A Novel Point Mutation in Prpf8 Causes Defects in Left-Right Axis Establishment in the Mouse

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List of Abbreviations:

3’Ss: 3’ splice site
5’Ss: 5’ splice site
ACT1: actin
Actc1: actin, alpha, cardiac muscle 1
Acvr1: activin A receptor, type 1
Akt1: RAC-alpha serine/threonine-protein kinase
Akt3: RAC-gamma serine/threonine-protein kinase
A-P: anterior-posterior
ASE: asymmetric enhancer region
ASF/SF2: arginine/serine-rich 1
ATM: ataxia telangiectasia mutated
Baf60c: BRG-1/Brm-associated factor 60C
Blm: Bloom syndrome, RecQ helicase like
BMP: bone morphogenetic protein
Bmp2: bone morphogenetic protein 2
Bmp4: bone morphogenetic protein 4
BMP5: bone morphogenetic protein 5
BMP7: bone morphogenetic protein 7
Bmpr1a: bone morphogenetic protein receptor, type 1A
Bmpr2: bone morphogenetic protein receptor, type II
BPS: branch point site
Ca^{2+}: calcium ion
CDK4: cyclin-dependent kinase 4
Cerl2: Cerberus-like protein 2
CHD: congenital heart disease
eNCC: cardiac neural crest cell
CNS: central nervous system
CUP1: copper metallothionein 1
CycD2: Cyclin D2
DIC: differential interference contrast
Dll-1: delta like 1
Dnahc11: dynein, axonemal, heavy chain 11
D-V: dorsal-ventral
Dvl1: dishevelled, dsh homologue 1
Dvl2: dishevelled, dsh homologue 2
Dvl3: dishevelled, dsh homologue 3
EMT: epithelial-mesenchymal transition
ENU: N-ethyl-N-nitrosourea
Epb4.1l5: erythrocyte membrane protein band 4.1 like 5
FGF: Fibroblast growth factor
FGF8: Fibroblast growth factor 8
FHF: first heart field
FOG2: friend of GATA protein 2
Foxa2: forkhead box a2
Foxh1: forkhead box h1
Foxj1: forkhead box j1
GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GATA1: globin transcription factor 1

GATA4: globin transcription factor 4

GATA6: globin transcription factor 6

GDF1: growth/differentiation factor 1

GFP: green fluorescent protein

Hh: hedgehog

Hox: homeobox

IKAP: IKK complex-associated protein

inv: inversion of embryonic turning

Irx4: Iroquois homeobox gene 4

Is11: islet-1

iv: inversus viscerum

LHF: late head fold

LPM: lateral plate mesoderm

L-R: left-right

MEF2: myocyte enhancer factor-2

Mef2c: myocyte enhancer factor 2c

MesP1: mesoderm posterior 1

MET: mesenchymal-epithelial transition

Mg$^{2+}$: magnesium ion

mLST8: MTOR associated protein, LST8 homologue

MPN: Mpr1/Pad1 N-terminal

mTORC2: mTOR complex 2
Myh10: myosin heavy chain 10
Myl4: myosin light chain 4
MyoD1: myogenic differentiation 1
NCC: neural crest cell
Nkx2.5: NK2 homeobox 5
Noto: notochord homeobox
Nppa: natriuretic peptide A
OT: outflow tract
p300: E1A binding protein p300
PCP: planar cell polarity
PCR: polymerase chain reaction
pHH3: phosphohistone-H3
Pitx2: pituitary homeobox 2
Pkd1l1: polycystic kidney disease 1 like 1
Pkd2: polycystin 2
PRC2: polycomb-repressive complex 2
Prpf3: pre-mRNA processing factor 3
Prpf31: pre-mRNA processing factor 31
Prpf8: pre-mRNA processing factor 8
qPCR: quantitative polymerase chain reaction
RA: retinoic acid
Rab23: Rab23, member RAS oncogene family
Rac1: Ras-related C3 botulinum toxin substrate 1
Rbpj: recombination signal binding protein for immunoglobulin kappa J region

