there are six classes of POU transcription factors (Class I, II, II, IV, V and VI) which all have important functions in regulating target genes that facilitate tissue specific cell differentiation and development (Herr et al., 1988, Rosenfeld, 1991).

The POU domain (150-160 amino acids in length) contains a POU specific domain (74-82 amino acids long) which is highly conserved within the POU family of transcription factors; and a POU homeodomain (60 amino acids long) which are connected by a variable linker sequence of approximately 15-27 amino acids (see Figure 1.15) (van der Vliet and Verrijzer, 1993). The POU homeodomain is highly conserved among the POU transcription factors and is related to the homeodomain present in homeotic genes. The POU homeodomain consists of a helix turn helix motif that facilitates high affinity DNA binding to A/T rich sequences within the target gene promoter, whereby the recognition helix contacts the base pairs within the major groove of DNA (see Figure 1.15). However, since the POU homeodomain binds to DNA with high affinity but low specificity, the POU specific domain (composed of 4 alpha helices) confers specificity by contacting the base pairs within the major groove of DNA (see Figure 1.15) (Budhram-Mahadeo et al., 2006a, van der Vliet and Verrijzer, 1993).
The POU specific domain amino acid sequence is highly conserved between each subclass of POU transcription factors, which suggests that the structure and function of the POU specific domain is also conserved within this TF family. Thus, the POU domain is a bi-partite DNA binding domain which facilitates high affinity, sequence specific binding by contacting the DNA sequence of the promoter/enhancer region of a target gene on both sides of the DNA molecule. In addition, the POU domain also facilitates DNA dependent and DNA independent protein-protein interactions with each other by forming heterodimers with co-factors or other TFs to further regulate target gene expression (Rosenfeld, 1991, van der Vliet and Verrijzer, 1993).
1.4 POU4F2/ Brn3b and the related POU4F1/ Brn3a Transcription Factor (TF)

The class 4 sub-family of POU (Pit-Oct-Unc) transcription factors (POU4F) include POU4F2/ Brn3b and the related but distinct POU4F1/ Brn3a genes. The related but distinct Brn3c, IPOU, tPOU and Unc-86 genes also belong to this subclass of transcription factors (Liu et al., 2000, Ninkina et al., 1993, Treacy et al., 1991).

Brn3b was first isolated from neuroblastoma cDNA whereas Brn3a was isolated from brain cDNA, which interestingly shares high sequence homology with the Unc-86 transcription factor (Lillycrop et al., 1992, Liu et al., 2000). The POU domain of Brn3b and Brn3a shares >95% sequence homology, and as such are able to regulate some similar target genes; e.g. both Brn3a and Brn3b can activate expression of HSP27 (see later for more detail). However, differences outside the POU domain means that Brn3b and Brn3a can give rise to antagonistic effects on other target genes (see 1.4.2).

Brn3b is encoded by a gene located on human chromosome 4q31.2 and on mouse chromosome 8; 8 C1. On the other hand, Brn3a is located on human chromosome 13q31.1 and mouse chromosome 14; 14 E2.3. Brn3b and Brn3a consist of two exons separated by an intron, which gives rise to two protein isoforms. Both isoforms contain the DNA binding POU domain which is encoded in exon 2 (see Figure 1.16). In this regard, the long (l) isoform of Brn3b (43kDa), contains both exon 1 and exon 2 whereas the short isoform (32kDa) only contains exon 2. Exon 1 codes for an additional domain found in the N-terminus of the protein, which makes it distinct from the short form of Brn3b (see Figure 1.16 (A)) (Budhram-Mahadeo et al., 2001, Budhram-Mahadeo et al., 2006a). Similarly, exon 2 of Brn3a encodes the POU domain and is present in both the long and short form of Brn3a, whereas the short isoform of Brn3a (32-35kDa) only contains exon 2. In addition, the N-terminal region of exon 2 encodes an activation domain that is also required for transcriptional activation by Brn3a (see Figure 1.16 (B)) (Latchman, 1998). Thus, exon 2 is important for the function of Brn3b and Brn3a since it encodes the DNA binding POU domain that is necessary for facilitating activation or repression of gene transcription (Latchman, 1999, Theil et al., 1993, Theil et al., 1994).
**Figure 1.16**: A: Schematic diagram showing the genomic and protein structure of the long and short form of the POU4F2/Brn3b transcription factor (Budhram-Mahadeo and Latchman, 2006). B: Diagram showing the genetic structure of the POU4F1/Brn3a transcription factor (Latchman, 1998).
1.4.1 Tissue specific gene expression and functions of POU4F2/Brn3b

Brn3b has been detected in a number of different tissues, including neuroblastoma cells, the testis, ovary and cervix, skeletal muscle, adipose tissue and heart (Bitsi et al., 2016, Budhram-Mahadeo et al., 2014, Budhram-Mahadeo et al., 2001, Farooqui-Kabir et al., 2008). Brn3b regulates the expression of a large number of target genes in specific tissues, and as such is able to drive different effects depending on the tissue that Brn3b is expressed in. In this regard, Brn3b plays an important role in controlling cell fate; from driving cell proliferation to regulating cell metabolism, survival and apoptosis.

For instance, Brn3b has been identified as a novel regulator of metabolic processes, since its expression has been detected in metabolically active insulin responsive tissues such as skeletal muscle, adipose tissue and liver (Bitsi et al., 2016). Type 2 diabetes is coupled with hyperglycaemia and insulin resistance. Therefore controlling blood glucose levels is essential for preventing the development of such conditions which are detrimental to life. Mice lacking Brn3b (Brn3b KO) are obese and contain a higher proportion of visceral fat when compared to WT controls. Moreover, Brn3b KO mice also exhibited hyperglycaemia and insulin resistance which was associated with decreased expression of GLUT4 (glucose transporter required for insulin mediated glucose uptake in metabolically active tissues) (Bitsi et al., 2016). This was interesting since GLUT4 heterozygous mice have been shown to develop insulin resistance (Rossetti et al., 1997). Brn3b was shown to activate GLUT4 expression in C2C12 myocyte (muscle) cells in co-transfection assays with the GLUT4 luciferase reporter. In addition, the GLUT4 promoter contains multiple Brn3b binding sites, and CHIP analysis showed that Brn3b binds to the GLUT4 promoter in C2C12 cells. Furthermore, overexpression of Brn3b in C2C12 cells triggered up-regulation of GLUT4, suggesting that Brn3b may facilitate glucose uptake in metabolically active tissues by regulating GLUT4 expression (Bitsi et al., 2016).

Brn3b expression in metabolically active tissues is also regulated by glucose and insulin. In skeletal muscle glucose triggered up-regulation of Brn3b (and increased Brn3b promoter activity in C2C12 cells), whereas insulin caused downregulation of Brn3b. In addition, WT mice fed on a high fat diet had reduced Brn3b protein levels. Consequently,
the complex regulation of Brn3b by glucose and insulin in insulin responsive tissues further suggests that Brn3b is important for maintaining glucose homeostasis in metabolically active tissues (Bitsi et al., 2016).

Brn3b is also necessary for retinal ganglion cell development, and as such is expressed in post mitotic retinal ganglion precursor cells (Xiang, 1998, Xiang et al., 1993). Brn3b was detected as early as E11.5 in the mouse retina, and at E12.5 was located in the centre of the retina. Between E13.5-E16.5, Brn3b was also identified in the ventricular zone of the retina at a stage of development when the majority of retinal ganglion cells are formed (Xiang, 1998). The importance of Brn3b in retinal ganglion cell development during development is indicated by blindness in Brn3b KO mice because the retinal ganglion cells undergo apoptosis before birth (Badea et al., 2009, Gan et al., 1996, Pan et al., 2008). Moreover, Brn3b KO mice display retinal ganglion cell defects and have thinner retinas when compared to control mice (Gan et al., 1996). Brn3b can trans-activate target genes independently that mediate retinal ganglion cell development such as Sonic Hedgehog (Shh), Brn3a, Olf1 and Ablim1 (Pan et al., 2008). Moreover, Brn3b also mediates its effects on retinal ganglion cell development/differentiation by interacting and forming a complex with Islet 1 (Isl1) which regulates the expression of other target genes including Ebf3 and Irx6 (additional transcription factors) which are important for the specification of subtypes of retinal cells (Li et al., 2014).

Brn3b also has several roles in a number of cancers, including mediating cell proliferation, migration and conferring resistance to cancer drugs. In this regard, Brn3b levels are increased in over 60% of breast cancers, which also correlates with elevated HSP27 expression. Interestingly, high HSP27 levels in breast cancers are associated with increased motility, metastasis and drug resistance (Lee and Budhram-Mahadeo, 2005, Lee et al., 2005). In addition, Brn3b has been shown to trans-activate the HSP27 promoter, either alone or through interaction with the oestrogen receptor (see later for more detail), and loss of Brn3b also caused HSP27 levels to decrease in breast cancer cells (Lee et al., 2005). This suggests that Brn3b may up-regulate the expression of HSP27 which in turn contributes to cell migration and drug resistance in breast cancers. Breast cancer cells that were resistant to the chemotherapeutic drug Doxorubicin also had elevated levels of Brn3b and HSP27 (Fujita et al., 2011). Similarly, this effect has also
been observed in ovarian cancer cells, whereby increased Brn3b levels in drug treated SKOV3 cells correlated with increased expression of HSP27, which interestingly is also associated with cell survival, drug resistance and cell metastasis in ovarian cancers (Arts et al., 1999, Maskell, 2018a). Furthermore, reduction of Brn3b levels in human ovarian cancer cells reduced HSP27 expression, cell viability and caused increased sensitivity to Cisplatin and Paclitaxel (drugs used to treat ovarian cancer). Increased Brn3b protein levels have also been observed in human ovarian cancer cells (SKOV3) (Budhram-Mahadeo et al., 2006a, Maskell, 2018a). As such, Brn3b may also alter the behaviour of ovarian cancer cells in a similar manner to breast cancers through regulation of HSP27.

However, Brn3b has also been shown to regulate other target genes in breast cancer cells which enable their progression and metastasis. For instance, plakoglobin which facilitates cell adhesion which is downregulated in several human cancers is repressed by Brn3b in breast cancer cells (Jiang, 1996). This in turn promotes their migratory properties and hence metastatic potential (Samady et al., 2006). Tumour growth is also mediated by Brn3b through repression of the tumour suppressor BRCA1 in breast cancer (Budhram-Mahadeo et al., 1999b). Brn3b also enhances tumour proliferation by activating the promoters of cyclin D1 and CDK4 in breast cancer cells, allowing cell cycle progression to occur (Budhram-Mahadeo et al., 2008).

As previously described, Brn3b was first identified in neuroblastoma cells, and has since been detected in neuroblastoma tumour samples (Budhram-Mahadeo et al., 2008). Since Brn3b was associated with enhancing cell proliferation, further studies were carried out by Irshad and colleagues whereby Brn3b was overexpressed in neuroblastoma cells to further determine the role of Brn3b in neuroblastoma cells in vitro and in vivo (Irshad et al., 2004). Concerning this, stable transfection of neuroblastoma cells with DNA constructs that induced overexpression of Brn3b in these cells caused increased proliferation within these cells. This has also been shown in vivo, whereby athymic nude mice were subcutaneously injected with neuroblastoma cells overexpressing Brn3b developed tumours faster when compared to nude mice injected with neuroblastoma cells that did not overexpress Brn3b. The tumours that developed in the nude mice injected with neuroblastoma cells overexpressing Brn3b were also more invasive into the surrounding tissue and skin, suggesting that Brn3b may also
Contribute to tumour invasion (Irshad et al., 2004). In addition, proliferation of neuroblastoma cells overexpressing Brn3b that were treated with retinoic acid was not affected. This is significant because treatment of neuroblastoma cells with retinoic acid usually causes them to stop proliferating and instead induces their differentiation or apoptosis. In this regard, Brn3b has been shown to repress the expression of alpha-internexin and neurofilament, which are necessary for neuronal differentiation, which further suggests that it may be involved in maintaining an undifferentiated and more proliferative phenotype in neuronal cells (Budhram-Mahadeo et al., 1995). Additionally, Brn3b has been shown to drive cyclin D1 expression (drives cell proliferation) since cyclin D1 protein levels were elevated in neuroblastoma tumour samples (expressing Brn3b) and neuroblastoma cells which overexpressed Brn3b, therefore Brn3b may also drive cell proliferation through transcriptional regulation of cyclin D1 (Budhram-Mahadeo et al., 2008). As such, Brn3b has been shown to be important for driving cell proliferation in neuroblastoma cells since neuroblastoma cells that did not overexpress Brn3b treated with retinoic acid did undergo differentiation and ceased proliferating (Irshad et al., 2004).

In summary, Brn3b is able to drive different cellular responses in different tissues by regulating specific subsets of target genes (see Table 1.0). As such, it is important to study the effects of Brn3b in a tissue specific context.
Table 1.0: Tissues where Brn3b has been identified and analysed, Brn3b target genes and examples of interacting factors that can regulate Brn3b effects on some genes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Brn3b target genes</th>
<th>Interacting partner</th>
<th>Function</th>
</tr>
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</table>
| Heart                       | Hsp27, MLC2, Bax, Noxa | p53                | • Adaptation after birth  
                              |                     |                     | • Cardiomyocyte apoptosis during development and in response to injury |
| Skeletal muscle             | GLUT4              | Direct effect       | Glucose uptake                                       |
| Cancers                     | CDK4, cyclin D1, BRCA1, HSP27, plakoglobin | Oestrogen receptor (ER), Isl1 | • Cell/Tumour proliferation  
                              |                     |                     | • Growth  
                              |                     |                     | • Drug resistance  
                              |                     |                     | • Tumour growth and invasion |
| Retinal Ganglion Cells      | Pax4               | Isl1                | Cell survival/development                             |
| Neural Crest and neuronal cells | α-internexin, neurofilament, HIPK1, Brn-3a |                     | • Neuronal cell development/differentiation  
                              |                     |                     | • Cell cycle arrest |
| Testis (spermatids)         | Unknown            | Unknown             | Germ cell development                                 |
1.4.2 Regulation of POU4F2/ Brn3b and POU4F1/ Brn3a expression gives rise to different effects on cell fate

Since Brn3b and Brn3a share over 95% homology within the DNA binding POU domain, both TFs are able to regulate some similar target genes. For example both Brn3b and Brn3a can trans-activate Heat Shock protein 27 (HSP27) expression, which itself is associated with cell survival (Farooqui-Kabir et al., 2004, Lee et al., 2005). Yet, Brn3b and Brn3a are complex since their own expression can be altered by external factors which can drive different effects on cell fate. Importantly, Brn3b and Brn3a can give rise to different effects because their amino acid sequences outside of the POU domain are not highly conserved and as such are different from one another. Regulation of Brn3b and Brn3a expression were first shown to be regulated in neuronal cells by serum, cyclic AMP (cAMP) and growth factors, which drove distinct processes in these cells.

Both Brn3b and Brn3a have been shown to be expressed in ND7 neuronal cells; but upon withdrawal of serum which stimulates neuronal differentiation, Brn3a mRNA levels increased whereas Brn3b mRNA levels decreased (Lillycrop et al., 1992). In addition, treatment of neuronal cells with cAMP alone and serum withdrawal alone caused up-regulation of Brn3a mRNA and downregulation of Brn3b mRNA. This also correlated with Brn3a promoter activation and repression of Brn3b promoter activity. Moreover, Brn3a mRNA levels decreased and Brn3b mRNA levels increased when neuronal cells cultured in serum free medium were treated with a mixture of growth factors (Budhram-Mahadeo et al., 1995, Budhram-Mahadeo et al., 1994). Consequently, the balance of Brn3a and Brn3b levels in specific tissues are important for driving specific cellular processes that determine cell fate.

In breast cancer, Brn3b expression has also been shown to be stimulated by oestrogen through its interaction with the oestrogen receptor (ERα). This stimulation of Brn3b expression was also shown to be mediated by MAPK signalling, since treatment of MCF7 breast cancer cells co-transfected with a Brn3b reporter construct with MAPK signalling inhibitors such as PD98059 strongly reduced Brn3b promoter activity (Ounzain, S, et al., 2011).
1.4.3 Differential regulation of gene expression by POU4F2/Brn3b and POU4F1/Brn3a can drive different effects on cell fate

Brn3b and Brn3a play important roles in cell fate determination through their ability to regulate the expression of target genes in a tissue specific manner. This may occur directly through directly binding to specific consensus DNA sequences within a target gene promoter. For example, Brn3b has been shown to activate GLUT4 expression in metabolically active tissues and has binding sites within the GLUT4 promoter; and Brn3a was shown to have binding sites within the Bcl-2 promoter, and as such activates Bcl-2 expression in neuronal cells (Bitsi et al., 2016, Budhram-Mahadeo et al., 1999a, Sugars et al., 2001). Importantly, Brn3a and Brn3b can indirectly regulate gene transcription since they contain protein-protein interaction domains within the POU homeodomain. Consequently, Brn3b and Brn3a are able to interact with other transcription factors and help recruit the basal transcriptional machinery to the promoter region of a target gene (Latchman and Latchman, 2007).

Brn3b has been shown to interact with the oestrogen receptor. The oestrogen receptor is a nuclear receptor that is expressed in a variety of tissues including the heart, reproductive tract and the brain and sensory neurons (Ciocca and Calderwood, 2005, Cooke et al., 1991, Holderegger and Keefer, 1986, Pasqualini and Sumida, 1986, Taylor and Al-Azzawi, 2000). Importantly, it acts to regulate gene transcription by forming a complex with other proteins which recognises a consensus sequence within a gene promoter/ enhancer known as the oestrogen response element (ERE) (Parker et al., 1991). As such, interaction of the oestrogen receptor with other transcription factors/ proteins is essential for mediating its function.

Brn3b (and Brn3a) is able to interact with the DNA binding C domain (amino acids 121-315) of the oestrogen receptor via its DNA binding POU domain since truncated forms of the oestrogen receptor that lacked the DNA binding C domain (amino acids 313-599) did not bind to the POU domain on Brn3b. This was demonstrated both in vitro and in vivo with protein extracts from rat brain and ovary using several techniques, including GST pull down assays and immunoprecipitation (Budhram-Mahadeo et al., 1998).
Moreover, gene reporter assays carried out in MCF7 breast cancer cells also showed that Brn3b is able to increase the activity of promoters that contained an ERE. Brn3b has also been shown to co-operate with the oestrogen receptor to activate the HSP27 promoter (via the ERE) in breast cancers, which requires Brn3 binding sites within the ERE of the HSP27 promoter (Lee et al., 2005).

Consequently, interaction of Brn3b with the oestrogen receptor can drive expression of ERE containing promoters (Budhram-Mahadeo et al., 1998). This is particularly interesting since HSP27 has also been shown to be associated with cardiomyocyte survival and differentiation after birth, the promoter of which has also been shown to be activated by Brn3b in cardiomyocytes (Davidson and Morange, 2000, Farooqui-Kabir et al., 2004). Additionally, HSP27 expression has also been shown to be increased by Brn3a and Brn3b (in the absence of Brn3a) in the developing heart, suggesting that Brn3b may also play a role in controlling the behaviour and survival of cardiomyocytes by regulating HSP27 expression (Farooqui-Kabir et al., 2008).

1.4.4. Interaction of POU4F2/ Brn3b and POU4F1/ Brn3a with p53 give rise to different effects on cell survival

Brn3a and Brn3b have also been shown to physically interact with the tumour suppressor p53 in ND7 cells via the POU domain (Budhram-Mahadeo et al., 1999a, Budhram-Mahadeo et al., 2006b). p53 is a transcription factor that plays an essential role in determining whether a cell undergoes cell cycle arrest or apoptosis in response to stress, such as DNA damage. For instance, p53 is able to mediate cell cycle arrest by activating the expression of p21Cip1/Waf1 which in turn binds to cyclin dependent kinase 2 (cdk2) and prevents cell cycle progression (Shaw, 1996). Yet, p53 activation by stress signals has also been shown to induce the expression of pro-apoptotic genes including Bax, which in turn feeds into the mitochondrial apoptotic pathway (will be discussed later) (Long et al., 1997). Importantly, Brn3b and Brn3a have antagonistic effects on gene expression when they are co-expressed with p53, and as such are able to drive different effects on cell survival/ apoptosis.
For instance, Brn3a has previously been shown to activate the Bcl-2 and Bcl-x gene promoters which are important anti-apoptotic proteins (Smith et al., 2001, Sugars et al., 2001). However, when Brn3a is co-expressed with p53 in neuronal cells, p53 inhibits the promoter activity of Bcl-2 in the presence of Brn3a (Smith et al., 2001). Moreover, Brn3a has been shown to interact with p53 to inhibit the expression of pro-apoptotic genes Bax and Noxa in neuronal cells; but also co-operate with p53 to induce cell cycle arrest by activating gene transcription of cyclin-dependent kinase inhibitor p21cip1/waf1 (Hudson et al., 2005).

In contrast, increased Brn3b levels in ND7 cells is associated with cell proliferation, however co-expression of Brn3b and p53 drives apoptosis in neuronal cells; this was also observed in MCF7 breast cancer cells (Budhram-Mahadeo et al., 2006a, Dennis et al., 2001). Elevated apoptosis in neuronal cells also correlated with increased Bax promoter activity and hence increased levels of Bax expression where Brn3b and p53 were co-expressed. On the other hand, promoter activity of p21cip1/waf1 was not increased when Brn3b was co-expressed with p53. This in turn suggests that in neuronal cells, Brn3b helps to drive apoptosis when it is co-expressed with p53 (by activating the expression of pro-apoptotic genes), but also inhibits cell cycle arrest by repressing the expression of cell cycle arrest genes (Budhram-Mahadeo et al., 2006a). Furthermore, studies in NRVM cultures showed that when Brn3b expression was reduced using siRNA, Bax expression also decreased even though p53 expression was unchanged. This further suggests that interaction of Brn3b with p53 is essential for driving expression of pro-apoptotic Bax (Farooqui-Kabir et al., 2004). Importantly, Brn3b and Brn3a have different regulatory effects on their target genes depending on its interacting partners.
1.4.5 Cardiac expression of POU4F2/Brn3b and POU4F1/Brn3a

1.4.5.1 Interaction of POU4F2/Brn3b and POU4F1/Brn3a with p53 drives different effects on cell fate in the stressed heart

Brn3a co-expression with p53 in neuronal cells drove cell cycle arrest, whereas Brn3b co-expression with p53 triggered apoptosis (Budhram-Mahadeo et al., 1999a, Budhram-Mahadeo et al., 2006a). Similarly, Brn3a and Brn3b have also been shown to be co-expressed with p53 within specific regions of the adult heart in response to acute stress (Budhram-Mahadeo et al., 2014).

Permanent coronary artery ligation was carried out in 7-14 week old mice in order to determine whether Brn3a and Brn3b play a role in responses of the heart to injury. qRT-PCR analysis of mRNA extracted from the infarct hearts showed that Brn3a and Brn3b were up-regulated by 1 day after surgery, and expression of Brn3b but not Brn3a was still elevated after 1 week. Co-expression studies showed that Brn3a expression was restricted to the non-injured myocardium and was not observed in the infarct zone, whereas Brn3b expression was localised to the infarct zone of the heart as well as in the non-injured areas. Brn3b expression was increased throughout the heart following coronary artery ligation, however its role in the heart by itself in response to injury is not yet known (Budhram-Mahadeo et al., 2014).

In addition, immunostaining with cleaved caspase 3 antibody showed that cells within the injured zone of the heart were undergoing apoptosis, suggesting that Brn3b may contribute to driving cardiomyocyte apoptosis in the stressed heart. To determine whether this was the case, co-immunostaining was undertaken with Brn3a, Brn3b and tumour suppressor p53 on the infarct hearts. Interestingly, Brn3a and Brn3b were co-expressed with p53 in the cellular regions in relation to the injured region of the heart. For instance, Brn3a and p53 were co-localised in cells adjacent to the injured myocardium, whilst Brn3b was co-localised with p53 within the infarct region of the injured heart. Furthermore, qRT PCR and western blotting showed increased levels of pro-apoptotic genes Bax and Noxa in cells within the infarct zone compared with the non-infarct zone, confirming that these cells were undergoing apoptosis when elevated...
levels of Brn3b and p53 were co-expressed following injury. In addition, co-expression of Brn3a and p53 in the non-infarct zone may suggest that Brn3a may be working with p53 to induce cell survival in the cells surrounding the injured myocardium (Budhram-Mahadeo et al., 2014).

Myocardial infarction was also simulated in vitro in isolated neonatal rat ventricular myocytes (NRVMs) using simulated ischemia/ re-oxygenation (sI/R) to further determine the role of Brn3b and Brn3a in injured cardiomyocytes. This study showed that cardiomyocyte cell viability was reduced, and that Brn3b mRNA levels increased following sI/R which correlated with induction of apoptotic genes Bax, Noxa and PUMA. This was also confirmed using immunostaining which showed that in sI/R injured cardiomyocytes, Brn3b was co-expressed with p53, Bax, and Noxa proteins which also showed morphological changes. In contrast, Brn3a and p53 mRNA levels were unchanged after sI but were increased following re-oxygenation and interestingly anti-apoptotic genes Bcl2 and Bcl-XL did not change following sI/R. Similar results were obtained when H9C2 cells were simulated with sI/R. Furthermore, reduction of Brn3b expression in NRVMs and H9C2 cells correlated with increased cell viability (MTT assay). Thus, these results further suggest that Brn3b and Brn3a play an important role in mediating cardiomyocyte responses to stress in the injured heart (Budhram-Mahadeo et al., 2014).

1.4.5.2 Expression of POU4F2/Brn3b and related POU4F1/Brn3a in the developing heart

Brn3b and Brn3a have complex patterns of gene expression in different tissues, the balance of which plays a critical role in driving specific distinct cellular processes. For instance, in neuronal cells, high Brn3b levels were associated with de-differentiation and cell proliferation. On the other hand, high Brn3a levels (and low Brn3b levels) in neuronal cells correlated with differentiation (Budhram-Mahadeo et al., 2006a, Lillycrop et al., 1992).
Moreover, Brn3b and Brn3a have overlapping expression at different levels in the developing rodent heart. Normal heart development is essential for sustaining life, and is regulated by changes in gene expression (regulated by transcription factors) that drive cell proliferation, differentiation and apoptosis. As such, these transcription factors may play an important role in cardiac development, but may also be unique foetal genes associated with the foetal gene programme that are re-expressed in response to chronic stress.

In this regard, Farooqui-Kabir and colleagues analysed Brn3a and Brn3b mRNA and protein levels in the developing mouse heart using western blot analysis and qRT-PCR in order to understand the potential functions of these TFs in the developing and neonatal heart (Farooqui-Kabir et al., 2008). Brn3a mRNA and protein was detected in the heart during development between stages E13.5-E17.5, which correlated with time points when the major stages of heart development are occurring (E11.5-E14.5). For example, immunostaining showed that at E13.0, Brn3a was expressed in the outflow tract and by E14.5, Brn3a was also detected within the atrioventricular valves and the myocardium. This suggests that Brn3a may play an important role during heart development (Farooqui-Kabir et al., 2008). Brn3a mRNA levels also increased between E18.5-E19.5 in line with a rise in Brn3a protein expression from postnatal stage P1 to P3, however from P9.5 to adult Brn3a mRNA levels decreased. On the other hand, Brn3b mRNA was also detected from E13.5, the levels of which increased until birth. However, Brn3b protein levels decreased during P1 to P3 in the neonatal heart which correlated with increased Brn3a expression (Farooqui-Kabir et al., 2008).

Brn3a and Brn3b protein was also detected in the adult and neonatal rat heart using western blot analysis. Immunostaining of cultured neonatal rat ventricular myocyte cells (NRVMs) with Brn3b and Brn3a also confirmed that they are expressed in the nucleus of cardiomyocytes. This may be expected since transcription factors act to regulate gene expression in the nucleus of cells (Farooqui-Kabir et al., 2008).

Intriguingly, previous studies have shown that Brn3a KO mice die at birth, which has previously been suggested to be due to failure of the pups to suckle due to a loss of sensory neuronal cells (Xiang et al., 1996). However, since these studies identified Brn3a expression in the mouse embryonic heart at critical stages of heart development,
morphological analysis was undertaken on hearts taken from Brn3a KO mice to determine the effects of loss of Brn3a on heart development. All Brn3a KO mice died by 1 day after birth, and a proportion of the mice (approximately 30%) showed heart defects including thickened endocardial cushion tissue and valve leaflets, and hypoplastic ventricular myocardium. This suggests that these mice had abnormal cardiac development, which may have instead led to their death after birth (Farooqui-Kabir et al., 2008).

Moreover, mRNA expression analysis at different embryonic stages was undertaken in the hearts of Brn3a KO and WT mouse embryos to determine gene expression changes associated with loss of Brn3a using qPCR. The results of this study showed that in the Brn3a KO embryos where there was no Brn3a expression (as expected), there was increased Brn3b mRNA expression. In addition, HSP27 is an important cardiac gene that is required for the survival and differentiation of cardiomyocytes after birth, the promoter of which is activated by both Brn3a and Brn3b (Davidson and Morange, 2000, Farooqui-Kabir et al., 2004). As such, HSP27 protein levels were first analysed in the WT developing mouse heart, which showed a similar expression pattern to Brn3a, suggesting that Brn3a is able to activate HSP27 expression in cardiomyocytes; this was further confirmed since Brn3a was also able to activate the HSP27 promoter in isolated cardiomyocytes. Brn3b was also shown to activate the HSP27 promoter in isolated cardiomyocytes, therefore HSP27 mRNA levels were analysed in the hearts of Brn3a KO and WT mice to determine the effect of loss of Brn3a on HSP27 expression. HSP27 mRNA levels were similar in both the WT and Brn3a KO embryos, which suggests that Brn3b up-regulation may compensate for loss of Brn3a expression. Furthermore, this compensatory regulation by Brn3b may enable normal levels of HSP27 to be maintained in the Brn3a KO heart that are required for normal development and survival of the embryonic heart (Farooqui-Kabir et al., 2008).
1.5 Studying heart development

Heart development is a complex process whereby cell proliferation, differentiation and apoptosis are co-ordinated in a timely manner to form the four chambered heart. For instance, cell proliferation ensures that the heart grows sufficiently to become the correct size to sustain life. In addition, differentiation is important for producing the specialised cells of the heart which all work together to enable the heart to efficiently pump blood around the body. On the other hand, apoptosis is also essential for heart development since this process maintains the correct number of cells in the heart in addition to contributing to key stages of heart development such as outflow tract septation (Poelmann et al., 1998).

Heart development begins when proliferation of pre-cardiac mesodermal cells give rise to the primary heart field and the secondary heart field to form the cardiac crescent. This occurs by E7.5 in the mouse, and by day 15 in the human embryo (see Figure 1.17) (Brand, 2003). The primary heart field eventually forms the left ventricle of the heart; and the secondary heart field gives rise to the right ventricle and the outflow tract (Brand, 2003, Black, 2007, Moorman et al., 2003, Sedmera and McQuinn, 2008, Sedmera et al., 2000). Next, fusion of the primary and secondary heart fields forms the linear heart tube (by embryonic stage E8 in the mouse and day 20 in the human embryo) which begins to beat and have pumping action. By E9 in the mouse and day 28 in the human embryo the linear heart tube undergoes rightward looping to form the early left and right ventricles (see Figure 1.17).

Next, differentiation of the cardiac stem cells within the second heart field forms the left ventricle, right ventricle, left atrium, and right atrium by E10 in the mouse and day 32 in the human embryo (Black, 2007). By 42 days in the human embryo and at embryonic stage E15.5 in the mouse embryo, ventricular trabeculation occurs which is an important part of development since it enables left ventricular (LV) mass to be increased in the absence of coronary circulation (see Figure 1.17) (Sedmera et al., 2000). Moreover, these ventricular trabeculations undergo compaction to form a compact myocardium, which is necessary for contractile function in the heart (Moorman et al., 2003, Sedmera and McQuinn, 2008, Sedmera et al., 2000). This also occurs in line with protrusion of the
coronary vasculature into the ventricle from the epicardium (Sedmera and McQuinn, 2008).

The outflow tract, which is formed of myocardial cells, endocardial cells (derived from the secondary heart field) and neural crest cells (which migrate to the outflow tract) also undergoes septation at this stage of heart development which is mediated by cell differentiation and apoptosis of neural crest cells (see Figure 1.17) (Poelmann et al., 1998). Finally, ventricular, atrial and further outflow tract septation occurs from E11-E13 in the mouse and day 50-60 in the human embryo to give rise to the four chambered heart (see Figure 1.17) (Brand, 2003, Black, 2007).
<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Schematic diagram</th>
<th>Milestones</th>
</tr>
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<tbody>
<tr>
<td>Cardiac crescent</td>
<td><img src="image1" alt="Image" /></td>
<td>• Cardiac differentiation</td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E7.5</strong></td>
<td></td>
<td>• Migration to midline</td>
</tr>
<tr>
<td>Human embryo day: <strong>Day 15</strong></td>
<td></td>
<td></td>
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<tr>
<td>Linear Heart tube</td>
<td><img src="image2" alt="Image" /></td>
<td>• Heart tube formation</td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E8.5</strong></td>
<td></td>
<td>• First heartbeat</td>
</tr>
<tr>
<td>Human embryo day: <strong>Day 20</strong></td>
<td></td>
<td>• Anterior-posterior and dorsal-ventral patterning</td>
</tr>
<tr>
<td>Looping Heart</td>
<td><img src="image3" alt="Image" /></td>
<td>• Early chamber formation</td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E9</strong></td>
<td></td>
<td>• Looping to the right</td>
</tr>
<tr>
<td>Human embryo day: <strong>Day 28</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chamber formation</td>
<td><img src="image4" alt="Image" /></td>
<td>• Chamber formation</td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E10</strong></td>
<td></td>
<td>• Trabeculation</td>
</tr>
<tr>
<td>Human embryo day: <strong>Day 32</strong></td>
<td></td>
<td>• Cushion formation</td>
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<tr>
<td></td>
<td><img src="image5" alt="Image" /></td>
<td>• Outflow tract septation</td>
</tr>
<tr>
<td>Ventricular septation</td>
<td></td>
<td>• Early conduction system formation</td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E11-E15</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human embryo day: <strong>Days 50 to 90</strong></td>
<td></td>
<td></td>
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<tr>
<td>Atrial septation</td>
<td><img src="image6" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E13 to birth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human embryo day: <strong>Days 60 to birth</strong></td>
<td></td>
<td></td>
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<tr>
<td>Outflow tract septation</td>
<td><img src="image7" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Mouse embryo day: <strong>E13 to birth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human embryo day: <strong>Days 60 to birth</strong></td>
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</table>

**Figure 1.17**: Timeline of cardiac development in the human and mouse. FHF: First heart field; SHF: Second heart field; AS: Atrial septum; CC: Cardiac crescent; PA: Pulmonary artery; VS: Ventricular septum; V: Ventricle; LA: Left atrium; RA: Right atrium; LV: Left ventricle; RV: Right ventricle; OT: Outflow tract; SV: Sinus venosus AO: Aorta [http://tube.medchrome.com/2011/04/development-of-heart-embryology-video.html](http://tube.medchrome.com/2011/04/development-of-heart-embryology-video.html)
1.5.1 Regulation of gene expression in the developing heart

To ensure that the structures of the heart are formed at the correct time and to prevent the development of heart defects, the cellular process that govern heart development are tightly regulated by changes in gene expression which determine cell fate. As such, cardiac transcription factors are essential for regulating the expression of a network of foetal genes (known as the foetal gene programme) that are expressed during foetal heart development and help the heart adapt to different physiological conditions and maintain normal function.

Examples of cardiac transcription factors that play important roles in heart development include GATA4, Nkx2.5, MEF2 and Islet1 which are required for formation and looping of the early heart tube. This has been shown by the fact that loss of these transcription factors in mice leads to looping defects in the heart. These transcription factors also have important roles in other aspects of heart development, such as formation of the right ventricle since their loss resulted in defects such as failure to form the right ventricle and embryonic lethality (Black, 2007, Cai et al., 2003, Lyons et al., 1995, Molkentin and Olson, 1997).

The GATA4 transcription factor is particularly important for facilitating heart development since GATA4 null mice also displayed cardiac defects including myocardial hypoplasia, double outlets of the right ventricle, and common atrioventricular canal (Pu et al., 2004). GATA4 is expressed in the pre-cardiac mesoderm and facilitates formation of the earliest heart tube. GATA4 is able to mediate key stages of heart development because it regulates the expression of many target genes through recognising and binding to the GATA motif in the promotor sequences of target genes. Such genes include the beta myosin heavy chain (ß-MHC) gene, Brain natriuretic peptide (BNP) and Atrial natriuretic factor (ANF). Consequently, regulation of expression of these target genes (among many others) by GATA4 has significant roles in cardiomyocyte survival, cardiac differentiation and morphogenesis (Akazawa and Komuro, 2003, Kuo et al., 1997, Molkentin et al., 1997). Activation of ß-MHC expression by GATA4 is particularly important since ß-MHC facilitates cardiomyocyte contraction and as such contributes to the beating action of the linear heart tube. ß-MHC plays an important role during foetal
heart development because it is expressed with alpha myosin heavy chain (α-MHC), which enables more efficient contraction of the developing heart muscle via interaction with actin. Moreover, β-MHC contributes to sarcomere, myofibril and muscle fibre formation and hence the structure and function of the developing heart. Since sarcomere shortening causes the heart muscle to contract, β-MHC plays an important role in mediating cardiomyocyte contraction and increasing cardiac contractility in the developing heart. In rodents, both α-MHC and β-MHC are expressed in the heart, however a larger proportion of α-MHC expression contributes to the effective contraction and hence fast heart rate in rodents. However, in the foetal heart, β-MHC is up-regulated in order to reduce the rate of contraction of the heart so that it is able to efficiently support foetal growth and development (Barry et al., 2008, Bernardo et al., 2010). Importantly, GATA4 is also able to indirectly regulate gene expression through interaction with other transcription factors. For example, GATA4 and homeotic transcription factor Nkx2.5 interact and up-regulate ANF expression, a marker of cardiac hypertrophy (Durocher et al., 1997). Similarly, interaction of other cardiac transcription factors is also important for normal heart development. For instance, MEF2 is co-expressed with Hand2, which is also important during heart development since this facilitates formation of the aortic arch arteries and the ventricles of the heart (Brand, 1997).

Similarly, several transcription factors are also involved in formation of the outflow tract and its septation. For example, Foxh1, Mef2c, and Hand2 knockout mice fail to develop the outflow tract and right ventricle (Brand, 2003, Buckingham et al., 2005, Kelly, 2005, Olson, 2006). Interestingly, Foxc1 and Foxc2 are also transcription factors that play a key role in this process. In this regard, Foxc1 and Foxc2 have been shown to be expressed in the secondary heart field and neural crest cells, and drive the expression of target genes such as Fgf8, Fgf10 and Tbx1. Importantly, loss of Foxc1/Foxc2 caused defects in outflow tract formation and downregulation of Fgf8, Fgf10 and Tbx1. Furthermore, Foxc1 and Foxc2 double knockout mice were embryonically lethal and died by E9.5, suggesting that these transcription factors may be important for heart development (Seo and Kume, 2006).
Immediate early genes such as proto-oncogenes c-fos, c-myc and c-jun are also associated with heart development. Expression of immediate early genes is induced by growth factors such as TGFβ, which was first discovered in a study whereby mouse embryonic fibroblasts were treated with growth factors, which showed rapid and transient upregulation of c-fos, c-myc, and c-jun (Greenberg and Ziff, 1984). They have also been shown to be expressed in the heart during development and play an important role during foetal heart development. In this regard, proto-oncogenes have been shown to transduce signals from growth factors to the cell nucleus where they regulate the expression of target genes that are involved in mediating cell growth and differentiation. For example, downregulation of c-myc correlated with reduced cardiomyocyte proliferation in the embryonic heart (Parker and Schneider, 1991, Sheng and Greenberg, 1990, Weinberg, 1989).

Interestingly, foetal heart development happens under hypoxic conditions since the developing foetus only receives oxygen from the mother’s placenta via the umbilical cord (Rajabi et al., 2007). Under such conditions, the ability to utilize fatty acids for ATP synthesis is restricted, therefore the main source of ATP synthesis to supply the energy demands of the developing foetus is the anaerobic glycolytic pathway which uses glucose as a substrate. In the foetal heart, glycogen occupies 30% of the total cardiomyocyte cell volume compared to approximately 2% in the adult cardiomyocyte. This in turn is hydrolysed to glucose by glycogenolysis and used as the substrate of glycolysis to generate ATP in the cell (Rajabi et al., 2007). Thus, enzymes and proteins that are involved in glycogen metabolism and glucose oxidation via the glycolytic pathway are up-regulated during foetal development. These include enzymes such as glycogen synthase, which catalyses the formation of glycogen polymers from glucose monomer subunits. One study showed that when glycogen synthase was knocked out in mice, the majority of the mice die before birth due to abnormal cardiac development (Rajabi et al., 2007).

Consequently, TFs play an important role in formation of the complex four chambered heart and the cellular and molecular changes that occur during development by regulating the expression of target genes.
1.6 Cardiac hypertrophy in response to stress

When the heart is put under stress such as exercise (physiological) or hypertension (pathological), the workload on the heart is increased. As such, cardiomyocytes of the heart undergo hypertrophy to enable the heart to work harder and more efficiently in response to stress, for example in response to increased haemodynamic load. Cardiomyocytes adapt in this manner since they are terminally differentiated and cannot replicate. Therefore they increase in size, which is known as cardiac hypertrophy (Rajabi et al., 2007). Cardiomyocyte hypertrophy is characterised by a number of features, including increased cell size, re-arrangement of the cytoskeleton, formation of new sarcomeres and increased protein synthesis (see Figure 1.18). These processes all contribute to an increased heart weight (HW): body weight (BW) ratio/ left ventricular (LV) mass: body weight ratio/ HW: Tibia length ratio in response to stress (Figure 1.18).

Different types of stresses can induce hypertrophy in the heart, however dependent on the type of stress, hypertrophy can either be beneficial or detrimental to cardiac function. For instance, physiological stress such as exercise results in cardiac hypertrophy that is reversible and beneficial to heart function. In contrast, prolonged pathological stress on the heart such as hypertension induces hypertrophy that is irreversible and detrimental to cardiac function since it results in cardiomyocyte loss and cardiac fibrosis which eventually leads to heart failure (see Table 1.1). Pathological cardiac hypertrophy is associated with a variety of cardiovascular diseases, including ischemic disease, hypertension, and valvular disease. Moreover, pathological hypertrophy is characterised by re-expression of foetal genes and as such is distinct from physiological hypertrophy (see Table 1.1) (McMullen and Jennings, 2007). As a result, understanding the cellular and molecular mechanisms behind pathological and physiological cardiac hypertrophy will be essential in order to provide better treatments and ways to prevent heart failure in patients with cardiovascular disease.
Figure 1.18: Diagram showing the differences between a normal and hypertrophic cardiomyocyte.