Rfx3: regulatory factor X3

RNP: ribonucleoprotein

RP: retinitis pigmentosa

SC35: splicing component 35 KDa

SELI: self-enhancement and lateral-inhibition system

SEM: scanning electron microscopy

Sf3b1: splicing factor 3b, subunit 1

SHF: secondary heart field

Shh: sonic hedgehog

snRNA: small nuclear RNA

snRNP: small nuclear ribonucleoprotein

T: brachyury

Tbx1: T-box 1

Tbx5: T-box 5

TEM: transmission electron microscopy

TGF-β: transforming growth factor β

Tie-1: tyrosine kinase with immunoglobulin-like and EGF like domains 1

Tie-2: tyrosine kinase with immunoglobulin-like and EGF like domains 2

Trp53: transformation related protein 53

URA3: Uracil requiring

Vangl1: vang-like 1

Vangl2: vang-like 2

Vcam1: vascular cell adhesion molecule 1
VEGF: vascular endothelial growth factor

Wnt: wingless-type MMTV integration site family

Wnt3a: wingless-type MMTV integration site family member 3a

Zic3: Zic family member 3

Zn$^{2+}$: zinc ion

ZO1: zonula occludens-1
Abstract

Human congenital heart disease (CHD) is the most common cause of non-infectious neonatal death affecting 1-2% of live births (Hoffman and Kaplan, 2002). Treatment of CHD requires major surgery and quality of life is often significantly reduced despite treatment. With the discovery of single gene mutations that cause CHD in model animals (Lyons et al., 1995), the role of genetics in CHD has become appreciated. The genetic basis of CHD is poorly understood, with different members of the same family presenting with different types of CHD (Schott et al., 1998), suggesting the causes of CHD are multifactorial. Cardiogenesis is intimately associated with the establishment of the left-right (L-R) body axis, with the two processes sharing several important transcription factors. Heart looping, in which the heart turns dextrally, is the earliest physical manifestation of L-R asymmetry. L-R patterning disorders are associated with an increased risk of CHD; heterotaxy (in which L-R asymmetry is neither normal nor mirror image) accounts for about 3% of all CHD (Zhu et al., 2006).

Investigating cardiogenesis and the causes of CHD necessitates the use of animal models, typically mice, chicks, zebrafish and Xenopus. Recently a strain of mouse with a mutation in a gene essential for cardiac development was isolated from an ENU mutagenesis screen (Kile et al., 2003) using mice carrying a balancer chromosome. It has been subsequently found that the most likely candidate gene codes for the protein Prpf8, an integral component of the spliceosome. The mutation is homozygous lethal, with homozygous mice having a grossly deformed heart, developmental delay and a high incidence of heart looping reversal, indicative of a L-R patterning disorder. In depth characterisation of homozygous mutant embryos revealed defects in the morphology of the embryonic node, nodal cilia and the expression pattern of L-R axis genes. We also investigated the expression of Prpf8 during embryogenesis, and the effect that the point mutation we found in our homozygous embryos has on splicing kinetics.
Declaration

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Chapter 1: Introduction
**Introduction: An overview of L-R axis establishment**

This introduction will attempt to cover all the stages from node formation, initiation of L-R axis establishment at the node, through to its translation to organogenesis relatively evenly. Unless otherwise indicated, laterality establishment in the mouse is the subject of this review as there are some significant differences between model organisms. Murine L-R axis establishment occurs at the node, a teardrop shaped depression at the anterior end of the primitive streak that consists of a ciliated epithelium in the depression and crown cells surrounding the nodal pit (reviewed in Lee and Anderson, 2008). L-R information is then transferred to the left lateral plate mesoderm (LPM), which determines the L-R symmetry in the developing organs of the embryo. Laterality establishment starts at E7.5 and organ directionality is fixed by E8.5.