Table 1.1: Features of physiological and pathological cardiac hypertrophy.

<table>
<thead>
<tr>
<th>Physiological hypertrophy</th>
<th>Pathological hypertrophy</th>
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<tbody>
<tr>
<td>Increase in myocyte size</td>
<td>Increase in myocyte size</td>
</tr>
<tr>
<td>Formation of new sarcomeres</td>
<td>Formation of new sarcomeres</td>
</tr>
<tr>
<td>Normal or enhanced cardiac function</td>
<td>Reduced heart function</td>
</tr>
<tr>
<td>No upregulation of the foetal gene programme</td>
<td>Increase in ANP, BNP and contractile proteins (foetal gene programme)</td>
</tr>
<tr>
<td>No myocyte apoptosis/necrosis</td>
<td>Myocyte apoptosis (upregulation of apoptotic genes Bax and p53) and necrosis</td>
</tr>
<tr>
<td>Reversible</td>
<td>Irreversible – Leads to heart failure</td>
</tr>
</tbody>
</table>
During the process of hypertrophy, the arrangement of new sarcomeres can affect the physiology and function of the heart and as such forms two sub-types of hypertrophy; known as concentric and eccentric hypertrophy (see Figure 1.19). Concentric hypertrophy occurs when the addition of sarcomeres in the cardiomyocytes happens in parallel. This in turn leads to an increase in ventricular wall thickness and a decrease in left ventricular chamber size (see Figure 1.19). Pathological stimuli such as hypertension and aortic stenosis, and physiological stimuli such as weight lifting can induce this form of hypertrophy (McMullen and Jennings, 2007). On the other hand, eccentric hypertrophy occurs when new sarcomeres form in series, which in turn leads to an increased chamber volume and thin ventricular walls (see Figure 1.19). Physiological hypertrophy that results from prolonged exercise such as walking, running, cycling and swimming induces an eccentric phenotype. In contrast, eccentric pathological hypertrophy is triggered by conditions such as aortic regurgitation and arteriovenous fistulas (Bernardo et al., 2010, McMullen and Jennings, 2007). In summary, these two sub-types of hypertrophy will have unique effects on cardiac output and the ability of the heart to function effectively. This in turn will be important for understanding how the heart adapts differently to different stresses and how to prevent progression to heart failure after the initial adaptive response.

Figure 1.19: Diagram showing the features of two subsets of hypertrophy; concentric and eccentric hypertrophy. Pathological and physiological stress are able to induce concentric or eccentric responses (Nature, 2017a).
1.6.1 Physiological and pathological hypertrophy

1.6.1.1 Cardiac hypertrophy in response to physiological stress

Physiological hypertrophy in the heart occurs in response to physiological stress, including stimuli such as growth, pregnancy and exercise (e.g. swimming, running and weightlifting). It is characterised by formation of new sarcomeres, increased protein synthesis and cytoskeletal reorganisation, however hearts do not undergo adverse remodelling. In addition, cardiomyocytes continue to use lipid metabolism as the main source of ATP production to main cardiac function and contractility. Furthermore, physiological hypertrophy is reversible and beneficial to cardiac function, and does not lead to heart failure since cardiomyocytes do not undergo apoptosis (Bernardo et al., 2010).

1.6.1.2 Hypertrophic signalling to physiological stress

Distinct cell signalling pathways involved in pathological hypertrophy have been well characterised (see 1.6.2.2), whereas one pathway has been shown to be associated with the response to physiological stress - the Insulin like growth factor (IGF1) pathway (see Figure 1.20) (Bernardo et al., 2010). IGF1 is essential for growth during development since IGF1 and IGF1 receptor knockout mice have reduced body weight and stunted growth compared to WT littermate controls (Baker et al., 1993). As such, IGF1 is required during foetal development and is expressed in the heart. IGF1 has been shown to be expressed in the developing human heart between 10-12 weeks of gestation, and IGF1 receptor expression was detected in the heart between 16-18 weeks of gestation (Iruretagoyena et al., 2014). IGF1 has also been shown to mediate cardiomyocyte proliferation in the developing sheep heart via Extracellular signal-regulated kinase (ERK) and phosphoinositol-3 kinase (PI3K) signalling (Sundgren et al., 2003). More importantly however, IGF1 has been shown to mediate hypertrophy in neonatal rat ventricular myocyte (NRVM) cultures treated with IGF1 (Ebensperger et al., 1998, Ito et al., 1991).

On binding of the IGF1 factor to its receptor (a receptor tyrosine kinase), receptor dimerization occurs which activates PI3 Kinase (PI3K). This in turn activates Protein
Kinase B/Akt which results in activation of mTOR (mechanistic target of Rapamycin). Activation of mTOR increases protein synthesis and hence facilitates the growth and hypertrophy of the cardiomyocyte (see Figure 1.20) (Bernardo et al., 2010). Overexpression of IGF1 in transgenic mice led to a physiological hypertrophic response that included increased heart mass and enhanced cardiac function (Delaughter et al., 1999). This also correlated with studies whereby the IGF1 receptor was overexpressed in transgenic mice which caused an increase in heart weight, increased ventricular wall thickness and myocyte size and improved cardiac function (McMullen et al., 2004).

Importantly, activation of different signalling pathways in response to physiological stress converge on gene expression changes, which drive downstream effects in cells that are important for mediating hypertrophic responses in the heart. Such gene expression changes are tightly regulated by transcription factors (TFs), therefore it will be important to identify additional transcription factors that mediate hypertrophic responses to physiological stress.

**Figure 1.20:** Diagram showing IGF signalling in the cardiomyocyte in response to physiological stress on binding of IGF1 to the IGF1 receptor. On binding of the IGF1 factor to its receptor (a receptor tyrosine kinase), receptor dimerization occurs which activates PI3 Kinase (PI3K). This in turn activates Protein Kinase B/Akt which results in activation of mTOR (mechanistic target of Rapamycin), which increases protein synthesis and hence facilitates the growth and hypertrophy of the cardiomyocyte (Bernardo et al., 2010).
1.6.2 Cardiac hypertrophy in response to chronic stress

Unlike physiological hypertrophy, pathological hypertrophy is associated with a number of cardiovascular diseases, including myocardial infarction, valvular disease and hypertension. Such chronic stresses are particularly detrimental to cardiac function since they cause constitutive activation of the Renin Angiotensin Aldosterone System (RAAS). Under normal physiological conditions the RAAS acts to regulate blood pressure. In this regard, a fall in blood pressure stimulates Renin secretion from the kidneys into the bloodstream. Renin is an enzyme which cleaves circulating angiotensinogen secreted by the liver into Angiotensin I, after which angiotensin converting enzyme (ACE) secreted by the lungs cleaves Angiotensin I to produce Angiotensin II (AngII). Following this, AngII acts to increase blood pressure in several ways. First, AngII causes vasoconstriction of blood vessels, increasing blood pressure. AngII also binds to the AT1 receptor and stimulates aldosterone release from the adrenal glands. In addition, aldosterone triggers reabsorption of water into the bloodstream, increasing blood volume and blood pressure (Iravanian and Dudley, 2008).

Chronic stress causes increased renin production by the kidneys which in turn results in increased AngII production, which acts to increase blood pressure and enables the heart to adapt to an increased workload. However, constitutive activation of the RAAS in response to chronic stress also causes release of reactive oxygen species which is associated with inflammation and cardiovascular damage (Ayada et al., 2015, Iravanian and Dudley, 2008). Cardiac hypertrophy in response to pathological stress also results in increased cardiomyocyte size, protein synthesis, formation of new sarcomeres and cytoskeletal reorganisation. Re-expression of the foetal gene programme also occurs as part of an early adaptive response to pathological stress. However, prolonged pathological stress triggers maladaptive responses that lead to cardiomyocyte cell death which in turn leads to long term irreversible damage to the heart and eventually heart failure (see Figure 1.21) (McMullen and Jennings, 2007).
Figure 1.21: Features of pathological cardiac hypertrophy that occurs in the cardiomyocyte and cardiac fibroblasts (Bernardo et al., 2010)
1.6.2.1 Hypertrophic signalling in response to chronic stress

Signal transduction is important for delivering signals from distant tissues, adjacent cells or individual cells (e.g. whereby signals secreted from a cell act on itself) from the plasma membrane to the cell nucleus which converge on changes in gene expression that can mediate diverse cellular processes that control cell fate, etc. Several cell signalling pathways are associated with mediating pathological cardiac hypertrophy, which are activated by different stimuli. For example, hypertrophic stimulus Angiotensin II (AngII) which triggers hypertension and an increased workload on the heart is a small peptide that mediates its effects through receptor tyrosine kinase and G-protein coupled receptor signalling (including Gs and Gaq/G11 signalling) (Frey and Olson, 2003).

Firstly, AngII activates Mitogen Activated Protein Kinase (MAPK) signalling, which is important for transducing signals to the nucleus of the cell from an external stimulus, which enables cell growth by activating protein synthesis (see Figure 1.22) (Frey and Olson, 2003). The MAPK pathway is stimulated when Angiotensin II binds to its AT1 receptor and activates Protein Kinase C, which in turn generates reactive oxygen species via activation of NAD(P)H oxidase. These reactive oxygen species then activate the Epidermal Growth Factor Receptor (EGFR), a receptor tyrosine kinase. Phosphorylation of EGFR recruits Grb2 and shc proteins to the receptor which in turn recruits the small G protein Ras to the receptor. Ras in turn then activates Raf, MAPK Kinase (MEK) which in turn phosphorylates and activates Extracellular signal Related Kinase (ERK). Activated ERK then translocates to the nucleus where it regulates the transcription of a number of genes involved in cardiomyocyte cell growth and survival (see Figure 1.22) (Mehta and Griendling, 2007). Overexpression of the MEK1 gene in transgenic mice has been shown to induce cardiac hypertrophy, suggesting that this pathway is important for mediating this response (Frey and Olson, 2003).
Calcineurin signalling is also important for mediating cardiac hypertrophy when stimulated with AngII, as was shown by Molkentin and colleagues (see Figure 1.23) (Molkentin et al., 1998). This pathway was first identified in T cells but since has been shown to be active in cardiomyocytes (Barry et al., 2008, Frey and Olson, 2003). Binding of AngII to the AT1 receptor also activates Phospholipase C, which hydrolyses 4, 5 bisphosphate into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Production of IP3 leads to muscle contraction by binding to its receptor on the sarcoplasmic reticulum, which allows calcium efflux from the sarcoplasmic reticulum into the cytoplasm. Calcium binds to calmodulin which then binds to the regulatory and catalytic

Figure 1.22: Diagram showing activation of MAP Kinase (MAPK) signalling on binding of AngII to the AT1 receptor. Briefly, on binding of AngII to the AT1 receptor, Protein Kinase C is activated which leads to the generation reactive oxygen species (ROS). These reactive oxygen species then activate the Epidermal Growth Factor Receptor (EGFR), a receptor tyrosine kinase. Phosphorylation (P) of EGFR recruits Grb2 and shc proteins to the receptor which in turn recruits the small G protein Ras to the receptor. Ras in turn then activates Raf, which activates MAPK Kinase (MEK) which in turn phosphorylates (P) and activates Extracellular signal Related Kinase (ERK). Activated ERK translocates to the nucleus where it regulates the transcription of a number of genes involved in cardiomyocyte cell growth and survival (Mehta and Griendling, 2007)
subunits of Calcineurin. Calcineurin is a calcium dependent protein phosphatase which dephosphorylates the NFAT transcription factor which leads to its activation (see Figure 1.23). Importantly, NFAT is an important cardiac transcription factor that regulates the expression of cardiac specific genes in the developing and hypertrophic heart in response to stress.

In this regard, in isolated neonatal rat ventricular cardiomyocytes activated Calcineurin has been shown to dephosphorylate and activate cardiac specific NFAT3. Upon activation of NFAT3, it translocates to the nucleus where it has been shown to interact with GATA4 to increase the promoter activity of BNP (a hypertrophic marker); suggesting that NFAT3 and GATA4 may be able to mediate and induce cardiac hypertrophy by up-regulating the expression of hypertrophic markers such as BNP. This was demonstrated by cytoskeletal reorganization and an increase in cardiomyocyte size on treatment of isolated neonatal rat ventricular myocytes with AngII (Molkentin et al., 1998).

Furthermore, Cyclosporine A (CsA) aCalcineurin inhibitor prevented the hypertrophic response in isolated neonatal rat ventricular cardiomyocytes that were treated with hypertrophic stimulus AngII. Consequently, this demonstrated that induction of the hypertrophic response in cardiomyocytes following AngII treatment was mediated by Calcineurin signalling (Molkentin et al., 1998). In addition, overexpression of Calcineurin in transgenic mice led to an increased heart size which eventually caused heart failure, which further suggests that Calcineurin signalling mediates pathological hypertrophy (Frey and Olson, 2003, Molkentin et al., 1998). Furthermore, Molkentin and colleagues generated mutant mice which expressed constitutively active NFAT3 (under control of the α-MHC promoter) which induced cardiac hypertrophy and cardiac fibrosis in these animals, which further demonstrates the importance of the NFAT3 transcription factor in mediating the hypertrophic response following pathological stress (Molkentin et al., 1998).
Figure 1.23: Activation of Calcineurin signalling on binding of AngII to the AT1 receptor. Briefly, on binding of AngII to the AT1 receptor, activation of Phospholipase C (PLC) leads to increased levels of intracellular calcium levels on binding of inositol-1,4,5-triphosphate (IP3) to its receptor on the sarcoplasmic reticulum of the cell. Calcium binds to calmodulin which then binds to the regulatory and catalytic subunits of Calcineurin. Calcineurin then dephosphorylates and activates the NFAT transcription factor which translocates to the nucleus to interact with GATA4 to regulate expression of hypertrophic genes such as Brain Natriuretic Peptide (BNP) (Molkentin et al., 1998). DAG: Diacylglycerol
Binding of AngII to the AT1 receptor also activates Phospholipase C, which hydrolyses 4, 5 bisphosphate into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). As previously described, on binding of AngII to the AT1 receptor, activation of Phospholipase C leads to increased levels of intracellular calcium levels on binding of IP3 to its receptor on the sarcoplasmic reticulum of the cell. Increased cytoplasmic calcium then binds to calmodulin and activates myosin light chain kinase (MLCK) which enables efficient muscle contraction of the heart muscle (see Figure 1.24). Overexpression of the Gαq/G11 receptor has also been shown to trigger cardiac hypertrophy which results in diminished cardiac function, indicating an additional role of this signalling pathway in the maladaptive response to stress on the heart (Frey and Olson, 2003).

Figure 1.24: Diagram showing activation of Phospholipase C (PLC) signalling on binding of AngII to the AT1 receptor. Briefly, Phospholipase C hydrolyses 4, 5 bisphosphate into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 then binds to its receptor on the sarcoplasmic reticulum, which allows calcium efflux from the sarcoplasmic reticulum into the cytoplasm. Increased cytoplasmic calcium then binds to calmodulin and activates myosin light chain kinase (MLCK) which enables efficient muscle contraction of the heart muscle, enabling cardiomyocyte hypertrophy and survival in response to pathological stress (Mehta and Griendling, 2007).
1.6.2.2 Regulation of gene expression in the heart in response to pathological stress

Transcriptional regulation of gene expression also plays an important role in response to chronic stress in the heart and as such is particularly important in regulating specific cellular and molecular changes during the adaptive phase of hypertrophy. Gene regulation in cardiomyocytes in response to stress is essential since they are terminally differentiated and can only enlarge by undergoing hypertrophy. Importantly, cardiomyocyte hypertrophy requires increased expression of genes that enable growth of the cardiomyocyte to occur through increased protein synthesis and elevating the protein content of the cardiomyocyte. As such, cardiac hypertrophy is coupled with increased phosphorylation and activity of RNA polymerase II (Abdellatif et al., 1998, Cutilletta et al., 1978, McDermott et al., 1991, McDermott et al., 1989). Importantly, the cell signalling pathways and hence the gene expression patterns that drive physiological and pathological cardiac hypertrophy are distinct. In particular, foetal genes that are expressed during heart development are re-expressed in response to pathological stress (e.g. hypertension), enabling the heart to adapt to an increased workload to maintain cardiac output. As such, cardiac transcription factors also play a pivotal role in mediating hypertrophic responses (Frey and Olson, 2003).

For instance, cardiac transcription factors such as Nkx2.5 and MEF2 are up-regulated in the heart during the early adaptive response of pathological hypertrophy. In this regard, Nkx2.5 is re-expressed as part of the pathological hypertrophic response to stress, and has been shown to be up-regulated in mice that underwent aortic banding and mice that were treated with isoproterenol and phenylephrine (which induce hypertrophy) (Saadane et al., 1999). In these mouse models, up-regulation of Nkx2.5 was associated with inducing and maintaining the hypertrophic response by regulating the expression of target genes that enable the heart to adapt to pathological injury (Saadane et al., 1999). In the hypertrophic heart, MEF2 expression is also increased where it helps to integrate several calcium calmodulin signals within the cardiomyocytes and is also associated with up-regulation of hypertrophic markers such as ANF and ß-MHC (Frey and Olson, 2003). During the early adaptive phase of pathological cardiac hypertrophy, ß-MHC is also up-regulated within the cardiomyocytes of the heart in order to contribute
to new sarcomere formation and increased cardiac contractility that enables the heart to adapt an increased workload (McMullen and Jennings, 2007).

ANF, a marker of cardiomyocyte differentiation that is increased in the cardiac atria, and BNP (which are both up-regulated by GATA4) are peptides that are important for regulating blood pressure. They allow the heart to cope with the increased haemodynamic load of the growing heart and also act to reduce hypertrophy and cardiac fibrosis. On a rise in blood pressure, increased ANF and BNP expression triggers cyclic GMP production causing reabsorption of water from the blood, reducing pressure and lowering the workload of the heart. Consequently, ANF and BNP re-expression in the early adaptive phase of pathological hypertrophy is required for adapting to increased haemodynamic load such as hypertension (Barry et al., 2008, Bernardo et al., 2010).

Proto-oncogenes have also been shown to be expressed on induction of cardiac hypertrophy, suggesting that they may also play an important role in mediating cardiomyocyte growth during the early adaptive response. For example, Komuro and colleagues demonstrated that c-myc was expressed in the foetal rat heart and was also re-expressed in the adult rat heart following induction of cardiac hypertrophy by aortic banding (Komuro et al., 1988). Furthermore, induction of cardiac hypertrophy using aortic banding in rats in a different study also showed induction of c-myc mRNA (Mulvagh et al., 1987). Induction of immediate early genes has also been demonstrated in vitro, whereby isolated neonatal rat ventricular myocytes treated with hypertrophic stimulus Angiotensin II (AngII) also demonstrated induction of immediate early genes c-fos, c-jun, Egr-1, and c-myc (Sadoshima et al., 1995). Cyclin D1 which is usually implicated in cell proliferation is another gene (and a Brn3b target gene) that is associated with the hypertrophic response in cardiomyocytes. For instance, overexpression of cyclin D1 in transgenic mice caused increased cardiomyocyte DNA synthesis and multinucleation (Soonpaa et al., 1997). Overexpression of cyclin D1 in isolated cardiomyocytes has also been shown to increase cardiomyocyte size which was accompanied by increased protein synthesis and protein content within the cardiomyocyte (Tamamori-Adachi et al., 2002).
In the adult heart, fatty acid oxidation is the main fuel source for generating ATP required for maintaining cardiac output (Bernardo et al., 2010). Lipid metabolism involves a number of enzymes that are required for uptake of fatty acids and their breakdown to release ATP, an example of which includes carnitine palmitoyl transferase 1 (CPT-1) (Bernardo et al., 2010). Importantly, these enzymes are also tightly regulated at the level of gene transcription by TFs such as peroxisome proliferator-activated receptors (PPARs) (Frey and Olson, 2003). When the heart is exposed to chronic stress, the metabolism of the heart switches from using lipids to metabolising glucose. This enables the cardiomyocytes of the heart to generate ATP whilst using less oxygen, since fatty acid oxidation requires more oxygen than glycolysis to generate ATP. This allows the heart to adapt to stress such as pressure overload induced hypertrophy, where it has been shown that genes involved in lipid metabolism such as PPAR and CPT-1 are downregulated (Frey and Olson, 2003). Consequently, due to this metabolic switch, enzymes and proteins that are involved in glycogen metabolism and glucose oxidation via the glycolytic pathway that are up-regulated during foetal development are re-expressed in the hypertrophic heart. Such genes include glycolytic enzymes such as phosphofructokinase, aldolase and hexokinase, and glycogen synthase, which catalyses the formation of glycogen polymers from glucose monomer subunits. One study showed that when glycogen synthase was knocked out in mice, the majority of the mice die before birth due to abnormal cardiac development (Rajabi et al., 2007). In addition, glucose transporters such as GLUT4 which mediate uptake of glucose into tissues are also up-regulated during pathological hypertrophy (Abel et al., 1999).
1.6.2.3 Cardiomyocyte apoptosis in the heart in response to pathological stress

As previously described, activation of cell signalling pathways converge on gene expression changes that determine cell fate in the heart in response to chronic stress (e.g. hypertension). Importantly, cell signals that promote cardiomyocyte survival are also tightly controlled and thus help to determine whether a cell under stress survives or undergoes programmed cell death if it cannot adapt to the stress. For example, activation of Calcineurin signalling is able to promote cell survival by activating NFAT expression, which upregulates expression of hypertrophic markers and cell survival genes such as MCIP1 (Molkentin, 2004). PI3 Kinase (PI3K)-Akt signalling also promotes cardiomyocyte survival and hypertrophy by activating mTOR which promotes protein synthesis, and activation of Akt also induces expression of cell survival and hypertrophic genes (Molkentin, 2004). In addition, in response to pathological stress, cytokines such as gp130 can activate Janus Kinase enzymes (JAKs) which in turn can activate STAT transcription factors that are able to induce expression of hypertrophic genes such as c-fos and ANP and cell survival genes such as Bcl-XL (van Empel and De Windt, 2004).

However, when the cardiomyocytes of the heart are put under prolonged pathological stress, maladaptive changes occur which eventually cause cardiomyocyte apoptosis since they can no longer cope with the stress. Apoptosis is also known as programmed cell death and usually occurs during the development of an organism to maintain cell populations within tissues. As such, it is a tightly regulated process which is characterised by a number of morphological changes within the cell. These include chromatin condensation, nuclear fragmentation, cell shrinkage, and membrane blebbing (Elmore, 2007). During this process, apoptotic bodies which contain cell cytoplasm and organelles are created which in turn get engulfed by macrophages, parenchymal cells, or neoplastic cells; importantly there is minimal inflammatory response associated with apoptosis (Elmore, 2007).

Due to the limited proliferative capacity of cardiomyocytes which are terminally differentiated in the adult heart, cardiomyocytes that undergo apoptosis do not get replaced which causes reduced cardiac function and contractility which eventually leads
to heart failure (McMullen and Jennings, 2007). A number of stresses induce apoptosis in the cardiomyocyte, including Angiotensin II signalling, oxidative stress/ hypoxia and mechanical stretch (Takemura and Fujiwara, 2004). Moreover, cardiomyocyte apoptosis can be mediated by two different pathways; the mitochondrial pathway and the death receptor pathway (see Figures 1.25 and 1.26).

For example, prolonged stimulation of G-protein coupled receptor signalling by AngII or overexpression of the AT1 receptor can cause activation of Protein Kinase C. This in turn causes up-regulation of pro-apoptotic Bcl-2 family genes such as Nix and Bax which translocate to the mitochondria. At the mitochondrial surface, competition between pro and anti-apoptotic signals determine whether the cardiomyocyte undergoes cell cycle arrest or apoptosis. If accumulation of pro-apoptotic genes (e.g. Bax and Noxa) at the mitochondrial surface outnumbers the proportion of anti-apoptotic genes (e.g. Bcl-XL) at the mitochondrial surface, cytochrome c is released from the mitochondria into the cytoplasm. Increased cytochrome c levels in the cytoplasm then forms the apoptosome upon binding with Apaf-1 and caspase 9 which in turn activates caspase 3 to drive cardiomyocyte apoptosis (see Figure 1.25) (van Empel and De Windt, 2004).
Figure 1.25: Activation of mitochondrial mediated apoptosis, for example through prolonged activation of G protein coupled receptor (GPCR) signalling (e.g. on binding of AngII to its AT1 receptor. Briefly, prolonged stimulation of GPCR signalling leads to activation of Protein Kinase C which in turn causes upregulation of pro-apoptotic Bcl-2 family genes which translocate to the mitochondria. Accumulation of pro-apoptotic genes at the mitochondrial surface causes cytochrome c to be released from the mitochondria into the cytoplasm, which then forms the apoptosome with Apaf-1 and caspase 9. Activation of caspase 3 then drives cardiomyocyte apoptosis. Diagram adapted from (van Empel and De Windt, 2004).
In addition, stress signals can also trigger cardiomyocyte apoptosis via activation of multiple members of the death receptor family including TNF-α, Fas and CD95 (see Figure 1.26). On binding of these activated ligands to their receptors in response to stress signals, death receptor clustering is triggered. Death receptor clustering then stimulates death receptor signalling and activates caspase 8 which in turn activates caspase 3 and drives cardiomyocyte apoptosis (van Empel and De Windt, 2004).

![Figure 1.26: Activation of apoptosis via the death receptor apoptotic pathway. Briefly, on binding of activated ligands such as Fas and TNF-α to their receptors in response to stress signals triggers death receptor clustering. Death receptor clustering then stimulates death receptor signalling and activates caspase 8 which in turn activates caspase 3 and drives cardiomyocyte apoptosis (van Empel and De Windt, 2004)](image)

In addition to signal transduction, transcriptional regulation of gene expression also plays an important role in mediating apoptosis. In this regard, p53 was first identified by Sir David Lane, and since has been shown to be a tumour suppressor gene that is mutated/ non-functioning in a wide variety of cancers (V, 1979).
Importantly, p53 is able to mediate its tumour suppressor effects through its function as a transcription factor. p53 contains a sequence specific DNA binding domain (from amino acid 100-300) that is necessary for activation of gene transcription (see Figure 1.27) (Long et al., 1997). For instance, p53 is able to activate the expression of p21cip1/waf1 which binds to cyclin dependent kinase 2 (cdk2) and prevents cell cycle progression (Shaw, 1996). The tumour suppressor action of p53 is also mediated by a non-specific DNA interaction domain at the C terminus of the protein that is able to bind to damaged DNA which can lead to cell cycle arrest or activation of apoptosis by regulating expression of its target genes. Furthermore, p53 also contains an activation domain (amino acids 1-43) which facilitates protein binding and hence interaction with members of the general transcription machinery, such as TFIID to enable gene transcription to occur (see Figure 1.27) (Long et al., 1997).

![Diagram showing the structure of the p53 transcription factor](image)

**Figure 1.27:** Diagram showing the structure of the p53 transcription factor, which contains an activation domain, a sequence specific DNA binding domain which binds to target gene promoters, and a non-specific DNA interaction domain that recognises damaged DNA (Long et al., 1997).

In addition to facilitating cell cycle arrest, p53 also plays an important role in mediating apoptosis in a variety of cell types, including cardiomyocytes in response to stress. For instance, stress signals including hypoxia and ischemia cause activation of p53 which can in turn induce expression of the Fas ligand. Consequently, on binding of Fas to its receptor, death receptor clustering and Caspase 3 activation triggers apoptosis. However, p53 activation by stress signals has also been shown to induce the expression of pro-apoptotic genes including Bax, which in turn feeds into the mitochondrial apoptotic pathway (Long et al., 1997).

Importantly, many studies have shown the importance of p53 in cardiomyocyte apoptosis in response to stress. For example, isolated neonatal rat cardiomyocytes that
were exposed to hypoxia for 24-48 hours demonstrated increased expression of apoptotic genes which correlated with increased levels of p53 (Long et al., 1997). In addition, a dog model of heart failure showed increased expression of active p53 within the injured region of the heart in apoptotic cardiomyocytes whereas inactive p53 was identified in non-injured regions of the heart (Sharov et al., 1996). Intriguingly, Budhram-Mahadeo and colleagues also showed the importance of p53 in cardiomyocyte apoptosis in the injured heart when co-expressed with the Brn3b transcription factor. In this regard, following left anterior descending (LAD) coronary artery ligation in C57BL/6 mice, Brn3b expression increased throughout the heart, including within the infarct zone of the heart. Interestingly, Brn3b was co-expressed with p53 within the infarct zone and border regions of the injured heart. This correlated with increased expression of Bax and Noxa and increased cardiomyocyte apoptosis within the infarct zone of the heart. Similar effects were also observed in isolated NRVM and H9C2 cells that underwent simulated ischemia/ reperfusion injury (sI/R) (Budhram-Mahadeo et al., 2014). Consequently, interaction of p53 with other transcription factors and proteins are important for facilitating its transcriptional function in triggering cardiomyocyte apoptosis in the stressed heart.

1.6.2.4 Cardiac remodelling in response to pathological stress

As previously described, other cell types in the heart are important for maintaining normal heart function. For example, under normal physiological conditions, cardiac fibroblasts produce collagen and extracellular matrix proteins (ECM). In addition, they surround the cardiomyocytes and help to support and maintain cardiomyocyte structure (Frey and Olson, 2003). The extracellular matrix also enables more efficient muscle contraction to occur via assisting cardiomyocyte shortening.

Under pathological conditions cardiac fibroblasts also contribute to the cardiac fibrosis that is observed in the hypertrophic heart. For example, increased synthesis of type 1 and type 3 collagen and ECM proteins by cardiac fibroblasts leads to increased collagen and ECM deposition around each cardiomyocyte. Overexpression of these proteins causes excessive collagen deposition around the cardiomyocytes which stiffens the
heart muscle (ventricles) and causes fibrosis. As a result, cardiomyocyte shortening and hence contraction is restricted which causes the ventricles of the heart to become stiff. This further contributes to diminished cardiac function and eventually heart failure following prolonged pathological stress (McMullen and Jennings, 2007).

Cardiac fibroblasts have also been shown to contribute to the hypertrophic response in cardiomyocytes via Fgf2 secretion. Fgf2 is a peptide that is secreted primarily by cardiac fibroblasts but also cardiomyocytes. Fgf2 secretion in turn activates a number of cell signalling pathways that drive changes in expression of genes that mediate the hypertrophic response (Fujiu and Nagai, 2014). For instance, secreted Fgf2 binds to the FGFR1 receptor and activates it. This in turn leads to activation the MAP Kinase pathway which causes activation of Ras which then activates Extracellular signal Related Kinase (ERK). ERK is an enzyme which translocates to the nucleus and activates cardiac transcription factors by phosphorylation. As such, cardiac transcription factors like GATA4 get activated which then up-regulate expression of their target genes that are associated with the hypertrophic response such as β-MHC (Fujiu and Nagai, 2014).

In addition, activation of the FGFR1 receptor by Fgf2 binding also leads to activation of Phospholipase C. This can result in Calcium release from the sarcoplasmic reticulum and activation of Calcineurin signalling. Consequently, this leads to activation of additional cardiac transcription factors such as MEF2 and NFAT that also regulate expression of hypertrophic markers, which results in increased protein synthesis and cardiomyocyte hypertrophy. Fgf2 is particularly important for mediating the hypertrophic response in cardiomyocytes since Fgf2 deficient mice did not undergo hypertrophy when they were treated with hypertrophic stimulus Angiotensin II (AngII) or following trans aortic constriction (Fujiu and Nagai, 2014).
1.7 Effects of cardiac hypertrophy on heart function

Physiological and pathological cardiac hypertrophy have been highly characterised at the molecular level. In order to better understand the mechanisms behind progression to heart failure from the early adaptive phase of hypertrophy to later maladaptive stages, understanding the impact of physiological and pathological hypertrophy on cardiac function is also important.

As previously described, physiological stress such as exercise is beneficial to cardiac function, whereas pathological stress such as hypertension is detrimental to cardiac function. As such, many studies have been undertaken to compare the heart function of humans exposed to physiological stress (trained running athletes) and patients suffering from hypertrophic cardiomyopathy (pathological stress) using echocardiography. Such studies have shown that following exercise training in athletes, the left ventricular (LV) septal thickness increased. Interestingly, in patients suffering with hypertrophic cardiomyopathy, the LV septal thickness was greater when compared to that of the athletes (Kreso et al., 2015, Venckunas and Mazutaitiene, 2007). In addition, the interventricular septal shape and motion was normal in athletes whereas it was irregular in patients with hypertrophic cardiomyopathy. The left ventricular chamber was also increased in the trained athletes, however pathological hypertrophic cardiomyopathy caused a decrease in left ventricular chamber diameter, suggesting that patients with this condition may have reduced ability to pump blood around the body due to having a smaller left ventricular chamber that is only able to pump a smaller volume of blood from the heart (Kreso et al., 2015, Venckunas and Mazutaitiene, 2007).

Furthermore, ejection fraction (the percentage volume of blood pumped from the heart to the rest of the body) was normal in athletes, but reduced in hypertrophic cardiomyopathy patients. Diastolic function (the ability of the ventricles to relax/fill with blood following systole) was also normal/ enhanced in athletes and was decreased in patients with hypertrophic cardiomyopathy, which was demonstrated by a decreased E/A ratio when compared to the athletes. The E/A ratio is equivalent to the early (E) diastolic filling velocity divided by the late (A) diastolic filling velocity is used to measure diastolic function in the heart, where the velocity is equal to the velocity of the blood
flowing through the mitral valve in the heart (Kreso et al., 2015, Venckunas and Mazutaitiene, 2007). Interestingly, there were also differences in the acoustic density of the myocardial wall of hearts exposed to physiological stress (athletes) versus pathological stress (hypertrophic cardiomyopathy). The acoustic density of a tissue represents its density and elastic properties of the tissue (e.g. myocardial wall) that enable ultrasound waves to penetrate it at a specific velocity (Iaizzo, 2015). The acoustic density of the myocardial wall was normal in the hypertrophic athletes’ hearts whereas it was increased in the hearts of patients with hypertrophic cardiomyopathy (due to increased collagen deposition) (Venckunas and Mazutaitiene, 2007, Kreso et al., 2015).

In summary, cardiac hypertrophy associated with pathological stress significantly reduces cardiac function and contractility, in addition to causing significant changes in the structure and morphology of the heart; whereas physiological stress induced by exercise does not significantly affect cardiac function in the hypertrophic heart.
1.8 Mouse models of physiological and pathological cardiac hypertrophy

It is not possible to use patients to study the effects of cardiac hypertrophy on heart morphology, function and gene expression changes. Therefore animal models of cardiac hypertrophy have and continue to be an important tool used to gain a better understanding behind the mechanisms that drive cardiac hypertrophy. As such, several animal models have been developed that induce both physiological and pathological hypertrophy which have been tested in a variety of animal species, including mice, rats, rabbits, pigs and dogs (see Table 1.2 and 1.3) (Blank et al., 1989, Sharov et al., 1996).

Mouse models are particularly important and widely used in cardiovascular research for a number of reasons. Firstly, many genes are highly conserved between mice and humans, and it is easy to genetically manipulate mouse embryos to generate transgenic mice. For instance, individual genes are either globally knocked out (to ablate gene expression) or overexpressed in the mouse in order to study gene function. Additionally, conditional knockout mice can also be generated which allows the function of genes within specific tissues to be studied, since ablation of gene expression is driven by a tissue specific promoter (Hall et al., 2009). In addition, mice have a short gestation time, meaning that high numbers of mice can be generated quickly which reach adult age by 2 months which allows experiments to be conducted within a relatively short space of time. Mouse breeding and housing is also low cost when compared to using larger animals for research.

Although mice are genetically similar to humans, it is important to note that there are similarities and differences between the development, morphology and structure of the mouse and human heart which need to be considered when interpreting data obtained from animal experiments. It is particularly relevant to identify similarities and differences in cardiac development between mice and humans since re-expression of foetal genes is a key feature of pathological cardiac hypertrophy. As such, there may be slight differences in response to pathological hypertrophy between mice and humans, meaning that data interpreted from mouse models of cardiac hypertrophy should be considered carefully. Interestingly, cardiac development is similar between mice and
humans, and the sequence of events during cardiac development is also similar. For example, septation of the atria of the heart begins at similar time points for both mice and humans and involves similar structures; and in both species begins during formation of the primary heart tube. Ventricular septation is also similar between mice and humans (Krishnan et al., 2014). In addition, formation of the semilunar valves of the heart, thinning of plate like structures and spiralling was comparable between mice and humans, as was the process of outflow tract septation (Krishnan et al., 2014). There are also some similarities in the morphology of the ventricles, such as similarities in ventricular trabeculation between mice and humans and the presence of two papillary muscles in the left ventricle in both species. However, there are also some differences in their morphology, which include the absence of a moderator band in the right ventricle of the mouse which is present in the human. The timeline in which cardiac development occurs in mice and humans is also different; in humans the heart is fully developed several months before birth whereas in the mouse the heart is only fully developed just a few days before birth (Krishnan et al., 2014).

In the adult heart, morphology is also comparable between mice and humans, (particularly the ventricles), however in humans there are clear differences between the trabeculation in the right and left ventricles of the heart whereas in the mouse heart the distinction is minimal (Doevendans et al., 1998). Importantly, the mouse heart is much smaller than the human heart, and since the body weight of a mouse is approximately 0.05% of the weight of a human, this has implications on the metabolism and energy requirements of the mouse heart. Firstly, the mouse has a much faster heart rate than humans (approximately 600 beats per minute in the mouse versus 60-90 beats per minute in the human) since it has a large surface area to volume ratio when compared to humans. In this regard, mice lose heat from their body more rapidly and hence have a higher energy demand than humans, and as such have increased myosin ATPase activity, increased oxygen capacity and increased SERCA activity when compared to humans (Doevendans et al., 1998). Since SERCA is a calcium dependent ATPase that plays an important role in muscle relaxation in cardiomyocytes, there may also be differences in calcium handling and muscle contraction between mouse and human hearts. Furthermore, although myofibril density has been shown to be similar between
mice and humans, mice have a greater mitochondrial density within their myocardium (37.9% in mice and 25.3% in humans), which may enable them to meet their increased energy demands within the body (Blank et al., 1989). Finally, there is a difference in the expression pattern of alpha myosin heavy chain (α-MHC) and beta myosin heavy chain (β-MHC) proteins in the mouse heart. β-MHC is expressed with alpha myosin heavy chain, and enables efficient contraction of the heart muscle via interaction with actin. Both species express α-MHC and β-MHC, however in mice a larger proportion α-MHC expression contributes to the effective contraction and hence fast heart rate (Barry et al., 2008, Bernardo et al., 2010, Iruretagoyena et al., 2014).

In summary, there are many similarities in the development, morphology and structure of the mouse and human heart. As such, mouse models are suitable for studying the mechanisms which drive cardiac hypertrophy in response to pathological and physiological stress. However, there are also key differences between the two species, including differences in size, metabolism and contractility that are important to consider when using mouse models to study physiological and pathological hypertrophy.
1.8.1 Mouse models of physiological hypertrophy

As previously described, growth, pregnancy and exercise all trigger physiological cardiac hypertrophy. Since pregnancy has a very short duration in the mouse, analysing the effects of hypertrophy of the heart in response to pregnancy is difficult. Therefore animal models of physiological hypertrophy usually involve exercising mice for a certain period of time and include methods such as swimming, treadmill and voluntary running (see Table 1.2).

For example, treadmill running involves training mice to run on a treadmill, after which they are made to run a set distance or for a set period of time per day for a number of weeks, either continuously or at intervals (Kemi, et al., 2001). There are some advantages to using treadmill running, including sufficient induction of cardiac hypertrophy in the mouse; ease of giving each animal the same amount and intensity of exercise; and ease of measuring and controlling the intensity and duration of exercise for each animal. However, motivating mice to run on a treadmill is difficult and can also cause them to become stressed which may alter the outcome of the experiment and make results difficult to interpret (Wang et al., 2010, Wisloff et al., 2001).

Swim training has also been shown to induce cardiac hypertrophy (increased HW: BW ratio), whereby mice are placed in a tank of water (with or without weights attached to the tail to vary the intensity of exercise) and allowed to swim for a specific period of time (e.g. between 1-6 hours per day) (Edwards, 2002, Evangelista et al., 2003, Kaplan et al., 1994, Medeiros et al., 2004). Although no motivation is required for this type of exercise due to fear of drowning, this fear is likely to induce additional stress and discomfort on the animal, which may influence the results obtained that may be caused by the stress of immersion in water alone. In addition, it is also difficult to measure the amount of exercise carried out by each animal. Moreover, several factors can influence the degree of cardiac hypertrophy induced in each mouse, such as water temperature, water tank depth, density of animals and water movement (Abel, 1994, Iemitsu et al., 2003).

For these studies, voluntary running was used to induce cardiac hypertrophy following physiological stress. Voluntary running involves using an exercise wheel that is placed
inside the animal cage, enabling the mouse to run on its own accord. Intensity of exercise can be adjusted by changing the resistance of the running wheel, and data collectors can also be attached to the running wheels to measure the distance and speed achieved by each mouse, making it easy to compare exercise ability between groups of mice (Wang et al., 2010). Typical studies allow the mice to run for 2 to 4 weeks to induce hypertrophy in the mouse. For example, a study performed by Allen and colleagues allowed adult male mice to voluntarily exercise for 2 to 4 weeks, during which time their running distances were recorded. This model was sufficient to induce moderate cardiac hypertrophy which was demonstrated by an increased heart weight to body weight ratio (Allen et al., 2001).