**Node Formation and Function**

The embryonic mouse node forms at E7.0 at the most anterior part of the primitive streak and contributes cells throughout the anterior-posterior axis including the brain, heart mesoderm and gut (reviewed in Beddington and Robertson, 1999). The node is homologous to the Spemann-Mangold organiser and Henson’s node in *Xenopus* and chickens, respectively, and as such is a major provider of tissue to the notochord (Beddington, 1994). Its patterning capacity changes through E7.0 and E8.0, and the node has been described as going through three stages: early-gastrula organiser, mid-gastrula organiser and node, each with its own genetic profile and contribution to the developing embryo (reviewed in Robb and Tam, 2004). However, surgical (Davidson *et al.*, 1999) or genetic ablation (Ang and Rossant, 1994) of the node disrupts A-P patterning to a much lesser degree than D-V and L-R patterning, suggesting it is a lesser player in establishing A-P polarity. Only the function of the node as an organ of laterality determination will be discussed further as its other functions are beyond the remit of this introduction.

The ventral node cells are derived from the anterior primitive streak. They undergo EMT and form rosette shaped clusters of *Noto<sup>+</sup>* cells that assemble long cilia on their apical surfaces in the centre of the cell cluster (Lee *et al.*, 2010; Yamanaka *et al.*, 2007).
These rosettes then move to the ventral surface of the embryo and can be seen beginning to emerge through the overlying visceral endoderm by E7.0 (Kwon et al., 2008; Lee et al., 2010; Sulik et al., 1994) to form the node (see Figure 1.1). At this point pockets of ciliated ventral node cells can be seen underneath the endoderm in confocal stacks, these form a continuous sheet prior to endoderm removal (Lee and Anderson, 2008). At the no-bud stage, when the overlying visceral endoderm is completely removed, the node is highly ciliated but completely flat; it assumes its stereotypical pit shape at the early headfold stage through an unknown process (Lee and Anderson, 2008; Sulik et al., 1994). At this stage, cells from the visceral endoderm flank the node cavity; this is disrupted in some mutants with disrupted node morphogenesis (Kwon et al., 2008; Migeotte et al., 2011). Ventral node cells are columnar and have a characteristically restricted apical surface that causes pit cells to protrude into the ventral cavity, giving a cobblestone appearance. Each cell has a long, highly motile cilium protruding into the ventral cavity that is positioned to the posterior of each cell; this localisation is controlled by the PCP pathway and is essential for L-R axis determination (Mahaffey et al., 2013). At the 6 somite stage the cells posterior to the node migrate and contribute to the posterior notochord (Yamanaka et al., 2007); the node disappears at the 7-8 somite stage.
Figure 1.1: Schematic showing the formation of the mouse node.

Cells from the dorsal epithelium undergo epithelial to mesenchymal transition (EMT) and migrate toward, but not into, the visceral endoderm. These cells then undergo mesenchymal to epithelial transition (MET) to form rosettes of now ciliated cells. These rosettes merge and delineate upon the epiblast, forming a new epithelium with the opposite apical-basal polarity to its origin. The overlying visceral endoderm is removed and the node forms its stereotypical pit. Once this is complete, the cilia begin to move, creating the nodal fluid flow.

Adapted from (Lee and Anderson, 2008)
While in mice what is often referred to as the node is specifying the indentation at the most posterior part of the notochord, it has been suggested that the mouse node is actually a condensation of cells immediately posterior to this indentation (Blum et al., 2007). Using morphological and gene expression analyses across species they find that the homologue to Spemann’s organiser is a wide plate of notochord, covered by visceral endoderm, and likely has no role in L-R axis determination. They suggest a renaming of what is commonly called the mouse node and suggest simply ‘posterior notochord;’ this change in nomenclature appears to be used haphazardly by other laterality researchers and to avoid confusion will not be used here.