In contrast to treadmill running, it is difficult to measure how much exercise each mouse has completed in voluntary running and to make sure every sure that every mouse receives the same amount of exercise. As such, in order to accurately validate results obtained from such studies, any phenotypes observed should be analysed relative to the amount of exercise that each individual mouse has completed since some mice may have a greater tendency to exercise than others. However, little training is required for this method since studies have shown that mice will spontaneously exercise when a wheel is put in their cage, in addition to the fact that they are naturally more active at night due to being nocturnal. Furthermore, animal welfare is not compromised using this model since the mice do not get stressed due to the exercise being voluntary (Wang et al., 2010).
Table 1.2: Animal models of physiological hypertrophy, their advantages and limitations

<table>
<thead>
<tr>
<th>Physiological hypertrophy model</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treadmill running</td>
<td>• Can accurately control and measure the amount of exercise</td>
<td>• May cause physical and psychological stress to the animal</td>
</tr>
<tr>
<td></td>
<td>• All animals within a group can be made to do the same amount and intensity of exercise</td>
<td>• Needs to be adapted to the physical fitness of each animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Difficult to motivate animals to exercise</td>
</tr>
<tr>
<td>Voluntary running</td>
<td>• Not stressful for the animal</td>
<td>• Difficult to quantify and measure the amount of exercise performed by each animal</td>
</tr>
<tr>
<td></td>
<td>• Animals are easily motivated to exercise</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Minimal technical requirements</td>
<td></td>
</tr>
<tr>
<td>Swim training</td>
<td>• Multiple animals can be exercised simultaneously</td>
<td>• Environment may cause discomfort and stress to the animal</td>
</tr>
<tr>
<td></td>
<td>• Little motivation required to exercise the animal</td>
<td>• Difficult to measure exercise intensity in for each animal</td>
</tr>
</tbody>
</table>
1.8.2 Animal models of pathological hypertrophy

In humans, pathological hypertrophy is associated with cardiovascular diseases such as myocardial infarction and hypertension. Therefore in order to study the effects of pathological hypertrophy in mice, cardiovascular disease must be simulated. In this regard, there are several mouse models of pathological hypertrophy which achieve this (see Table 1.3), including left anterior descending artery ligation (mimics myocardial infarction). For instance, Yang and colleagues performed coronary artery ligation in 10 to 12 week old mice which displayed an increase in HW: BW ratio and cardiac remodelling, demonstrating that this method can be used to induce hypertrophy (Yang et al., 2002). However, coronary artery ligation surgery is difficult to perform, and it is difficult to generate an identical infarct size in all experimental animals. In addition, the complexity of the coronary artery ligation surgery can lead to mice becoming poorly quickly and high rates of mortality in mice, which must be monitored carefully in order to ensure that animal welfare is preserved in such experiments.

Experimental methods that induce pressure overload to the heart and cardiac hypertrophy have also been used in the mouse. For example, trans-aortic banding was a model that was first developed in the mouse by Rockman and colleagues (Rockman et al., 1991). Although this model increases the HW: BW ratio and expression of hypertrophic markers such as ANF, it is difficult to make the constricted diameter of the aorta consistent between mice. Moreover, this model requires significant training due to the complexity of the surgery (Rockman et al., 1991).

Transgenic mouse models have also been used to induce cardiac hypertrophy. For instance, genes that are associated with cell signalling pathways that mediate hypertrophy following pathological stress have been overexpressed in mice to induce cardiac hypertrophy, including Calmodulin, Interleukin 6 and MEK1 (Frey and Olson, 2003, Gruver et al., 1993, Hirota et al., 1996). Whilst these models successfully induce hypertrophy, it is important to note that these models are not truly representative of human cardiovascular disease, and that hypertrophic responses may vary depending on the mouse strain used to develop the models.
Isoproterenol (beta adrenergic receptor agonist) treatment has also been used to induce cardiac hypertrophy in rodents through injection or administration via osmotic pumps. This has been shown to quickly and reproducibly increase cardiomyocyte and hence heart size (characterised by cardiomyocyte enlargement) and trigger gene expression changes that are associated with cardiac remodelling following pathological stress (Brooks and Conrad, 2009, Errami et al., 2008). However, the mechanism by which Isoproterenol induces cardiac hypertrophy is also unclear and it does not mimic cardiovascular disease in humans, therefore using it to study pathological hypertrophy may not be a suitable model (Oudit et al., 2003, Rose et al., 2007).

Mouse models which trigger hypertension have also been implemented for inducing cardiac hypertrophy, since increased blood pressure (and volume) to the heart increases its workload, causing it to adapt by undergoing hypertrophy. In addition, these models are particularly relevant since humans can also develop hypertension which can lead to cardiac hypertrophy. Endothelin 1 for example is a peptide that induces hypertension by causing vasoconstriction of the blood vessels and through stimulation of the renin angiotensin aldosterone system, and as such has been widely used to study the effects of pathological hypertrophy (Gomes et al., 2012). For example, treatment of isolated rabbit and neonatal rat ventricular cardiomyocytes with Endothelin 1 induced hypertrophy, which was demonstrated by an increase in cell size and width (Bupha-Intr et al., 2012, Ito et al., 1991). However, although Endothelin 1 has been shown to induce hypertrophy in cardiomyocytes and in transgenic mice overexpressing Endothelin 1, infusion of Endothelin 1 into mice has not been used as a model to study cardiac hypertrophy (Yang et al., 2004). One study showed that intrathecal injection of Endothelin 1 in rats caused increased blood pressure, cardiovascular toxicity and death due to respiratory arrest (Poulat et al., 1994). Furthermore, other studies have shown that injection of Endothelin 1 into the cheek or nape of the neck in mice stimulated itching and pain (Gomes et al., 2012, McQueen et al., 2007, Trentin et al., 2006).
Angiotensin II

Angiotensin II (AngII) is a peptide which causes hypertrophy by generating an increased workload on the heart by inducing hypertension. AngII is able to mediate its effects on binding to the AT1 receptor by activating a number of signalling cascades, including MAP kinases (e.g. ERK 1/2, JNK, p38MAPK), receptor tyrosine kinases (e.g. PDGF, EGFR, insulin receptor), and non-receptor tyrosine kinases (e.g. Src, JAK/STAT, focal adhesion kinase (FAK)). Activation of AngII signalling in response to pathological stress results in increased transcription of a number of genes involved in protein synthesis, cardiomyocyte cell growth and survival (Mehta and Griendling, 2007).

AngII has previously been shown to induce hypertrophy in vascular smooth muscle cells, however despite this AngII models that have been used to induce hypertension also induced cardiac hypertrophy in mice. For instance, numerous studies have shown that treatment of mice with AngII induced ventricular hypertrophy, suggesting that AngII may be a good model for inducing hypertrophy by stimulating hypertension (Crowley et al., 2006, Tsuruda et al., 2016, Wang et al., 2014). Furthermore, another study was carried out whereby WT and Thromboxane receptor C57BL/6 knockout mice were treated with AngII determine the role of Thromboxane receptors in hypertension, which caused increased HW: BW ratios and hence cardiac hypertrophy in these mice (Francois et al., 2004). In addition, another study in which the role of fibroblast growth factor 6 (Fgf6) protein in the heart was being determined, cardiac hypertrophy was also induced (shown by increased cardiomyocyte size) in Fgf6 knockout mice using AngII infusion. Interestingly this correlated with expression of hypertrophic markers ANP, BNP and β-MHC and increased cardiac fibrosis (Matsumoto et al., 2013).

To determine the cellular and molecular effects of AngII treatment on cardiomyocytes, Sadoshima and colleagues treated isolated rat ventricular cardiomyocytes with AngII for 48 hours (Sadoshima et al., 1995). Cardiomyocyte hypertrophy was induced which was demonstrated by increased cardiomyocyte protein synthesis but not increased cardiomyocyte proliferation. In addition, induction of immediate early genes that are usually associated with the hypertrophic response was observed, including c-fos, c-jun, jun B, Egr-1, and c-myc (Sadoshima et al., 1995).
These studies have shown that AngII infusion is a suitable model for inducing pathological cardiac hypertrophy in mice. It is a particularly good model to use since it mimics hypertension that leads to pathological hypertrophy in humans, in addition to it being easy to administer (via Alzet osmotic mini pumps). However, some caveats of this model include that induction of hypertrophy may take longer when compared to other mouse models (2-4 weeks), and that sometimes the Alzet osmotic mini pumps used to administer the AngII may malfunction (however this is rare) which can make it difficult to determine whether AngII has been administered effectively.
### Table 1.3: Animal models of pathological hypertrophy, their advantages and limitations

<table>
<thead>
<tr>
<th>Pathological hypertrophy model</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
</table>
| Angiotensin II (AngII)         | • Mimics hypertrophy induced by hypertension in humans  
• Easy to administer | • Difficult to determine if the AngII has worked |
| Endothelin I                   | • Causes vasoconstriction and mimics hypertension observed in humans  
• Induces hypertrophy in cardiomyocytes | • Not suitable for treating mice since it causes pain and itching at site of injection  
• Intrathecal injection causes cardiotoxicity in rats |
| Isoproterenol infusion         | • Induces hypertrophy quickly (10 days maximum)  
• Reproducible  
• Easy to administer | • Mechanism by which hypertrophy is induced is unclear  
• Doesn’t mimic human model of cardiovascular disease induced hypertrophy  
• High mortality rate |
| Trans aortic constriction      | • Mimics aortic stenosis disease in humans  
• Low mortality rate  
• Induces hypertrophy quickly | • Difficult to perform  
• Difficult to maintain exact diameter of constriction in all mice |
| Left anterior descending artery (LAD) ligation | • Representative model of myocardial infarction in humans | • Difficult to perform  
• High mortality rate  
• Difficult to generate identical infarct size in all mice |
| Transgenic mice                | • Can identify specific genes and pathways that are involved in mediating pathological hypertrophy | • Doesn’t fully mimic pathological hypertrophy in humans  
• Hypertrophic responses may be strain specific |

Table 1.3: Animal models of pathological hypertrophy, their advantages and limitations
1.9 Analysing cardiac function in the mouse

Mouse models of pathological and physiological cardiac hypertrophy have been essential in cardiovascular research and have enabled a better understanding of the cellular and molecular mechanisms that lead to morphological, functional, structural and gene expression changes in the heart during the hypertrophic response. In this regard, analysis of cardiac function in the mouse is also important for analysing the effects of pathological and physiological stress on cardiac function. Consequently, several imaging techniques have been used for imaging and analysing the function of the rodent heart, including fluorodeoxyglucose positron emission tomography (FDG-PET) and Magnetic Resonance Imaging (MRI) (see Table 1.5).

Echocardiography and ultrasound is also an important imaging technique that can be used to analyse the effects of pathological and physiological cardiac hypertrophy on heart function in the mouse (and hence was used for the following studies – see Materials and Methods). Murine echocardiography is an established non-invasive technique that allows detailed analysis of cardiac function in the mouse in real time. Echocardiography uses high frequency ultrasound waves to generate detailed images of the heart in the mouse which can be used to asses cardiac morphology, for example by using the Visualsonics Vevo 2100 system (see Figure 1.28). A wide variety of cardiovascular functional parameters can also be measured, from heart anatomy (e.g. Left Ventricular (LV) mass, heart length, etc.), heart functions (e.g. cardiac output, ejection fraction, fractional shortening, etc.) and blood flow parameters, including ascending and descending aorta velocities and common carotid artery velocities (see Table 1.4).
M. Murine echocardiography can be carried out in either conscious mice or anaesthetised mice. However, it is more commonly performed on anaesthetised mice since conscious mice are more difficult to restrain, have a higher heart rate and are more likely to be stressed which may affect the heart functional parameters measured. The anaesthetised mouse can be manipulated into several positions to allow acquisition of several images using either the parasternal long axis view (PSLAX) (e.g. of the left ventricle), short axis view (SAX) (e.g. to image the ventricular wall and papillary muscles) or apical 4 chamber view (Apic4) (e.g. to image the mitral valve). In addition, cardiac functional parameters can also be measured such as cardiac output, ejection fraction and fractional shortening using the B-mode (Two dimensional imaging for quantifying and visualising heart anatomy), M-mode (visualisation and quantification of heart wall motion) or pulse wave Doppler (PW) mode (to quantify blood flow velocity).

Whilst scanning the mouse, several variables must be maintained as fluctuations in them can influence the heart rate and other heart functional parameters, including temperature, duration of scanning and type of anaesthesia used. Importantly, using anaesthesia has previously been shown to depress cardiac functional parameters, including heart rate, fluctuations in which also influence other heart functional parameters. For instance, use of ketamine with xylazine to anaesthetize C57BL/6 mice significantly reduced their cardiac output index, ejection fraction and fractional

<table>
<thead>
<tr>
<th>Heart anatomy</th>
<th>Cardiac function</th>
<th>Blood flow parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Heart length</td>
<td>• Cardiac output</td>
<td>• Ascending aorta velocity</td>
</tr>
<tr>
<td>• Heart area</td>
<td>• Ejection fraction</td>
<td>• Descending aorta velocity</td>
</tr>
<tr>
<td>• LV mass</td>
<td>• Fractional shortening</td>
<td>• LCCA PSV</td>
</tr>
<tr>
<td>• Aortic root</td>
<td>• Fractional area change</td>
<td>• RCCA PSV</td>
</tr>
<tr>
<td>• Interventricular septum (IVS) thickness</td>
<td>• Diastolic Volume</td>
<td>• Mitral valve velocity</td>
</tr>
<tr>
<td></td>
<td>• Systolic Volume</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4: Examples of cardiovascular functional parameters that can be measured using echocardiography/ultrasound
shortening. Different types of anaesthesia may also generate differences in cardiac function. For example, a study performed by Roth and colleagues tested the effects of different anaesthetic agents on cardiac function in C57BL/6J mice, including isofluorane, TBE, ketamine with midazolam, and ketamine with xylazine (Roth et al., 2002). Interestingly, isofluorane caused the least reduction in cardiac function and was also able to generate the most reproducible heart function data in each mouse strain used (and as such was used as the method of anaesthesia for our studies – see Materials and Methods). For example, isofluorane was able to generate a consistent heart rate in multiple scans of both C57BL/6J and C57BL/6N mice used in the study (Roth et al., 2002).

Furthermore, different mouse strains may also display differences in cardiac function. Previous studies have demonstrated differences in heart weight in different mouse strains, e.g. in FVB mice, heart weight was approximately 150mg whereas in Swiss mice it was approximately 180mg, and differences in ventricular wall septal thickness were also observed between Swiss mice and C57BL/6 mice (Doevendans et al., 1998). These differences in heart anatomy between different mouse strains may contribute to their differences in cardiac function, which may be further enhanced when using different types of anaesthesia. For example, Roth and colleagues also showed differences in heart rate and ejection fraction in C57BL/6J and C57BL/6N mice anesthetized with ketamine with midazolam; in particular C57BL/6J mice showed significantly increased heart rate and fractional shortening when compared with C57BL/6N mice (Roth et al., 2002).

In this regard, although murine echocardiography is a powerful non-invasive tool that allows detailed analysis of heart anatomy and cardiovascular parameters in normal and genetically modified mice, it is essential to consider the influence of internal and external factors that can cause changes in cardiac function including mouse strain and duration and types of anaesthesia used.
Figure 1.28: Visualsonics Vevo 2100 system – an example of the equipment used to perform murine echocardiography.
Table 1.5: Summary of imaging techniques used to analyse heart structure and function in rodents, their advantages and limitations

<table>
<thead>
<tr>
<th>Imaging technique</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echocardiography/ultrasound</td>
<td>• Non-invasive and not stressful for the animal</td>
<td>• Animal needs to be anaesthetised which may affect heart function</td>
</tr>
<tr>
<td></td>
<td>• Produces high quality images of the heart</td>
<td>• Training required to obtain reproducible images</td>
</tr>
<tr>
<td></td>
<td>• Large number of heart function parameters can be measured</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Repeated measurements can be conducted on the same animal over time</td>
<td></td>
</tr>
<tr>
<td>Magnetic resonance imaging (MRI)</td>
<td>• Non-invasive and causes minimal stress to the animal</td>
<td>• Animal needs to be anaesthetised to keep the body still during scanning which may affect heart function</td>
</tr>
<tr>
<td></td>
<td>• Generates high resolution images of the heart</td>
<td>• Training required to use the MRI scanner</td>
</tr>
<tr>
<td></td>
<td>• Heart function can also be measured</td>
<td>• Expensive</td>
</tr>
<tr>
<td>Fluorodeoxyglucose Positron Emission Tomography (FDG-PET)</td>
<td>• Can be used to measure blood flow to and from the heart</td>
<td>• Heart function parameters cannot be measured</td>
</tr>
<tr>
<td></td>
<td>• Can be used to assess differences in healthy and diseased tissue in the heart (e.g. following a myocardial infarction)</td>
<td>• Injection of FDG may cause stress and discomfort to the animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Animal may have an allergic reaction to the FDG used</td>
</tr>
</tbody>
</table>
1.10 Brn3b and cardiac hypertrophy

Since Brn3b is expressed in the foetal heart and is increased in response to stress, e.g. following coronary artery ligation, it was important to consider if Brn3b could be involved in driving responses seen during pathological hypertrophy (Budhram-Mahadeo et al., 2014). Preliminary studies were carried out in vitro and in vivo to analyse for changes in Brn3b expression during the hypertrophic response (Maskell, 2018c).

In this regard, Brn3b expression increased in rat neonatal ventricular myocyte cells (NRVMs) and rat foetal heart derived cell line H9C2 cells (see Figure 1.29); this in turn correlated with high induction of hypertrophic marker genes β-MHC and ANP. Co-immunostaining of Brn3b and phalloidin (stains actin filaments) showed that cells with increased Brn3b expression underwent cytoskeletal remodelling, another feature of cardiac hypertrophy (Maskell, 2018c). AngII treatment also stimulated Cyclin D1 expression, which has been shown to mediate the hypertrophic response in addition to being regulated by Brn3b (Budhram-Mahadeo et al., 2008, Soonpaa et al., 1997, Tamamori-Adachi et al., 2002).

Furthermore, transfection of H9C2 cells with a Brn3b reporter construct and MAPK inhibitor PD98059 indicated that the MAPK signalling pathway may be required for up-regulation of Brn3b following AngII treatment. This was demonstrated by induction of Brn3b promoter activity following AngII treatment which was blocked by PD98059. Interestingly, Calcineurin signalling which induces cardiac hypertrophy also induced Brn3b expression in H9C2 cells which was blocked by the Calcineurin inhibitor Cyclosporine A (CsA) (Maskell, 2018c). As a result, Calcineurin signalling may also be involved in up-regulating Brn3b expression during the hypertrophic response following AngII treatment.
In vivo studies were also carried out using Angiotensin II (AngII) to induce hypertrophy in Brn3b KO and WT littermate control mice (Saline was used as a control), using Alzet osmotic pumps for a period of 2 weeks. Firstly, AngII treatment increased Brn3b mRNA and protein levels in WT hearts. In addition, cardiac function assessed by echocardiography showed that cardiac hypertrophy was induced in WT mice, whereas Brn3b KO mice showed no significant induction of the hypertrophic response. There were also some changes in cardiac function (including cardiac output, ejection fraction and fractional shortening) in Brn3b KO mice, suggesting that loss of this TF might influence responses to pathological injury.
1.11 Aims and Hypothesis

Previous studies have shown that the POU4F2/Brn3b TF is highly expressed in the foetal mouse heart. Since transcription factors are essential for determining cell fate and normal heart development, one of the aims of this study was to determine the potential role of POU4F2/Brn3b in this process. POU4F2/Brn3b is also expressed in the adult mouse heart under normal conditions, and is re-expressed in response to injury or stress (Farooqui-Kabir et al., 2008, Budhram-Mahadeo et al., 2014). In addition, preliminary studies have shown that following pathological stress which induced cardiac hypertrophy (AngII treatment), Brn3b mRNA and protein expression was induced in H9C2 cells and Neonatal Rat Ventricular Myocytes (NRVMs) (Maskell, 2018c). Importantly, foetal genes are re-expressed during pathological hypertrophic responses; therefore it was also important to analyse the potential function of POU4F2/Brn3b in mediating hypertrophic responses in the heart since it is expressed during heart development. As such, the observations described above have led to the following hypothesis being proposed:

**Hypothesis:** The foetal TF POU4F2/Brn3b is re-expressed in the stressed heart where it may play a role in the early adaptive hypertrophic response, but when co-expressed with p53 at later stages may induce cell death thereby leading to heart failure.

**Aims:**

1. To further characterise the role of Brn3b (and Brn3a) in the developing and adult heart by analysing changes in cardiac morphology and gene expression changes in Brn3a KO mouse embryos; and by analysing cardiac and vascular function in the hearts of Brn3b knockout (KO) and WT control male and female mice at baseline.

2. To assess the roles of Brn3b in driving cardiac hypertrophic responses either under pathological conditions (e.g. AngII infusion) or physiological stress (e.g. exercise) and determine functional, physiological and morphological changes using Brn3b knockout (KO) mutant mice and wild type (WT) controls.
3. To analyse for gene expression changes associated with Brn3b in stressed hearts and identify molecular mechanisms by which Brn3b mediates its effects in cardiomyocytes using isolated Neonatal Rat Ventricular Myocytes (NRVMs) and H9C2 cells.
Chapter Two

Materials and Methods
2.0 Materials

Unless otherwise stated, all chemicals and reagents of analytical grade were purchased from Sigma Aldrich (Poole, Dorset, UK) and Merck (Poole, Dorset, UK). All tissue culture reagents were supplied by Gibco/Life Technologies (Paisley, UK). Tissue culture plastic ware was obtained from Starstedt (Nümbrecht, Germany). Reagents for immunohistochemistry were obtained from Vector Laboratories (Peterborough, UK) and Invitrogen/Life Technologies (Paisley, UK).

Additional laboratory materials were provided by the following suppliers:

Cell culture
Collagenase Type 2 Invitrogen
Gelatine Invitrogen
Laminin Invitrogen

Cell lines
H9C2 Myoblast cell line derived from the embryonic rat heart

DNA Preparations (Cloning and Genotyping)
Agarose Lonza
dNTP Mix (10mM) Promega
Direct PCR Lysis Reagent Viagen
GoTaq DNA Polymerase Promega
100bp and 1kb DNA Ladders New England Biolabs
Proteinase K Invitrogen
Restriction enzymes and buffers New England Biolabs
JM109 competent E.coli cells Promega

Kits
Dead End Colorimetric TUNEL Kit Promega
**Immunohistochemistry**

- Glass coverslips: Scientific Laboratories Ltd
- Superfrost Plus microscope slides: VWR International
- Goat Serum: Abcam
- Avidin/Biotin Kit: Vector Laboratories
- MOM Blocking Kit: Vector Laboratories
- AB Complex: Vector Laboratories

**In-vivo mouse models**

- Angiotensin II: Sigma Aldrich
- Osmotic mini pumps (model 2004): Alzet
- Isofluorane: Abbvie Ltd
- Ultrasound acoustic gel: Eco-Med Pharmaceutical
- Running wheels and data collection software: Lafayette

**Transfection**

- Fugene Transfection reagent: Promega

**Western blotting**

- PBS Tablets: Gibco/Life Technologies
- Pre-stained Colour Broad range Protein Ladder: New England Biolabs
- 30% Polyacrylamide: Bio-Rad
- X-ray films: Thermofisher Scientific
- ECL substrate: Bio-Rad
### Standard Buffers and Solutions (Table 2.0)

<table>
<thead>
<tr>
<th>Buffer Decoded</th>
<th>Components of Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1X Phosphate Buffered Saline (PBS)</strong></td>
<td>2x PBS Tablets (Life Technologies) dissolved in 1 litre of ddH2O to give a final concentration of 20mM Phosphate, 280mM Sodium Chloride and 5.36mM Potassium Chloride</td>
</tr>
<tr>
<td><strong>10X Running Buffer (For WB)</strong></td>
<td>ddH2O (6.6ml), Tris-Base (8.0ml), Glycine (5.0ml), 10% SDS (100ml)</td>
</tr>
<tr>
<td><strong>10X Transfer Buffer (For WB)</strong></td>
<td>ddH2O (4.0ml), Tris-Base (9.6ml), Glycine (5.0ml), 10% SDS (0.37g), Methanol (200ml)</td>
</tr>
<tr>
<td><strong>1X Tris Buffered Saline (TBS) pH 7.5</strong></td>
<td>6.05g Tris-base and 8.76 g NaCl in 800 mL of H2O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1 litre with H2O.</td>
</tr>
<tr>
<td><strong>1X Tris-Acetate (TAE) Buffer</strong></td>
<td>400mM Tris-Base, 200mM Sodium Acetate and 20mM EDTA pH 8.0</td>
</tr>
<tr>
<td><strong>Blocking Buffer (For IHC)</strong></td>
<td>Western blot: 4% Skimmed Milk Powder in 0.1% PBST IHC: 10% Goat serum in 0.1% PBST (tissue sections on microscope slides) or 20% Goat serum in PBS/0.1% Triton-X-100 (cells on coverslips)</td>
</tr>
<tr>
<td><strong>Luria Bertani (LB) Agar</strong></td>
<td>1% (w/v) Bacto-tryptone, 1% (w/v) NaCl, 0.5% Bacto-yeast extract, 1.5% Bacto-agar and ddH2O to 1 litre</td>
</tr>
<tr>
<td><strong>Luria Bertani (LB) Media</strong></td>
<td>1% (w/v) Bacto-tryptone, 1% (w/v) NaCl, 0.5% Bacto-yeast extract and ddH2O to 1 litre</td>
</tr>
<tr>
<td><strong>RIPA Buffer</strong></td>
<td>50mM Tris HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 2mM EDTA containing 1 tablet of protease inhibitor cocktail</td>
</tr>
<tr>
<td><strong>Wash Buffer (For WB and IHC)</strong></td>
<td>Western blot and IHC (tissue sections on microscope slides): 0.1% Tween-20 in 1X PBS IHC (cells on coverslips): 0.1% Triton-X-100 in 1X PBS</td>
</tr>
<tr>
<td><strong>2X Laemmli Buffer</strong></td>
<td>10% SDS, Glycerol, 1% Bromophenol blue, Tris-HCl pH 6.8, ddH2O</td>
</tr>
<tr>
<td><strong>1X ADS Buffer</strong></td>
<td>116mM NaCl, 20mM HEPES, 0.8mM NaH2PO4, 5.6mM glucose, 5.4mM KCl, and 0.6mM MgSO4, pH 7.35</td>
</tr>
</tbody>
</table>

Table 2.0: Standard Buffers and solutions
### List of Antibodies (Table 2.1)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Dilutions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actinin</td>
<td>Rabbit pAb</td>
<td>IHC 1:500</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>α-actinin</td>
<td>Mouse mAb</td>
<td>IHC 1:500</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Mouse mAb</td>
<td>WB 1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Bax</td>
<td>Rabbit pAb</td>
<td>WB 1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>β-MHC</td>
<td>Mouse mAb</td>
<td>WB: 1:1000</td>
<td>Genetech</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Rabbit</td>
<td>WB 1:2000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>Brn3a</td>
<td>Rabbit pAb</td>
<td>WB 1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Brn3b</td>
<td>Rabbit pAb</td>
<td>WB 1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Collagen 1</td>
<td>Mouse mAb</td>
<td>WB 1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Mouse mAb</td>
<td>WB:1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Gamma (γ) tubulin</td>
<td>Mouse mAb</td>
<td>WB:1:1000</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Mouse mAb</td>
<td>WB 1:2000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>p53</td>
<td>Sheep pAb</td>
<td>WB 1:1000</td>
<td>Calibochem</td>
</tr>
<tr>
<td>p53</td>
<td>Mouse mAb</td>
<td>WB: 1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Noxa</td>
<td>Rabbit pAb</td>
<td>IHC 1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>VWF</td>
<td></td>
<td>WB:1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Goat anti-rabbit HRP secondary antibody</td>
<td>Goat pAb</td>
<td>WB 1:2000</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti-mouse HRP secondary antibody</td>
<td>Rabbit pAb</td>
<td>WB 1:2000</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti-sheep HRP secondary antibody</td>
<td>Rabbit pAb</td>
<td>WB 1:2000</td>
<td>Dako</td>
</tr>
<tr>
<td>Goat anti-rabbit biotinylated secondary antibody</td>
<td>Goat pAb</td>
<td>IHC 1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Primary Antibody</td>
<td>Dilution</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>Rabbit anti-goat biotinylated secondary antibody</td>
<td>Rabbit pAb</td>
<td>IHC 1:200</td>
<td>Dako</td>
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<tr>
<td>Anti-mouse biotinylated secondary antibody</td>
<td>N/A</td>
<td>IHC 1:200</td>
<td>Vector Laboratories</td>
</tr>
<tr>
<td>Alexa Fluor 555 Donkey anti-mouse secondary antibody</td>
<td>Donkey pAb</td>
<td>IHC 1:2000</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
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*Table 2.1: List of antibodies*
### List of Primers (Table 2.2)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Oligonucleotide sequences</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB F</td>
<td>Mouse</td>
<td>5’ GGCTGTATTCCCCCTCATCG 3’</td>
<td>58</td>
</tr>
<tr>
<td>ACTB R</td>
<td>Mouse</td>
<td>5’ CCAGTTGGTAACAATGCCATGT 3’</td>
<td></td>
</tr>
<tr>
<td>ANF F</td>
<td>Mouse</td>
<td>5’ TGGGCTCTTCTCCATCAC 3’</td>
<td>58</td>
</tr>
<tr>
<td>ANF R</td>
<td>Mouse</td>
<td>5’ GCCAAAAGGCCAGGAGAG 3’</td>
<td></td>
</tr>
<tr>
<td>β-MHC F</td>
<td>Mouse</td>
<td>5’ GCCTACCTCATGGGAAGAA 3’</td>
<td>58</td>
</tr>
<tr>
<td>β-MHC R</td>
<td>Mouse</td>
<td>5’ ACTATTCTGCTGGTAC 3’</td>
<td></td>
</tr>
<tr>
<td>Bax F</td>
<td>Mouse</td>
<td>5’ CTGCAGAGGATATTGCTGA 3’</td>
<td>60</td>
</tr>
<tr>
<td>Bax R</td>
<td>Mouse</td>
<td>5’ GATCAGCTCGGGCACTTTAG 3’</td>
<td></td>
</tr>
<tr>
<td>B2M F</td>
<td>Mouse</td>
<td>5’ TTGCTTTTCTGTGCTTCAAC 3’</td>
<td>58</td>
</tr>
<tr>
<td>B2M R</td>
<td>Mouse</td>
<td>5’ GTTCGCCCTCCATTCCAC 3’</td>
<td></td>
</tr>
<tr>
<td>Brn3a F</td>
<td>Mouse</td>
<td>5’ TCACGCTCTCGCAACAACAAA 3’</td>
<td>59</td>
</tr>
<tr>
<td>Brn3a R</td>
<td>Mouse</td>
<td>5’ TCCGGCTTTTCATTTTTCA 3’</td>
<td></td>
</tr>
<tr>
<td>Brn3b F</td>
<td>Mouse</td>
<td>5’ GAGAGAGGCTACACATTC 3’</td>
<td>60</td>
</tr>
<tr>
<td>Brn3b R</td>
<td>Mouse</td>
<td>5’ ATGTTGCTGTGGGCTTCA 3’</td>
<td></td>
</tr>
<tr>
<td>Collagen 1 F</td>
<td>Mouse</td>
<td>5’ TGGAAGACGCGGACGAG 3’</td>
<td>54</td>
</tr>
<tr>
<td>Collagen 1 R</td>
<td>Mouse</td>
<td>5’ GCGCAGGAAGTCAGTGC 3’</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1 F</td>
<td>Mouse</td>
<td>5’ AACACTTCCTCTCAAA 3’</td>
<td>52</td>
</tr>
<tr>
<td>Cyclin D1 R</td>
<td>Mouse</td>
<td>5’ GAACCTCATTGGTGGA 3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH F</td>
<td>Mouse</td>
<td>5’ CTTGATGCGATACTCTTG 3’</td>
<td>58</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>Mouse</td>
<td>5’ GCGGAGGCTGAGCTGTA 3’</td>
<td></td>
</tr>
<tr>
<td>GLUT4 F</td>
<td>Mouse</td>
<td>N/A – Standard primers (Quantitect: Catalogue no: QTO1538334)</td>
<td>60</td>
</tr>
<tr>
<td>GLUT4 R</td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin F</td>
<td>Mouse</td>
<td>5’ AGACAATCGGCTGATGAT 3’</td>
<td>60</td>
</tr>
<tr>
<td>Neomycin R</td>
<td>Mouse</td>
<td>5’ ATACCTTCGCCAGGACGA 3’</td>
<td></td>
</tr>
<tr>
<td>Noxa F</td>
<td>Mouse</td>
<td>5’ TCGAAAAGAGACAGGAG 3’</td>
<td>60</td>
</tr>
<tr>
<td>Noxa R</td>
<td>Mouse</td>
<td>5’ CACTTTGCTCCAAATCTG 3’</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2**: List of primers
2.1 Generation of the Brn3a KO mouse

Brn3a KO mice were obtained from Xiang’s research group who generated the mutants using a targeting construct and standard recombination techniques (Xiang et al., 1996). Briefly, the Brn3a gene was knocked out with a targeting vector in which the Neomycin gene cassette (pNeoTK) was used to replace the mouse Brn3a coding region (4.0kb). In this regard, the Neomycin targeting vector is flanked by 3.0kb of 5’ Brn3a sequence and 1.6kb of the Brn3a 3’ sequence, and contains restriction sites which were used to ligate the targeting vector into the wild-type (WT) Brn3a gene (see Figure 2.0) (Xiang et al., 1996). This new Brn3a/Neomycin construct was then linearized and inserted into AB-1 embryonic stem cells. Next, the embryonic stem cells were selected for resistance to G418 and 1-(2-deoxy-2-fluoro/β-D-arabinofuranosyl)-5-iodouracil (FIAU). Embryonic stem cells which contained the Brn3a/Neomycin gene targeting construct (identified using Southern blotting) were then injected into C57BL/6J mouse blastocysts. Targeted embryonic stem cell clones were injected into C57BL/6J blastocysts to generate chimeric mice, which were bred/ backcrossed to C57BL/6J mice to produce Brn3a Heterozygous and Brn3a KO progeny on a pure C57BL/6J background (Xiang et al., 1996).

Brn3a KO (-/-) and WT (+/+) control mice were generated by crossing Brn3a heterozygous mice (+/-) which gave rise to Mendelian ratio, i.e. 50% +/- mice, 25% +/+ mice and 25% -/- mice. The genotypes of litters were determined by others in the laboratory who performed PCR on DNA extracted from weaned mouse pup ear tissue biopsies (using Proteinase K digestion – see 2.2) using PCR primers designed to amplify Brn3a and Neomycin fragments. Timed mating of Brn3a heterozygous mice to generate Brn3a KO mouse embryos at stages E16.5, E17.5 and E18.5 (and hence genotyping of these embryos) used for studying Brn3a and Brn3b expression in the developing heart was also undertaken by others in the laboratory. Figure 2.0 below shows a diagram of the Brn3b/Neomycin targeting construct used to generate the Brn3a KO mouse:
WT Brn3a gene:

Targeting vector containing Neomycin cassette:

Brn3b plus the targeting vector inserted to inhibit expression and function:

**Figure 2.0:** Diagram showing the targeting vector used to inhibit Brn3a expression and function in the Brn3b KO mouse and the restriction sites used to ligate it into the wild-type gene (B; BamHI Bs; BstXI H; HindIII K; KpnI N; NotI S; SalI X; XhoI). Briefly, a PGK-neo cassette (Neo) targeting construct flanked by 3.0 kb of Brn3a 5' and 1.6 kb of Brn3a 3' sequences (thick blue lines) replaced a segment containing the complete Brn3a coding region (4.0kb) (Xiang et al., 1996).
2.2 Generation of the Brn3b KO mouse

Brn3b KO mice were obtained from Xiang’s research group who generated the mutants using a targeting construct and standard recombination techniques (Gan et al., 1996). Briefly, the Brn3b gene was knocked out with a targeting vector in which the Neomycin gene cassette (pNeoTK) was used to replace the mouse Brn3b coding region (4.4kb) (see Figure 2.1). In this regard, the Neomycin targeting vector is flanked by 2.7kb of 5’ Brn3b sequence and 4.5kb of the Brn3b 3’ sequence, and contains BamHI and HindIII restriction sites which were used to ligate the targeting vector into the wild-type (WT) Brn3b gene (see Figure 2.1) (Gan et al., 1996). This new Brn3b/ Neomycin construct was then linearized and inserted into AB-1 embryonic stem cells. The embryonic stem cells were then selected for resistance to G418 and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU). Southern blot analysis identified embryonic stem cells which contained the Brn3b/ Neomycin gene targeting construct, which were then injected into C57BL/6J mouse blastocysts. As a result, chimeric mice were generated which in turn were bred and backcrossed over a number of generations to generate Brn3b Heterozygous and Brn3b KO progeny on a pure C57BL/6J background (Gan et al., 1996). Figure 2.1 below shows a diagram of the Brn3b/Neomycin targeting construct used to generate the Brn3b KO mouse:
Wild-type Brn3b gene:

[Diagram showing wild-type Brn3b gene with restriction sites and exons]

Targeting vector containing Neomycin cassette:

[Diagram showing targeting vector with BamHI, HindIII, and Not1 restriction sites and Neo and TK cassettes]

Brn3b plus the targeting vector inserted to inhibit expression and function:

[Diagram showing Brn3b gene with the targeting vector inserted, showing restriction sites and Neo cassette]

**Figure 2.1:** Diagram showing the targeting vector used to inhibit Brn3b expression and function in the Brn3b KO mouse and the restriction sites used to ligate it into the wild-type gene. Briefly, a PGK-neo cassette (Neo) targeting construct flanked by 2.7 kb of Brn3b 5' and 4.5 kb of Brn3b 3' sequences (thick blue lines) replaced a segment containing the complete Brn3b coding region (4.4kb) (Gan et al., 1996).
2.3 Brn3b/Neomycin genotyping PCR

Brn3b KO (-/-) and WT (+/+) control mice were generated by crossing Brn3b heterozygous mice (+/-) which gave rise to Mendelian ratio, i.e. 50% +/- mice, 25% +/- mice and 25% -/- mice.

To determine the genotypes of litters, DNA was extracted from ear tissue biopsies obtained from each pup after weaning by overnight digestion at 55°C in 100µl Direct PCR Lysis Reagent (Tail) and 1µl 20mg/ml Proteinase K. The following day, Proteinase K was inactivated by incubating samples at 85°C for 45 minutes. Next, 5µl of each sample was used to amplify either a Brn3b fragment (150bp in size, present in WT and Heterozygous mice), or a Neomycin fragment (250bp in size, present in Heterozygous and KO mice) using polymerase chain reaction (PCR). The following PCR primers were used to amplify the Brn3b and Neomycin fragments, and Brn3b/Neomycin DNA fragments were amplified using the BRN3K001 PCR programme (see below):

**Brn3b fragment (150bp):**

Forward primer:
5' GAGAGAGCGCTCACAATTCC 3'

Reverse primer:
5' ATGGTGGTGGTGGCTCTTTAC 3'

**Neomycin fragment (250bp):**

Forward primer:
5' AGACAATCGGCTGCTCTGAT 3'

Reverse primer:
5' ATACTTTCTCGGCAGGAGCA 3'

**BRN3K001 PCR programme:**

1. 95°C 15 minutes
2. 94°C 30 seconds
3. 60°C 30 seconds
4. 72°C 30 seconds
5. 72°C 5 minutes
6. 4°C HOLD.

x45
PCR products were resolved by gel electrophoresis on a 2% agarose gel containing Gel Red (for visualisation of DNA fragments using UV light) alongside a 100bp DNA Ladder to separate the Brn3b and Neomycin fragments. PCR products were resolved at 100V for 30 minutes in 1X TAE Buffer, after which Brn3b/ Neomycin fragments were visualised using a UV trans illuminator. Figure 2.2 below shows an example of an agarose gel image containing WT, Heterozygous and Brn3b KO samples:

![Figure 2.2](image)

**Figure 2.2:** An example of a 2% agarose gel showing the results of a Brn3b/Neomycin genotyping PCR reaction. WT mice only have one band; the 150bp Brn3b fragment (lanes 4, 6, 7, 8, 11, 13, 14 & 16). Brn3b KO mice on the other hand contain one band that represents the Neomycin fragment (250bp – lanes 2, 3, 15 & 17). Finally, Heterozygous mice have both the Brn3b (150bp) and Neomycin (250bp) fragments, represented by 2 bands on the agarose gel (lanes 1, 5 (faint Neo band), 9, 10 & 12). M = 100bp Ladder; - = Negative control

### 2.4 Animal subjects

All studies using mouse models were undertaken in accordance with Home Office guidelines (Animals Scientific Procedures Act 1986) and approved by local Ethics Review Board. Brn3b KO (−/−) and WT (+/+) control mice generated by crossing Brn3b heterozygous mice (+/−) were on a C57BL/6J background strain, and outbred WT mice (C57BL/6J) used in preliminary studies (see Chapter Four) were obtained from approved commercial sources (Harlan UK or Charles River). Mice were housed in an animal unit with a 12 hour light/ dark cycle and had *ad libitum* access to standard chow and water.
2.5 Pathological cardiac hypertrophy mouse model (Angiotensin II infusion)

Several animal models have been successfully used to induce pathological hypertrophy, including trans-aortic constriction, Endothelin 1 and Isoproterenol infusion, transgenic mouse models and LAD ligation. However, each of these models have limitations that make them unsuitable for this study, including lethal side effects (cardiotoxicity and itching symptoms from using Endothelin 1); difficulty to perform and maintain consistency of induced hypertrophy within individual animals (LAD and trans-aortic constriction); and not fully mimicking cardiovascular diseases which induce hypertrophy in humans (transgenic mice).

In contrast, Angiotensin II (AngII) infusion is easy to perform and mimics cardiac hypertrophy induced by hypertension in humans. Furthermore, previous studies have shown induction of Brn3b expression and activation of MAPK and Calcineurin signalling (characterised pathways for pathological hypertrophy) by AngII in hypertrophic cardiomyocytes (Maskell, 2018c). Therefore for this study, pathological cardiac hypertrophy was induced in Brn3b KO and age matched WT mice by treatment with Angiotensin II, which causes hypertension and cardiac hypertrophy over longer periods (see Figure 2.3 for more information). Saline was used as a control since saline does not induce hypertension. Since surgery is an experimental variable that could potentially cause stress to the animal independently of hypertrophic stimuli; control animals must be exposed to the same surgery as the AngII treated mice to ensure that the cardiac hypertrophy induced in the AngII treated animals is specifically caused by AngII (and not the stress of surgery).

Briefly, Alzet osmotic mini pumps (model 2004) were filled with either saline (control) or AngII at a concentration of 5µg/µl made in 0.9% saline (generating a release rate of 4.5mg/kg/day in the mice) and primed by overnight incubation in 0.9% saline at 37°C. The following day, pumps were subcutaneously inserted into Brn3b KO and age matched WT mice that were 2 months old and weighed at least 20g (surgery was performed by Valerie Taylor). Before surgery, mice were injected with 0.05 mg/kg of the analgesic Buprenorphine, and during surgery mice were maintained under anaesthesia using 1.5-
2% Isofluorane. At baseline, 2 weeks and 4 weeks post-surgery, cardiac function was assessed by echocardiography/ultrasound using the Visualsonics Vevo 2100 System. For these *in vivo* studies, a minimum of 5-6 WT mice were used for saline controls and 5-6 WT mice were used for AngII treatment (see Chapter Four). However, since some hearts (n=3) were used for morphological studies, only 3 WT hearts from each group were able to be used for Western blot/qPCR analysis.

**Effects of Angiotensin II:**

- Causes vasoconstriction of blood vessels, increasing blood pressure
- Binds to the AT1 receptor and stimulates aldosterone release from the adrenal glands
- Aldosterone triggers reabsorption of water into the bloodstream, increasing blood volume and blood pressure
- This causes pressure and volume overload to the heart, causing it to work harder and undergo hypertrophy

**Figure 2.3:** Diagram showing the effects of Angiotensin II on the heart
2.6 Physiological cardiac hypertrophy mouse model (Exercise)

Several models have also been used to induce physiological cardiac hypertrophy, including treadmill running, voluntary running and swim training. Since treadmill running and swim training cause stress and discomfort in the animal and require a high amount of skill, time and optimisation for training the mice to run on the treadmill/swim, voluntary running was chosen for this study. To simulate physiological stress on the heart, Brn3b KO and age matched WT control mice (2 months old) were voluntarily exercised on igloo running wheels for 4 weeks (see Figure 2.4). During the fourth week of exercise their average speed and cumulative distances were recorded using running wheels attached to Lafayette wizard data collection software. Cumulative distance is defined as increasing distance upon addition of multiple distance measurements. For these studies, the measurements from day 1 were added to the measurements recorded for day 2, which were then added to the measurements for day 3 (continued until day 7) increasing the cumulative distance measurement at each time point. At 4 weeks post exercise cardiac function was assessed by echocardiography/ultrasound using the Visualsonics Vevo 2100 system.

Figure 2.4: Example of running wheel used to voluntarily exercise the mice
2.7 Assessment of cardiac function using echocardiography/ultrasound

Cardiac function was analysed at baseline and 4 weeks post exercise/ AngII infusion in Brn3b KO and WT age matched control mice using echocardiography and ultrasound, (using the Visualsonics Vevo 2100 system). This is a well-established non-invasive technique and hence popular method for analysing cardiac function in the mouse (Barisione et al., 2006, Doevendans et al., 1998, Roth et al., 2002). This system uses ultrasound to analyse the morphology and function of the heart in real time in anaesthetised mice. In brief, mice were anaesthetised and maintained under anaesthesia using 1-2% isofluorane. Whilst under anaesthesia, mice were taped to a heated ECG platform via their paws, and acoustic gel was used to provide electrical conductance between the platform and the animal (via the paws) (see Figures 2.5-2.8). Before scanning, the chest of the animal was shaved (using hair removal cream) to eliminate attenuation of the ultrasound signal by animal fur. Finally, ultrasound gel was applied to the chest of the animal which was then scanned using a 40MHz ultrasound probe. The animal was then manipulated into several positions to allow acquisition of the parasternal long axis view (PSLAX), short axis view (SAX) and apical 4 chamber view (Apic4) in order to measure cardiac functional parameters such as cardiac output, ejection fraction and fractional shortening using the B-mode (Two dimensional imaging for quantifying and visualising heart anatomy), M-mode (visualisation and quantification of heart wall motion) or pulse wave Doppler (PW) mode (to quantify blood flow velocity) (see Figures 2.5 – 2.8 for representative images). Cardiac function was calculated from short axis M-mode images using the Teicholtz method and from 1 long axis and 3 short axis B-mode images using the Simpson’s slice method (Lohr, 2005).

For example, the ultrasound probe was placed in a vertical position onto the chest area (with the notch pointing towards the head of the mouse) and rotated approximately 35 degrees anti-clockwise in order to acquire the parasternal long axis view (see Figure 2.5), which in turn can be used to visualise a number of anatomical parameters (see Table 2.3). Once the left ventricle was visible with the apex of the heart on the left of the monitor and the aorta on the right of the monitor, images were acquired in B-mode and
M-mode which were used by the software in the Visualsonics Vevo 2100 system to calculate a number of measurements and functional parameters (e.g. heart length) using Simpson’s slice and/or Teicholtz method (see Table 2.4 and 2.6.1 for more information) (Lohr, 2005).

Parasternal short axis view was achieved by rotating the ultrasound probe (in the vertical PSLAX position) 90 degrees anti-clockwise, enabling left ventricular area and left ventricular mass to be calculated from B-mode/ M-mode measurements (see Figure 2.6, Table 2.3 and Table 2.4). In particular, to measure the left ventricular area, three slices/diameters of the left ventricle (in B-mode) were obtained: one just below the aorta (proximal); one in the middle of the heart (where the papillary muscles are visible); and one near the apex of the heart (distal). These three measurements were then used to calculate the area of the heart and functional parameters including cardiac output, ejection fraction, fractional area change, stoke volume, end diastolic and end systolic volume using Simpson’s slice method by the Visualsonics software (see Figure 2.6) (Lohr, 2005).

Figure 2.5: Left: Image showing the position of the ultrasound probe in the parasternal long axis view. Right: Ultrasound image showing the reference points for capturing the correct image in the parasternal long axis view (apex on the left and aorta on the right) (Lohr, 2005).
To obtain the apical four chamber view, the ECG platform was manipulated so that the head of the mouse faced downwards and the top of the ultrasound probe was also pointed downwards so that it was in contact with the apex of the heart (see Figure 2.7). Once the left ventricle was visible with the apex of the heart at the top of the monitor in B-mode, Colour Doppler Pulse Wave mode was used to obtain images which enabled blood flow velocity through the mitral valve to be calculated (Lohr, 2005).

**Figure 2.6:** Left: Image showing the position of the ultrasound probe in the parasternal short axis view. Right: Ultrasound image showing the reference point for capturing the correct middle slice image in the parasternal short axis view (Lohr, 2005).
Next, the mouse on the ECG platform was manipulated so that the left side of the animal faced downwards with the ultrasound probe parallel to the right side of the chest of the animal and just below the chin of the animal (see Figure 2.8). From this position the aortic arch could be visualised (see Figure 2.8) whereby colour Doppler Pulse Wave view was then used to obtain images used to measure blood flow velocity through the ascending and descending aorta (Lohr, 2005).

**Figure 2.7:** Left: Image showing the position of the ultrasound probe in the apical four chamber view. Right: Ultrasound image showing the reference point for capturing the correct apical four chamber view image (B-mode) (Lohr, 2005).

**Figure 2.8:** Left: Image showing the position of the ultrasound probe in the aortic arch view. Right: Ultrasound image showing the reference point for capturing the correct aortic arch view image (B-mode) (Lohr, 2005).
Finally, for measurement of blood flow velocity through the left and right common carotid arteries (using Doppler Pulse Wave mode), the mouse was manipulated so that its ventral side was facing upwards (i.e. with the ECG platform horizontal, as in the PSLAX position), and the ultrasound probe was placed into a vertical position (with the notch pointing to the head of the mouse) just below the chin of the animal (Lohr, 2005).

Table 2.3: Cardiac anatomical parameters visible in the parasternal long axis view, parasternal short axis view and apical four chamber view:

<table>
<thead>
<tr>
<th>Anatomical parameters visible in parasternal long axis view (PSLAX)</th>
<th>Anatomical parameters visible in parasternal short axis view (SAX)</th>
<th>Anatomical parameters visible in apical four chamber view (Apic4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Left ventricle (LV)</td>
<td>• Left ventricle</td>
<td>• Left ventricle</td>
</tr>
<tr>
<td>• Aorta</td>
<td>• Right ventricle</td>
<td>• Left atria</td>
</tr>
<tr>
<td>• Right ventricle</td>
<td>• Anterior wall</td>
<td>• Left atria</td>
</tr>
<tr>
<td>• Pulmonary artery</td>
<td>• Posterior wall</td>
<td>• Right ventricle</td>
</tr>
<tr>
<td>• Left atrium</td>
<td>• Intraventricular septum</td>
<td>• Right atria</td>
</tr>
<tr>
<td>• Aortic valve</td>
<td>• Papillary muscles</td>
<td>• Mitral valve</td>
</tr>
<tr>
<td>• LV Anterior wall</td>
<td></td>
<td>• Tricuspid valve</td>
</tr>
<tr>
<td>• Mitral valve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• LV Posterior wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Pulmonary veins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Intraventricular septum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Papillary muscles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Table showing cardiac anatomical parameters visible in the parasternal long axis view, parasternal short axis view and apical four chamber view:
Table 2.4: Cardiac functional parameters that were measured by ultrasound and the modes/views that were used to obtain them:

<table>
<thead>
<tr>
<th>Functional parameter</th>
<th>Method</th>
<th>B-mode, M-mode or Doppler PW mode?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>PSLAX</td>
<td>B-mode</td>
</tr>
<tr>
<td>Area</td>
<td>SAX</td>
<td>B-mode</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>Simpson’s and Teicholtz</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>Ejection Fraction</td>
<td>Simpson’s and Teicholtz</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>Fractional Shortening</td>
<td>Teicholtz</td>
<td>M-mode</td>
</tr>
<tr>
<td>FAC</td>
<td>Simpson’s</td>
<td>B-mode</td>
</tr>
<tr>
<td>Stroke Volume</td>
<td>Simpson’s and Teicholtz</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>Diastolic Volume</td>
<td>Simpson’s and Teicholtz</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>Systolic Volume</td>
<td>Simpson’s and Teicholtz</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>LV Mass</td>
<td>SAX</td>
<td>B-mode and M-mode</td>
</tr>
<tr>
<td>IVS Diastole/Systole</td>
<td>PSLAX</td>
<td>M-mode</td>
</tr>
<tr>
<td>Aortic root</td>
<td>PSLAX</td>
<td>M-mode</td>
</tr>
<tr>
<td>Ascending aorta velocity</td>
<td>Aortic arch</td>
<td>Doppler PW</td>
</tr>
<tr>
<td>Descending aorta velocity</td>
<td>Aortic arch</td>
<td>Doppler PW</td>
</tr>
<tr>
<td>LCCA PSV</td>
<td>Carotid</td>
<td>Doppler PW</td>
</tr>
<tr>
<td>RCCA PSV</td>
<td>Carotid</td>
<td>Doppler PW</td>
</tr>
<tr>
<td>Mitral valve velocity</td>
<td>Apic 4</td>
<td>Doppler PW</td>
</tr>
</tbody>
</table>

Table 2.4: Table to show the cardiac functional parameters that were measured using the Visualsonics Vevo 2100 system and the modes/views that were used to obtain them

2.7.1 Simpson’s slice and Teicholtz method

As previously described, Simpson’s slice and Teicholtz formulae were used by the Visualsonics Vevo 2100 software to calculate functional parameters from B-mode and M-mode images in the parasternal long axis or short axis view (see Table 2.4). In this regard, Simpson’s slice method is a geometric method which uses the principle whereby the shape of the heart is assumed to be a hemispherical cylinder (i.e. an ellipse with one end cut off) (see Figure 2.9). Simpson’s uses the ventricle length and 3 ventricular diameters (one measurement from just below the aorta, one from the middle of the
heart near the papillary muscles, and one from near the apex) to calculate the area of the heart and the chamber volume, which in turn are used to calculate functional parameters including cardiac output, ejection fraction and stroke volume (see Table 2.4) (Lohr, 2005). However, it is important to note that the fractional shortening Simpson’s measurement is incorrect; therefore for results chapter five the fractional shortening measurements displayed were obtained from fractional shortening calculated using M-mode/ Teicholtz method. Teicholtz method is another geometric method used to calculate the area of the heart and hence functional parameters; however only one ventricular diameter measurement and the ventricular length are required for calculating the area of the ventricle and hence functional parameters (Lohr, 2005). As such, it is less accurate than the Simpson’s slice method. Therefore where parameters could be measured using both Simpson’s and Teicholtz method (see Table 2.4), only Simpson’s measurements were included in this thesis since these are considered the most accurate and reliable. Importantly, for accurate calculations of functional parameters, multiple repeated images/ measurements are acquired (for reproducibility) at both diastole and systole (which is achieved because the Visualsonics Vevo 2100 system captures multiple frames/ cardiac cycles for each image). Furthermore, intra user variability was previously tested by others in Daniel Stuckey’s research group who showed that there was little difference between multiple measurements made on the same mouse. For instance, they demonstrated a percentage error of only 2% when measuring left ventricular mass when using the 2D Simpsons method; and a percentage error of just 5% when measuring ejection fraction using the 2D Simpsons method (Stuckey, et al., unpublished).
Figure 2.9: Images of the cardiac functional parameters that were measured/obtained using the Visualsonics Vevo 2100 system and the modes/views that were used to acquire them:

- A: Long axis view (B mode)
- B: Short axis view (B mode)
- C: Short axis view (M mode)
- D: Aortic flow (B mode)
- E: Carotid flow (B mode)
- F: Carotid flow (M mode)

Figure 2.9: Images of the cardiac functional parameters that were measured using the Visualsonics Vevo 2100 system and the modes/views that were used to obtain them. A: Long axis view (B mode), B: Short axis view (B mode), C: Short axis view (M mode), D: Aortic view (B mode), E: Carotid flow (B mode), F: Carotid flow (M mode)
2.8 Collection and processing of Brn3b KO and WT age matched control mouse tissues for molecular and morphological analysis

At the end of each experiment (Exercise or Angiotensin II treatment), Brn3b KO and age matched WT mice were sacrificed using CO$_2$ and cervical dislocation, after which tissues including hearts were either snap frozen in liquid nitrogen and stored at -80°C, or fixed overnight in 4% Paraformaldehyde (PFA).

For later studies, snap frozen hearts were ground up into a fine powder using liquid nitrogen and a mortar and pestle which was then used to prepare total RNA or cellular protein. PFA fixed hearts were processed overnight using the Leica TP1050 tissue processor and then embedded in paraffin wax, after which 5µm serial sections were cut from them using a microtome. Heart sections were then used for molecular and morphological analysis (e.g. Immunostaining).
2.9 RNA extraction, cDNA synthesis and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

Total RNA was isolated from Brn3b KO and age matched WT whole hearts using Trizol Reagent (Invitrogen). Snap-frozen whole hearts were first homogenized in liquid nitrogen before re-suspending in Trizol, whereas isolated cardiomyocyte cells were immediately harvested in Trizol reagent. RNA was then extracted according to the manufacturer’s protocol. To remove genomic DNA contaminants, DNAse1 treatment was performed in a 100µl reaction using RNAse-free DNase1, (37°C, 2 hours – after 1st hour 1µl DNase1 was added). DNAse1 enzyme was removed by Phenol: Chloroform extraction after which RNA was precipitated using Ethanol. The quantity and quality of RNA was assessed using the Nano drop 1000 and 100ng-1µg of total RNA was used for cDNA synthesis. cDNA synthesis was carried out in a 50µl reaction (see below) using RNA Superscript II Reverse Transcriptase with 100ng-1µg RNA. Before cDNA synthesis, RNA was prepared by linearization at 65°C for 10 minutes, then incubation on ice for 2 minutes. Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out using SYBR Green chemistry on the Opticon 2 DNA engine thermal cycler (Bio-Rad). 2-4µl of cDNA was amplified in a 20µl reaction (see below) using unique primers to each gene. The housekeeping gene, GAPDH, was used to correct for variability between samples. Relative mRNA levels were normalised to GAPDH mRNA and calculated using the ΔΔCt method. Shown below are the components of the cDNA synthesis and qPCR reactions, and their cycle conditions:

**cDNA synthesis (50µl reaction):**

- RNA: 5-10µl
- RNAse Inhibitor: 1µl
- 5X First Strand Buffer: 5µl
- dNTP Mix (10mM): 2.5µl
- 0.1M DTT: 2.5µl
- Random Primers: 1.25µl
- RNA Superscript II Reverse Transcriptase: 0.5µl
- RNAse-free water: 27.25-32.25µl
‘cDNA2’ programme:
1. 25°C  10 minutes
2. 42°C  50 minutes
3. 70°C  15 minutes
4. 4°C   Hold.

qRT-PCR (20µl reaction):
cDNA:                     2-4µl
Power SYBR qPCR Master Mix:  10µl
Forward primer:           1µl
Reverse primer:           1µl
RNAse-free water:         4-6µl

qRT-PCR programme:
1. 95°C  15 minutes
2. 95°C  30 seconds
3. 54-60°C 30 seconds
4. 60-72°C 30 seconds
5. 70-80°C 10 seconds
6. Plate read
7. Melting curve from 65°C to 95°C, read every 0.3°C, hold for 1 second between reads.
8. END.
2.10 Analysis of protein expression and localisation

2.10.1 Western blotting

To analyse expression levels of proteins of interest in the hearts of Brn3b KO and WT age matched mice following physiological (exercise) or pathological stress (AngII treatment), total protein was first isolated from them. Briefly, at the end of exercise/AngII treatment, hearts were excised and snap frozen in liquid nitrogen to prevent protein degradation by proteases. Next, snap frozen hearts were ground up into a fine powder using liquid nitrogen and a mortar and pestle. The ground up frozen tissue was then homogenized in 300µl 1X RIPA Buffer (Table 2.0). Homogenized protein samples were then centrifuged at 13,000rpm for 10 minutes to remove cell debris. 10-50µl of the supernatant was then diluted 1:1 in 2X Laemmli Buffer (containing 1% β-mercaptoethanol) and boiled at 100°C for 10 minutes.

For analysis of protein expression levels within NRVM and H9C2 cell lines treated with AngII, cells were briefly washed with PBS and then homogenized in 250µl 2X Laemmli Buffer. Similar to protein extracts isolated from mouse heart tissue, 1% β-mercaptoethanol was added to each sample which was then boiled for 10 minutes at 100°C.

Protein extracts (10-50µl) were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) alongside a pre-stained protein ladder using a 12% or 15% SDS-PAGE gel depending on the molecular weight of the proteins of interest being detected (see Table 2.4 below). This was achieved by running the SDS-PAGE gel in 1X Running Buffer (see Table 2.5) for 2 hours at 125V. To enable immunodetection of the proteins, the resolved proteins were transferred from the gel to a charged PVDF membrane overnight at 30V at 4°C in 1X Transfer Buffer (Table 2.5).

The following day, the PVDF membrane was first blocked in 4% milk for 1 hour at room temperature (RT), after which it was incubated in the appropriate primary antibody (1:1000/ 1:2000 dilution in 2% milk/PBS/T) for 2 hours at room temperature. Next, the PVDF membrane was washed 5 times in PBS/T (5 min, RT) and then incubated in the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (1:2000 dilution in 1% milk/PBS/T) for 1 hour at RT. The membrane was then washed 10 times for 5 minutes in PBS/T and incubated in HRP substrate for 5 minutes at RT which results in a colour reaction that can be visualized under UV light. The intensity of the bands on the PVDF membrane was measured using a densitometer and compared to the pre-stained protein ladder to determine the molecular weight of the proteins of interest.
dilution in 2% milk/PBS/T) for 1 hour at room temperature. The PVDF membrane was then washed another 5 times in PBS/T (5 min, RT) after which it was incubated in Enhanced Chemiluminescence (ECL) substrate for 1 minute. Finally, proteins were detected by exposing the membranes to X-ray film for varying amounts of time (between 1 second and 15 minutes) which was developed using the SRX-101A film processor (see Figure 2.10). Protein quantification was carried out using Fiji (ImageJ) software and was adjusted with β-tubulin (housekeeping protein).

**Figure 2.10:** Schematic diagram showing the principles of western blotting
### Table 2.5: Recipes for a 12% resolving gel, 15% resolving gel and 10% stacking gel used for SDS-PAGE electrophoresis of protein samples:

<table>
<thead>
<tr>
<th></th>
<th>12% Resolving Gel (For Middle molecular weight proteins: 10-70kDa)</th>
<th>15% Resolving Gel (For Low molecular weight proteins: 12-45kDa)</th>
<th>10% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>6.6ml</td>
<td>4.0ml</td>
<td>6.8ml</td>
</tr>
<tr>
<td>30% Polyacrylamide</td>
<td>8.0ml</td>
<td>9.6ml</td>
<td>1.7ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl pH 8.8</td>
<td>5.0ml</td>
<td>5.0ml</td>
<td>N/A</td>
</tr>
<tr>
<td>1M Tris-HCl pH 6.8</td>
<td>N/A</td>
<td>N/A</td>
<td>1.25ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2ml</td>
<td>0.2ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.2ml</td>
<td>0.2ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.008ml</td>
<td>0.008ml</td>
<td>0.01ml</td>
</tr>
</tbody>
</table>

Table 2.5: Table showing the recipes for a 12% resolving gel, 15% resolving gel and 10% stacking gel

### Table 2.6: Recipes for 10X Running Buffer and 10X Transfer Buffer used for SDS-PAGE electrophoresis of protein samples:

<table>
<thead>
<tr>
<th></th>
<th>10X Running Buffer (1 litre)</th>
<th>10X Transfer Buffer (1 litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>6.6ml</td>
<td>4.0ml</td>
</tr>
<tr>
<td>Tris-Base</td>
<td>8.0ml</td>
<td>9.6ml</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.0ml</td>
<td>5.0ml</td>
</tr>
<tr>
<td>SDS</td>
<td>100ml (10%)</td>
<td>0.37g</td>
</tr>
<tr>
<td>Methanol</td>
<td>N/A</td>
<td>200ml</td>
</tr>
</tbody>
</table>

Table 2.6: Table showing the recipes for 10X Running Buffer and 10X Transfer Buffer used for SDS-PAGE electrophoresis of protein samples
2.11 Immunostaining

2.11.1 Immunostaining of formalin fixed, paraffin embedded embryonic and adult heart sections

To assess for differences in protein levels and protein localisation in heart sections of embryonic Brn3a KO and WT mice and adult Brn3b KO and WT mice (at baseline or following AngII treatment/exercise), dewaxed and rehydrated sections were processed as follows (see Figure 2.11):

Antigen retrieval was carried out by boiling tissue sections in 0.01M Sodium Citrate (pH 6.0) for 10 minutes in a microwave. After cooling to RT, endogenous peroxidases were blocked by incubation in 0.3% hydrogen peroxide solution (30 min, RT), washed twice in 0.1% PBS/T (5 min, RT) and then blocked in 0.1% PBS/T/10% goat serum (30 min, RT). Next, sections were incubated in the appropriate primary antibody (1:100-1:1000 dilution in 0.1% PBS/T – see Table 2.1) either overnight at 4°C or for 1-2 hours at room temperature (see Figure 2.11). Slides were kept in a sealed humidified chamber to prevent drying. A secondary Ab only negative control was also included in each immunostaining experiment.

Following primary antibody incubation, tissue sections were washed 5 times in 0.1% PBS/T (5 min, RT) and incubated in the appropriate biotinylated secondary antibody (1:200 dilution in PBS/T - see Table 2.1) in a sealed humidified chamber (1 hour, RT). Sections were then washed another 5 times in 0.1% PBS/T (5 min, RT). For colorimetric detection using DAB substrate, sections were incubated in AB Complex (Vectastain Elite ABC Kit (Vectorlabs) – 1 drop Reagent A + 1 drop Reagent B in 5ml 0.1% PBS/T) in a sealed humidified chamber (30 min, RT). Finally, heart sections were washed another 5 times in 0.1% PBS/T (5 min, RT) before being incubated in 3,3'-Diaminobenzidine (DAB) substrate (1 Sigmafast DAB tablet + 1 Urea H₂O₂ tablet in 1ml ddH₂O) until they turned brown in colour (approximately 5 – 20 minutes depending on the primary antibody used) (see Figure 2.11).

After immunostaining, all slides were dehydrated in a graded ethanol series (from 50% -100% Ethanol – 5 minutes per ethanol grade), washed twice in Xylene (5 minutes per
wash), then mounted using the Xylene cover slipper. Slides were imaged using the NDP Nanozoomer.

**Figure 2.11**: Schematic diagram showing the principles of DAB immunostaining
2.11.2 Immunostaining of cultured cells

At the start of experiments, cells were plated onto glass coverslips in a 6 well plate at a density of $1 \times 10^5$ - $1 \times 10^6$ cells per well and allowed to settle for 24 hours before the start of the experiment. For immunostaining of plated cells at the end of experiments (see Figure 2.12), cells were first washed twice in PBS, after which they were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT). Next, cells were washed 3 times in PBS (5 minutes, RT) and then permeabilized in PBS/0.1% Triton-X-100 for 5 minutes at room temperature. Cells were then washed another 3 times in PBS (5 minutes, RT), after which they were blocked in PBS/ 20% goat serum for 1 hour at room temperature, followed by incubation in the appropriate primary antibody (1:100-1:1000 dilution – see Table 2.1) for 1-2 hours at room temperature. Next, cells were washed 5 times in PBS (5 minutes, RT) and incubated in the appropriate fluorescent secondary antibody (1:2000 dilution (see Table 2.1) for 1 hour at room temperature. For fluorescent detection of immunostained cells, cells were washed another 5 times in PBS (5 minutes, RT) before being mounted onto microscope slides using mounting medium with DAPI (Vectorlabs). Mounted slides were stored in dark conditions (wrapped in foil) at 4°C and were imaged using the Zeiss Axioskop 2 microscope.

**Figure 2.12:** Schematic diagram showing the principles of fluorescent immunostaining in cells
2.12 Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) staining of Brn3a KO and WT embryonic heart sections to analyse cardiomyocyte apoptosis

TUNEL staining identifies cells that are undergoing apoptosis by detecting fragmentation of DNA within tissues that generates single stranded ends (3’-OH), which is a key feature of apoptosis. Briefly, nucleotides that are labelled with Biotin bind to the single stranded ends of fragmented DNA in apoptotic cells via the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Next, tissues are incubated in Streptavidin (which binds to the biotinylated nucleotides) which is conjugated to Horseradish Peroxidase (HRP), which in turn catalyses the production of a brown product from 3,3’ Diaminobenzidine (DAB) substrate. Consequently, all apoptotic nuclei within the tissue are stained dark brown. In this protocol, apoptotic cells are detected by colorimetric labelling of fragmented DNA, a key feature of apoptosis (see Figure 2.13).

The DeadEnd Colorimetric TUNEL System (Promega, Southampton, UK) was used according to the manufacturer’s protocol. In brief, dewaxed and rehydrated formalin fixed, paraffin embedded Brn3a KO and WT embryonic heart sections were incubated in 0.85% NaCl (5 minutes, RT) and washed in PBS (5 minutes, RT). Next, sections were fixed using 4% PFA (15 minutes, RT), washed twice in PBS (5 minutes, RT) and permeabilized in 20µg/ml Proteinase K (15 minutes, RT). Heart sections were then washed in PBS (5 minutes, RT), fixed again using 4% PFA (5 minutes, RT) and washed twice in PBS (5 minutes, RT). Following the PBS washes, sections were incubated in Equilibration Buffer for 5 minutes at room temperature, after which they were incubated in rTdT reaction mix (60 minutes, 37°C) in a sealed humidified chamber to prevent drying of the sections. The labelling reaction was stopped by incubating sections in 2X SSC (15 minutes, RT), after which the sections were washed three times in PBS (5 minutes, RT) and incubated in 0.3% hydrogen peroxide for (5 minutes, RT). Next, sections were incubated in Streptavidin HRP (diluted 1:500 in PBS) for 30 minutes at room temperature, washed three times in PBS (5 minutes, RT), and finally incubated in DAB substrate until the sections turned brown in colour. Finally, sections were washed in ddH₂O and were then dehydrated in a graded ethanol series (from 50% -100% Ethanol – 5 minutes per ethanol
grade), washed twice in Xylene (5 minutes per wash), then mounted using the Xylene cover slipper. Slides were imaged using the NDP Nanoczoomer.

**Figure 2.13**: Diagram showing the mechanism behind TUNEL staining in apoptotic cells within a tissue

1. Fragmented DNA (blue) in apoptotic nuclei of cells is labelled with biotinylated nucleotides (red)

2. Streptavidin conjugated to HRP binds to biotinylated nucleotides via Biotin (B) incorporated into the fragmented DNA

3. HRP catalyses the production of a brown product from 3,3'-Diaminobenzidine (DAB) substrate. Brown spots (brown triangle) within the tissue (e.g. heart) represent the location of apoptotic nuclei.
2.13 Haematoxylin and Eosin (H&E) staining of Brn3a KO and WT embryonic heart sections

To analyse for differences in morphology in the embryonic hearts of Brn3a KO and WT mice, formalin fixed paraffin embedded heart sections from Brn3a KO and WT mouse embryos were stained with Haematoxylin and Eosin using the SAKURA Tissue Tek DRS 2000 automatic slide stainer.

In brief, dewaxed and rehydrated heart sections were incubated in Haematoxylin solution (5 minutes, RT), rinsed in tap water (20 seconds, RT) and then rinsed in 1% HCl 70% EtOH (6 seconds, RT). Next, sections were incubated in Eosin solution (4 minutes, RT) and then rinsed in tap water (2 minutes, RT). Finally, sections were dehydrated in a graded ethanol series, incubated twice in Xylene (2 minutes, RT) and then cover slipped using a Xylene cover slipper.

Results of H&E staining:

Blue staining = Cell nuclei
Red/ pink staining = Cell cytoplasm
2.14 Masson’s trichrome staining of Brn3b KO and WT heart sections for collagen and ECM deposition

To analyse for differences in collagen and ECM deposition in the hearts of Brn3b KO and WT mice at baseline and following physiological (exercise) and pathological (AngII infusion) stress, formalin fixed paraffin embedded heart sections from Brn3b KO and WT mice were stained with Masson’s trichrome using the SAKURA Tissue Tek DRS 2000 automatic slide stainer.

In brief, dewaxed and rehydrated heart sections were incubated in Lugol’s Iodine (5 minutes, RT), after which they were incubated in 3% sodium thiosulphate (3 minutes, RT) and rinsed in tap water (1 minute, RT). Next, sections were incubated in Celestine blue solution (10 minutes, RT), rinsed in tap water (30 seconds, RT) and incubated in Haematoxylin solution (5 minutes, RT). After rinsing in water (30 seconds, RT), sections were incubated in 1% HCl 70% EtOH (20 seconds, RT) and rinsed twice in tap water (30 seconds, 2 minutes, RT). Next, sections were incubated in 100% EtOH (30 seconds, RT) and 0.2% Orange G Picric alcohol (8 minutes, RT), after which they were rinsed in tap water and incubated in Red Mixture (7 minutes, RT). Following rinsing in distilled water (20 seconds, RT), sections were incubated in 1% Phosphotungstic acid (30 seconds, RT), rinsed in distilled water (20 seconds, RT) and then incubated in 0.5% Chicago Sky Blue solution (5 minutes, RT). Finally, sections were incubated in 1% acetic acid and then dehydrated in a graded ethanol series, incubated twice in Xylene (1 minute, RT) and then cover slipped using a Xylene cover slipper.

Results of Masson’s trichrome staining:

- **Blue staining** = Collagen/ECM
- **Red/Pink staining** = Cytoplasm
- **Dark Purple/Black** = Nuclei
2.15 Cell Culture

2.15.1 Maintenance, long term storage and recovery of cell lines

Using cell lines such as the myoblast cell line derived from the embryonic rat heart (H9C2) are valuable in cardiovascular research. In particular, they are a close representation of cardiomyocytes in vivo; however in contrast they are not terminally differentiated meaning that they can be maintained in culture and can hence be used for longer experiments, etc. In addition, using cell lines is important for compliance with the 3 R’s (reduction, refinement and replacement) which allows fewer animals to be used within a study. However importantly, H9C2 cells which have been used in this study also express endogenous Brn3b and undergo hypertrophic changes in response to AngII treatment (Watkins et al., 2011). This allows the potential role of Brn3b in the hypertrophic response to be investigated in more detail in cardiomyocytes.

Cell lines were routinely cultured in monolayers in 75cm² tissue culture flasks in growth medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) antibiotic. Cells were kept in a tissue culture incubator maintained at 37°C with 5% CO₂. When cells reached 80% confluence (approximately every 3 days), they were passaged by incubation in 0.05% Trypsin-EDTA solution (following a PBS wash) for 5 minutes. To detach the cells, tissue culture flasks were tapped after which the trypsin was neutralised by re-suspending cells in an appropriate volume of growth medium (dependent on how often the cells needed to be passaged).

For long term storage of cell lines, trypsinised cells were pelleted by centrifugation at 1000rpm for 5 minutes. The cell pellet was then re-suspended in 1ml of growth medium containing 20% DMSO and aliquotted into a 1ml cryovial which was wrapped in tissue and stored at -80°C. The next day, the cryovial was transferred to liquid nitrogen for long term storage. To recover cell lines that had been stored long term in liquid nitrogen, cryovials were immediately thawed in a 37°C water bath. Next, the thawed cells were added to a T25 tissue culture flask containing 10ml growth medium containing 10% FBS and 1% P/S. The next day, the growth medium was changed to remove any residual DMSO which may be toxic to the cells.
2.16 Preparation of Neonatal Rat Ventricular Myocytes (NRVMs)

NRVMs are a primary cell culture and are the closest representation of cardiomyocytes in vivo, and can partially replace the use of animals. Similarly, NRVMs also have limited proliferative capacity therefore experiments can only be conducted within a short timescale. However importantly, NRVMs express endogenous Brn3b and closely represent in vivo cardiomyocytes and have been shown to undergo hypertrophic changes following AngII treatment (Watkins et al., 2011). For in vitro analysis of changes in gene expression in cardiomyocytes following pathological stress (AngII treatment), neonatal rat ventricular myocytes (NRVMs) were isolated from P0-P2 neonatal rat pup hearts (see Figure 2.14).

Briefly, rat pups were sacrificed using cervical dislocation and decapitation, after which hearts were quickly removed into ice cold ADS Buffer (116mM NaCl, 20mM HEPES, 0.8mM NaH$_2$PO$_4$, 5.6mM glucose, 5.4mM KCl, and 0.6mM MgSO$_4$, pH 7.35) and cut into small pieces to aid digestion. The minced hearts were placed in a 15ml Falcon tube with 7ml digestion buffer (ADS Buffer containing 0.5mg/ml Collagenase Type II and 0.6mg/ml Pancreatin) which was incubated at 37°C for 15 minutes with gentle agitation. The presence of Collagenase Type II and Pancreatin enabled breakdown of collagen and other extracellular matrix proteins to release the cardiomyocytes from the heart tissue into solution (see Figure 2.14).

Following digestion, the supernatant was removed, added to another Falcon tube containing 2ml FBS and centrifuged at 1000rpm for 6 minutes to pellet the cardiomyocytes. Next, the cell pellet was re-suspended in plating medium (4:1 DMEM: M199 medium, 10% Horse serum, 5% FBS and 1% P/S) plus 2ml FBS and incubated at 37°C until all digests were completed. The minced heart tissue was digested, centrifuged and re-suspended in plating medium (see above) seven times. The seven re-suspended digests were pooled together and centrifuged at 1000rpm for 6 minutes, after which the cell pellet was re-suspended in 10ml plating medium. To remove fibroblasts from the cell suspension, the re-suspended cardiomyocytes were pre-plated twice in a 10cm tissue culture dish, where they were incubated at 37°C for 30 minutes. Following pre-plating, isolated cardiomyocytes were plated onto 1% gelatine coated 6-well plates at a
density of $5 \times 10^5$ cells per well and were incubated at 37°C for 24h (see Figure 2.14). The next day, the media was changed to maintenance medium (4:1 DMEM: M199, 10% FBS and 1% P/S), and cells were cultured for another 48h before the start of experiments.

![Diagram showing the method of NRVM isolation from neonatal rat pups](image)

**Figure 2.14:** Diagram showing the method of NRVM isolation from neonatal rat pups

### 2.17 Treatment of NRVM and H9C2 cell lines with Angiotensin II to induce hypertrophy

To further analyse for changes in gene/protein expression in cardiomyocytes following a hypertrophic response, hypertrophy was induced in NRVM and H9C2 cell lines. Before the start of the experiments, cells were plated onto 6-well plates at a density of $5 \times 10^5$ cells per well and allowed to acclimatise for 24h. After plating, cells were treated with 10µg/ml Angiotensin II for 2h, 4h, 8h and 24h, after which cells were harvested for protein/RNA which was then analysed using Western blotting (protein) or qPCR (RNA).
2.18 Statistical analysis

All data presented in tables and graphs in results chapters’ three to five represents the mean and standard error/ standard deviation. Statistical analysis was assessed using Prism 5.0 software (GraphPad Inc., San Diego, CA, USA). Comparisons of mean values between experimental groups (e.g. WT Saline vs WT AngII) were performed using the Mann-Whitney Test or Students T-Test. Statistical significance was defined as p<0.05. This was because the cardiovascular phenotype of the Brn3b KO mouse is unknown and has not yet been fully characterised. Consequently, the Brn3b KO mouse may not have the same phenotype and hence functional parameter measurements as age-matched WT mice. As such, WT AngII treated mice were compared with WT saline control mice and Brn3b KO AngII treated mice were compared with Brn3b KO saline control mice. Where statistical significance was observed, ANOVA and Bonferroni post hoc tests were undertaken for analysis of variation between experimental groups.
Chapter Three

Essential but partially redundant roles for POU4F1/Brn3a and POU4F2/Brn3b transcription factors in the developing heart
3.0 Introduction

The heart is the first functioning organ to develop and is essential for development of the rest of the embryo and life after birth. Heart development is a tightly regulated process, therefore defects or errors in the cellular processes that govern development of the heart can be detrimental. Failure of the heart to develop can lead to still births or give rise to congenital heart disease in babies, which is one of the most common types of birth defects that occurs every 9 in 1000 births in the UK. Examples include ventricular septal defect, Tetralogy of Fallot and coarctation of the aorta (Brand, 2003, NHS, 2015). As such, it is essential to understand the cellular and molecular mechanisms which cause congenital heart disease in order to provide better treatments and quality of life for patients living with this condition.

Proliferation of pre-cardiac mesodermal cells during early heart development is essential for formation of the beating linear heart tube, the cells of which then undergo differentiation to have specialised functions which in turn contribute to the main structures of the heart; including the left and right atria and ventricles and the outflow tract (Black, 2007, Brand, 2003). Cell apoptosis is also necessary during heart development for removing unnecessary cells from the heart and for forming the final structure of the four chambered heart. For example, cell apoptosis is required for ventricular and outflow tract septation during the later stages of heart development (Poelmann et al., 1998). These cellular processes are tightly regulated by cardiac transcription factors which drive the expression of specific target genes that mediate these processes and are hence essential for controlling key processes of heart development.

Consequently, it is highly likely that alterations in such regulators, e.g. abnormal expression (such as loss of or over expression), mutations or loss of their function may contribute to errors in heart development and hence the occurrence of heart defects and congenital heart disease. For instance, loss of transcription factors MEF2C, GATA4, Nkx2.5 and Baf60c which mediate cardiac stem cell differentiation within the second heart field to form the atria, right ventricle and outflow tract of the heart leads to defects in heart development (Black, 2007). For example, Nkx2.5 mutant mice have disrupted
heart development (as it is important for formation of the heart tube), causing them to die before birth (Lyons et al., 1995). MEF2, which is expressed during all stages of heart development is another necessary cardiac transcription factor since MEF2 knockout mice fail to form the right ventricular region and do not undergo rightward looping of the heart tube during development (Brand, 1997). Furthermore, protein-protein interaction between transcription factors is also important for regulating expression of target genes involved in heart development. For example, GATA4 and Nkx2.5 interact and up-regulate ANF expression, which facilitates regulation of blood pressure in the developing heart (Barry et al., 2008, Bernardo et al., 2010, Durocher et al., 1997).

The POU4F2/Brn3b and the related but distinct POU4F1/Brn3a POU transcription factors have important roles in determining cell fate in the heart following injury. Moreover, in the injured heart Brn3a and Brn3b mediate different effects on cardiomyocyte survival when co-expressed with tumour suppressor p53 (Budhram-Mahadeo et al., 2014). For example, following coronary artery ligation in mice, Brn3b expression increased throughout the heart, the role of which is yet to be elucidated. However, increased Brn3b and p53 were co-expressed in the region of injury where they correlated with up-regulation of pro-apoptotic genes Bax and Noxa. Consequently, cardiomyocyte apoptosis also increased within the injured myocardium. In contrast, Brn3a was only detected in the non-injured myocardium and was co-localised with p53 in cells that were adjacent to the infarct zone. Co-expression of Brn3a and p53 within this region correlated with downregulation of pro-apoptotic genes Bax and Noxa and up-regulation of the p21<sup>cip1/waf1</sup> cell cycle inhibitor, which is associated with cell survival (Budhram-Mahadeo et al., 2014).

Brn3a and Brn3b have also been shown to be expressed in the developing heart, however their functions are yet to be elucidated. In the developing mouse heart, Brn3a mRNA and protein were detected between stages E13.5-E17.5, i.e. time points when the major stages of heart development were occurring, and was localised to the outflow tract, myocardium and atrioventricular valves by E14.5 (shown by immunostaining). Brn3b mRNA was also detected from E13.5, and steadily increased until birth. However, Brn3b protein levels decreased during P1 to P3 in the neonatal heart whereas Brn3a expression was elevated at this time point. Brn3a and Brn3b were also detected in
primary cultures of neonatal rat ventricular myocytes (NRVMs) (Farooqui-Kabir et al., 2008).

Interestingly, Brn3a KO mouse hearts during mid-gestation (E14.5) showed changes in cardiac morphology, such as hyperplastic valve cushions, which correlated with increased Brn3b mRNA expression (Farooqui-Kabir et al., 2008). This is particularly significant since increased Brn3b expression has previously been shown to drive cell proliferation in breast cancer and neuroblastoma cells (Budhram-Mahadeo et al., 2008, Fujita et al., 2011, Irshad et al., 2004, Lee and Budhram-Mahadeo, 2005). Additionally, Brn3a and Brn3b have both been shown to trans activate the HSP27 promoter in cardiomyocytes, which is also associated with cardiomyocyte survival; and in Brn3a KO mice increased Brn3b expression maintained HSP27 expression in the absence of Brn3a in the heart (Farooqui-Kabir et al., 2004, Farooqui-Kabir et al., 2008). Therefore this may suggest that the balance of Brn3a and Brn3b expression in the heart may play important roles in heart development and cardiomyocyte survival. Since all Brn3a KO mutant embryos died by 0.5 days after birth and cardiac morphology has not yet been elucidated at later stages of gestation, it was important to determine the complex roles of Brn3a and Brn3b in heart development and cardiomyocyte survival during later gestational stages in the mouse embryo (Farooqui-Kabir et al., 2008). In this regard, the aims of this chapter are as follows:

**Aims:**

- Analyse the expression of Brn3b in the hearts of Brn3a KO mouse embryos (i.e. in the absence of Brn3a) and determine the potential regulatory relationship between Brn3b and Brn3a in the developing heart.