Establishment of L-R axis occurs in the node

The establishment of L-R axis in the node occurs at embryonic day (E) 7.5 (Collignon et al., 1996). Around the node, fluid flows towards the future left side of the node, driven by the clockwise (when viewed from the ventral side, see Figure 1.2) motion of 9+0 cilia (Sulik et al., 1994). It has been shown that as few as two cilia can establish correct L-R asymmetry, demonstrating the high sensitivity of this system (Shinohara et al., 2012). The leftward movement of fluid is essential for proper L-R axis formation and its absence results in the randomisation of the L-R axis; its artificial reversal results in situs inversus (Nonaka et al., 2002). This is conserved in humans; human congenital diseases that are caused by loss of cilia function, such as Kartagener syndrome, often display L-R patterning defects (reviewed in Kosaki and Casey, 1998, and in Norris and Grimes, 2012). Mouse models with mutations in genes essential for nodal cilia function, such as inversin (Watanabe et al., 2003), cause L-R axis defects by disrupting nodal flow (Okada et al., 1999). This directional flow is conserved in zebrafish (Sampaio et al., 2014), rabbits (Okada et al., 2005) and Xenopus (Schweickert et al., 2007) though significant differences between these animals in L-R asymmetry establishment indicate divergence in the establishment of laterality (reviewed in Ramsdell, 2005).

It is important to consider that there is strong evidence to suggest that either laterality is established very early in all species, or that rodents may have a non-representative
method of breaking left-right symmetry. This last point requires urgent attention, because it may yet be seen that mice are a poor model for human laterality establishment, potentially reducing the biomedical relevance of research. Both the chick and pig (Gros et al., 2009) do not appear to use motile cilia for laterality establishment and rely on much earlier cues. Unlike mice, chicken Shh is expressed preferentially on the left side of Hensen’s node and plays an active role in establishing L-R asymmetry, as well as the midline (Levin et al., 1995; Levin et al., 1997). Asymmetric Shh expression is not driven by inducing Shh expression in adjacent cells, but by asymmetric movement of the cells of Hensen’s node (Gros et al., 2009). Xenopus and zebrafish (Kawakami et al., 2005), which both do have a cilia driven ‘nodal’ flow, appear to have a much earlier initial symmetry breaking event using H+/K+-ATPase ion channel activity, as do chicks (Levin et al., 2002). All these animals seem to establish laterality long before the appearance of the embryonic organiser. This has been interpreted as indicative of a universal, highly conserved symmetry breakage event that is later reinforced by a cilia dependant mechanism in many, but not all organisms (reviewed in Vandenberg and Levin, 2013). This initial symmetry breakage may use the inherent chirality of the cytoskeleton to segregate the left and right body axis. Importantly, this review and others emphasise that proteins involved in cilia function may also be important in cytoskeletal function, potentially confounding interpretation. Whether or not mice adhere to this model has not been resolved, perhaps due to the difficulty in manipulating very early mouse embryos. There is evidence to suggest manipulating mouse embryos at the 8 cell stage disturbs some aspects of laterality establishment; the direction of turning and the position of the vitelline vessels was reversed in some embryos but reversal of the viscera or cardiac looping was never observed (Gardner, 2010). It may emerge that the nodal flow is used to reinforce previously established laterality in many species, or that mice have dispensed with earlier symmetry breaking steps entirely.

There are two models of leftward fluid flow sensing. The determinant molecule model suggests that the flow of fluid results in an increase in concentration of signalling molecules, possibly sonic hedgehog (Shh) or retinoic acid (RA), which initiates left-sided specific signalling (Tanaka et al., 2005) though Shh has since been shown to be dispensable in the node (Tsiairis and McMahon, 2009). The mechanical stress model suggests that the movement of the fluid is physically sensed by immotile cilia on the
crown cells that express polycystin-2 (Pkd2), a Ca^{2+} permeable channel (McGrath et al., 2003; Yoshiba et al., 2012). Pkd2, and its binding partner Pkd1l1, have been shown to be essential for initiating the laterality signalling cascade in a manner conserved between mice and medeka fish (Field et al., 2011; Kamura et al., 2011). Current evidence appears to favour the mechanical stress model as the most parsimonious explanation for how nodal flow initiates laterality signalling. What is clear is that a left-sided calcium ion (Ca^{2+}) signal transduction cascade, mediated by the inositol triphosphate pathway, is initiated and this specifies left-sided specific signalling (McGrath et al., 2003; Yoshiba et al., 2012). This occurs not in the node crown cells but in the endoderm (Yoshiba et al., 2012). Through an unknown mechanism this signal is translated into asymmetric laterality gene expression around the node.