- Investigate changes in expression of Brn3b target genes in the hearts of Brn3a KO mouse embryos and whether changes in these target genes is associated with changes in cell fate (e.g. cell proliferation, apoptosis) and cardiac morphology during heart development.
3.1 Results

3.1.1 Reciprocal expression of Brn3a and Brn3b mRNA and protein in the developing heart

To further determine the relationship between Brn3a and Brn3b in the developing mouse heart, RNA was prepared from hearts which were isolated from WT embryonic (E11.5–E18.5) and postnatal (P1–P3) mice, and Brn3a and Brn3b levels were measured using qRT-PCR. Figure 3.0 (A) shows that at embryonic stages E11.5 to E15.5, high Brn3a mRNA levels in the heart correlated with low Brn3b mRNA levels. However, from embryonic stage E17.5 to postnatal stage P1, Brn3a mRNA expression was reduced in the heart when high expression of Brn3b mRNA was detected. Finally, from postnatal stage P1 to P3 in the mouse heart, the pattern of Brn3a and Brn3b mRNA expression recapitulated the pattern which was observed between embryonic stages E11.5 to E15.5, whereby high Brn3a mRNA expression was associated with reduced levels of Brn3b mRNA (see Figure 3.0 (A)) (Maskell et al., 2017).

In addition, Brn3a and Brn3b protein localisation was analysed in WT E18.5 mouse embryonic hearts using DAB immunostaining, which showed relatively low Brn3a protein expression (top) and higher levels of Brn3b protein expression (bottom) in the ventricular myocardium (dark brown spots – Figure 3.0 (B)) (Maskell et al., 2017).
Figure 3.0: A: Graph showing high levels of Brn3a and low levels of Brn3b mRNA expression in the hearts of WT mice between E11.5 and E15.5. In contrast, low levels of Brn3a expression and high levels of Brn3b mRNA expression were observed between stages E17.5-P1. From P1 to P3, Brn3a mRNA levels increased whereas Brn3b mRNA levels decreased. Data represents mean and standard deviation obtained from 4 hearts. mRNA levels were measured using qRT-PCR and were calculated relative to the expression of the housekeeping gene GAPDH. B: Representative images showing DAB Immunostaining of WT E18.5 mouse embryonic hearts showing localised expression of Brn3a (top) and Brn3b (bottom) (indicated by dark brown spots) (Maskell et al., 2017).
To further determine the relationship between Brn3a and Brn3b in the developing heart, RNA was also extracted from Brn3a KO and WT mouse hearts at embryonic stages E14.5 to E18.5, which was then used to measure levels of Brn3b mRNA expression using qRT-PCR. Between stages E14.5-E16.5, Brn3b mRNA levels were low in the WT mouse heart, whereas in the Brn3a KO mouse heart they were significantly increased (Student’s T-Test, p < 0.0005) (Figure 3.1 (A)). In contrast, at E18.5 Brn3b mRNA levels were elevated in the WT heart whereas they were significantly decreased in the Brn3a KO mouse heart (Student’s T-test, p < 0.0005).

DAB immunostaining was also carried out in E16.5 Brn3a KO and WT mouse embryonic hearts to analyse Brn3b protein localisation. In this regard, figure 3.1 (B) showed DAB immunostaining of Brn3b protein in Brn3a KO and WT E16.5 mouse embryonic hearts. In the WT heart (left), Brn3b protein expression was minimal in the ventricular myocardium, however in the Brn3a KO heart Brn3b protein levels were increased (indicated by increased dark brown spots) (Maskell et al., 2017).
Figure 3.1: A: Graph showing increased Brn3b mRNA expression in Brn3a KO and WT mouse embryonic hearts (using qRT-PCR) relative to the housekeeping gene GAPDH. Data represents mean and standard error of 5 individual hearts for each genotype at each time point ** Statistical significance (Student’s T-Test p < 0.0005) B: Representative images showing DAB immunostaining of Brn3a KO and WT mouse embryonic hearts (n=3) showing increased Brn3b expression in the Brn3a KO heart (dark brown spots) (Maskell et al., 2017).
3.1.2 Brn3a KO mouse embryonic hearts showed morphological changes during late gestation which correlated with up-regulation of Brn3b target genes

Increased Brn3b expression in the Brn3a KO mouse heart during mid gestation (E14.5) was associated with changes in cardiac morphology, including hyperplastic septum. Interestingly, increased cell proliferation has been observed in breast cancer and neuroblastoma cells that had elevated levels of Brn3b (Budhram-Mahadeo et al., 2008, Farooqui-Kabir et al., 2008, Irshad et al., 2004). Additionally, Brn3b has been shown to activate HSP27 expression in cardiomyocytes in the absence of Brn3a, which plays an important role in mediating cardiomyocyte survival and differentiation after birth (Davidson and Morange, 2000, Farooqui-Kabir et al., 2008). As such, this may suggest that the balance between Brn3a and Brn3b expression may play an important role in driving specific responses during heart development. Therefore it was important to identify whether Brn3a and Brn3b have additional roles in heart development during the later stages of gestation (E16.5 onwards).

As a result, hearts from Brn3a KO and WT E18.5 mice were fixed (using 4% PFA), embedded in paraffin wax and cut into 5µm-10µm sections and then stained with Haematoxylin and Eosin (H&E) to assess differences in cardiac morphology. Analysis of E18.5 heart sections stained with H&E showed differences in cardiac morphology in the Brn3a KO mouse heart, including increased trabeculation, reduced myocardial compaction, and appearance of myocardial crypts/fissures when compared with age matched WT controls (Figure 3.2). Reduction in heart length, ventricular wall and ventricular septal thickness was also observed in E18.5 Brn3a KO mice, which may suggest that loss of Brn3a affects the size of the developing heart (Maskell et al., 2017).
In addition, it was important to determine whether differences in cardiac morphology were due to changes in expression of Brn3b target genes such as cyclin D1 (associated with cell cycle progression) since aberrant expression of such genes can alter cell fate. Therefore mRNA levels of Brn3b target gene cyclin D1 were analysed using qRT-PCR using RNA isolated from Brn3a KO and WT E16.5 mouse embryonic hearts. As shown by figure 3.3 (A), cyclin D1 mRNA levels were elevated in the Brn3a KO heart at E16.5 when compared to WT controls. Moreover, DAB immunostaining of Brn3a KO and WT E17.5 mouse embryonic hearts confirmed increased cyclin D1 protein localisation in the Brn3a KO heart when compared to WT controls (Figure 3.3 (B)).

**Figure 3.2:** A: Representative images of H&E stained WT E18.5 mouse embryonic hearts (n=3). B: Representative images of H&E stained Brn3a KO E18.5 mouse embryonic hearts (n=3). Images were captured at 2.5x and 5x magnification. LV = Left ventricle; RV = Right ventricle; IVS = Interventricular septum (Maskell et al., 2017)
Figure 3.3: Representative images of H&E stained Brn3a KO (ii) and WT (i) E18.5 mouse embryonic hearts. A: Graph showing expression of cyclin D1 mRNA in Brn3a KO and WT E16.5 hearts. GAPDH was used as the housekeeping gene. Data represents means and standard error of multiple hearts (WT; n=6, Brn3a KO; n=5). *Statistical significance p<0.05 B: Representative images showing DAB immunostaining of Brn3a KO and WT E17.5 hearts (n=3) with cyclin D1 (dark brown spots) Images were captured at 2.5x and 10x (boxed images) magnification (Maskell et al., 2017).
3.1.3 Brn3b target genes which affect cell fate were increased in Brn3a KO hearts which correlated with evidence for cardiomyocyte apoptosis

Previous studies have shown that following acute injury, Brn3b levels increased in the heart (the role of which is unknown) and in the injured myocardium. However, when increased Brn3b levels were co-expressed with p53 in the injured myocardium, up-regulation of pro-apoptotic genes Bax and Noxa occurred which was associated with cardiomyocyte apoptosis (Budhram-Mahadeo et al., 2014). In addition, figure 3.2 (B) showed changes in cardiac morphology upon loss of Brn3a (Brn3a KO E18.5 mouse hearts), including increased trabeculation and an apparent loss of compaction in the myocardium. Consequently, it was important to elucidate whether (increased) Brn3b may also mediate cardiomyocyte apoptosis in the absence of Brn3a in the developing heart by regulating expression of pro-apoptotic genes Bax and Noxa upon co-expression with p53. In this regard, mRNA levels of Bax, Noxa and p53 were analysed using qRT-PCR of RNA isolated from Brn3a KO and WT E16.5 mouse embryonic hearts. Analysis of mRNA levels in E16.5 hearts showed increased levels of Brn3b and pro-apoptotic genes Bax and Noxa in Brn3a KO mice, whereas p53 mRNA levels remained relatively unchanged when compared to WT controls (Figure 3.4 (A), (B) and (C)).

Protein localisation of Bax was also assessed in Brn3a KO and WT E17.5 mouse embryonic hearts using DAB immunostaining, which confirmed increased Bax protein expression in the trabeculated myocardium of Brn3a KO E17.5 mouse embryonic hearts (Figure 3.4 (C)). In addition, formalin fixed, paraffin embedded sectioned hearts from Brn3a KO and WT E17.5 mice were analysed for differences in cardiomyocyte apoptosis using TUNEL staining (which detects and marks cells undergoing apoptosis) to determine whether loss of Brn3a may cause cardiomyocyte loss during late gestation (Maskell et al., 2017). Figure 3.5 shows increased TUNEL positive cells (dark brown spots) in the Brn3a KO E17.5 mouse heart (top) when compared to WT controls, which indicates that increased cardiomyocyte apoptosis may be occurring in the Brn3a KO E17.5 mouse embryo. In this regard, these results may suggest that loss of Brn3a may lead to
cardiomyocyte loss, and as a result may be important for cardiomyocyte survival during heart development in the later stages of gestation (Maskell et al., 2017).

**Figure 3.4:** A: Graph showing differences in Brn3b and p53 mRNA expression in Brn3a KO and WT E16.5 hearts (mean and standard error of multiple independent hearts: WT n=6 and Brn3a KO n=5) B: Graph showing increased mRNA expression of Bax and Noxa in Brn3a KO E16.5 hearts when compared to WT control hearts. GAPDH was used as the housekeeping gene (data represents mean and standard error of several independent hearts - WT; n=6, Brn3a KO; n=5). * Statistical significance p < 0.05 C: Representative images showing DAB immunostaining of WT (n=3) and Brn3a KO (n=3) E17.5 hearts showing increased Bax expression (dark brown spots) in the Brn3a KO heart (top). Images were captured at 2.5x and 10x (boxed images) magnification (Maskell et al., 2017).
Figure 3.5: Representative images showing TUNEL stained (dark brown spots which stain apoptotic cells) WT (i) (n=3) and Brn3a KO (ii) (n=3) E17.5 mouse embryonic hearts, showing increased staining in Brn3a KO hearts (Maskell et al., 2017).
3.1.4 Brn3a and Brn3b are expressed in the developing zebrafish heart and the effects of their loss can be studied by using morpholino injections

Zebrafish are a popular animal model in the field of cardiovascular research because it is easy to reduce the expression of multiple genes in the zebrafish embryo without causing embryonic lethality, which allows the effects of loss of specific genes on heart development to be studied in vivo over time. The zebrafish embryo is also transparent, which means that heart morphology, structure and development can be seen clearly since the heart is visible. Although the zebrafish heart morphology and structure differs from the mouse and human heart as it only consists of two chambers (one atrium and one ventricle), the cellular and molecular mechanisms of zebrafish heart development are highly conserved between zebrafish and mammals (Goldstein et al., 1998, Harvey, 1999). In addition, Brn3a and Brn3b are highly conserved between mammals and zebrafish, which means that they may be a suitable model for investigating the effects of loss of Brn3a and Brn3b on cardiac development (Maskell et al., 2017). Furthermore, the zebrafish is a good model for heart development studies since it is externally fertilised, which means that gene expression can be manipulated easily by injecting morpholino constructs that target knockdown of specific genes.

Therefore initial studies aimed to confirm Brn3a and Brn3b expression in the zebrafish heart. Briefly, western blot analysis was performed using protein extracted from isolated adult zebrafish hearts or zebrafish embryos from 24, 48 and 72 hours post fertilisation (hpf). Figure 3.6 (A) shows that single isoforms of both Brn3a (30kDa) and Brn3b (43kDa) proteins were expressed in adult zebrafish heart extracts when compared with mouse heart protein samples (positive control) which expressed two isoforms.

Brn3a and Brn3b protein localisation was also analysed in the zebrafish heart by performing DAB and fluorescent immunostaining (fluorescent immunostaining was performed by Kashif Qamar) with antibodies specific for Brn3a, Brn3b and Tropomyosin (cardiomyocyte marker) on zebrafish embryos from 72 hours post fertilisation (hpf) (Maskell et al., 2017). Brn3a and Brn3b proteins were localised to the ventricle of the zebrafish heart at 72h hpf, which interestingly displayed a similar expression pattern to
Tropomyosin (cardiomyocyte marker), which may suggest that Brn3a and Brn3b are expressed in the cardiomyocytes of the developing zebrafish heart (Figure 3.6 B)).

It was also important to determine the potential roles of Brn3a and Brn3b in zebrafish heart development. As such, initial studies were performed to determine whether Brn3a and Brn3b expression could be reduced specifically in the zebrafish heart. This was achieved by injecting morpholino antisense oligonucleotides (MOs) designed to block the translation of Brn3a and Brn3b messenger RNA (mRNA). Morpholinos were injected into fertilised eggs to target either Brn3a or Brn3b or both simultaneously (performed by Thomas Hawkins). Preliminary studies used to optimise dose (2ng, 3ng and 4ng) and time of treatment showed that using 2ng of Brn3a and 2ng of morpholino caused optimal knockdown of Brn3a and Brn3b without causing toxicity to the embryos when compared to non-silencing (NS) morphants which was confirmed by western blot analysis (Figure 3.6 (C)). This was further confirmed by equal levels of housekeeping protein gamma tubulin in both NS and Brn3a/Brn3b morpholino protein samples (used as a loading control) (Figure 3.6 (C)) (Maskell et al., 2017).
Figure 3.6: A: Representative western blot image showing expression of Brn3a (30kDa) and Brn3b (43kDa) protein in zebrafish adult hearts (Z) (n=3), which is recognised by the same Brn3a and Brn3b antibodies used to detect Brn3a and Brn3b protein in the adult mouse heart (M). B: Representative images of DAB immunostained 72 hpf zebrafish hearts showing Brn3a and Brn3b protein localisation in the ventricle of the heart (dark brown spots), which correlated with Tropomyosin (cardiomyocyte marker) localisation in the ventricle. C: Representative western blot image showing reduction of Brn3a and Brn3b protein in total protein extracted from zebrafish embryos that were injected with morpholinos targeting Brn3a and Brn3b (morpholino injections performed by Thomas Hawkins) (n=3). Gamma (γ) tubulin was used as a loading control. A = Atrium, V = Ventricle P = Pericardium (Maskell et al., 2017).
3.2 Discussion

In this results chapter, we have provided further evidence to support that Brn3a and Brn3b are important cardiac transcription factors in the developing heart. Additionally, the results displayed in this chapter provide a better understanding of the effects of their loss on the later stages of heart development. Consequently, this may provide more detailed molecular mechanisms behind heart development, which in turn may enable the prevention of embryonic lethality/provision of better treatments for people living with congenital heart defects (e.g. ventricular septal defect) in the future.

In this regard, we have shown a reciprocal pattern of Brn3a and Brn3b expression throughout heart development (Figure 3.1), suggesting that the balance of these transcription factors may be important for the correct determination of cell fate in a temporal and spatial manner. We also showed that Brn3b expression was elevated in the absence of Brn3a in Brn3a KO mouse embryonic heart which correlated with morphological changes, elevated cyclin D1, Bax and Noxa expression and increased apoptosis during late gestation (Figures 3.2-3.5). Furthermore, we have also demonstrated expression of Brn3a and Brn3b in the developing zebrafish heart (Figure 3.6 (Maskell et al., 2017)).

Interestingly, POU4F1/ Brn3a and the related but distinct POU4F2/ Brn3b are DNA binding POU transcription factors which drive changes in cell fate in a tissue specific manner. The results from this study have shown that Brn3a and Brn3b may have important functions during heart development, and may regulate expression of target genes directly or indirectly by interaction with other transcription factors (such as p53). As such, Brn3a and Brn3b may have important roles in determining cell fate in the heart during development.

Firstly, we have shown that expression of these transcription factors in the developing mouse heart has been detected as early as E9.5 (Figure 3.1 (A)). Both Brn3a and Brn3b are also expressed in neonatal rat ventricular myocytes, which suggests a potential role for these transcription factors in cardiomyocyte development and function, which are as yet not known (Farooqui-Kabir et al., 2008). Additionally, previous studies have shown increased Brn3b expression in developing Brn3a KO mouse hearts at E14.5 which was
associated with changes in cardiac morphology (Farooqui-Kabir et al., 2008). This suggested a complex relationship between Brn3a and Brn3b during heart development, therefore it was important to determine the regulatory relationship between Brn3a and Brn3b in the developing heart. Quantitative analysis showed that during mid-gestation, high Brn3a levels correlated with low Brn3b expression, whereas during the later stages of gestation, Brn3a expression was reduced but Brn3b expression was increased. After birth however, high Brn3a expression correlated with low Brn3b expression, suggesting that Brn3a and Brn3b have a reciprocal pattern of gene expression during development (Figure 3.1 (A)). Furthermore, increased Brn3b mRNA and protein levels in Brn3a KO mouse hearts (Figure 3.1 (B)) suggests that Brn3a may repress expression of Brn3b in the heart. In contrast, sensory neurons isolated from Brn3a KO mice had low Brn3b mRNA levels, which suggests that there may be a complex and tissue specific relationship between Brn3a and Brn3b (Maskell et al., 2017, McEvilly et al., 1996). Interestingly, reporter assays carried out by others in the laboratory showed that Brn3a reduced Brn3b promoter activity in NRVMs, suggesting that Brn3a may repress Brn3b expression in cardiomyocytes and that the observed reciprocal pattern of Brn3a and Brn3b expression may be specific to the heart (see Figure 1C in (Maskell et al., 2017)).

Cyclin D1 expression was analysed in Brn3a KO hearts since ectopic expression of Brn3b target genes such cyclin D1 can drive changes in cell fate. Cyclin D1 mRNA and protein levels were elevated in the Brn3a KO mouse heart at E16.5 and E17.5 in the ventricle when compared to WT control hearts (Figure 3.3). Cyclin D1 is a known Brn3b target gene and has been shown to be elevated in neuroblastoma and breast cancer cells that have increased Brn3b expression (Budhram-Mahadeo et al., 2008, Farooqui-Kabir et al., 2008, Irshad et al., 2004). Since elevated Brn3b and cyclin D1 is associated with increased cell proliferation, the increased cyclin D1 levels may be a result of increased transcriptional activation by elevated Brn3b in the absence of Brn3a. Interestingly, cyclin D proteins play an important role in driving cell cycle progression and supporting myocardial proliferation during cardiac development but are downregulated when cells become terminally differentiated, therefore the hyperplasia of the heart that was observed at E14.5 in the Brn3a KO mouse heart may be due to increased cyclin D1 levels (Budhram-Mahadeo V. S, et al., 2008).
Interestingly, loss of all Brn3a KO mice soon after birth may be linked to the changes in cardiac morphology observed at E14.5, therefore further histological analysis of E18.5 Brn3a KO mouse hearts was undertaken to determine whether cardiac defects during the later stages of gestation may have been responsible for neonatal death (Farooqui-Kabir et al., 2008). As such, E18.5 Brn3a KO mouse hearts had increased ventricular trabeculation, reduced myocardial compaction and reduced ventricular wall and septal thickness, suggesting a loss of cardiomyocytes at this stage (Figure 3.2). At E16.5, Brn3b mRNA levels increased in Brn3a KO hearts whereas levels of p53 remained unchanged compared to WT hearts, however at E18.5 Brn3b levels were reduced in the Brn3a KO heart (Figure 3.4). This further suggests the loss of cardiomyocytes during the later stages of gestation; this may be linked to co-expression of Brn3b with p53 which in turn up-regulates the expression of Bax to drive apoptosis. This is evidenced by Budhram-Mahadeo and colleagues who showed that following coronary artery ligation in mice, overall Brn3b expression increased in the heart (the function of which is not known); however within the infarct zone its increased expression with p53 correlated with induction of apoptotic genes such as Bax and increased cardiomyocyte apoptosis (Budhram-Mahadeo et al., 2014). In contrast, Brn3a expression was localised to the non-injured myocardium, however its co-expression with p53 in cells adjacent to the infarct zone correlated with induction of genes such as p21^cip1/waf1 cell cycle inhibitor, genes associated with cell survival (Budhram-Mahadeo et al., 2014).

Bax and Noxa mRNA levels were elevated in the E16.5 Brn3a KO heart with no significant change in p53 expression. This also correlated with increased TUNEL positive cells in the Brn3a KO E17.5 heart, which provides evidence that more cells were undergoing apoptosis (Figures 3.4 and 3.5). Consequently this may contribute to the cell loss and changes in cardiac morphology observed in the Brn3a KO heart at E18.5. Moreover, since previous studies have shown that aberrant expression of Brn3b with p53 correlated with increased Bax and Noxa expression and increased cardiomyocyte cell death in the injured myocardium, it is likely that the increased TUNEL positive cells observed in the Brn3a KO E17.5 heart may also be apoptotic cardiomyocytes (Budhram-Mahadeo et al., 2014). It will be important to validate these observations by quantifying the TUNEL positive cells observed in the Brn3a KO mouse heart sections. For instance,
this could be achieved by using software such as ImageJ. In this regard, identical regions of WT and Brn3a KO heart sections could be selected, after which ImageJ could be used to count the number of TUNEL positive cells within the selected regions. Furthermore, it will be important to use other techniques to confirm increased apoptosis in the Brn3a KO mouse embryonic hearts, for example by detecting increased levels of Annexin V or Caspase 3, a protein which has been well characterised in both the mitochondrial and death receptor mediated apoptotic pathways (Elmore, 2007). Detecting cytochrome c release (component of mitochondrial mediated apoptosis) in Brn3a KO and WT heart sections and visualising these sections in more detail by using transmission electron microscopy (to detect characteristics of apoptosis such as membrane blebbing and nuclear fragmentation) could also be used to analyse differences in cardiomyocyte apoptosis in more depth (Elmore, 2007). Additionally, reducing Brn3b expression has also been shown to reduce Bax expression in cardiomyocytes where p53 levels were maintained, and sensory neurons from Brn3b KO mice which had unchanged p53 levels also had reduced levels of Bax and were resistant to apoptotic stimuli, which further suggests that Brn3b is required for induction of Bax expression (rather than p53 alone) (Ensor et al., 2003, Budhram-Mahadeo et al., 2014, Budhram-Mahadeo et al., 2006b). Additionally, Brn3a has previously been shown to inhibit p53 mediated apoptosis in neurones, therefore loss of Brn3a as well as elevated Brn3b may contribute to the increased cardiomyocyte apoptosis observed in the Brn3a KO heart at E18.5 (Hudson et al., 2005, Hudson et al., 2004). This is significant because apoptosis is a tightly regulated process during heart development and is normally responsible for removing unnecessary cells during development and for septation of the outflow tract, etc. (Poelmann et al., 1998). As such, increased Brn3b levels being co-expressed with p53 in the Brn3a KO heart may cause aberrant apoptosis to occur during the later stages of heart development. Therefore the lack of regulation of apoptosis may be responsible for the increased trabeculation and ventricular septal thinning observed in the Brn3a KO E18.5 heart; and hence may contribute to congenital heart defects such as ventricular septal defect observed in new born babies, which are common causes of neonatal mortality (Brand, 2003). Therefore the balance and tight regulation of Brn3a and Brn3b expression will be important for regulating the cellular process that govern normal
cardiac development (Maskell et al., 2017). However, to elucidate the specific roles of Brn3a and Brn3b in cardiac development, additional studies need to be completed.

Consequently, these results suggest that during mid-gestation increased levels of Brn3a may promote cardiomyocyte differentiation during cardiac development by repressing Brn3b and hence blocking p53 mediated apoptosis, but may also co-operate with p53 to up-regulate cell cycle inhibitor p21\(^{cip1/waf1}\) which is associated with cell survival. In contrast, increased Brn3b upon loss of Brn3a may drive cell proliferation in the heart during mid gestation but at later stages may also be co-expressed with increased p53, which in turn drives up-regulation of Bax and Noxa. This in turn may result in increased cardiomyocyte loss and cardiac morphological changes during the later stages of heart development (Maskell et al., 2017).

Although Brn3a KO and Brn3b KO mice survive until birth, suggesting that individually loss of these transcription factors is not embryonically lethal; further studies undertaken by others in the laboratory showed that no Brn3a/Brn3b double KO or triple allele mutant (Brn3a \(-/-\) / Brn3b \(+/-\)) mice were recovered from Brn3a/Brn3b heterozygote crosses at E9.5 (see Table 1 in (Maskell et al., 2017)). This suggests that loss of both Brn3a and Brn3b causes embryonic lethality in mice, and that Brn3a and Brn3b may partially compensate for each other during early embryonic development (Maskell et al., 2017). Similar compensatory effects have been observed with Msx1 and Msx2, which are homeobox transcription factors that have important functions in mediating endothelial to mesenchymal transformation during formation of the atrioventricular valves in the heart (Chen et al., 2008). Whilst single Msx1 KO (\(-/-\)) and Msx2 KO (\(-/-\)) mice do not display atrioventricular valve defects, Msx1/ Msx2 double KO mutant mice between E14.5 and E16.5 displayed atrial septal defect and hypoplastic atrioventricular valves. Moreover, Msx1/ Msx2 double KO mice were able to be recovered between E14.5 and E16.5; in contrast no Brn3a/ Brn3b double KO mutant mice could be recovered at E9.5, which further highlights the importance of Brn3a and Brn3b in embryogenesis and their potential roles in cardiac development (Chen et al., 2008). Compensatory effects of related transcription factors occur if they regulate the expression of similar target genes. Importantly, Brn3a and Brn3b both trans activate expression of heat shock protein HSP27 which is important for cardiomyocyte
differentiation. In this regard, loss of Brn3a and Brn3b may also affect foetal heart development through downregulation of HSP27, which may in turn affect cardiomyocyte differentiation (Farooqui-Kabir et al., 2004, Farooqui-Kabir et al., 2008, Fujita et al., 2011, Lee et al., 2005).

Molkentin and colleagues similarly showed that when heterozygote GATA4 mice were crossed, no GATA4 KO mice were found in the litters, suggesting that loss of this transcription factor caused embryonic lethality (Molkentin and Olson, 1997). This was also demonstrated by the fact that following analysis of a large number of embryos taken from GATA4 heterozygote crosses, no GATA4 KO embryos were found at stage E10.5 (Molkentin et al., 1997). Therefore GATA4 is an important cardiac transcription factor that has significant roles in cardiomyocyte survival and cardiac differentiation and morphogenesis, loss of which causes cardiac defects including myocardial hypoplasia, double outlets of the right ventricle, and common atrioventricular canal (Kuo et al., 1997, Pu et al., 2004). Since Brn3a and Brn3b have also been shown to be expressed in the developing heart and that their loss causes embryonic lethality, it is likely that Brn3a and Brn3b are also important cardiac transcription factors that may play an important role during heart development, since double Brn3a/Brn3b KO mice are not viable, and cardiac defects are observed in Brn3a KO mouse embryos at E18.5. Interestingly, mutations in GATA4 and Nkx2.5 have also been identified in patients with congenital heart disease (including ventricular septal defect) and have also been associated with increased risk of congenital heart disease, therefore it would be interesting to analyse Brn3a and Brn3b expression in patients with congenital heart disease (Granados-Riveron et al., 2012, Yang et al., 2013).

Unfortunately, because Brn3a/ Brn3b double KO mice were not viable, it was not possible to determine whether loss of Brn3a and Brn3b caused heart defects in the mouse during development. However, Brn3a and Brn3b are highly conserved between mammals and zebrafish (87% and 76% respectively), and are encoded by single genes in the zebrafish genome, which means that morpholinos can be designed for injection into externally fertilised eggs to reduce protein expression of both Brn3a and Brn3b. Furthermore, during early development, zebrafish embryos can survive with cardiac defects because they extract oxygen and nutrients by diffusion, which can be visualised
in live embryos since they are transparent (Hu et al., 2000, Monteiro et al., 2008). It is important to note that although zebrafish have a two chambered heart and mammals have a four chambered heart, the cellular and molecular processes that drive heart development in both species are highly conserved. For example, cardiac development in both zebrafish and higher vertebrates is initiated from the lateral plate mesoderm and hearts undergo rightward looping to align the atria and ventricular chambers (Goldstein et al., 1998, Harvey, 1999).

The zebrafish animal model is also good for studying the effects of loss of Brn3a and Brn3b on cardiac development since both Brn3a and Brn3b mRNA and protein were detected in the adult zebrafish heart by qRT-PCR and western blotting (Figure 3.6 (A)). Importantly, DAB and fluorescent immunostaining (see figure 4C in (Maskell et al., 2017)) also showed that Brn3a and Brn3b protein was localised to the ventricle of the embryonic (24-72 hpf) zebrafish heart where expression of cardiomyocyte marker Tropomyosin was also observed, which may also suggest that Brn3a and Brn3b are expressed in the cardiomyocytes of the zebrafish heart (Figure 3.6 (B)).

Injection of morpholino antisense oligonucleotides (MOs) designed to block the translation of Brn3a and Brn3b messenger RNA (mRNA) into zebrafish embryos at 24, 48 and 72hpf caused successful knockdown of Brn3a and Brn3b protein (demonstrated by western blotting) (Figure 3.6 (C)). This is important because these data demonstrate that the effects of loss of Brn3a and Brn3b expression on heart development can be easily studied in the zebrafish. Furthermore, others in the laboratory showed that knockdown of Brn3a and Brn3b protein in zebrafish embryos (using morpholino injection) caused striking cardiac defects in double morphant hearts. Concerning this, reduction of both Brn3a and Brn3b expression caused failure to loop resulting in a linear heart morphology, atrioventricular valve defects (demonstrated by retrograde blood flow) and contractile dysfunction (see figure 5 in (Maskell et al., 2017)). These results suggest that loss of Brn3a and Brn3b is likely to be responsible for the cardiac defects observed in the double morphant zebrafish embryo and that Brn3a and Brn3b may be important for cardiac development (Maskell et al., 2017). This is particularly interesting since these defects associated with loss of Brn3a and Brn3b are similar to the defects observed in Nkx2.5 KO and MEF2 KO mice which also show defects in rightward looping of the heart.
tube (Brand, 1997, Lyons et al., 1995). As such, these results further suggest that Brn3a and Brn3b are also important cardiac transcription factors that facilitate normal heart development.

In summary, the results obtained from this study in both mice and zebrafish provide strong evidence for key roles of Brn3a and Brn3b during cardiac development with limited redundancy (Maskell et al., 2017). Moreover, increased Brn3b in the absence of Brn3a which correlated with cardiomyocyte apoptosis may cause cardiac defects and post.birth lethality in Brn3a KO mice; which provides a more convincing explanation for complete loss of mutant Brn3a KO mice within 0.5 days after birth rather than behavioural and suckling defects, as proposed by earlier studies (McEvilly et al., 1996, Xiang et al., 1996).
Chapter Four

Regulation of Brn3b in cardiomyocytes by hypertrophic stimuli
4.0 Introduction

When the heart is put under stress (e.g. through disease or physical demand), the workload of the heart increases. As such, contractile cardiomyocytes which facilitate beating of the heart enlarge and undergo cytoskeletal reorganisation in order adapt to maintain cardiac function. Cardiomyocyte hypertrophy occurs since they are terminally differentiated cells which cannot replicate. Importantly, different types of stresses on the heart can either be beneficial or detrimental to heart function (Bernardo et al., 2010, McMullen and Jennings, 2007).

For instance, physiological stress such as exercise or pregnancy causes heart function to be maintained and is reversible. Pathological stress however, such as hypertension and neurohumoral activation with vasoactive peptides, e.g. Angiotensin II (AngII) or Endothelin 1 (ET1) which generates pressure and volume overload to the heart can drive changes that become irreversible if sustained. During the early adaptive response, pathological hypertrophy is characterised by re-expression of foetal genes which enable the heart to adapt and maintain cardiac output. For example, re-expression of β-MHC facilitates cardiomyocyte contraction and new sarcomere formation (Barry et al., 2008, Bernardo et al., 2010, McMullen and Jennings, 2007). Proto-oncogenes such as c-myc, c-fos and c-jun which usually facilitate cardiomyocyte growth in the developing heart are also induced by pathological stimuli such as AngII and pressure overload (Izumo et al., 1988, Mulvagh et al., 1987). Cell cycle regulators including cyclin D1 have also been implicated in the hypertrophic response and facilitate cardiomyocyte growth (Soonpaa et al., 1997, Tamamori-Adachi et al., 2002). Furthermore, following pathological stress, cardiomyocytes switch from using fatty acids to metabolising glucose to generate ATP, which requires additional genes such as the GLUT4 glucose transporter (Abel et al., 1999, Bernardo et al., 2010).

Importantly however, prolonged pathological stress leads to maladaptive changes including cardiac fibrosis and remodelling which result in poor systolic function (Barry et al., 2008, Bernardo et al., 2010, McMullen and Jennings, 2007). Moreover, stress signals and activation of AngII signalling results in activation of p53 which activates expression of pro-apoptotic genes such as Bax, which in turn drives cardiomyocyte cell death (Long
et al., 1997, van Empel and De Windt, 2004). Since cardiomyocytes are terminally differentiated, apoptosis leads to cardiomyocyte loss, which when combined with fibrosis as a result of pathological stress, reduces cardiac function and systolic function which eventually leads to heart failure. This in turn is responsible for a high rate of mortality in patients suffering from cardiovascular disease (Cowie et al., 2000, Savarese and Lund, 2017).

The cell signalling pathways that converge on changes in gene expression and mediate pathological hypertrophy have been well characterised. For example, Angiotensin II (AngII) which induces hypertrophy by triggering hypertension and hence an increased workload on the heart is a small peptide that mediates its effects through multiple signalling pathways, including MAP Kinase, Calcineurin and G-protein coupled receptor signalling. This in turn leads to transcriptional activation of hypertrophic markers in the nucleus such as β-MHC, ANF, and BNP which leads to increased protein synthesis, cardiomyocyte growth and contraction in response to pathological stress (Mehta and Griendling, 2007, Molkentin et al., 1998). However, the molecular mechanisms underlying the transition from adaptive responses to irreversible pathological changes are not fully understood. Increased transcriptional activity and hence changes in gene expression that lead to protein synthesis are essential for mediating hypertrophic growth because cardiomyocytes cannot replicate to help the heart adapt to stress (Cutilletta et al., 1978, Abdellatif et al., 1998, McDermott et al., 1991, McDermott et al., 1989). Therefore it is important to identify the molecular regulators that alter the expression of genes which can determine hypertrophic responses in the stressed heart. Re-expression of foetal genes such as ANF, BNP and β-MHC are tightly regulated and controlled by cardiac transcription factors such as GATA4, Mef2, Hand2 and NKX2.5 (Saadane et al., 1999). Consequently it will be essential to identify and characterise other cardiac transcription factors that drive expression of other genes during the hypertrophic response in order to understand the molecular switch from the early adaptive response to later pathological changes that lead to heart failure.

Interestingly, the POU4F2/Brn3b transcription factor has important functions in determining cell fate by regulating the expression of target genes in a tissue specific manner. For example, Brn3b can drive cell proliferation/growth by activating cyclin D1
and CDK4 in cancers (Budhram-Mahadeo et al., 2008). In addition, Brn3b mediates glucose uptake in metabolically active tissues such as skeletal muscle by up-regulating the expression of the GLUT4 glucose transporter (Bitsi et al., 2016). Brn3b has also been detected in cardiomyocytes at the mRNA and protein level in the developing mouse heart. Since re-expression of foetal genes occurs following pathological stress, it may be possible that Brn3b could also be re-expressed during pathological hypertrophy to mediate hypertrophic responses in the cardiomyocyte (Farooqui-Kabir et al., 2008, Maskell et al., 2017). Moreover, in the injured mouse heart, e.g. following coronary artery ligation, Brn3b expression was elevated throughout the heart in both the injured and non-injured myocardium. Although the role of Brn3b in the uninjured myocardium is unknown, increased Brn3b expression within the infarct zone of the heart was co-expressed with tumour suppressor p53 which correlated with induction of pro-apoptotic Bax and increased cardiomyocyte apoptosis (Budhram-Mahadeo et al., 2014). Similarly, aberrant Brn3b expression in Brn3a KO developing hearts also correlated with increased Bax expression and induction of apoptosis (Maskell et al., 2017). As such, Brn3b has been shown to drive cardiomyocyte apoptosis in the heart in response to injury. Since sustained pathological stress leads to activation of p53 and Bax which is associated with progression to heart failure, Brn3b may also play an important role in mediating cardiomyocyte apoptosis during pathological cardiac hypertrophy through co-operation with p53 to up-regulate pro-apoptotic Bax expression (Chatterjee et al., 2011, Condorelli et al., 1999, Del Re et al., 2007). Therefore Brn3b may play an important role in the heart when under stress.

In addition, preliminary studies consisting of transfection assays of H9C2 cells have shown that the MAPK pathway activated the Brn3b promoter (reporter construct), since the MAPK inhibitor PD98059 blocked its activity following AngII treatment (which activates the MAPK pathway) (Maskell, 2018b). This is particularly interesting since MAPK signalling is a well characterised pathway that is required for mediating pathological cardiac hypertrophy (Mehta and Griendling, 2007). Additionally, others in the laboratory previously showed that Calcineurin signalling which induces pathological cardiac hypertrophy via activation of the NFAT transcription factor was also shown to induce Brn3b promoter activity in transfection assays in H9C2 cells; since the Calcineurin
inhibitor Cyclosporine A (CsA) blocked its activity (Maskell, 2018b). Furthermore, bioinformatics analysis identified multiple NFAT binding sites within the proximal 6 kb Brn3b promoter, suggesting that Calcineurin signalling may act via regulation of Brn3b by NFAT (Maskell, 2018b). As a result, Calcineurin signalling may also be involved in up-regulating Brn3b expression during the hypertrophic response following AngII treatment. Since CnA transgenic mice also develop cardiac hypertrophy, adverse remodelling, heart failure and sudden death it is likely that Brn3b plays an important role in driving specific hypertrophic responses in the stressed heart (Molkentin, 2004, Molkentin et al., 1998). Consequently, these preliminary studies further suggest that Brn3b may play an important role in pathological hypertrophy since cell signalling pathways associated with pathological hypertrophy (MAPK, Calcineurin, etc.) caused up-regulation of Brn3b expression.

Since Brn3b can drive complex effects on gene expression and cell fate that depends on cell type, growth conditions and co-expression of other regulators, and to determine whether Brn3b may also play a role in mediating the hypertrophic response (as a component of the foetal gene programme) studies were undertaken to analyse the functions of Brn3b in response to pathological stress driving hypertrophic responses. In this regard, the aims of this chapter are as follows:

**Aims:**

- Analyse expression and localisation of Brn3b and expression of hypertrophic markers (e.g. β-MHC) in AngII treated WT mouse hearts and cardiomyocyte cultures to investigate whether Brn3b is required for mediating hypertrophic responses to AngII treatment.

- Analyse expression of Brn3b target genes in AngII treated WT mouse hearts and cardiomyocyte cultures to investigate how Brn3b may be involved in mediating hypertrophic responses to AngII treatment.
4.1 Results

4.1.1 AngII treatment caused up-regulation of Brn3b in the hypertrophic hearts of WT mice

Since Brn3b is expressed in cardiomyocytes in the developing and stressed heart in response to injury, it was important to understand whether Brn3b may also play a role in mediating cardiac hypertrophy in response to chronic pathological stress (Budhram-Mahadeo et al., 2014, Farooqui-Kabir et al., 2008, Maskell et al., 2017). To achieve this and to determine how Brn3b may be regulated in response to pathological hypertrophy, pressure and volume overload was induced in 2 month old WT female mice by treatment with AngII for 4 weeks (via Alzet osmotic pumps – saline was used as a control). Outbred WT mice (C57BL/6J) used in preliminary studies were obtained from approved commercial sources (Harlan UK or Charles River) and additional WT (+/+) control mice used to complete these studies were generated by crossing Brn3b heterozygous mice (+/-) on a C57BL/6J background strain. Following treatment, cardiac hypertrophy was measured in the hearts of WT female mice treated with AngII (and saline controls) by calculating the LV Mass (using ultrasound) and the LV mass: body weight (LV Mass: BW) ratio. Concerning this, there was a trend towards elevated LV mass: BW ratio and elevated LV Mass (Mann-Whitney Test WT saline vs WT AngII *p=0.0317) in WT female mice treated with AngII when compared to saline controls (see Figure 4.0).

Brn3b expression was also measured at the protein level in isolated WT hearts following AngII treatment using western blotting. Figure 4.1 shows that following 4 weeks of AngII treatment, there were trends towards increased Brn3b protein levels in WT hearts when compared to saline controls, which appeared to correlate with increased levels of the hypertrophic marker β-MHC, confirming induction of cardiac hypertrophy, but the changes were not statistically significant (Figure 4.1). Similarly, previous studies undertaken by others in the laboratory showed increases in Brn3b and β-MHC mRNA in the hearts of WT mice following AngII treatment (Maskell, 2018b). This suggests that induction of cardiac hypertrophy correlated with increased Brn3b expression. Moreover, Brn3b protein localisation was assessed in formalin fixed, paraffin embedded WT hearts treated with AngII (or saline control) using DAB immunostaining which
confirmed increased Brn3b expression in the ventricles of WT AngII treated hearts when compared to saline controls (Increased dark brown spots - Figure 4.2).

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<td></td>
<td>Baseline</td>
<td>4 weeks</td>
<td>Baseline</td>
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<tr>
<td>LV Mass (mg)</td>
<td>96.60 ± 15.87</td>
<td>84.95 ± 5.67</td>
<td>93.91 ± 7.89</td>
<td>*110.32 ± 22.66</td>
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<tr>
<td>LV Mass: BW ratio</td>
<td>3.95 ± 0.61</td>
<td>4.00 ± 0.42</td>
<td>4.55 ± 0.31</td>
<td>4.73 ± 1.38</td>
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<tr>
<td>IVS thickness (mm)</td>
<td>1.10 ± 0.26</td>
<td>1.26 ± 0.09</td>
<td>1.21 ± 0.20</td>
<td>1.35 ± 0.41</td>
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**Figure 4.0:** Table showing increased LV Mass and LV Mass: BW ratio and intraventricular septal (IVS thickness) following 4 weeks of AngII treatment in the hearts of WT mice when compared to saline controls. Data represents mean and standard error of 5 individual hearts (Student’s T-Test was used to determine significance between WT Saline and WT AngII groups) *Mann-Whitney Test WT saline vs WT AngII 4 weeks p=0.0317.
Figure 4.1: A: Representative western blot image showing increased expression of Brn3b protein (32 kDa) following 4 weeks of AngII treatment in the hearts of WT female mice (n=3) when compared to saline control mice (n=3). Increased Brn3b levels correlated with increased levels of hypertrophic marker β-MHC (200 kDa). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of Brn3b protein in WT AngII treated female mice adjusted with β-tubulin. C: Graph showing quantification of β-MHC protein in WT AngII treated female mice adjusted with β-tubulin. Data represents mean and standard error (n=3).
Figure 4.2: Representative images of DAB immunostaining showing increased expression of Brn3b protein (dark brown spots) following 4 weeks of AngII treatment in the hearts of WT C57BL/6 mice (n=3) (B) compared to WT saline control mice (A) (n=3).
4.1.2 Hypertrophic stimulus AngII induced Brn3b expression in NRVM cells

There were trends towards increased Brn3b protein expression in the hearts of WT mice treated with hypertrophic stimulus AngII (Figures 4.1 and 4.2). However, cardiomyocytes only make up approximately one third of the total cell number in the heart, with several other cell types contributing to heart morphology, structure and function including fibroblasts and endothelial cells. Therefore it was important to confirm that increased Brn3b expression upon induction of hypertrophy by AngII treatment occurred in cardiomyocytes rather than other cell types. To achieve this, primary neonatal rat ventricular cardiomyocyte cultures (NRVMs - prepared from 1-2 day old rat pups) were used as a model of isolated cardiomyocyte cells since these cultures are obtained from neonatal rat pup hearts and are not yet differentiated which allows them to be maintained in culture.

Briefly, isolated NRVM cells were treated with AngII for 24 hours after which they were harvested for protein which was used to analyse changes in Brn3b and hypertrophic marker β-MHC expression using western blotting (2 independent experiments were carried out). Figure 4.3 shows a representative western blot image (of 3 independent experiments) (Figure 4.3 (A)) and protein quantification graph (Figure 4.3 (B)) analysing Brn3b protein expression in NRVMs which indicate a trend towards increased Brn3b protein levels following 24 hours of AngII treatment when compared to untreated cells. β-tubulin was used as a protein loading control. However, to confirm and validate these results, sample numbers must be increased. Interestingly, preliminary studies carried out by others in the laboratory showed significant increases in Brn3b mRNA in line with elevated β-MHC mRNA (Maskell, 2018b).
Figure 4.3 A: Representative western blot image showing increased expression of Brn3b following 24 hours of AngII treatment in NRVM cells (n=2 experiments). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of Brn3b protein in AngII treated NRVMs adjusted with β-tubulin. Data represents mean and standard error (n=2). Therefore to confirm and validate these results, sample numbers must be increased.
Additionally, it was important to establish Brn3b localisation in hypertrophic cardiomyocytes to determine how it may mediate hypertrophic responses. Therefore NRVM cells were plated onto glass coverslips and then treated with AngII for 24 hours, after which immunofluorescence was carried out to analyse expression and localisation of Brn3b. Alpha actinin (red) was also used to stain the cytoskeletal protein to assess changes in cytoskeletal reorganisation (associated with cardiac hypertrophy).

Figure 4.4 shows representative images of immunofluorescent staining for Brn3b (green) and alpha actinin (red) of AngII treated (Figure 4.4 (B)) or untreated control NRVM cells (Figure 4.4 (A)). In this regard, Brn3b was localised to the cell nucleus which was as expected of a transcription factor since transcription factors act to regulate target gene expression in the nucleus. Clear changes in cytoskeletal reorganisation were observed in NRVMs following AngII treatment (increased red staining), indicating that these cells were undergoing hypertrophy. This also correlated with increased Brn3b expression (green) following AngII treatment in NRVM cells (shown by increased green staining), suggesting that AngII treatment caused up-regulation of Brn3b expression (Figure 4.4 (B)).
Figure 4.4: Representative immunofluorescent images of NRVMs stained for Brn3b (green) and α-actinin (red) A: Untreated NRVMs showing low levels of Brn3b expression (green) and the organisation of the cytoskeleton using α-actinin (red) and DAPI stain (blue - stains nuclei). B: NRVMs treated with AngII (24 hours) showed significant increases in Brn3b expression (green) that is localised to the nucleus of the NRVM cells. Increased α-actinin staining (red) shows changes in the cytoskeletal reorganisation of the NRVMs with increased Brn3b expression (which is associated with cardiac hypertrophy). All images were obtained at 100X magnification (n=3 independent experiments)
4.1.3 AngII also induced Brn3b expression in H9C2 cells

To further confirm that induction of Brn3b expression following AngII treatment was specific to hypertrophic cardiomyocytes, H9C2 cells were used. H9C2 cells are a rat foetal heart derived immortalised cell line that also closely resemble cardiomyocytes in vivo, and express endogenous Brn3b (Budhram-Mahadeo et al., 2014). Importantly, H9C2 cells have been used for these studies since they have been shown to undergo gene expression changes that mimic changes in cardiomyocytes following AngII treatment (Watkins et al., 2011). Moreover, H9C2 cells help to reduce the number of animals being used, and hence contribute to compliance with the 3R’s (reduction, refinement and replacement of animals) within a study by conducting in vitro experiments. To confirm changes in Brn3b protein expression over time following AngII treatment, western blot analysis was undertaken using proteins harvested from H9C2 cells treated with AngII for 2, 4, 8 and 24 hours. Interestingly, there was also a trend towards increased Brn3b protein expression following 2, 4, 8 and 24 hours of AngII treatment in H9C2 cells, which correlated with increased protein levels of hypertrophic marker β-MHC (β-tubulin was used as a protein loading control) (see Figure 4.5). However, to confirm and validate these results, sample numbers must be increased. Previous studies carried out by others in the laboratory also showed increased Brn3b and β-MHC mRNA in AngII treated H9C2 cells, further indicating the requirement of Brn3b in mediating hypertrophic responses in cardiomyocytes (Maskell, 2018b).
Figure 4.5: A: Representative western blot image showing increased expression of Brn3b protein (32 kDa) following 2 hours, 4 hours, 8 hours and 24 hours of AngII treatment in H9C2 cells (n=2 independent experiments). Increased Brn3b expression correlated with increased expression of hypertrophic marker β-MHC (200 kDa). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of Brn3b protein in AngII treated H9C2 cells adjusted with β-tubulin. C: Graph showing quantification of β-MHC protein in AngII treated H9C2 cells adjusted with β-tubulin. Data represents mean and standard error (n=2). Therefore to confirm and validate these results, sample numbers must be increased.
To further analyse the localisation of Brn3b expression in hypertrophic cardiomyocytes, H9C2 cells were plated onto glass coverslips followed by treatment with AngII for 24 hours. Immunofluorescence was then carried out using antibodies for Brn3b. Phalloidin (red) was used to stain the cytoskeleton in order to identify changes in cytoskeletal organisation in the cells.

Representative immunofluorescent images (see Figure 4.6) showed relatively low levels of Brn3b expression (green staining) in untreated H9C2 cells, which was increased within the nucleus of the cells following AngII treatment. Additionally, changes in cytoskeletal reorganisation (characteristic of hypertrophy) shown by red Phalloidin staining demonstrated that Brn3b was increased in cells undergoing hypertrophic changes.

Figure 4.6: Representative images showing H9C2 cells stained with Brn3b (green) and Phalloidin (red) A: Untreated H9C2 cells showing low levels of Brn3b expression (green) and the organisation of the cytoskeleton using Phalloidin (red) and DAPI stain (blue - stains nuclei). Images were obtained at 40X magnification. B: AngII treated H9C2 cells (24 hours) show significant increases in Brn3b expression (green) that is localised to the nucleus of the H9C2 cells. Phalloidin staining (red) shows significant cytoskeletal reorganisation of the H9C2 following AngII treatment. DAPI (blue) stains the nuclei of the H9C2 cells. Images were obtained at 100X magnification (n=3 independent experiments).
4.1.4 Expression of Brn3b target genes cyclin D1 and GLUT4 and pro-apoptotic Bax were increased following AngII treatment in the hearts of WT mice

Brn3b plays important roles in determining cell fate by regulating transcription of multiple target genes. Since Brn3b was up-regulated in hypertrophic cardiomyocytes, it was important to determine whether Brn3b target genes such as cyclin D1 and GLUT4 which are implicated in hypertrophic responses were also up-regulated when Brn3b is increased in hypertrophic cells. Therefore protein harvested from hearts of WT mice treated with AngII for 4 weeks were used for western blot analysis to measure changes in expression of Brn3b target genes cyclin D1 and GLUT4. Figure 4.7 shows that following AngII treatment in WT mice, there was a trend towards increased protein levels of cyclin D1 (37kDa) and GLUT4 (50kDa) in the heart when compared to WT saline control mice; but the changes were not statistically significant. β-tubulin was used as a protein loading control. Importantly, it will be necessary to confirm and validate these results by increasing sample numbers.

Since co-expression of p53 with increased Brn3b expression was associated with up-regulation of the pro-apoptotic gene Bax (in the heart following acute injury) which is implicated in the progression to heart failure following pathological stress, changes in pro-apoptotic Bax and p53 expression were also analysed. As shown in figure 4.8, AngII treatment caused increased protein levels of Bax (21kDa) but no significant changes in p53 (53kDa) levels in the hearts of WT mice, which correlated with elevated levels of Brn3b (Figure 4.1) when compared to control hearts (but the changes were not statistically significant). β-tubulin was used as a protein loading control. Similarly, sample numbers must be increased to confirm and validate these results.
Figure 4.7: A: Representative western blot image showing increased expression of GLUT4 (50kDa) and cyclin D1 (37kDa) following 4 weeks of AngII treatment in the hearts of WT mice (n=3) when compared to WT saline controls (n=3). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of GLUT4 protein in AngII treated WT female hearts adjusted with β-tubulin. C: Graph showing quantification of cyclin D1 protein in AngII treated WT female hearts adjusted with β-tubulin. Data represents mean and standard error (n=3).
Figure 4.8: A: Representative western blot image showing increased expression of p53 (53kDa) and pro-apoptotic Bax (21kDa) following 4 weeks of AngII treatment in the hearts of WT female mice (n=3) when compared to WT saline controls (n=3). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of p53 protein in AngII treated WT female hearts adjusted with β-tubulin. C: Graph showing quantification of Bax protein in AngII treated WT female hearts adjusted with β-tubulin. Data represents mean and standard error (n=3).
4.1.5 AngII up-regulation of Brn3b correlated with increased expression of its target genes cyclin D1 and GLUT4 and pro-apoptotic Bax in NRVM and H9C2 cells

Similar studies were carried out to analyse for changes in Brn3b target genes in NRVM and H9C2 cells which express endogenous Brn3b and undergo hypertrophic changes following AngII treatment (Budhram-Mahadeo et al., 2014, Watkins et al., 2011). Concerning this, protein extracted from isolated NRVM and H9C2 cells that were treated with hypertrophic stimulus AngII for 24 hours were used for western blotting to analyse for changes in cyclin D1 and GLUT4 which are associated with hypertrophic changes (Abel et al., 1999, Soonpaa et al., 1997, Tamamori-Adachi et al., 2002). As shown in figures 4.9 and 4.10, there was a trend towards increased cyclin D1 protein levels in NRVMs and H9C2 cells after AngII treatment when compared to untreated cells (C). Similarly, there was also a trend towards increased GLUT4 protein levels following 24 hours AngII treatment in NRVM and H9C2 cells; β-tubulin was used as a protein loading control. However, to confirm and validate these results, sample numbers must be increased.

Western blotting also showed that increased Brn3b protein expression in AngII treated NRVM and H9C2 cells (Figure 4.1) correlated with increased levels of pro-apoptotic Bax when compared to untreated controls. β-tubulin was used as a protein loading control (see Figures 4.11 and 4.12). Moreover, p53 protein levels remained relatively unchanged suggesting that increased Bax expression was due to co-expression of p53 with increased Brn3b (see Figures 4.11 and 4.12). Similarly, sample numbers must be increased in order to confirm and validate these results.
Figure 4.9: A: Representative western blot image showing increased expression of GLUT4 (50kDa) and cyclin D1 (37kDa) following AngII treatment in NRVM cells when compared to untreated controls (n=2). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of GLUT4 protein in AngII treated NRVMs adjusted with β-tubulin. C: Graph showing quantification of cyclin D1 protein in AngII treated NRVMs adjusted with β-tubulin. Data represents mean and standard error (n=2). Therefore to confirm and validate these results, sample numbers must be increased.
Figure 4.10: A: Representative western blot image showing increased expression of p53 (55kDa) and Bax (21kDa) following AngII treatment in NRVM cells when compared to untreated controls (n=2). β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of p53 protein in AngII treated NRVMs adjusted with β-tubulin. C: Graph showing quantification of Bax protein in AngII treated NRVMs adjusted with β-tubulin. Data represents mean and standard error (n=2). Therefore to confirm and validate these results, sample numbers must be increased.
Due to unlimited supply, H9C2 cells were useful to undertake more detailed time course studies to analyse changes in expression of Brn3b target genes over time. Therefore plated H9C2 cells were treated with AngII for 2, 4, 8 and 24 hours, after which they were harvested for protein which was used to analyses changes in cyclin D1 and GLUT4. As seen in figure 4.15, low cyclin D1 and GLUT4 protein levels were observed at baseline, which steadily increased following 2, 4, 8 and 24 hours AngII treatment in H9C2 cells when compared to untreated cells (see Figure 4.11). β-tubulin was used as a protein loading control. Interestingly, cyclin D1 levels appeared to decrease in H9C2 cells at 24 hours after AngII treatment (see Figure 4.11). Similar to cyclin D1 and GLUT4, Bax expression was low in untreated cells, but increased from 2 to 24 hours of AngII treatment in H9C2 cells; in contrast Bax protein levels were low in untreated cells (see Figure 4.12) (β-tubulin was used as a protein loading control). Similar to AngII treated NRVMs, p53 levels also remained relatively unchanged in AngII treated H9C2 cells, further suggesting that co-expression of p53 with increased Brn3b caused up-regulation for Bax. Importantly, in order to confirm and validate these results, sample numbers must be increased.
Figure 4.11: A: Representative western blot image (n=2 experiments) showing increased expression of GLUT4 (50kDa) and cyclin D1 (37kDa) following 2 hours, 4 hours, 8 hours and 24 hours of AngII treatment in H9C2 cells when compared to untreated controls. β-tubulin (55kDa) was used as a loading control.

B: Graph showing quantification of GLUT4 protein in AngII treated H9C2 cells adjusted with β-tubulin.

C: Graph showing quantification of cyclin D1 protein in AngII treated H9C2 cells adjusted with β-tubulin. Data represents mean and standard error (n=2). Therefore to confirm and validate these results, sample numbers must be increased.
Figure 4.12: A: Representative western blot image (n=2 experiments) showing increased expression of pro-apoptotic Bax (21kDa) and p53 (53kDa) following 2 hours, 4 hours, 8 hours and 24 hours of AngII treatment in H9C2 cells when compared to untreated controls. β-tubulin (55kDa) was used as a loading control. B: Graph showing quantification of p53 protein in AngII treated H9C2 cells adjusted with β-tubulin. C: Graph showing quantification of Bax protein in AngII treated H9C2 cells adjusted with β-tubulin. Data represents mean and standard error (n=2). To validate these results, sample numbers must be increased.
4.2 Discussion

In the previous results chapter, we showed that Brn3b was expressed in the developing heart and preliminary studies undertaken by others in the laboratory indicated that Brn3b may be up-regulated by hypertrophic signalling pathways MAP Kinase and Calcineurin (Maskell et al., 2017, Maskell, 2018c). Since re-expression of foetal genes occurs during pathological hypertrophy, it was important to determine whether Brn3b may be another foetal gene that is re-expressed to mediate hypertrophic responses to pathological stress (McMullen and Jennings, 2007). This is particularly important because the molecular mechanisms that cause the switch from the early adaptive response to later detrimental changes (e.g. cardiomyocyte death, fibrosis, and adverse remodelling) associated with progression to heart failure are not fully understood. Changes in gene expression are responsible for driving changes in cellular processes that contribute to cardiac hypertrophy. Therefore it is likely that transcription factors (such as Brn3b) which regulate the expression of target genes (e.g. hypertrophic markers) that drive changes in cell fate will also have important roles in driving hypertrophic responses in the heart following stress.

In this chapter, we have shown that following pathological stress (AngII treatment), Brn3b expression was elevated in WT hearts/ cardiomyocytes that had undergone hypertrophic changes. Interestingly, there were also trends towards elevated levels of Brn3b target genes GLUT4 and cyclin D1 which are associated with adaptive hypertrophic responses, such as metabolic switching and growth (Busk et al., 2002). Moreover, trends towards elevated Bax protein levels which is associated with cardiomyocyte loss and progression to heart failure were observed in hypertrophic cardiomyocytes. This has also been observed in the hearts of rodents following AngII treatment (Condorelli et al., 1999). As such, we have gained a better understanding of the potential molecular mechanisms that facilitate cardiac hypertrophy in response to pathological stress, and how regulation of gene expression by Brn3b may be important for this process.

In this study, we have shown that the transcription factor POU4F2/ Brn3b may be required for mediating hypertrophic responses in the heart. Concerning this, there was a trend towards elevated Brn3b protein levels in the hearts of WT female mice treated
with AngII (known to induce hypertrophy), which displayed hypertrophic changes characterised by increased LV mass, LV Mass: BW ratio, intraventricular septal thickness and induction of hypertrophic marker β-MHC (Figures 4.0-4.2). Similarly, others in the laboratory also showed induction of Brn3b and β-MHC mRNA in WT AngII treated mouse hearts (Maskell, 2018b). Importantly, it will be necessary to use additional methods to confirm and quantify the level of hypertrophy in the hearts of these WT mice. For instance, increases in HW: BW and HW: Tibia length ratios could also be measured to determine hypertrophic changes. Additionally, other foetal genes are re-expressed during pathological hypertrophy, such as ANF and BNP (Bernardo et al., 2010). Therefore it will also be important to analyse the hearts of WT AngII treated mice for changes in other hypertrophic markers such as ANF and BNP. Furthermore, cardiac hypertrophy is also characterised by increased cardiomyocyte size. As such, to further determine cardiomyocyte hypertrophy, formalin fixed, paraffin embedded AngII treated WT hearts could be stained with a cardiomyocyte marker, e.g. cardiac troponin or Nkx2.5. Following this, the dimensions of the identified cardiomyocytes could be measured using software such as NDP Nanorzoomer or ImageJ. Similar studies in primary NRVM cultures and H9C2 cells also showed trends towards increased expression of Brn3b protein following AngII treatment (Figures 4.3-4.6) (elevated Brn3b and β-MHC mRNA was also observed in AngII treated NRVM and H9C2 cells by Maskell and colleagues) (Maskell, 2018b). NRVMs are a suitable in-vitro model to accompany these studies since they closely resemble cardiomyocytes in vivo which express endogenous Brn3b. Similarly, H9C2 cells also mimic cardiomyocytes in vivo, express endogenous Brn3b and have been shown to undergo hypertrophic responses following treatment with AngII (Budhram-Mahadeo et al., 2014, Watkins et al., 2011). Increased Brn3b levels also appeared to correlate with changes in cytoskeletal re-organisation (shown by alpha actinin/ Phalloidin staining) and induction of hypertrophic markers in NRVM and H9C2 cells (Figures 4.4 and 4.6); therefore these results suggest that the effects of AngII on inducing Brn3b expression was acting in cardiomyocytes. Since the cytoskeleton is composed of proteins such as actin and desmin, it would also be important to confirm changes in cytoskeletal re-organisation in AngII treated NRVMs and H9C2 cells by measuring increases in expression of these proteins, e.g. by western blotting or immunofluorescence. Immunofluorescence staining of NRVM and H9C2 cells also
showed that increased Brn3b protein in hypertrophic cardiomyocytes was localised to the nucleus. Brn3b is a transcription factor which acts primarily in the nucleus to regulate expression of target genes. Therefore these results further suggest that Brn3b may play a role in the hypertrophic response following pathological stress by regulating the expression of its target genes in the nucleus of hypertrophic cardiomyocytes. Increased Brn3b expression in breast cancer and neuroblastoma caused increased cell proliferation, therefore Brn3b is known to have strong oncogenic potential (Dennis et al., 2001, Irshad et al., 2004, Ounzain et al., 2011). Similarly, foetal genes including c-fos and c-myc which are re-expressed in hypertrophic hearts in response to stress also have oncogenic potential (Komuro et al., 1990). Thus, these results suggest that Brn3b may be a novel oncogenic regulator that is also required to mediate hypertrophic responses in the stressed heart. However, Brn3b is increased after 8 and 24 hrs of AngII treatment (in H9C2 cells) whereas c-fos and c-myc expression are elevated very early during the hypertrophic response (30–60 min respectively), suggesting that the effects of Brn3b may be distinct from oncogenes that are re-expressed during very early stages of the hypertrophic response (Izumo et al., 1988).

Interestingly, there was also a trend towards elevated GLUT4 protein levels following AngII treatment in WT female mice which correlated with increased Brn3b expression (Figure 4.7). These observations were particularly interesting because GLUT4 is regulated by Brn3b in metabolically active tissues, and is also increased in the heart in response to stress (Abel et al., 1999, Bitsi et al., 2016). Furthermore, a key feature of pathological cardiac hypertrophy is metabolic switching from fatty acid metabolism to glucose metabolism, which is associated with heart development (Frey and Olson, 2003, McMullen and Jennings, 2007). Consequently, these results suggest that Brn3b may mediate the metabolic switch to glucose during the hypertrophic response by up-regulating expression of GLUT4. However, the role for Brn3b in regulating GLUT4 expression in cardiomyocytes needs to be tested. GLUT4 protein levels also appeared to increase following AngII treatment in NRVM and H9C2 cells, which may suggest that the changes in GLUT4 following AngII treatment may be regulated by increased Brn3b in cardiomyocytes, however sample numbers must be increased to validate these results.
There were also trends towards elevated cyclin D1 protein levels in the hearts of WT mice following AngII treatment in line with increased Brn3b protein expression (Figure 4.7). Cyclin D1 is another Brn3b target gene and is also associated with cardiac hypertrophy since hypertrophic stimuli have been shown to strongly induce its expression in cardiomyocytes (Soonpaa et al., 1997, Tamamori-Adachi et al., 2002). In addition, transgenic cyclin D1 overexpressing mice undergo cardiac hypertrophy and have enlarged hearts. In contrast, cyclin D1 KO mice display attenuated cardiac hypertrophy, suggesting that cyclin D1 may be important for mediating the hypertrophic response in the heart (Busk et al., 2002, Irshad et al., 2004). Since increased Brn3b levels correlated with trends towards elevated cyclin D1 in hypertrophic cardiomyocytes, these results suggest that Brn3b may be facilitating the hypertrophic response by up-regulating cyclin D1 expression. Cyclin D1 protein levels also appeared to be increased following AngII treatment in both NRVM and H9C2 cells (Figures 4.9 and 4.11), suggesting that the effects of AngII on increased Brn3b and cyclin D1 was acting in hypertrophic cardiomyocytes. Furthermore, in H9C2 cells, cyclin D1 protein levels increased from 2 hours to 8 hours of AngII treatment, which decreased by 24 hours which may suggest that cyclin D1 is required for the early adaptive hypertrophic response, but not during the later stages of cardiac hypertrophy (Figure 4.11). As a result, this may also mean that cyclin D1 expression may be regulated in a time specific manner by Brn3b. To confirm and validate these results sample numbers must be increased and additional time course studies would need to be completed in NRVM and other cardiomyocyte cell cultures. As such, the role for Brn3b in regulating cyclin D1 expression needs to be investigated in more detail.

Trends towards increased Bax expression and unchanged p53 expression were also observed in line with increased Brn3b levels in AngII treated WT mice (Figure 4.8). Interestingly, Brn3b co-expression and interaction with other transcription factors can also regulate changes in gene expression and drive different effects on cell fate depending on the interacting partner. For example, Brn3b interacts and co-operates with the oestrogen receptor to activate heat shock protein HSP27 which is associated with survival (Farooqui-Kabir et al., 2008). In contrast, co-expression of Brn3b with p53 has been shown to drive Bax expression and apoptosis in the heart during development.
and in response to injury (Budhram-Mahadeo et al., 2014, Maskell et al., 2017). Moreover, Brn3b alone repressed the promoter activity of Bax, which demonstrates that Brn3b is able to regulate distinct target genes in the absence of p53, however when co-expressed with p53 is able to drive programmed cell death (Budhram-Mahadeo et al., 2006a). Reduction of Brn3b expression in NRVM cultures also caused reduction of Bax expression even though p53 expression was unchanged, which further suggests that Brn3b is required for driving expression of pro-apoptotic Bax in cardiomyocytes (Farooqui-Kabir et al., 2004).

As such, these results suggest that co-expression of Brn3b with p53 may also be involved in facilitating cardiomyocyte cell death (and loss) during the later stages of pathological cardiac hypertrophy by up-regulating Bax expression since increased levels of p53 and Bax are associated with progression to heart failure (Ahuja et al., 2007, Frey and Olson, 2003). To confirm this however, it would be important to confirm that cardiomyocyte apoptosis was occurring in the WT mouse heart following AngII treatment, for example by using TUNEL staining. Similarly, there were also trends towards elevated Bax protein levels but unchanged p53 protein levels following AngII treatment in NRVM and H9C2 cells in line with increased expression of Brn3b (Figures 4.10 and 4.12), which may also suggest that the changes in p53 and Bax expression observed in WT mice were acting in cardiomyocytes. In addition, it would be important to confirm that the effects of AngII on increased Brn3b expression were associated with cardiomyocyte apoptosis by staining AngII treated NRVM and H9C2 cells with apoptotic markers such as Caspase 3 or Annexin V. Interestingly, in H9C2 cells high levels of Bax expression were only observed following 8 hours and 24 hours of AngII treatment (Figure 4.12), which may indicate that Bax expression may also be regulated in a time specific manner (similar to cyclin D1).

In summary, these results suggest a potential model whereby Brn3b may drive distinct target genes in hypertrophic cardiomyocytes during the early adaptive response (GLUT4 and cyclin D1); however during the later maladaptive stages of hypertrophy may drive induction of Bax expression when co-expressed with p53 which in turn may drive pathological changes which result in progression to heart failure.
Chapter Five

Response of Brn3b KO mice to pathological and physiological hypertrophic stimuli
5.0 Introduction

When the heart becomes stressed, it must adapt in order to maintain cardiac output. Such adaptation to physiological (e.g. exercise) or pathological (e.g. hypertension) stress occurs through hypertrophic growth of terminally differentiated cardiomyocytes which have limited proliferative capacity. Cardiomyocyte hypertrophy is characterised by increased cell size and surface area, new sarcomere formation and cytoskeletal reorganisation (Ahuja et al., 2007, Frey and Olson, 2003, McMullen and Jennings, 2007). Additionally, these cellular process are mediated by increased rates of gene transcription and protein synthesis which increase the protein content of the hypertrophic cardiomyocyte (Abdellatif et al., 1998, McDermott et al., 1989, Morgan et al., 1987).

Whilst cardiomyocytes adapt in a similar manner to both physiological and pathological stress, the outcomes of physiological and pathological cardiac hypertrophy are different. In this regard, physiological hypertrophy is reversible and is associated with normal heart function. For example, trained running athletes had normal diastolic function and ejection fraction (Kreso et al., 2015, Venckunas and Mazutaitiene, 2007). Physiological hypertrophy is also mediated via IGF1 signalling and as such is distinct from pathological stress which is mediated by MAP Kinase, Calcineurin and G-protein coupled receptor signalling (Bernardo et al., 2010, Frey and Olson, 2003, Mehta and Griendling, 2007, Molkentin et al., 1998). When pathological stress on the heart is prolonged, signal transduction pathways are activated that drive changes in expression of pro-apoptotic genes such as p53 and Bax which results in cardiomyocyte apoptosis. Induction of these genes is associated with progression to heart failure since loss of terminally differentiated cardiomyocytes in this manner leads to reduction of heart mass and hence poor heart function (Long et al., 1997, McMullen and Jennings, 2007). For instance, patients with hypertrophic cardiomyopathy had reduced ejection fraction and diastolic dysfunction (Kreso et al., 2015, Venckunas and Mazutaitiene, 2007). Moreover, cardiomyocyte apoptosis triggers continuous proliferation and activation of cardiac fibroblasts which produce excess collagen and ECM proteins which surround the cardiomyocytes. This stiffens the heart muscle and reduces its systolic function (McMullen and Jennings, 2007). Heart failure causes a high rate of mortality in patients,
therefore it is essential to understand the molecular mechanisms that cause the early beneficial adaptive response to lead to later maladaptive changes that progress to heart failure (Cowie et al., 2000, Savarese and Lund, 2017).

Importantly, hypertrophic responses to physiological and pathological stress in the heart are driven by changes in gene expression. Such gene expression changes are tightly regulated by DNA binding transcription factors. Hence, it is likely that cardiac transcription factors will also play an important role in mediating hypertrophic responses to pathological and physiological stress. Therefore it will be necessary to identify the molecular regulators that are responsible for mediating the molecular switch from the early adaptive response to later pathological changes in the heart in order to provide better treatments for patients living with heart failure. Thus, it will also be important to determine the effects of loss of these molecular regulators on the hypertrophic response and cardiac function in order to analyse the role of these factors in the heart in response to stress.

As shown in Chapter Four, the POU4F2/Brn3b transcription factor was up-regulated by hypertrophic stimulus AngII in WT mice and is activated by MAP Kinase and Calcineurin signalling, suggesting that it may be required for mediating the pathological hypertrophic response. Interestingly, this also correlated with increased protein levels of Brn3b target genes cyclin D1 and GLUT4 (Maskell, 2018c). Moreover, increased Brn3b levels in hypertrophic hearts/ cardiomyocytes correlated with elevated Bax expression whereas p53 levels remained unchanged. These data suggest that Brn3b may also drive cardiomyocyte apoptosis in the later maladaptive stages of pathological hypertrophy when co-expressed with p53 by up-regulating Bax expression which may lead to heart failure (Maskell, 2018c). This is supported by previous studies which showed that increased Brn3b co-expressed with p53 in the injured myocardium correlated with induction of Bax and Noxa and cardiomyocyte apoptosis (Budhram-Mahadeo et al., 2014). As such, Brn3b may represent a novel foetal gene that is re-expressed during pathological hypertrophy.

As a result it will be important to analyse the effects of loss of Brn3b on the hypertrophic response to pathological stress and confirm its requirement in the stressed heart. Importantly, the cellular and molecular mechanisms that mediate the hypertrophic
response to physiological stress are not fully understood. Brn3b has been shown to mediate the early adaptive hypertrophic response to pathological stress which is similar to hypertrophic responses to physiological stress. Consequently, it will be necessary to determine whether Brn3b is also required for mediating hypertrophic responses to physiological stress and hence the effects of loss of Brn3b in response to physiological stress in the heart. Therefore studies were undertaken in male and female Brn3b KO and WT mice to determine the effects of loss of Brn3b on heart morphology, structure and function in response to pathological and physiological stress. In this regard the aims of this chapter are as follows:

Aims:

- Further determine the role of Brn3b for mediating hypertrophic responses in the heart to pathological stress (AngII treatment) by analysing hypertrophic responses (e.g. changes in LV Mass and HW: BW ratio) to AngII treatment in the hearts of Brn3b KO (and WT littermate controls) male and female mice.

- Investigate whether Brn3b may be required for mediating hypertrophic responses in the heart to physiological stress by analysing hypertrophic responses (e.g. changes in LV Mass and HW: BW ratio) to voluntary exercise in the hearts of Brn3b KO (and WT littermate controls) male and female mice.

- Investigate the effects of loss of Brn3b on cardiac function following pathological and physiological stress by analysing cardiac function in AngII treated and exercised Brn3b KO (and WT littermate controls) male and female mice.
5.1 Results

For all graphs displayed in the following results section, the colour code described below applies:

- Black = WT Male
- Red = Brn3b KO Male
- Purple = WT Female
- Green = Brn3b KO Female

5.1.1 Comparison of baseline cardiac function in Brn3b KO mice with WT controls

To analyse for differences in cardiac function under normal conditions, echocardiography was carried out in 2 month old Brn3b KO and WT control male and female mice (WT Male n=13, Brn3b KO Male n=12, WT Female n=11, Brn3b KO Female n=13) where a variety of functional parameters were analysed (see Materials and Methods – section 2.5).

Figure 5.0 and 5.1 showed that LV Mass was significantly lower in WT female mice when compared to WT male mice (Mann-Whitney Test *p=0.0277). In Brn3b KO male and female mice, LV Mass was slightly lower at baseline when compared to WT controls, but was not statistically significant. Additionally, there were no statistically significant differences between functional parameters in Brn3b KO and WT male and female mice. For instance, similarities in cardiac output, ejection fraction, stroke volume and fractional shortening were observed (see Figure 5.0).
### Table A

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Baseline (M)</th>
<th>Brn3b KO Baseline (M)</th>
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<tr>
<td>Body weight (g)</td>
<td>26.25 ± 2.43</td>
<td>23.65 ± 2.36</td>
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<tr>
<td>LV Mass (mg)</td>
<td>135.20 ± 30.39</td>
<td>118.70 ± 9.62</td>
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<td>Cardiac Output (ml/min)</td>
<td>17.53 ± 8.74</td>
<td>16.27 ± 4.58</td>
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<td>Stroke volume (µl)</td>
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<tr>
<td>Ejection Fraction (%)</td>
<td>50.31 ± 5.24</td>
<td>50.80 ± 10.21</td>
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<tr>
<td>Fractional Shortening (%)</td>
<td>10.58 ± 2.77</td>
<td>11.27 ± 3.67</td>
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<td>Fractional Area Change (%)</td>
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<td>End Systolic volume (µl)</td>
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<td>Aortic root (mm)</td>
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<td>1.33 ± 0.13</td>
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<td>753.62 ± 217.71</td>
</tr>
<tr>
<td>Descending Aorta velocity (mm/s)</td>
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<td>-805.92 ± 155.68</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.48 ± 0.15</td>
<td>1.49 ± 0.25</td>
</tr>
<tr>
<td>LCCA PSV (mm/s)</td>
<td>-265.89 ± 46.73</td>
<td>-262.60 ± 77.61</td>
</tr>
<tr>
<td>RCCA PSV (mm/s)</td>
<td>-233.11 ± 64.31</td>
<td>-242.98 ± 70.92</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Baseline (F)</th>
<th>Brn3b KO Baseline (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>20.15 ± 1.18</td>
<td>19.43 ± 1.25</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>107.94 ± 16.35</td>
<td>105.45 ± 10.88</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>10.43 ± 2.77</td>
<td>11.49 ± 2.54</td>
</tr>
<tr>
<td>Stroke volume (µl)</td>
<td>28.03 ± 6.36</td>
<td>29.46 ± 5.62</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>52.25 ± 8.03</td>
<td>49.50 ± 8.81</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>12.00 ± 4.65</td>
<td>10.70 ± 4.20</td>
</tr>
<tr>
<td>Fractional Area Change (%)</td>
<td>42.92 ± 11.38</td>
<td>41.22 ± 9.93</td>
</tr>
<tr>
<td>End Diastolic volume (µl)</td>
<td>54.08 ± 10.50</td>
<td>60.11 ± 7.94</td>
</tr>
<tr>
<td>End Systolic volume (µl)</td>
<td>26.05 ± 7.19</td>
<td>30.65 ± 8.47</td>
</tr>
<tr>
<td>Aortic root (mm)</td>
<td>1.24 ± 0.10</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>Ascending Aorta velocity (mm/s)</td>
<td>606.70 ± 219.79</td>
<td>607.02 ± 232.41</td>
</tr>
<tr>
<td>Descending Aorta velocity (mm/s)</td>
<td>-656.91 ± 148.33</td>
<td>-662.97 ± 122.55</td>
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<tr>
<td>E/A ratio</td>
<td>1.58 ± 0.39</td>
<td>1.72 ± 0.36</td>
</tr>
<tr>
<td>LCCA PSV (mm/s)</td>
<td>-231.33 ± 78.14</td>
<td>-286.66 ± 56.37</td>
</tr>
<tr>
<td>RCCA PSV (mm/s)</td>
<td>-218.41 ± 78.07</td>
<td>-215.32 ± 54.78</td>
</tr>
</tbody>
</table>

**Figure 5.0:** A: Table showing echocardiography functional parameters of 2 month old Brn3b KO and WT male (M) mice measured at baseline. B: Table showing baseline functional parameters of 2 month old Brn3b KO and WT female (F) mice. A and B: Data represents mean and standard deviation of multiple animals (WT Male n = 13, Brn3b KO Male n = 12, WT Female n = 11, Brn3b KO Female n = 13)
Figure 5.1: Graph showing a slight reduction in LV Mass at baseline in 2 month old Brn3b KO male and female mice when compared to WT controls. LV Mass was significantly lower in WT female mice when compared to WT males (Mann-Whitney Test \( *p=0.0277 \)). Data represents mean and standard error of multiple animals (WT Male n = 13, Brn3b KO Male n = 12, WT Female n = 11, Brn3b KO Female n = 13) ns = not significant.
5.1.2 Cardiac hypertrophy was attenuated in the Brn3b KO male and female mouse following pathological stress

Brn3b has been implicated in hypertrophic responses in the heart (see Chapter Four), therefore similar studies were undertaken in Brn3b KO male and female mice to analyse the effects of loss of Brn3b on the hypertrophic response to pathological stress. Cardiac hypertrophy was induced by treating 2 month old Brn3b KO and WT mice with AngII (4.5mg/kg/day) for 4 weeks (saline was used as a control.) At baseline and 4 weeks following AngII treatment, LV Mass, LV Mass: BW ratio, HW: BW ratio and HW: Tibia length ratios were measured in the hearts of Brn3b KO and WT male/ female mice using echocardiography to determine whether cardiac hypertrophy had been induced (see Figures 5.1-5.4).

As expected, hypertrophy was induced in the hearts of WT male mice following AngII treatment when compared to saline controls, as demonstrated by significant increases in LV Mass, LV Mass: BW ratio and HW: BW ratio (Mann-Whitney test **p=0.0062, **p=0.0062 and *p=0.0451 respectively) (Figure 5.3, 5.4 and 5.5). There was no significant change in LV Mass following AngII treatment in the hearts of Brn3b KO male mice when compared to saline controls but the LV Mass: BW ratio of Brn3b KO AngII treated mice did increase when compared to Brn3b KO saline controls (Mann-Whitney test *p=0.0303). However, it was attenuated when compared to WT AngII treated male mice (Figure 5.2 and 5.3). Therefore these results may suggest that Brn3b is required for the hypertrophic response since attenuated hypertrophy was observed in mice lacking Brn3b.

LV Mass also significantly increased as expected following AngII treatment in the hearts of WT female mice when compared to saline controls, indicating cardiac hypertrophy (Figure 5.3, 5.4 and 5.5) (Mann-Whitney test *p=0.0317). LV Mass: BW ratio also increased in WT female mice following AngII treatment, but was not statistically significant, and there was also no significant change in HW: BW ratio in the hearts of WT AngII treated female mice (Figure 5.5) (this may be due to the variability in HW: BW ratios in the female WT saline control mice). In contrast, Brn3b KO female mice did not appear to undergo hypertrophy following AngII treatment since there was no change in
LV Mass or LV Mass: BW ratio when compared to Brn3b KO saline controls (see Figures 5.2 and 5.3). Consequently, this further suggests that Brn3b may also be required for mediating hypertrophic responses in the hearts of female mice.