**Genes Involved in Establishment of Laterality at the Node**

The cardinal signalling molecule involved in L-R axis establishment is the transforming growth factor-β (TGF-β) protein superfamily signalling molecule Nodal (Collignon et al., 1996). Nodal is initially expressed on both sides of the node but becomes preferentially expressed on the left side of the node. This asymmetric expression in the node is needed for left-sided Nodal expression in the lateral plate mesoderm (LPM), which in turn directs expression of other L-R patterning genes (Saijoh et al., 2003). Nodal expression is directed to the node by a 350bp upstream enhancer and is likely induced by FoxA2 (Brennan et al., 2002). Mice with nonfunctional inversin consistently express Nodal on the right, rather than the left, of the LPM (Lowe et al., 1996) and consistently display situs inversus (Yokoyama et al., 1993), demonstrating the link between L-R patterning of the node, the LPM and the whole organism. Nodal expression and Nodal activity is controlled by a number of upstream genes and proteins, such as Cerl2, an antagonist of Nodal (Marques et al., 2004). Like Nodal, Cerl2 is initially expressed bilaterally but is instead then preferentially expressed on the right side of the node (Marques et al., 2004). This ensures that Nodal activity occurs only on the left side of the node. Canonical Wnt signalling is important for controlling correct Nodal and Cerl2 expression around the node, with Wnt 3 and Wnt3a being directly implicated in repressing Cerl2 expression (Kitajima et al., 2013; Nakamura et al., 2012; Nakaya et al., 2005). Both Wnt3a expression and β-catenin protein are preferentially localised to the left of the node at E7.75-E8.0. Wnt3a expression is, in turn, controlled
by Notch signalling and is spatially restricted to crown cells by Foxa2 and Rbpj (Kitajima et al., 2013). Fluid flow appears to induce asymmetric expression of Cerl2 by degrading Cerl2 mRNA on the left side of the node; this is then maintained by left-sided Wnt signalling to ensure Cerl2 expression is lower to the left of the node (Nakamura et al., 2012). This removes Cerl2 mediated inhibition of Nodal activity (Marques et al., 2004); Nodal can now upregulate Nodal expression (Norris et al., 2002), increasing Nodal expression on the left side of the node, and continue the laterality signalling cascade.

Figure 1.2: Cilia driven fluid flow in the node.

Diagram showing the leftward flow of fluid in the node. Cilia in the nodal pit beat to create a directional movement of fluid. This is sensed by immotile cilia, which then initiates the preferentially sided expression of genes such as Cerl2 and Nodal. This initiates laterality establishment.
Asymmetry established in the node is transferred to LPM

Once increased left sided Nodal concentrations in the node have been established, *Nodal* is then expressed in the left lateral mesodermal plate (LPM) (See Figure 1.3). *Nodal* expression in the LPM is dependent upon *Nodal* expression in the node (Brennan *et al.*, 2002; Saijoh *et al.*, 2003); mice without *Nodal* expression in the node do not develop proper L-R asymmetry in the LPM (Brennan *et al.*, 2002; Lowe *et al.*, 2001), resulting in visceral right isomerism. Quite how this information is conveyed is not fully understood. The most favoured hypothesis is that the Nodal produced in the node is transported to the LPM where it induces its own expression; this may be through gap junctions in the gut endoderm (Saund *et al.*, 2012; Viotti *et al.*, 2012). Nodal can travel long ranges (Yamamoto *et al.*, 2003) and it induces its own expression in the LPM (Saijoh *et al.*, 2000). GDF1 in the node is necessary for asymmetric *Nodal* and Pitx2 expression in the LPM; GDF1 is also needed for *Nodal* expression to expand in the LPM (Tanaka *et al.*, 2007). While it has been previously suggested that GDF1 can initiate signalling alone (Wall *et al.*, 2000), it is now known that GDF1 dimerises with Nodal to allow long range signalling but cannot itself initiate signalling (Tanaka *et al.*, 2007). Kawasumi and colleagues showed that *Nodal* expression, but not Nodal autocrine or paracrine signalling, in the perinodal cells is needed for correct L-R patterning (Kawasumi *et al.*, 2011). Perinodal cells apparently have the internal machinery to respond to Nodal, but cannot detect extracellular Nodal. This suggests that Nodal is produced within these cells for transport to the LPM, and that Nodal is not secreted in the node pit. However, it still remains to be shown that Nodal protein travels from the node to the LPM, instead of downstream signalling components conveying signalling, despite all this circumstantial evidence.
Figure 1.3: Nodal signalling in the LPM.