<table>
<thead>
<tr>
<th></th>
<th>WT Saline 4 weeks</th>
<th>WT AngII 4 weeks</th>
<th>Brn3b KO Saline 4 weeks</th>
<th>Brn3b KO AngII 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW: Tibia length ratio (Male)</td>
<td>N/A</td>
<td>16.48</td>
<td>N/A</td>
<td>9.61</td>
</tr>
<tr>
<td>HW: Tibia length ratio (Female)</td>
<td>10.83</td>
<td>7.68</td>
<td>8.03 ± 1.82</td>
<td>9.62 ± 1.37</td>
</tr>
</tbody>
</table>

**Figure 5.2: A:** Table showing the available HW: Tibia length BW ratio of Brn3b KO and WT male and female mice following 4 weeks of AngII treatment (mean and standard deviation where multiple values were available). N/A = Not available. Due to low sample numbers (n=1-3 per group) statistical analysis could not be performed, therefore sample numbers need to be increased.
Figure 5.3: A: Graph showing LV Mass following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test **p=0.0062). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=5, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing LV Mass following 4 weeks of AngII treatment in Brn3b KO and WT female mice. (Mann-Whitney Test *p=0.0317). Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
Figure 5.4: A: Graph showing LV Mass: BW ratio following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test **p=0.0062; Mann Whitney Test *p=0.0303) Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=5, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing LV Mass: BW ratio following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
Figure 5.5: A: Graph showing HW: BW ratio following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test *p=0.0451) Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=5, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing HW: BW ratio following 4 weeks of AngII treatment in Brn3b KO and WT female mice (Mann-Whitney Test *p=0.0350) Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
<table>
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<tr>
<th></th>
<th>WT Saline (M)</th>
<th>WT AngII (M)</th>
<th>Brn3b KO Saline (M)</th>
<th>Brn3b KO AngII (M)</th>
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<tr>
<td>Body weight (g)</td>
<td>27.26 ± 2.21</td>
<td>27.94 ± 1.49</td>
<td>27.46 ± 2.05</td>
<td>25.73 ± 1.90</td>
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<tr>
<td>LV Mass (mg)</td>
<td>98.88 ± 9.94</td>
<td>152.54 ± 32.44</td>
<td>101.55 ± 24.52</td>
<td>132.83 ± 20.34</td>
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<tr>
<td>Cardiac Output (ml/min)</td>
<td>14.45 ± 4.32</td>
<td>18.62 ± 4.46</td>
<td>23.08 ± 7.88</td>
<td>17.25 ± 2.22</td>
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<tr>
<td>Stroke volume (µl)</td>
<td>38.21 ± 10.78</td>
<td>38.90 ± 6.31</td>
<td>45.46 ± 8.90</td>
<td>36.84 ± 3.89</td>
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<tr>
<td>Ejection Fraction (%)</td>
<td>50.75 ± 11.01</td>
<td>42.75 ± 9.52</td>
<td>54.65 ± 5.12</td>
<td>*45.53 ± 4.57</td>
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<tr>
<td>End Diastolic volume (µl)</td>
<td>75.34 ± 12.68</td>
<td>*92.85 ± 14.80</td>
<td>83.34 ± 15.63</td>
<td>81.43 ± 10.23</td>
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<tr>
<td>End Systolic volume (µl)</td>
<td>37.13 ± 11.36</td>
<td>53.95 ± 14.00</td>
<td>37.88 ± 9.33</td>
<td>44.59 ± 7.98</td>
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<td>Fractional Shortening (%)</td>
<td>35.67 ± 4.16</td>
<td>24.09 ± 3.72</td>
<td>32.75 ± 4.03</td>
<td>27.19 ± 5.76</td>
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<tr>
<td>Fractional Area Change (%)</td>
<td>45.59 ± 10.79</td>
<td>36.06 ± 8.66</td>
<td>42.85 ± 9.50</td>
<td>40.07 ± 8.01</td>
</tr>
<tr>
<td>Aortic root (mm)</td>
<td>1.36 ± 0.13</td>
<td>*1.60 ± 0.21</td>
<td>1.39 ± 0.18</td>
<td>1.57 ± 0.30</td>
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<tr>
<td>Ascending Aorta velocity (mm/s)</td>
<td>762.37 ± 170.76</td>
<td>1382.72 ± 628.37</td>
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<td>1134.74 ± 397.43</td>
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<tr>
<td>Descending Aorta velocity (mm/s)</td>
<td>-813.43 ± 88.58</td>
<td>-846.96 ± 141.10</td>
<td>-859.49 ± 38.57</td>
<td>-861.28 ± 112.50</td>
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<td>E/A ratio</td>
<td>1.40 ± 0.16</td>
<td>1.68 ± 0.43</td>
<td>1.52 ± 0.14</td>
<td>1.50 ± 0.31</td>
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<tr>
<td>LCCA PSV (mm/s)</td>
<td>-269.21 ± 115.20</td>
<td>-205.45 ± 47.47</td>
<td>-298.35 ± 83.75</td>
<td>-255.01 ± 78.63</td>
</tr>
<tr>
<td>RCCA PSV (mm/s)</td>
<td>-230.58 ± 45.15</td>
<td>*-161.16 ± 39.65</td>
<td>-221.05 ± 30.43</td>
<td>-263.18 ± 115.31</td>
</tr>
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</table>

Figure 5.6: Table showing functional parameters of Brn3b KO and WT male (M) mice following 4 weeks of AngII treatment measured using echocardiography and ultrasound (Ejection fraction *Mann Whitney Test Brn3b KO saline vs Brn3b KO AngII p=0.0101) (Diastolic volume *Mann Whitney Test WT Saline vs WT AngII p=0.0451) (Fractional shortening *Mann Whitney Test Brn3b KO Saline vs Brn3b KO AngII p=0.0480) (Aortic root diameter *Mann Whitney Test WT saline vs WT AngII p=0.0451) (RCCA PSV *Mann Whitney Test WT saline vs WT AngII p=0.0109) Data represents mean and standard deviation of multiple animals: WT and Brn3b KO saline n=5; WT AngII n=8; Brn3b KO AngII n=7
<table>
<thead>
<tr>
<th></th>
<th>WT Saline (F)</th>
<th>WT AngII (F)</th>
<th>Brn3b KO Saline (F)</th>
<th>Brn3b KO AngII (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>21.32 ± 1.10</td>
<td>23.90 ± 1.68</td>
<td>22.30 ± 1.38</td>
<td>21.40 ± 1.84</td>
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<tr>
<td><strong>LV Mass (mg)</strong></td>
<td>84.95 ± 5.67</td>
<td>110.32 ± 22.66</td>
<td>93.19 ± 19.50</td>
<td>95.69 ± 27.24</td>
</tr>
<tr>
<td><strong>Cardiac Output (ml/min)</strong></td>
<td>14.17 ± 3.91</td>
<td>13.31 ± 1.64</td>
<td>14.06 ± 1.63</td>
<td>15.04 ± 2.94</td>
</tr>
<tr>
<td><strong>Stroke volume (µl)</strong></td>
<td>30.17 ± 6.15</td>
<td>31.58 ± 3.87</td>
<td>29.79 ± 3.43</td>
<td>31.04 ± 4.57</td>
</tr>
<tr>
<td><strong>Ejection Fraction (%)</strong></td>
<td>53.85 ± 3.51</td>
<td>47.82 ± 5.88</td>
<td>49.46 ± 9.94</td>
<td>46.80 ± 5.68</td>
</tr>
<tr>
<td><strong>End Diastolic volume (µl)</strong></td>
<td>55.82 ± 9.00</td>
<td>66.20 ± 4.71</td>
<td>61.71 ± 10.77</td>
<td>67.97 ± 17.94</td>
</tr>
<tr>
<td><strong>End Systolic volume (µl)</strong></td>
<td>25.66 ± 3.67</td>
<td>*34.62 ± 5.23</td>
<td>31.92 ± 10.81</td>
<td>36.93 ± 13.93</td>
</tr>
<tr>
<td><strong>Fractional Shortening (%)</strong></td>
<td>34.03 ± 6.30</td>
<td>29.76 ± 2.50</td>
<td>32.57 ± 2.23</td>
<td>27.64 ± 7.84</td>
</tr>
<tr>
<td><strong>Fractional Area Change (%)</strong></td>
<td>46.43 ± 5.75</td>
<td>38.22 ± 7.06</td>
<td>45.99 ± 8.58</td>
<td>41.10 ± 8.13</td>
</tr>
<tr>
<td><strong>Aortic root (mm)</strong></td>
<td>1.37 ± 0.13</td>
<td>1.45 ± 0.10</td>
<td>1.21 ± 0.05</td>
<td><strong>1.44 ± 0.13</strong></td>
</tr>
<tr>
<td><strong>Ascending Aorta velocity (mm/s)</strong></td>
<td>657.88 ± 96.03</td>
<td>825.18 ± 342.55</td>
<td>640.40 ± 75.93</td>
<td><strong>1089.64 ± 363.28</strong></td>
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<tr>
<td><strong>Descending Aorta velocity (mm/s)</strong></td>
<td>-733.84 ± 64.03</td>
<td>-612.74 ± 60.27</td>
<td>-817.40 ± 84.61</td>
<td>-819.52 ± 164.57</td>
</tr>
<tr>
<td><strong>E/A ratio</strong></td>
<td>1.51 ± 0.13</td>
<td>1.52 ± 0.24</td>
<td>1.36 ± 0.06</td>
<td>1.61 ± 0.31</td>
</tr>
<tr>
<td><strong>LCCA PSV (mm/s)</strong></td>
<td>-379.32 ± 95.31</td>
<td>-236.71 ± 93.75</td>
<td>-377.13 ± 102.61</td>
<td>-346.96 ± 122.59</td>
</tr>
<tr>
<td><strong>RCCA PSV (mm/s)</strong></td>
<td>-262.78 ± 17.80</td>
<td>-195.40 ± 60.24</td>
<td>-333.09 ± 60.21</td>
<td>-254.92 ± 65.53</td>
</tr>
</tbody>
</table>

**Figure 5.7:** Table showing functional parameters of Brn3b KO and WT female (F) mice following 4 weeks of AngII treatment measured using echocardiography and ultrasound (End Systolic volume Mann-Whitney Test: WT saline vs WT AngII *p=0.0159) (Aortic root diameter Mann-Whitney Test: Brn3b KO saline vs Brn3b KO AngII **p=0.0047) (Ascending aorta velocity Mann-Whitney Test: Brn3b KO saline vs Brn3b KO AngII **p=0.0012) Data represents mean and standard deviation of multiple animals: WT Saline n=5; Brn3b KO saline n=6; WT AngII n=5; Brn3b KO AngII n=7 ns = not significant
5.1.3 Cardiac function and systolic function were reduced in the Brn3b KO male mouse, but not in the Brn3b KO female mouse following AngII treatment

Cardiac hypertrophy was attenuated in Brn3b KO male and female mice following treatment with pathological stimulus AngII. Therefore echocardiography and ultrasound was undertaken in AngII treated male and female Brn3b KO and WT mice to determine the effects of loss of Brn3b on heart function following pathological stress.

Following AngII treatment, cardiac output and stroke volume were unchanged in the hearts of WT male mice when compared to saline controls, whereas these parameters were reduced in Brn3b KO males, (Stroke volume Mann-Whitney Test Brn3b KO saline vs Brn3b KO AngII *p=0.0379) but the changes were not statistically significant (see Figure 5.6). In addition, there were trends towards decreased ejection fraction in the hearts of WT mice following AngII treatment, but was not statistically significant. Similarly, ejection fraction significantly decreased in the hearts of Brn3b KO male mice following AngII treatment when compared to Brn3b KO saline controls (Mann-Whitney Test *p=0.0070) (Figure 5.6 and 5.8). Moreover, end diastolic volume significantly increased in the hearts of WT male mice following AngII treatment when compared to saline controls (Mann-Whitney Test *WT saline vs WT AngII p=0.0289), whereas there was no significant change in end diastolic volume in Brn3b KO AngII treated male mice (Figure 5.10). As such, these results suggest that loss of Brn3b may affect the ability of male mice to adapt their heart in response to pathological stress.

Cardiac output and stroke volume were also unchanged in the hearts of WT female mice following AngII treatment. Similarly, these parameters were also unchanged in the hearts of AngII treated Brn3b KO female mice (Figure 5.7). Whereas there were trends towards decreased ejection fraction in the hearts of WT AngII treated female mice (which was not statistically significant), there was no significant change in ejection fraction following AngII treatment in Brn3b KO female mice when compared to Brn3b KO saline controls. Ejection fraction in the hearts of Brn3b KO AngII treated female mice was also comparable to the ejection fraction of WT female mice following AngII treatment (see Figure 5.9 (B)). Moreover, similar to WT female mice, end diastolic
volume increased following AngII treatment in the hearts of Brn3b KO female mice when compared to saline controls (Figure 5.7 and 5.10 (B)), however these changes were not statistically significant. Thus, these results suggest that the hearts of female mice are able to adapt to pathological stress in the absence of Brn3b.
Figure 5.8: A: Graph showing stroke volume following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test Brn3b KO saline vs Brn3b KO AngII \( *p=0.0379 \)). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline \( n=7 \), WT AngII \( n=8 \) Brn3b KO AngII \( n=7 \)). B: Graph showing ejection fraction following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Data represents mean and standard error of multiple animals (WT saline \( n=5 \), Brn3b KO saline \( n=6 \) WT AngII \( n=5 \) Brn3b KO AngII \( n=7 \)). ns = not significant
**Figure 5.9:**

**A:** Graph showing ejection fraction following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test Brn3b KO saline vs Brn3b KO AngII **p=0.0070**). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=7, WT AngII n=8 Brn3b KO AngII n=7) ns = not significant.

**B:** Graph showing ejection fraction following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant.
Figure 5.10: A: Graph showing end diastolic volume following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test WT saline vs WT AngII *p=0.0279). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=7, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing end diastolic volume following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
End systolic volume also increased following AngII treatment in the hearts of WT male mice (Mann Whitney test WT saline vs WT AngII p=0.0401) which correlated with trends towards reduced fractional shortening and fractional area change, but the changes were not statistically significant. Increased end systolic volume is linked to cardiac systolic function because it means that the heart is inefficient at ejecting blood from the heart since the majority of blood in the heart remains in the ventricle and has not been pumped to the rest of the body during systole. End systolic volume also increased in the hearts of Brn3b KO AngII treated male mice, but was not statistically significant (see Figures 5.6 and 5.11). As such, these results suggest that loss of Brn3b affects systolic function in male mice following pathological stress.

However, in the hearts of WT female mice, end systolic volume significantly increased following AngII treatment when compared to saline controls (Mann-Whitney Test *p=0.0159). Additionally, there were trends towards reduced fractional shortening and fractional area change in the hearts of WT AngII treated female mice, but the changes were not statistically significant. Interestingly, there was no change in end systolic volume, fractional shortening and fractional area change in the hearts of Brn3b KO female mice following AngII treatment when compared to saline controls (see Figures 5.6 and 5.10). Interestingly, these results indicate that systolic function may be preserved in the Brn3b KO female mouse following pathological stress.
Figure 5.11: A: Graph showing end systolic volume following 4 weeks of AngII treatment in Brn3b KO and WT male mice. Mann-Whitney Test WT Saline vs WT AngII *p=0.0401 Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=7, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing fractional shortening following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Mann-Whitney Test WT Saline vs WT AngII *p=0.0159. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
5.1.4 AngII treatment also caused changes in blood flow in the Brn3b KO mouse

Activation of AngII signalling triggers pathological cardiac hypertrophy by inducing hypertension and as such has multiple effects on blood flow. For instance, AngII causes dilation of the lumen of the aorta and hence elevation of blood flow velocity through the aorta; however it reduces blood flow velocity through the carotid arteries (Barisione et al., 2006, Loquet et al., 1991). Moreover, earlier studies using PV loop analysis and tail cuff plethysmography showed increased blood pressure in Brn3b KO mice under normal conditions (Budhram-Mahadeo, unpublished data). Therefore following 4 weeks of AngII treatment, blood flow velocity was measured in Brn3b KO and WT male and female mice using ultrasound (with the Doppler pulse wave mode) to determine the effects of loss of Brn3b on blood flow velocity in response to pathological stress. Evidently, changes in blood flow parameters (velocity, etc.) were also used to confirm elevation of blood pressure by AngII.

Following AngII treatment, the diameter of the aortic root lumen significantly increased in the hearts of WT male mice when compared to saline controls (Mann-Whitney Test *p=0.0451) (Figure 5.10). Blood flow velocity through the ascending and descending aorta also increased in in the hearts of WT AngII treated male mice, but the changes were not statistically significant (Figure 5.6 and 5.11). Interestingly, the blood flow velocity through the right common carotid artery (RCCA) significantly decreased following AngII treatment in WT male mice when compared to saline controls (Mann-Whitney Test *p=0.0109) (Figure 5.6 and 5.12). Conversely, there was no change in the diameter of the aortic root lumen or blood flow velocity through the ascending aorta in the hearts of Brn3b KO male mice following AngII treatment when compared to saline controls. However, blood flow velocity through the ascending aorta and RCCA increased in Brn3b KO AngII treated male mice when compared to saline controls (Figure 5.10).

In WT female mice, there was no change in the diameter of the aortic root lumen following AngII treatment. Blood flow velocity through the ascending aorta increased whereas it significantly decreased through the descending aorta in the hearts of WT AngII treated female mice (Mann-Whitney Test: *p=0.0317). Blood flow velocity through
the RCCA also decreased following AngII treatment in WT female mice (but was not statistically significant). On the other hand, the diameter of the aortic root lumen increased in the hearts of Brn3b KO female mice following AngII treatment (Figure 5.10) (Mann-Whitney Test **p=0.0047). Blood flow velocity through the ascending aorta also significantly increased in the hearts of Brn3b KO AngII treated female mice when compared to saline controls (Mann-Whitney Test **p=0.0012) (Figure 5.11). Moreover, blood flow through the descending aorta velocity remained unchanged in the hearts of Brn3b KO AngII treated female mice, whereas blood flow through the RCCA decreased (but was not statistically significant) (Figure 5.12).
Figure 5.12: A: Graph showing aortic root diameter following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test WT saline vs WT AngII *p=0.0278). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=7, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing aortic root diameter following 4 weeks of AngII treatment in Brn3b KO and WT female mice. (Mann-Whitney Test Brn3b KO saline vs Brn3b KO AngII **p=0.0047. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant
Figure 5.13: A: Graph showing ascending aorta velocity following 4 weeks of AngII treatment in Brn3b KO and WT male mice. Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=6, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing ascending aorta velocity following 4 weeks of AngII treatment in Brn3b KO and WT female mice. (Mann-Whitney Test Brn3b KO saline vs Brn3b KO AngII **p=0.0012). Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant.
**Figure 5.14:** A: Graph showing Right Common Carotid Artery Peak Systolic velocity (RCCA PSV) following 4 weeks of AngII treatment in Brn3b KO and WT male mice. (Mann-Whitney Test WT saline vs WT AngII **p=0.0093). Data represents mean and standard error of multiple animals (WT and Brn3b KO saline n=7, WT AngII n=8 Brn3b KO AngII n=7) B: Graph showing RCCA PSV following 4 weeks of AngII treatment in Brn3b KO and WT female mice. Data represents mean and standard error of multiple animals (WT saline n=5, Brn3b KO saline n=6 WT AngII n=5 Brn3b KO AngII n=7) ns = not significant.
5.15 Analysis of ECM deposition in the heart and surrounding blood vessels of Brn3b KO mice following pathological stress

Changes in heart function and blood flow velocity following AngII treatment were observed in both male and female Brn3b KO mice. Therefore morphological changes in the heart were analysed on formalin fixed 5-10µm Brn3b KO and WT AngII treated male and female heart sections using Masson’s trichrome staining (for collagen and ECM deposition). Collagen and ECM deposition is represented by blue staining (see Materials and Methods).

There was minimal collagen and ECM deposition in the hearts of WT saline control male mice, with some staining surrounding the blood vessels (BV) of the heart as expected. In addition, low collagen and ECM deposition was observed in the ventricles and surrounding the blood vessels of Brn3b KO saline male hearts (Figure 5.15).

On the other hand, collagen and ECM deposition appeared to be increased in the ventricles and surrounding the blood vessels in the hearts of WT mice following AngII treatment (Figure 5.16). Interestingly, collagen and ECM deposition also appeared increased in the ventricles of Brn3b KO AngII treated male hearts when compared to Brn3b KO saline and WT littermate controls treated with AngII. However, while collagen and ECM deposition was visible in the hearts of Brn3b KO and WT AngII treated mice, it was impossible to quantify this (e.g. by using ImageJ). This was because there were no focal regions of ECM in these hearts and since there was minimal detectable ECM in the hearts of Brn3b KO and WT saline control hearts (and hence could not be quantified). As such it is not possible to compare ECM deposition accurately between the hearts of AngII treated and saline control mice. Therefore additional images have been included which highlight the uniform distribution of ECM deposition in the hearts of Brn3b KO and WT AngII treated and saline control mice (produced using ImageJ – Figure 5.17).

Collagen and ECM deposition was also visible around the blood vessels in the hearts of Brn3b KO AngII treated male mice, suggesting that loss of Brn3b may result changes in morphology in the Brn3b KO male heart and blood vessels in response to pathological stress which may cause reduced systolic function (Figure 5.16)
**Figure 5.15:** A: Representative image of a WT saline control male heart (n=3) stained with Masson’s trichrome showing low levels of collagen and ECM deposition (blue) in the ventricle and surrounding blood vessels (BV). B: Representative image of a Brn3b KO saline control male heart (n=3) stained with Masson’s trichrome showing collagen and ECM deposition in the ventricle and surrounding blood vessels (BV).
Figure 5.16: A: Representative image of a WT AngII treated male heart (n=3) stained with Masson’s trichrome showing increased levels of collagen and ECM deposition (blue) in the ventricle and surrounding blood vessels (BV). B: Representative image of a Brn3b KO AngII treated male heart (n=3) stained with Masson’s trichrome showing higher levels of collagen and ECM deposition in the ventricle and surrounding blood vessels (BV).
Figure 5.17: A: Representative colour threshold images (acquired and transformed using ImageJ) of a WT saline (top left), Brn3b KO saline (top right), WT AngII (bottom left) and Brn3b KO AngII male heart (n=3) stained with Masson’s trichrome highlighting collagen and ECM deposition (blue) in the ventricle since this was unquantifiable.
Similarly, there was no significant ECM deposition in the hearts of WT and Brn3b KO Saline control female mice, but it was detected surrounding the blood vessels of these hearts. Following AngII treatment, ECM deposition was evident in the hearts of WT female mice (as expected) since collagen and ECM deposition appeared increased in the ventricles of these mice when compared to WT saline controls (Figures 5.18 and 5.19). This was also evident surrounding the blood vessels in the hearts of WT female AngII treated mice.

In the hearts of Brn3b KO female mice treated with AngII, collagen deposition also appeared increased in the ventricles when compared to Brn3b KO saline control female mice, but was comparable to the amount of collagen and ECM deposition observed in WT AngII treated female mice (Figure 5.18 and Figure 5.19). This may suggest that loss of Brn3b in female mice may not result in cardiac fibrosis following AngII treatment.

Unfortunately however, there were no focal regions of ECM deposition in the ventricles of the AngII treated Brn3b KO and WT hearts; and there was minimal detectable ECM in the hearts of Brn3b KO and WT saline control hearts (and hence could not be quantified). As such it is not possible to quantify and compare ECM deposition accurately between the hearts of AngII treated and saline control mice (e.g. by using ImageJ). Therefore additional images have been included which highlight the uniform distribution of ECM deposition in the hearts of Brn3b KO and WT AngII treated and saline control mice (produced using ImageJ – Figure 5.20). However, increased collagen and ECM deposition was evident surrounding the blood vessels in the hearts of Brn3b KO AngII treated female mice when compared to Brn3b KO saline control females, suggesting that like males, loss of Brn3b may result in increased collagen deposition in the vasculature of female mice following AngII treatment.
Figure 5.18: A: Representative image of a WT saline control female heart (n=3) stained with Masson’s trichrome showing low levels of collagen and ECM deposition (blue) in the ventricle and surrounding blood vessels (BV). B: Representative image of a Brn3b KO saline control female heart (n=3) stained with Masson’s trichrome showing collagen and ECM deposition in the ventricle and surrounding blood vessels.
Figure 5.19: A: Representative image of a WT AngII treated female heart (n=3) stained with Masson’s trichrome showing increased levels of collagen and ECM deposition (blue) in the ventricle and surrounding blood vessels (BV). B: Representative image of a Brn3b KO AngII treated female heart (n=3) stained with Masson’s trichrome showing higher levels of collagen and ECM deposition in the surrounding blood vessels (BV) of the left ventricle.
**Figure 5.20: A:** Representative colour threshold images (acquired and transformed using ImageJ) of a WT saline (top left), Brn3b KO saline (top right), WT AngII (bottom left) and Brn3b KO AngII female heart (n=3) stained with Masson’s trichrome highlighting collagen and ECM deposition (blue) in the ventricle since this was unquantifiable.
**5.1.6 Changes in Brn3b target genes in the hearts of Brn3b KO mice following AngII treatment**

AngII treatment up-regulated Brn3b mRNA and protein in the hearts of WT mice, which correlated with elevated levels of Brn3b target genes GLUT4, cyclin D1 and Bax and p53 protein which are all expressed during the hypertrophic response to pathological stress (Tamamori-Adachi et al., 2002, Abel et al., 1999, Long et al., 1997, McMullen and Jennings, 2007). Therefore Brn3b target genes were assessed by analysing mRNA levels of GLUT4, cyclin D1, Bax and hypertrophic marker β-MHC in female Brn3b KO and WT AngII treated hearts using qRT-PCR.

As previously observed, Brn3b mRNA levels were significantly elevated in the hearts of WT mice following 4 weeks of AngII treatment (Mann-Whitney Test WT saline vs WT AngII *p=0.0283), whereas Brn3b mRNA was not detected in the hearts of Brn3b KO AngII treated and Saline control female mice (Figure 5.21). In addition, the hypertrophic hearts of WT AngII treated female mice had significantly elevated levels of hypertrophic marker β-MHC mRNA (*Mann Whitney WT saline vs WT AngII p=0.0219) (Figure 5.22). Moreover, there were trends towards increased mRNA levels of GLUT4, cyclin D1 (not statistically significant) and Bax (Figures 5.23-5.25) in the hearts of WT female mice following AngII treatment when compared to Saline controls (Mann-Whitney test Bax WT saline vs WT AngII *p=0.0126).

Conversely, the hearts of Brn3b KO AngII treated female mice had reduced mRNA levels of hypertrophic marker β-MHC (Figure 5.22). These data provide further evidence that the hearts of these mice did not undergo hypertrophy since reduced β-MHC levels correlated with reduced LV Mass and LV Mass: BW ratios in Brn3b KO AngII treated female mice. Therefore these results further suggest that Brn3b may be required for the hypertrophic response to pathological stress in females. Interestingly, there were trends towards elevated GLUT4 and cyclin D1 mRNA levels in the hearts of Brn3b KO AngII treated female mice when compared to saline controls (but the changes were not statistically significant) (Figure 5.23 and 5.24). However, Bax mRNA levels were reduced in the hearts of Brn3b KO AngII treated mice whereas they were significantly increased in WT AngII treated female mice (*Mann Whitney WT saline vs WT AngII p=0.034)
(Figure 5.25). Whilst these results are interesting, it is important to note that the data presented for GLUT4 and cyclin D1 are preliminary studies, therefore ‘n’ numbers must be increased in order to validate these observations.

**Figure 5.21:** Graph showing Brn3b mRNA levels in the hearts of Brn3b KO and WT AngII/ saline treated mice. GAPDH was used as a housekeeping gene. (Mann-Whitney Test WT saline vs WT AngII *p=0.0283). Data represents mean and standard error of multiple animals: WT saline n=5; WT AngII n=5; Brn3b KO saline n=5; Brn3b KO AngII n=5)

**Figure 5.22:** Graph showing β-MHC mRNA levels in the hearts of Brn3b KO and WT AngII/ saline treated mice. GAPDH was used as a housekeeping gene. (Mann-Whitney Test WT saline vs WT AngII *p=0.0219). Data represents mean and standard error of multiple animals: WT saline n=5; WT AngII n=5; Brn3b KO saline n=5; Brn3b KO AngII n=5)
Figure 5.23: Graph showing GLUT4 mRNA levels in the hearts of Brn3b KO and WT AngII treated/ saline control mice. GAPDH was used as a housekeeping gene. Data represents mean and standard error of multiple animals: WT saline n=3; WT AngII n=1; Brn3b KO saline n=3; Brn3b KO AngII n=3). These data are preliminary results therefore ‘n’ numbers must be increased to validate these observations.

Figure 5.24: Graph showing cyclin D1 mRNA levels in the hearts of Brn3b KO and WT AngII treated/ saline control mice. GAPDH was used as a housekeeping gene. Data represents mean and standard error of multiple animals: WT saline n=3; WT AngII n=1; Brn3b KO saline n=3; Brn3b KO AngII n=3). These data are preliminary results therefore ‘n’ numbers must be increased to validate these observations.

Figure 5.25: Graph showing Bax mRNA levels in the hearts of Brn3b KO and WT AngII/ saline treated mice. GAPDH was used as a housekeeping gene. (Mann-Whitney Test WT saline vs WT AngII *p=0.0126. Data represents mean and standard error of multiple animals: WT saline n=5; WT AngII n=5; Brn3b KO saline n=5; Brn3b KO AngII n=5)
5.1.7 Exercise performance was reduced in Brn3b KO male mice but was enhanced in Brn3b KO female mice

The involvement of Brn3b in response to physiological stress is not yet known. Therefore this was investigated by voluntarily exercising 2 month old Brn3b KO and WT male and female mice for 4 weeks. During the 4th week of exercise, average speed and cumulative distance were recorded for 7 days using Lafayette software to determine whether loss of Brn3b affected running speed and distance during voluntary exercise.

Intriguingly, there were striking differences in exercise performance between Brn3b KO male and female mice when compared to WT littermate controls. In WT male mice following 4 weeks of exercise, cumulative distance increased from day 1 to day 7 of the 4th week of exercise and average speed was unchanged (Average speed from day 1 to day 7 was approximately 10m/min). However, following 4 weeks of voluntary exercise in Brn3b KO male mice, average speed was significantly reduced when compared to WT littermate controls (Mann-Whitney Test: WT vs Brn3b KO Exercise Day 1 **p=0.0079, Day 2 *p=0.0159, Day 3 *p=0.0159, Day 4 *p=0.0079, Day 5 **p=0.0079, Day 6 **p=0.0079, Day 7 *p=0.0317) (Figure 5.26). Cumulative distance was also reduced in Brn3b KO male mice during days 1 to 7 of voluntary exercise when compared to WT controls (was not statistically significant during days 2 to 7), and was significantly lower at day 1 (Mann-Whitney Test: **Brn3b KO Exercise vs WT Exercise p=0.0079) (Figure 5.27).

In WT female mice following 4 weeks of voluntary exercise, cumulative distance also increased from day 1 to day 7 of the 4th week of exercise and average speed was unchanged (Average speed from day 1 to day 7 was approximately 10m/min). In contrast, there was no significant difference in the average speed recorded during days 1 to 5 between Brn3b KO and WT female mice during the 4th week of exercise. However, higher average running speeds were recorded during days 6 and day 7 of the 4th week of exercise when compared to WT controls (but the differences were not statistically significant) (Figure 5.26). Interestingly, cumulative distance was higher in Brn3b KO female mice during days 1 to 7 of the 4th week of voluntary exercise when compared to WT controls, however the differences were not statistically significant (Figure 5.27).
summary, these results may suggest that Brn3b KO male mice are not able to adapt to exercise as well as their WT littermates, whereas Brn3b KO females are able to adapt to physiological stress. Thus, loss of Brn3b may affect adaptation to exercise in male mice but not in female mice.

Figure 5.26: A: Graph showing the average speed of Brn3b KO and WT male mice during days 1 to 7 of the 4th week of exercise. Brn3b KO male mice displayed significantly lower average running speeds when compared to WT littermate controls (Mann-Whitney Test: WT vs Brn3b KO Exercise Day 1 **p=0.0079, Day 2 *p=0.0159, Day 3 *p=0.0159, Day 4 *p=0.0079, Day 5 **p=0.0079, Day 6 **p=0.0079, Day 7 *p=0.0317). B: Graph showing the average speed of Brn3b KO and WT female mice during days 1 to 7 of the 4th week of exercise. Data represents mean and standard error of multiple animals: WT Saline male n=5; WT Exercise Male n=6; Brn3b KO Saline n=5; Brn3b KO Exercise male n=6; WT Saline female n=5; WT Exercise female n=6; Brn3b KO Saline female n=6; Brn3b KO Exercise female n=6.
Figure 5.27: A: Graph showing cumulative distance of Brn3b KO and WT male mice during days 1 to 7 of the 4th week of exercise. Brn3b KO male mice displayed significantly lower cumulative distance at Day 1 when compared to WT littermate controls (**Mann-Whitney Test p=0.0079)

B: Graph showing cumulative distance of Brn3b KO and WT female mice during days 1 to 7 of the 4th week of exercise. Data represents mean and standard error of multiple animals: WT Saline male n=5; WT Exercise Male n=6; Brn3b KO Saline n=5; Brn3b KO Exercise male n=6; WT Saline female n=5; WT Exercise female n=6; Brn3b KO Saline female n=6; Brn3b KO Exercise female n=6.
5.1.8 Analysis of cardiac hypertrophy in the Brn3b KO mouse following exercise

The potential role of Brn3b in mediating hypertrophic responses in the heart to physiological stress is unknown. Therefore to determine whether Brn3b may also be required for mediating the hypertrophic response following physiological stress, echocardiography and ultrasound was performed on 2 month old Brn3b KO and WT male and female mice that were subjected to 4 weeks of voluntary exercise to assess cardiac function. At the end of the experiment LV Mass and LV Mass: BW ratio were also measured (using echocardiography and ultrasound) to determine whether cardiac hypertrophy had taken place. Exercised mice were compared to Saline treated control mice, which were sedentary and age matched to the exercised mice and were used as a control in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice.

Following 4 weeks of exercise, there was no significant increase in LV Mass and LV Mass: BW ratio in WT male mice when compared to saline controls. Similarly, the hearts of Brn3b KO male mice did not undergo hypertrophy following 4 weeks of exercise since there was no change in LV Mass or LV Mass: BW ratio when compared to Brn3b KO saline controls (Figure 5.28 (A)).

Similarly, there was no significant change in LV Mass or LV Mass: BW ratio in WT female mice following 4 weeks of exercise, suggesting that the hearts of these mice had not undergone hypertrophy. Similar to males, the hearts of Brn3b KO female mice also did not undergo cardiac hypertrophy following 4 weeks of exercise since LV Mass and LV Mass: BW ratio were also unchanged when compared to Brn3b KO saline control mice, (Figure 5.28 (B)).

In the literature, the expected degree of hypertrophy following 4 weeks of voluntary exercise in C57BL/6 has been shown to be between 5% and 15% (Allen et al., 2001, Konhilas et al., 2015). In addition, studies undertaken by Allen and colleagues showed that the average daily distance run by C57BL/6 mice was 6.8km and their average speed during the 4th week of exercise was 32 meters per minute (Allen et al., 2001). However,
the average speed of the Brn3b KO and WT male and female mice during the 4th week of voluntary exercise was between 3 and 15 metres per minute (Figure 5.25). As such, the reason for the lack of cardiac hypertrophy observed in these mice may be because the intensity of the voluntary exercise may not have been as high as in the literature due to the lower running speeds of the mice in these studies and since the exercise was voluntary. Therefore it will be important to optimise the duration of voluntary exercise for the mice in these studies (e.g. increase the length of voluntary exercise) in order to achieve the same exercise intensity and hypertrophic responses as reported in the literature (Allen et al., 2001, Konhilas et al., 2015). This will also enable the requirement of Brn3b in the hypertrophic response following physiological stress to be analysed in more detail.
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<td>WT Exercise 4 weeks</td>
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**Figure 5.28:** A: Table showing LV Mass LV Mass: BW ratio, HW: BW ratio and HW: Tibia length ratios of Brn3b KO and WT male mice following 4 weeks of voluntary exercise. B: Table showing LV Mass, LV Mass: BW ratio, HW: BW ratio and HW: Tibia length of Brn3b KO and WT male mice following 4 weeks of voluntary exercise of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. A and B: Exercised mice were compared to Saline treated control mice, which were sedentary and age matched to the exercised mice and were used as a control in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard deviation of multiple animals (when available): WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5. Mann-Whitney test was performed between WT saline and WT Exercise and Brn3b KO saline and Brn3b KO Exercise groups. ** Mann-Whitney test Brn3b KO saline vs Brn3b KO Exercise male HW: BW ratio p=0.0087 N/A = Not available. Due to low sample numbers for tibia length (n=1-3 per group) statistical analysis could not be performed, therefore sample numbers need to be increased.
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Figure 5.29: Table showing functional parameters of Brn3b KO and WT male (M) mice following 4 weeks of voluntary exercise (*Mann-Whitney Test: WT Saline vs WT Exercise p=0.0173). Exercised mice were compared to sedentary age matched Saline control mice in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard deviation of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5.
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</tr>
<tr>
<td><strong>LV Mass (mg)</strong></td>
<td>84.95 ± 5.67</td>
<td>103.22 ± 19.23</td>
<td>93.19 ± 19.50</td>
<td>89.80 ± 18.49</td>
</tr>
<tr>
<td><strong>Cardiac Output</strong></td>
<td>14.17 ± 3.91</td>
<td>18.03 ± 11.56</td>
<td>14.06 ± 1.63</td>
<td>21.13 ± 9.80</td>
</tr>
<tr>
<td><strong>Stroke volume</strong></td>
<td>30.17 ± 6.15</td>
<td>34.39 ± 8.78</td>
<td>29.79 ± 3.43</td>
<td><strong>40.19 ± 7.23</strong></td>
</tr>
<tr>
<td><strong>Ejection Fraction (%)</strong></td>
<td>53.85 ± 3.51</td>
<td>47.26 ± 10.70</td>
<td>49.46 ± 9.94</td>
<td>53.71 ± 5.78</td>
</tr>
<tr>
<td><strong>Fractional Shortening (%)</strong></td>
<td>12.67 ± 3.62</td>
<td>10.52 ± 5.44</td>
<td>10.80 ± 4.17</td>
<td>11.02 ± 4.48</td>
</tr>
<tr>
<td><strong>Fractional Area Change (%)</strong></td>
<td>46.43 ± 5.75</td>
<td>38.08 ± 12.82</td>
<td>45.99 ± 8.58</td>
<td>47.46 ± 7.40</td>
</tr>
<tr>
<td><strong>End Diastolic volume (µl)</strong></td>
<td>55.82 ± 9.00</td>
<td>*72.51 ± 6.63</td>
<td>61.71 ± 10.77</td>
<td>74.85 ± 10.92</td>
</tr>
<tr>
<td><strong>End Systolic volume (µl)</strong></td>
<td>25.66 ± 3.67</td>
<td>38.12 ± 7.58</td>
<td>31.92 ± 10.81</td>
<td>34.66 ± 6.74</td>
</tr>
<tr>
<td><strong>Aortic root (mm)</strong></td>
<td>1.37 ± 0.13</td>
<td>1.42 ± 0.14</td>
<td>1.21 ± 0.05</td>
<td>*1.31 ± 0.09</td>
</tr>
<tr>
<td><strong>Ascending Aorta velocity (mm/s)</strong></td>
<td>657.88 ± 96.03</td>
<td>729.59 ± 337.08</td>
<td>640.40 ± 75.93</td>
<td>589.27 ± 143.83</td>
</tr>
<tr>
<td><strong>Descending Aorta velocity (mm/s)</strong></td>
<td>-733.84 ± 64.03</td>
<td>-648.34 ± 124.62</td>
<td>-817.40 ± 84.61</td>
<td><strong>-638.25 ± 120.95</strong></td>
</tr>
<tr>
<td><strong>E/A ratio</strong></td>
<td>1.51 ± 0.13</td>
<td>1.33 ± 0.21</td>
<td>1.36 ± 0.06</td>
<td>1.73 ± 0.20</td>
</tr>
<tr>
<td><strong>LCCA PSV (mm/s)</strong></td>
<td>-379.32 ± 95.31</td>
<td>-316.70 ± 114.95</td>
<td>-377.13 ± 102.61</td>
<td>-351.90 ± 41.92</td>
</tr>
<tr>
<td><strong>RCCA PSV (mm/s)</strong></td>
<td>-262.78 ± 17.80</td>
<td>-268.92 ± 129.45</td>
<td>-333.09 ± 60.21</td>
<td><strong>-251.51 ± 58.64</strong></td>
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</table>

**Figure 5.30:** Table showing functional parameters of Brn3b KO and WT female (F) mice following 4 weeks of voluntary exercise. Stroke Volume **Mann-Whitney Test: Brn3b KO Saline vs Brn3b KO Exercise p=0.0087; End Diastolic volume *Mann-Whitney Test: WT Saline vs WT Exercise p=0.0173; Aortic root *Mann-Whitney Test: Brn3b KO Saline vs Brn3b KO Exercise p=0.0349; Descending aorta velocity **Mann-Whitney Test: Brn3b KO Saline vs Brn3b KO Exercise p=0.0022; RCCA PSV *Mann-Whitney Test: Brn3b KO Saline vs Brn3b KO Exercise p=0.0260. Exercised mice were compared to sedentary age matched Saline control mice in order to minimise the number of animals used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard deviation of multiple animals: WT Saline male n=5; WT Exercise Male n=5; Brn3b KO Saline n=5; Brn3b KO Exercise male n=5; WT Saline female n=5; WT Exercise female n=5; Brn3b KO Saline female n=6; Brn3b KO Exercise female n=5.
5.1.9 Analysis of cardiac function in Brn3b KO male and female mice following exercise

Cardiac function was analysed in Brn3b KO and WT male and female mice following 4 weeks of exercise using echocardiography and ultrasound to determine the effects of loss of Brn3b on heart function following physiological stress. Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study.

In the hearts of WT male mice following 4 weeks of exercise, cardiac output and stroke volume increased whereas ejection fraction was reduced when compared to WT saline controls, but the changes were not statistically significant. In addition, end diastolic volume significantly increased in the hearts of WT male mice following 4 weeks of exercise when compared to saline controls (Mann-Whitney Test: WT saline vs WT Exercise *p=0.0173) (Figure 5.29). Similar to AngII treatment, cardiac function was reduced in the hearts of Brn3b KO male mice following 4 weeks of exercise. For instance, Brn3b KO male mice displayed reduced cardiac output, stroke volume and ejection fraction following 4 weeks of exercise when compared to WT controls (Figure 5.29). In addition, there was no significant change in end diastolic volume in the hearts of Brn3b KO male mice following exercise when compared to Brn3b KO saline controls. As such, these results suggest that the hearts of Brn3b KO male mice may have more difficulty adapting to physiological stress. Interestingly, physiological stress may cause some changes in blood flow in in Brn3b KO and WT male mice. For example, in the hearts of WT mice following exercise, blood flow velocity through the ascending aorta, descending aorta velocity and RCCA increased (but was not statistically significant). Conversely, there was no significant change in the diameter of the aortic root lumen in the hearts of Brn3b KO male mice when compared to WT saline controls. Similarly, blood flow velocity through the ascending aorta and descending aorta and RCCA also increased in Brn3b KO male mice following 4 weeks of exercise, however the changes were not statistically significant. Brn3b KO male mice also showed no changes in the diameter of the aortic root lumen following 4 weeks of exercise (Figure 5.30).
On the other hand, cardiac function was not significantly affected in the hearts of Brn3b KO female mice following 4 weeks of exercise. In the hearts of WT female mice following 4 weeks of exercise, cardiac output and stroke volume increased, whereas ejection fraction was reduced when compared to WT saline controls. Additionally, end diastolic volume significantly increased in the hearts of WT female mice following 4 weeks of exercise (*Mann-Whitney Test: WT saline vs WT Exercise p=0.0173). In contrast to Brn3b KO male mice, cardiac output and ejection fraction increased in the hearts of Brn3b KO female mice following 4 weeks of exercise when compared to Brn3b KO saline controls (but the changes were not statistically significant). Moreover, these changes correlated with significantly increased stroke volume in the hearts of Brn3b KO female mice following 4 weeks of exercise when compared to Brn3b KO saline controls (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise **p=0.0087) (Figure 5.29 and 5.32).

End diastolic volume also increased in the hearts of Brn3b KO female mice following 4 weeks of exercise, but was not statistically significant when compared to Brn3b KO saline controls. As such, this may suggest that diastolic function in Brn3b KO female mice is not affected by exercise (Figure 5.29).

Following 4 weeks of exercise, changes in blood flow were observed in the hearts of WT female mice. For example, blood flow velocity through the ascending aorta increased in WT female mice following 4 weeks of exercise. However, blood flow velocity through the descending aorta decreased in WT female mice following exercise when compared to WT Saline controls (but these changes were not statistically significant). In addition, there was no change in the diameter of the aortic root lumen or in blood flow velocity through the RCCA in WT female mice following exercise. Exercise also caused some changes in blood flow in Brn3b KO female mice since blood flow velocity through the descending aorta significantly decreased in Brn3b KO female mice following exercise when compared to saline controls (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise **p=0.0022) (Figure 5.29). Blood flow velocity through the RCCA also significantly decreased in the hearts of Brn3b KO female mice following 4 weeks of exercise when compared to saline controls (Mann-Whitney Test: female Brn3b KO saline vs Brn3b KO AngII *p=0.0260), (Figure 5.30). Blood flow velocity through the ascending aorta was also reduced in the hearts of Brn3b KO female mice following 4 weeks of
exercise, which also displayed a significantly increased aortic root lumen diameter when compared to saline controls (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise *p=0.0349) (Figures 5.29 and 5.32). In summary, these results suggest that loss of Brn3b may cause differences in cardiac function in response to physiological stress in male vs. female mice, with male mice not being able to adapt as effectively as females. Moreover, physiological stress may also induce vascular changes that are distinct in Brn3b KO male and female mice which result in their differences in cardiac function following exercise.
Figure 5.31: A: Graph showing stroke volume of Brn3b KO and WT male mice following 4 weeks of voluntary exercise. B: Graph showing stroke volume of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. (**Mann-Whitney Test: Brn3b KO Saline vs Brn3b KO Exercise p=0.0087) A and B: Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study. Data represents mean and standard error of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5. ns = not significant
Figure 5.32: A: Graph showing end diastolic volume of Brn3b KO and WT male mice following 4 weeks of voluntary exercise (*Mann-Whitney Test WT saline vs WT Exercise p=0.0173). B: Graph showing end diastolic volume of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. (*Mann-Whitney Test WT saline vs WT Exercise p=0.0173). A and B: Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard error of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5. ns = not significant
Figure 5.33: A: Graph showing descending aorta velocity of Brn3b KO and WT male mice following 4 weeks of voluntary exercise. B: Graph showing descending aorta velocity of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise **p=0.0022). A and B: Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard error of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5. ns = not significant
Figure 5.34: A: Graph showing RCCA peak systolic velocity of Brn3b KO and WT male mice following 4 weeks of voluntary exercise. B: Graph showing RCCA peak systolic velocity of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise *p=0.0260). A and B: Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard error of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5. ns = not significant
Figure 5.35: A: Graph showing aortic root diameter of Brn3b KO and WT male mice following 4 weeks of voluntary exercise. B: Graph showing aortic root diameter of Brn3b KO and WT female mice following 4 weeks of voluntary exercise. (Mann-Whitney Test: Brn3b KO saline vs Brn3b KO Exercise *p=0.0349). A and B: Exercised mice were compared to sedentary age matched saline control mice in order to minimise the number of animals being used in the study due to the difficulty of obtaining Brn3b KO mice. Data represents mean and standard error of multiple animals: WT saline male n=5; WT Exercise Male n=5; Brn3b KO saline n=5; Brn3b KO Exercise male n=5; WT saline female n=5; WT Exercise female n=5; Brn3b KO saline female n=6; Brn3b KO Exercise female n=5.
5.2 Discussion

Different stresses (i.e. pathological and physiological) drive the same early adaptive hypertrophic response in the cardiomyocyte, however the molecular mechanisms behind the differences between physiological and pathological hypertrophy are poorly understood (McMullen and Jennings, 2007). In the previous chapters, we have provided evidence that the transcription factor Brn3b may be an important foetal cardiac gene that may be required for mediating hypertrophic responses to pathological stress (AngII treatment). As such, it was important to further determine the potential importance of this transcription factor in mediating pathological cardiac hypertrophy by studying the effects of loss of Brn3b on hypertrophic responses and by analysing subsequent gene expression changes in the hearts of Brn3b KO mice. Since the molecular mechanisms behind physiological hypertrophy have not been fully characterised, it was also important to determine whether Brn3b may also be required for mediating hypertrophic responses to physiological stress by analysing hypertrophic responses to physiological stress in Brn3b KO mice following voluntary exercise. Furthermore, since pathological and physiological hypertrophy lead to differences in cardiac function, it was also important to characterise the effects of loss of Brn3b (following pathological and physiological stress) on cardiac function using ultrasound.

In this chapter, we have provided further evidence that the POU4F2/Brn3b transcription factor may be required for mediating adaptive hypertrophic responses in the heart following pathological and physiological stress since Brn3b KO mice showed trends towards attenuated responses. Moreover, we have shown that Brn3b may have sex specific effects in mediating hypertrophic responses in the heart and maintaining heart function in response to pathological and physiological stress in males and females since Brn3b KO male and female mice displayed differences in cardiac function. Interestingly, we also showed that Brn3b KO male and female mice have different responses to exercise, with Brn3b KO female mice appearing to cope better with exercise in comparison to Brn3b KO male mice.

At baseline, echocardiography and ultrasound analysis of 2 month old Brn3b KO and WT male and female mice showed that although there was trend towards lower LV Mass in
Brn3b KO mice, cardiac function in these animals was preserved since functional parameters including ejection fraction and fractional shortening were similar between Brn3b KO mice and WT controls. This was similar to previous data obtained by Wang and colleagues (Wang et al., 2010)

Both Brn3b KO male and female mice displayed attenuated cardiac hypertrophy in response to AngII treatment and exercise as demonstrated by reduced LV Mass and LV Mass: BW ratios when compared to WT controls. This was further confirmed in the hearts of Brn3b KO AngII treated female mice which had reduced expression of hypertrophic marker β-MHC. Importantly β-MHC expression needs to be investigated in the hearts of Brn3b KO AngII treated male mice, and it would also be useful to analyse changes in expression of other hypertrophic markers such as ANF in both male and female Brn3b KO mice to confirm attenuated hypertrophic responses in these animals. While we previously showed that Brn3b was increased in hypertrophic WT mouse hearts following AngII treatment (see Chapter Four), Brn3b has also been shown to have oncogenic potential since it drives growth and proliferation in some cancers (Irshad et al., 2004) (Budhram-Mahadeo et al., 2008). Other oncogenes including c-fos and c-myc are also expressed in the stressed heart (Komuro et al., 1988) (Mulvagh et al., 1987). Therefore these results suggest that Brn3b may be an oncogene that is re-expressed in hypertrophic hearts that is required for adaptive responses to pathological and physiological stress. Similarly, Bueno and colleagues showed that Calcineurin deficient mice (CnAβ−/−) displayed reduced heart size at baseline, but had attenuated cardiac hypertrophy following AngII infusion (Bueno et al., 2002). In addition, transgenic mice lacking the p85 subunit of PI3K specifically in the muscle (p85α mKO p85β−/−) also had reduced heart size but preserved cardiac function at baseline and displayed attenuated cardiac hypertrophy following 4 weeks of swim training (Luo et al., 2005). Since these effects are very similar to those observed in Brn3b KO mice at baseline and following 4 weeks of AngII treatment and exercise, these data presented further suggest that Brn3b may be a novel regulator that is required for mediating hypertrophic responses in the heart in response to physiological and pathological stress.

Interestingly, Brn3b KO male and female mice displayed differences in cardiac function in response to pathological and physiological stress. In this regard, hearts from Brn3b
KO male mice had elevated cardiac output and stroke volume at baseline. However, stroke volume and ejection fraction decreased following 4 weeks of AngII treatment and exercise when compared to WT controls. As such, these results suggest that the hearts of Brn3b KO are having to work harder in order to maintain cardiac output, however following pathological stress they are unable to adapt to an increased workload. Following AngII treatment and exercise, the hearts of Brn3b KO male mice showed no changes in end diastolic volume, whereas it increased in WT male mice. During diastole, the left ventricle relaxes to allow the chamber to fill with blood which is then pumped from the heart to the rest of the body during systole (Rik Kapila, 2009). As such, the data presented suggests that the hearts of Brn3b KO male mice may be failing to relax and fill with blood efficiently during diastole, and hence may display diastolic dysfunction. In patients, diastolic dysfunction is associated with stiffening of the heart and failure to increase stroke volume, which interestingly was observed in Brn3b KO male mice following AngII treatment and Exercise. Diastolic dysfunction is also associated with heart failure, therefore these results are significant since prolonged pathological stress leads to heart failure, therefore Brn3b may be required for the adaptive responses of the heart to pathological stress (Rik Kapila, 2009). Diastolic dysfunction in patients is also associated with reduced exercise capacity, which is also interesting because since this was observed in Brn3b KO male mice following 4 weeks of exercise (Rik Kapila, 2009). As such, these results indicate an important role for Brn3b in maintaining heart function and adapting to pathological and physiological stress in males, and suggest that loss of Brn3b may result in diastolic dysfunction in male mice in response to stress.

There were also increases in the diameter of the aortic root lumen and ascending aorta velocity in the hearts of WT male mice following AngII treatment. This was as expected since these are characteristics of elevated blood pressure and since previous studies have shown that AngII treatment via osmotic mini pumps into mice caused dilation of the aorta (Barisone et al., 2006, Haskett et al., 2012). Reduced RCCA peak systolic velocity was also observed in AngII treated WT male mice which was similar to data obtained by Loquet and colleagues who demonstrated reduced carotid blood flow velocity in humans following AngII infusion (Loquet et al., 1991). In contrast, there was no change in the diameter of the aortic root lumen, ascending aorta velocity or RCCA
peak flow velocity in Brn3b KO AngII treated male mice. As such, these results may suggest that Brn3b KO male mice may not be responding to AngII in the same manner as WT controls and hence may not be able to adapt their hearts in response to an increased workload; however this hypothesis and the effects of AngII on blood flow in Brn3b KO male mice needs to be investigated further.

Since prolonged pathological stress results in cardiac fibrosis and stiffening of the heart muscle, functional parameters that assess systolic function were also measured in Brn3b KO and WT mice following 4 weeks of AngII treatment and exercise using echocardiography (McMullen and Jennings, 2007). Following AngII treatment, systolic function (e.g. fractional shortening, fractional area change) was also reduced in Brn3b KO male mice, which correlated with apparent ECM deposition in the hearts and surrounding blood vessels. However, since ECM deposition could not be quantified reliably it will be important to undertake additional studies to investigate whether loss of Brn3b may result in cardiovascular remodelling associated with the later stages of pathological hypertrophy (McMullen and Jennings, 2007). Intriguingly, ongoing studies by Dr. Budhram-Mahadeo and colleagues have shown differences in vascular structure in Brn3b KO mice, including increased collagen deposition and calcification in the aortas of Brn3b KO mice at baseline. Furthermore, Dr. Budhram-Mahadeo and colleagues have also identified Brn3b binding sites on the collagen I promoter, suggesting that collagen I expression may be regulated by Brn3b. As such, the role of Brn3b on regulating collagen expression in the heart needs to be investigated further. However, it would be interesting to determine whether increased collagen deposition in Brn3b KO male mice observed following pathological stress may be due to potential de-repression of collagen expression in the absence of Brn3b.

Brn3b KO male mice had reduced average speed and cumulative distance following 4 weeks of exercise when compared to WT controls. The hearts of Brn3b KO also failed to undergo hypertrophy following 4 weeks of exercise, which may be due to these animals not doing enough exercise due to their inability to do so. Consequently, other exercise methods will need to be tested in future studies (e.g. treadmill or timed exercise) in order to induce hypertrophy in Brn3b KO mice since other studies have shown that 8 weeks of voluntary exercise is sufficient to induce cardiac hypertrophy (Dworatzek et
al., 2014). These exercise capacity results of Brn3b KO exercised mice are also particularly interesting since Brn3b has previously been shown to be expressed in skeletal muscle (Bitsi et al., 2016). While the potential function of Brn3b is not known and hence needs to be elucidated, several studies have shown the importance of transcription factors in skeletal muscle structure, function and exercise performance. For instance, skeletal muscle specific Liver Kinase B1 (LKB1) knockout mice showed reduced exercise capacity following voluntary wheel running and treadmill running, in line with decreased muscle fibre area and decreased mitochondrial and mitochondrial protein content (Thomson et al., 2010, Thomson et al., 2007). Therefore it would be interesting to determine whether Brn3b plays a role in skeletal muscle structure and function and hence whether its loss may be responsible for the poor exercise capacity observed in Brn3b KO male mice. For instance, it would be interesting to investigate whether Brn3b regulates expression of mitochondrial proteins in skeletal muscle.

In contrast, heart function was not significantly affected by AngII treatment and exercise in Brn3b KO female mice. For instance, there was no significant difference in functional parameters such as cardiac output, stroke volume, ejection fraction and fractional shortening in the hearts of Brn3b KO female mice following exercise and AngII treatment when compared to Saline controls. While there were no changes in diameter of the aortic root lumen, ascending aorta velocity and RCCA peak systolic velocity, Brn3b KO female mice demonstrated significant increases in the diameter of the aortic root lumen and ascending aorta velocity; whereas RCCA peak systolic velocity decreased. Since AngII has been shown to cause dilation of the aorta and increase blood flow velocity through the ascending aorta (and hence increase blood pressure) and decrease blood flow velocity through the RCCA, these results suggest that AngII may be causing increased blood pressure in these animals (Barisone et al., 2006, Haskett et al., 2012, Loquet et al., 1991). Conversely, these results were not observed in Brn3b KO male mice, therefore this may also suggest that Brn3b may be required for adaptive changes in the vasculature in response to AngII. As such, these results suggest that the hearts and of female mice may be able to adapt to hypertension induced by AngII in the absence of Brn3b. Importantly, this hypothesis needs to be investigated in more detail. In addition, the hearts of Brn3b KO AngII treated female mice did not show cardiac fibrosis which
correlated with unchanged fractional shortening in these animals. However, increased collagen and ECM deposition was observed surrounding the blood vessels in the hearts of Brn3b KO AngII treated female mice, suggesting that loss of Brn3b may result in thickening of the blood vessels in response to stress, however this needs to be investigated further since this could not be reliably quantified in the Masson’s trichrome stained hearts. As such, these results suggests that female mice are able to adapt to pathological and physiological stress in the absence of Brn3b. Similar effects have been observed in other transgenic mouse models. For instance, α-myosin heavy chain transgenic female mice displayed cardiac hypertrophy but had preserved systolic function (Olsson et al., 2001). In addition, transient receptor potential canonical (TRPC1) knockout mice also displayed attenuated cardiac hypertrophy but had preserved fractional shortening when compared to WT controls following myocardial infarction (Seth et al., 2009).

Interestingly, there were trends towards elevated GLUT4 and cyclin D1 mRNA levels in the hearts of Brn3b KO AngII treated female mice (however ‘n’ numbers must be increased in order to validate results). GLUT4 and cyclin D1 are Brn3b target genes that are expressed in hypertrophic hearts and were elevated at the mRNA and protein level (see Chapter Four) in WT female mice following AngII treatment in line with elevated Brn3b expression (Abel et al., 1999, Tamamori-Adachi et al., 2002). Consequently, it may be expected that Brn3b target genes GLUT4 and cyclin D1 levels would be reduced in Brn3b KO AngII treated mice due to the absence of Brn3b expression in these animals, however this was not the case. Previous studies have shown that Brn3b is able to interact with the oestrogen receptor and trans-activate its promoter (Budhram-Mahadeo et al., 1998). Brn3b has also been shown to co-operate with the oestrogen receptor to activate the HSP27 promoter in breast cancers, which also activates HSP27 expression in cardiomyocytes which is associated with cardiomyocyte survival and differentiation after birth (Davidson and Morange, 2000, Farooqui-Kabir et al., 2004, Lee et al., 2005). This is of particular interest since pre-menopausal women are better protected against cardiovascular disease due to the presence of oestrogen (Murphy, 2011). Since Brn3b and the oestrogen receptor have been shown to interact and activate HSP27 expression, it may be possible that these two factors interact and regulate the
expression of other target genes such as GLUT4 and cyclin D1, however further experiments need to be carried out to confirm this hypothesis. Moreover, since cardiac function is not affected in Brn3b KO female mice in response to physiological and pathological stress, it could also be possible that the oestrogen receptor may be compensating for loss of Brn3b expression and contributing to maintained heart function, and may also be responsible for elevated levels of GLUT4 and cyclin D1 in the absence of Brn3b. Such compensatory effects may occur due to regulation of similar target genes by Brn3b and the oestrogen receptor. For example, Brn3b and the oestrogen receptor both trans activate HSP27 expression and similarly, both Brn3a and Brn3b have been shown to partially compensate for one another in the developing heart, which both activate the HSP27 promoter as well (Budhram-Mahadeo et al., 1998, Lee et al., 2005, Maskell et al., 2017). Therefore it will be important to investigate whether Brn3b and the oestrogen receptor interact to regulate expression of additional target genes such as GLUT4 and cyclin D1 in the stressed heart.

In contrast to Brn3b KO male mice, exercise performance was unaffected in Brn3b KO female mice following 4 weeks of exercise since there was no difference in average speed or cumulative distance between Brn3b KO and WT female mice during the fourth week of voluntary exercise. This is also interesting since oestrogen receptors ERα and ERβ are expressed in skeletal muscle, and have also been shown to be elevated following endurance exercise in humans (Wiik et al., 2005). Moreover, Riba and colleagues demonstrated the importance of ERα in muscle oxidative metabolism and mitochondrial morphology and function since skeletal muscle specific ERα knockout mice had reduced muscle oxidative metabolism and aberrant mitochondrial morphology and function; including enlarged and fused mitochondria, and reduced mitochondrial oxygen consumption (Ribas et al., 2016). Since Brn3b has previously been shown to interact with the oestrogen receptor to regulate gene expression (e.g. HSP27), it would be interesting to determine whether Brn3b may also interact with the oestrogen receptor to regulate gene expression. Moreover, similar to the heart, the oestrogen receptor may compensate for loss of Brn3b in the skeletal muscle and hence contribute to maintenance of skeletal muscle morphology and function in Brn3b KO mice, which in turn may explain the reason why Brn3b KO female mice have similar average speed and
cumulative distance following 4 weeks of exercise when compared to WT control mice. Importantly however, this hypothesis needs to be investigated in more detail.

qRT-PCR analysis of Brn3b KO and WT AngII treated female mice also showed that pro-apoptotic Bax expression was reduced in Brn3b KO AngII treated female mice when compared to WT controls. As such, these results suggest that less apoptosis may be occurring in Brn3b KO AngII treated female mice in response to pathological stress. We have previously shown that following AngII treatment Bax expression increased in the hypertrophic hearts of WT mice which correlated with elevated Brn3b and p53 (see Chapter Four); and in the injured heart elevated Brn3b within the infarcted myocardium co-expressed with p53 also correlated with increased Bax expression and apoptosis (Budhram-Mahadeo et al., 2014). Therefore these results further suggest that Brn3b may co-operate with p53 to drive Bax expression and cardiomyocyte apoptosis in the later stages of pathological hypertrophy. Since Brn3b KO male mice displayed poor cardiac function and fibrosis following AngII treatment, Bax and p53 expression will need to be analysed in the hearts of Brn3b KO and WT AngII treated male mice.

In summary, we have provided further evidence that Brn3b is essential for driving hypertrophic responses to pathological stress, but may also facilitate cardiomyocyte apoptosis in the later maladaptive stages when co-expressed with p53 by driving pro-apoptotic Bax expression. We have also shown that there are sex differences in response to pathological stress in Brn3b KO mice, therefore Brn3b may also have distinct roles in mediating hypertrophic responses in males and females via potential interaction with other proteins such as the oestrogen receptor. Similarly, Brn3b may also be essential for driving hypertrophic responses to physiological stress and have distinct roles in males and females that affect cardiac function and exercise capacity. In order to confirm these findings and hypotheses, additional experiments must be undertaken.
Chapter Six

General Discussion & Future Directions
6.0 General Discussion

The heart is an essential organ during development since it is required for embryonic survival; and in adult life the heart plays an important role in adapting to stress under different conditions, e.g. hypertension. The cellular processes which govern normal heart development and function (e.g. proliferation, differentiation and apoptosis) are regulated by cell signalling pathways which converge on changes in expression of genes which help to determine cell fate in the heart (Black, 2007, McMullen and Jennings, 2007, Poelmann et al., 1998). In this regard, DNA binding transcription factors (TFs) are important for cell fate determination in the developing and adult heart by regulating expression of cardiac genes which drive normal cellular processes in the heart (Latchman and Latchman, 2007). For instance, cardiac transcription factors (e.g. GATA4) regulate expression of foetal genes such as β-MHC that are required for processes such as muscle contraction and growth in the developing heart (Brand, 2003). Re-expression of such foetal genes are also essential for mediating adaptive hypertrophic responses in the heart in response to pathological stress (Barry et al., 2008) Consequently imbalances of levels of cardiac transcription factors may be responsible for driving defects in developmental processes or hypertrophic responses to stress. This in turn may result in congenital heart disease in babies or heart failure in patients with cardiovascular disease which can be detrimental to life. Therefore identifying cardiac transcription factors involved in maintaining cardiac homeostasis in the developing and stressed heart will be essential for providing further knowledge in this field and for providing better treatments for patients with congenital heart disease and cardiovascular disease (Cowie et al., 2000, Savarese and Lund, 2017).

POU4F2/ Brn3b is a unique transcription factor which is important for determining cell fate in a tissue specific context, and can drive different effects on cell fate depending on its interacting partners. For instance, in breast cancer and neuroblastoma, Brn3b is associated with driving cell proliferation by regulating target genes such as cyclin D1 and BRCA1 (Budhram-Mahadeo et al., 2008, Irshad et al., 2004). However, while increased Brn3b levels in ND7 cells are associated with cell proliferation, co-expression of Brn3b with p53 drives apoptosis in neuronal cells (Budhram-Mahadeo et al., 2006a). Importantly, both Brn3b and the related but distinct Brn3a transcription factors are
expressed in cardiomyocytes during heart development and have unique spatial and temporal patterns of expression (Farooqui-Kabir et al., 2008). In addition, Brn3b was up-regulated in the hearts of Brn3a KO mouse embryos, providing evidence that it may compensate for loss of Brn3a expression in the heart (Farooqui-Kabir et al., 2004, Farooqui-Kabir et al., 2008). Following coronary artery ligation in the mouse, Brn3b expression also increased throughout the heart; however within the injured myocardium its co-expression with p53 correlated with up-regulation of pro-apoptotic Bax and cardiomyocyte apoptosis (Budhram-Mahadeo et al., 2014). Taken together, these data suggest that Brn3b may have essential roles in the heart during development and in response to stress.

In this thesis, we have provided further evidence that the correct expression of Brn3b is essential for normal heart development, and that it may be a unique foetal gene that is expressed in response to pathological stress. In this regard, for the first time we have provided further evidence that Brn3b may be required for mediating hypertrophic responses in the heart since its elevated expression in hypertrophic hearts/cardiomyocytes was associated with increased expression of Brn3b target genes (GLUT4 and cyclin D1) that drive adaptive hypertrophic changes such as metabolic switching and growth (Abel, 1994, Soonpaa et al., 1997). Furthermore, we have shown the potential importance of Brn3b in cardiac hypertrophy to pathological stress since loss of Brn3b causes attenuated hypertrophic responses in Brn3b KO mice. Finally we have also shown that Brn3b may be important for cardiac adaptation to stress in males since Brn3b KO male and female mice display differences in cardiac function following pathological and physiological stress.

Importantly, we have extended the studies undertaken by Farooqui-Kabir and colleagues and have provided further evidence to support that Brn3b is an important transcription factor in the developing heart. In particular, for the first time we have shown that aberrant Brn3b expression in the hearts of Brn3a KO mouse embryos drives changes in cell fate and behaviour and hence heart development (Maskell et al., 2017). We first demonstrated a reciprocal pattern of Brn3b and Brn3a expression in the heart throughout development; when Brn3a expression was high, Brn3b levels were reduced (and vice versa). Additionally, elevated Brn3b levels in the hearts of Brn3a KO mice
correlated with increased levels of Brn3b target gene cyclin D1. Since the hearts of Brn3a KO mice displayed changes in cardiac morphology during mid gestation including hyperplasia, these results may suggest that up-regulation of cyclin D1 as a result of elevated Brn3b levels may contribute to the hyperplasia of the heart that was observed at E14.5 in the Brn3a KO mouse heart (Maskell et al., 2017, Budhram-Mahadeo et al., 2008, Farooqui-Kabir et al., 2008, Irshad et al., 2004). In particular, cardiomyocyte proliferation is regulated by cyclin D1 and its interacting partner cyclin dependent kinase 4 (CDK4) through phosphorylation of retinoblastoma and hence de-repression of the E2F transcription factor which regulates cell cycle genes (CDK4) (Hotchkiss et al., 2012, Johnson and Walker, 1999, Matsushime et al., 1994, Quelle et al., 1993). The results of this study have also further highlighted how Brn3b can drive different effects on cell fate depending on its interacting partners since elevated Brn3b (and unchanged p53) correlated with increased Bax and Noxa levels and cardiomyocyte apoptosis at E17.5 in Brn3a KO mouse hearts. This was in line with morphological changes in Brn3a KO mouse hearts at E16.5, e.g. increased ventricular trabeculation in Brn3a KO hearts at E16.5. These results are interesting since previous studies undertaken by Budhram-Mahadeo and colleagues have shown similar changes in p53 and Bax expression in the injured heart (and hence cardiomyocyte apoptosis) following chronic stress (Budhram-Mahadeo et al., 2014). Consequently, aberrant Brn3b expression in the developing heart in the absence of Brn3a may be responsible for driving aberrant apoptosis and hence increased ventricular trabeculation and non-compaction observed in the Brn3a KO E18.5 heart and the death of Brn3a KO mice 0.5 days after birth. This research is important because we have provided further evidence for how the incorrect regulation of Brn3b expression may also lead to changes in cardiac morphology, and potentially congenital heart defects in babies which are common causes of neonatal mortality (Brand, T, 2003). This is further evidenced by additional studies undertaken in our laboratory which showed that loss of Brn3a and Brn3b in mouse embryos caused embryonic lethality; which is similar to double Msx1/ Msx2 KO mutant mice which displayed cardiac defects between E14.5 and E16.5 and died before birth (Bei et al., 2000, Chen et al., 2008, Ishii et al., 2005, Maskell et al., 2017, Satokata et al., 2000)
We have also provided evidence that zebrafish can be used as another animal model to study the importance of Brn3a and Brn3b expression in heart development since we showed expression of Brn3a and Brn3b protein in the ventricles of the developing zebrafish heart, which are highly conserved between mammals and zebrafish (87% and 76% respectively). Additional studies undertaken by others in the laboratory whereby zebrafish embryos were injected with morpholinos to knock down both Brn3a and Brn3b expression caused striking cardiac defects in double morphants such as looping and atrioventricular valve defects. As such, these results provide further evidence that Brn3a and Brn3b hence may be important TFs for cardiac development (Goldstein et al., 1998, Harvey, 1999, Maskell et al., 2017).

Taken together, these findings are also important because these Brn3b target genes such as cyclin D1 are also (foetal genes) required for mediating adaptive hypertrophic growth in the stressed heart; as well as Bax which is increased at later pathological stages of hypertrophy which eventually leads to heart failure. Therefore these studies have also provided evidence that Brn3b may be important for driving re-expression of foetal genes in response to pathological stress. Consequently, further studies were carried out to determine the potential role of Brn3b in facilitating cardiac hypertrophy.

We have also shown for the first time that Brn3b may be an important transcription factor that is required for mediating hypertrophic responses to pathological stress. In this regard, Brn3b protein levels were elevated in the hearts of WT mice and cardiomyocyte cultures treated with AngII. This correlated with characteristic hypertrophic changes including increased LV Mass, LV Mass: BW ratio and induction of hypertrophic marker β-MHC. These findings are important because re-expression of foetal genes is a key feature of pathological hypertrophy (Barry et al., 2008). For these studies, AngII treatment was used since it mimics cardiac hypertrophy induced by hypertension in humans (Ayada et al., 2015). Increased workload is induced since activation of AngII signalling (upon binding of AngII to the AT1 receptor) causes vasoconstriction and increased aldosterone release which causes increased water reabsorption into the blood vessels which acts to increase blood volume, blood flow velocity and hence blood pressure. As a result, AngII causes the terminally differentiated cardiomyocytes of the heart to undergo hypertrophy in order to increase its mass and
hence enable increased contraction to pump the elevated blood volume around the body and maintain cardiac output. This is facilitated by increased rates of transcription and synthesis of foetal genes/ proteins (e.g. β-MHC, ANF and BNP) by cardiac transcription factors (Frey and Olson, 2003, Mehta and Griendling, 2007). As such, tight regulation of expression of foetal genes is essential for normal adaptive hypertrophic responses. However, prolonged pathological stress induces aberrant expression of pro-apoptotic genes such as Bax which leads to loss of terminally differentiated cardiomyocytes and eventually heart failure (Chatterjee et al., 2011, van Empel and De Windt, 2004).

Importantly, studies undertaken by others in the laboratory showed that hypertrophic signalling pathways activated by AngII such as MAP Kinase and Calcineurin caused increased expression of Brn3b mRNA and protein. Moreover, Budhram-Mahadeo and colleagues have demonstrated activation of the Brn3b promoter by MAP Kinase signalling; and additional studies undertaken by others in the laboratory have also shown that in AngII treated cardiomyocytes this activation is blocked specifically by the MAP Kinase inhibitor PD98059 but not the p38 inhibitor SB203580. This in turn demonstrated that Brn3b is activated specifically by the p42/44MAPK/ERK1 pathway and not by p38 MAP Kinase in AngII treated cardiomyocytes. Therefore Brn3b may represent a novel foetal gene that is also re-expressed as part of the foetal gene programme during pathological hypertrophy which is activated by MAP Kinase or Calcineurin signalling since Brn3b has been shown to be expressed in the developing heart (Barry et al., 2008, Farooqui-Kabir et al., 2008, Maskell et al., 2017). This was further evidenced by chromatin immunoprecipitation studies undertaken in our laboratory (by myself and other colleagues) which demonstrated binding of the transcription factor NFAT to the Brn3b promoter in AngII treated cardiomyocytes which in turn is activated by Calcineurin signalling in response to pathological stress (Molkentin, 2004, Maskell, 2018c).

Brn3b target genes such as GLUT4 and cyclin D1 which are associated with mediating hypertrophic responses may also be up-regulated in the hearts of WT mice and in cardiomyocyte cultures treated with AngII (Abel et al., 1999, Busk et al., 2002, Soonpaa et al., 1997, Tamamori-Adachi et al., 2002). These target genes have been implicated in
hypertrophic responses since the heart reverts to a foetal phenotype and uses glucose metabolism during pathological cardiac hypertrophy which requires glucose transporters such as GLUT4 (Frey and Olson, 2003, McMullen and Jennings, 2007). Brn3b has also been shown to activate GLUT4 expression in metabolically active tissues such as skeletal muscle, therefore Brn3b may also activate GLUT4 expression in the stressed heart and facilitate metabolic switching to glucose following pathological stress (Bitsi et al., 2016). Additionally, cyclin D1 KO mice display attenuated cardiac hypertrophy, therefore Brn3b may also facilitate the hypertrophic response by up-regulating cyclin D1 expression, thereby facilitating cardiomyocyte growth (Busk et al., 2002, Irshad et al., 2004). Consequently, in this thesis we have for the first time shown that Brn3b may be a unique cardiac transcription factor that is required for mediating adaptive hypertrophic responses through regulating expression of target genes that are necessary for these processes, e.g. GLUT4 and cyclin D1. However, since Brn3b is a transcription factor it is likely that it may also regulate other unknown target genes in response to pathological stress. As such it will be important to identify additional genes that may be regulated by Brn3b following pathological stress (e.g. by using RNA sequencing).

Increased Bax and p53 expression also correlated with increased Brn3b expression in hypertrophic hearts of WT mice, which is an important finding because during the later maladaptive stages of cardiac hypertrophy elevated p53 and Bax expression is associated with progression to heart failure (Ahuja et al., 2007, Frey and Olson, 2003). Moreover, previous studies have already shown that when elevated Brn3b is co-expressed with p53, elevated Bax expression drives cardiomyocyte apoptosis in the developing heart and in response to injury (Budhram-Mahadeo et al., 2014, Maskell et al., 2017). Interestingly, reduction of Brn3b expression in cardiomyocytes caused reduction of Bax expression (Budhram-Mahadeo et al., 2006a). Therefore since reduced Bax expression was also observed in the hearts of Brn3b KO AngII treated female mice, these results further suggest the importance of Brn3b for driving Bax expression and cardiomyocyte apoptosis upon co-expression with p53 in the stressed heart. Therefore we have also provided evidence that Brn3b may be involved in driving the maladaptive hypertrophic responses which eventually lead to heart failure.
Moreover, cardiac hypertrophy was attenuated in both Brn3b KO male and female mice following 4 weeks of AngII treatment, as shown by reduced LV Mass and LV Mass: BW ratio. These results show the importance of cardiac transcription factors such as Brn3b in mediating hypertrophic responses in the heart since similar effects were observed in other transgenic mouse models, e.g. in mice lacking Calcineurin (CnAβ−/−) which displayed attenuated cardiac hypertrophy following AngII infusion (Bueno et al., 2002). As such, the data presented in this thesis further indicate an important role for Brn3b in adaptation of the heart to pathological stress. Loss of Brn3b also resulted in different cardiac functional responses to pathological and physiological stress in male and female mice. Reduced heart function such as decreased ejection fraction were observed in Brn3b KO male mice following 4 weeks of AngII treatment. Therefore these results suggest that Brn3b may also be required for adaptation of the heart and maintenance of heart function in response to pathological stress since its loss in mice caused defects in cardiac function (Kreso et al., 2015). Such functional parameters are also important for maintaining cardiac output, therefore these results also provide further evidence that Brn3b may also be important for maintaining cardiac output in early adaptive hypertrophic responses since changes in functional parameters occur in order to maintain cardiac output in the stressed heart (McMullen and Jennings, 2007). Importantly, the results of this study have shown that cardiac protection was not observed in Brn3b KO male mice following AngII treatment which is interesting since previous studies have shown that co-expression of Brn3b and p53 (which in turn is increased following activation of AngII signalling) drives cardiomyocyte apoptosis by inducing Bax and Noxa expression in the injured heart. As such, the results of this study suggest that Brn3b may be required for the adaptive hypertrophic response in male mice, since its loss in males results in poor heart function (Budhram-Mahadeo et al., 2014).

Additionally, male Brn3b KO mice had poor exercise capacity and reduced heart function following 4 weeks of exercise, including decreased cardiac output and ejection fraction. This may suggest that Brn3b may also play an important role in adaptation to physiological stress in the heart in males. In addition, since Brn3b is a transcription factor it may also regulate unknown target genes that are involved in adaptation of the heart.
to stress, therefore the poor cardiac function observed in these mice may be a result of loss of expression of such unknown genes, however this hypothesis needs to be investigated further. Interestingly, 90% of athletes that die from sudden cardiac death are male (Sharma et al., 2015). As such, it would be interesting to analyse Brn3b expression in trained athletes in order to determine whether low Brn3b expression may partially be responsible for lack of cardiac protection and hence sudden cardiac death in athletes. However, further experiments need to be carried out to investigate the role of Brn3b in mediating hypertrophic responses to physiological stress in more detail. Brn3b KO males also displayed poor exercise capacity, with reduced average running speed and cumulative distance when compared to WT controls. This is particularly interesting since Brn3b has previously shown to be expressed in skeletal muscle therefore these results also suggest that Brn3b may be required for normal skeletal muscle function and hence adaptation to exercise in males (Bitsi et al., 2016). As such, it would also be interesting to analyse the morphology of skeletal muscle in Brn3b KO male mice to determine whether the observed poor exercise capacity and poor heart function observed may be due to a skeletal muscle defect in these animals.

Conversely, exercise capacity was unchanged in Brn3b KO female mice and cardiac function and contractility were not affected in Brn3b KO female mice following 4 weeks of exercise/ AngII treatment. These observations were similar to those observed other transgenic mouse models, including α-myosin heavy chain transgenic female mice which displayed cardiac hypertrophy but had preserved systolic function; whereas transgenic male mice displayed systolic impairment (Olsson et al., 2001). Interestingly, previous studies have shown that Brn3b is able to interact with the oestrogen receptor and drive expression of promoters that contain oestrogen response elements (ERE), e.g. HSP27, which has in turn been shown to be expressed in cardiomyocytes (Budhram-Mahadeo et al., 1998, Farooqui-Kabir et al., 2004, Lee et al., 2005). As such, it may be possible that Brn3b may interact with the oestrogen receptor and regulate the expression of other target genes such as GLUT4 and cyclin D1 following pathological stress. However, further experiments need to be carried out to confirm this hypothesis. Moreover, since the oestrogen receptor is expressed in the heart, and cardiac function is not affected in Brn3b KO female mice in response to physiological and pathological stress; the
oestrogen receptor may also be able to compensate for loss of Brn3b expression in the stressed heart by regulating expression of Brn3b target genes such as GLUT4 and cyclin D1 in Brn3b KO AngII treated mice (Taylor and Al-Azzawi, 2000). This hypothesis needs to be investigated further, however several studies have provided evidence for this hypothesis, whereby the oestrogen receptor has been shown to regulate GLUT4 and cyclin D1 expression (Barros et al., 2006, Lamb et al., 2000, Neuman et al., 1997, Sabbah et al., 1999). Taken together, the results presented in this thesis suggest that Brn3b may also be a novel regulator that mediates hypertrophic responses in the heart to pathological and physiological stress in females, but may not be required for cardiac protection in females, however this needs to be investigated further. Since exercise capacity was also unaffected in Brn3b KO female mice, these results may also suggest that Brn3b may not be essential for normal skeletal muscle function in females; which may be mediated through compensation by the oestrogen receptor which has been shown to be expressed in skeletal muscle (Wiik et al., 2005).

In summary, we have shown that transcription factors Brn3b and the related but distinct Brn3a TFs may play an essential role in the developing heart and may partially compensate for one another since their loss caused embryonic lethality and heart looping defects (Maskell et al., 2017). During the later stages of heart development, elevated Brn3b expression in the absence of Brn3a correlated with up-regulation of pro-apoptotic Bax and cardiomyocyte apoptosis, which in turn was associated with heart defects such as ventricular trabeculation. Consequently, the balance of Brn3a and Brn3b expression will be important for normal heart development. Additionally, we have shown that Brn3b may be a novel foetal gene that is re-expressed during pathological hypertrophy in the heart. Importantly, we have shown that Brn3b may be required for mediating early adaptive hypertrophic responses to pathological stress in by up-regulating target genes such as GLUT4 and cyclin D1 that enable metabolic switching and cardiomyocyte growth. However, during the later maladaptive phases of pathological hypertrophy we have shown that elevated levels of Brn3b may contribute to cardiomyocyte apoptosis when co-expressed with p53 by increasing Bax expression. Finally, we have shown that Brn3b may be required for the early adaptive response to stress in males, but not in females since Brn3b KO females displayed preserved cardiac
function following pathological and physiological stress. Taken together, these results provide a base for future work to investigate the role of Brn3b in the developing heart and in response to stress in more detail.

6.1 Future directions

In this study, we have shown that Brn3b and the related but distinct Brn3a TF may play an essential role in the developing heart and may partially compensate for one another using mouse and zebrafish models (Maskell et al., 2017). Whilst the zebrafish model is a useful tool that enables heart development to be studied in live embryos over time, it is important to analyse these observations with caution since zebrafish have a two chambered heart whereas mammals have a four chambered heart (Goldstein et al., 1998, Harvey, 1999). Therefore to confirm the potential roles of Brn3a and Brn3b in the developing heart, it will be important to generate cardiac specific double Brn3a/ Brn3b knockout mice in order to assess how loss of Brn3a and Brn3b specifically in the heart affects cardiac development.

We have also shown that Brn3b is an important transcription factor that may be required for mediating hypertrophic responses in the heart to pathological and physiological stress. Although elevated levels of Brn3b target genes (e.g. GLUT4 and cyclin D1) were observed in hypertrophic WT hearts and cardiomyocytes, the molecular mechanism behind how Brn3b may mediate hypertrophic responses is not yet known. Therefore it will be important to establish the specific molecular mechanisms of how Brn3b is involved in mediating hypertrophic responses. For example, chromatin immunoprecipitation analysis could be performed in AngII treated WT mouse hearts/ cardiomyocytes to determine whether Brn3b is directly regulating expression of its target genes. It will also be important to identify additional unknown Brn3b target genes that may also mediate the hypertrophic response to pathological stress.

The results of this study have importantly shown that Brn3b KO male mice are unable to adapt to pathological stress, therefore it will be important to analyse molecular and gene expression changes in the hearts/ cardiomyocytes of Brn3b KO male mice and hence investigate why Brn3b KO male mice are unable to adapt to pathological stress.
We have also shown that Brn3b KO female mice have preserved cardiac function and elevated levels of Brn3b target genes in response to pathological stress, which may be due to compensation by the oestrogen receptor since studies undertaken by Budhram-Mahadeo and colleagues have shown that Brn3b interacts with the oestrogen receptor (Budhram-Mahadeo et al., 1998). As such, it would be interesting to analyse the effects of loss of the oestrogen receptor in Brn3b KO female mice in hypertrophic responses to pathological and physiological stress, for instance by overectomizing these animals or by treating them with Tamoxifen (followed by AngII infusion or exercise). It would also be interesting to investigate the potential interaction of Brn3b with ERα and ERβ in the heart in response to stress since ERβ is also present in the heart and since previous studies undertaken by Budhram-Mahadeo and colleagues investigated Brn3b interaction with ERα (Taylor and Al-Azzawi, 2000).

The importance of Brn3b in mediating hypertrophic responses in the heart to pathological and physiological stress has also been demonstrated through attenuated hypertrophic responses in Brn3b KO male and female mice. Using transgenic Brn3b KO mice in this project has been a powerful tool that has allowed the potential role of Brn3b in hypertrophic responses to pathological and physiological stress to be determined in more detail. However, it is important to note that these Brn3b KO mice are constitutive knockouts, and are characterised by several phenotypes which may also contribute to the cardiac responses to pathological stress (Bitsi et al., 2016, Goran et al., 2003, Grundy et al., 1999, Kamide et al., 1996). Therefore it will be important to generate cardiac specific conditional knockout mice whereby Brn3b expression is specifically knocked out in the myocardium. Cardiac specific Brn3b inducible knockout mice could also be used in order to analyse the effects of loss of Brn3b specifically in the heart in response to stress.

Importantly, Brn3b KO male mice had reduced exercise capacity and cardiac function following exercise, whereas Brn3b KO female mice had preserved exercise capacity and cardiac function. Unfortunately, cellular and molecular characterisation of these animals has not yet been completed (due to time constraints) and the cellular and molecular mechanisms that mediate physiological hypertrophy are not well understood. Therefore the potential role of Brn3b in adaptation to exercise and physiological stress in the heart
in males and females needs to be investigated in more detail (e.g. by analysing morphological and gene expression changes in the hearts of Brn3b KO exercised mice). Brn3b has also been shown to be expressed in skeletal muscle and regulate target gene (e.g. GLUT4) expression in skeletal muscle (Bitsi et al., 2016). Therefore it would also be interesting to investigate whether Brn3b KO male mice fail to adapt to exercise due to the absence of Brn3b in skeletal muscle; and similarly whether the oestrogen receptor may compensate for loss of Brn3b in skeletal muscle in Brn3b KO female mice (enabling them to cope with physiological stress). In addition, IGF1 signalling has been characterised for mediating physiological hypertrophy, therefore it would be interesting to determine whether IGF1 signalling activates Brn3b expression and hence whether Brn3b may be associated with this pathway and mediating hypertrophic responses to physiological stress (Bernardo et al., 2010).

Finally, there are several animal models that have been used to induce pathological and physiological hypertrophy, including trans aortic constriction, myocardial infarction and treadmill running (Skavdahl et al., 2005, Wisloff et al., 2001, Yang et al., 2002). Therefore it will also be necessary to further investigate the effects of loss of Brn3b on hypertrophic responses to pathological and physiological stress by testing these models in Brn3b KO (and WT) male and female mice.
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