Preferentially left sided expression of Nodal around the node induces Nodal expression in the left LPM. This in turn induces the expression of Nodal inhibitors Lefty1 and Lefty2 in the midline and left LPM respectively. This prevents ectopic expression of Nodal in the right LPM. Nodal also induces the expression of Pitx2c in the left LPM (not shown).
Patterning of the LPM

Once the left-side determinant signal is received in the left LPM, it interacts with the protein cofactor Cryptic and is detected by activin type II receptors (reviewed in Schier and Shen, 2000). Nodal signalling activates the transcription factor Foxh1, which initiates Nodal expression in the LPM (Saijoh et al., 2000; Yamamoto et al., 2003) via an intronic asymmetric enhancer region (ASE) (Adachi et al., 1999; Norris and Robertson, 1999). Cells which receive Nodal signalling will adopt a left-sided fate; cells which do not, such as the right LPM, will adopt a right-sided fate. To confine the left-sided expression to the left LPM, Nodal induces the expression of its own inhibitors, the TGF-β proteins Lefty-1 and Lefty-2 (Yamamoto et al., 2003). Lefty-2 is produced in the left LPM itself, under control of an ASE and Foxh1, while Nodal diffuses to the midline and induces expression of Lefty-1 (Meno et al., 2001) (see Figure 1.3) with the help of GDF1 (Tanaka et al., 2007). This regulatory feedback loop precisely controls Nodal expression to ensure it occurs in the correct time and place. There are also other signalling pathways that modulate Nodal signalling and help determine L-R asymmetry. Rab23 functions in a Hh independent manner to allow Nodal signalling to propagate through the LPM; loss of Rab23 inhibits production of Nodal in the LPM (Fuller et al., 2014). The BMP/Smad1 pathway sets a threshold on Nodal signalling bilaterally by limiting the availability of Smad4; this protects against noise in the system and allows for the generation of a strong signal (Furtado et al., 2008). This system allows Nodal to robustly and specifically induce the expression of Pitx2c in the left LPM, which ultimately specifies a left identity there (Ryan et al., 1998).

Nakamura and colleagues put forward a model in which the nodal flow generates a subtle L-R asymmetry that must then be amplified by a self-enhancement and lateral-inhibition system (SELI) to determine the embryonic L-R axis, rather than nodal flow being the sole determining factor and downstream events blindly following its lead (Nakamura et al., 2006). They suggest the interaction between Nodal and its inhibitors Lefty-1 and -2 are what generate the robust L-R asymmetry signal in the LPM. Lefty-1 and Lefty-2 diffuse to the right LPM and repress Nodal activity, preventing it from adopting the left-sided fate. This model successfully predicted their finding that Nodal
is briefly expressed in the right LPM at a lower level than in the left since both sides receive the activating signal from the node. They also demonstrated that if the left LPM is removed or disabled, the right LPM will readily adopt the left-handed fate if the node is still intact. They assert that without this SELI mechanism both sides would still adopt a left-handed fate, even with an intact nodal flow.

### L-R axis and its importance in organogenesis

While the left and right sides of the body may have been defined biochemically, organogenesis must be able to translate this into correct anatomical development. Obvious anatomical asymmetry only becomes apparent after Nodal expression stops. The subsequent processes are poorly understood, though the information bestowed by Nodal must presumably be maintained through downstream transcription factors. The best known is Pitx2c which is essential for correct L-R axis patterning and is needed in the developing heart, foregut and dorsal mesentery (Shiratori et al., 2001); the other 2 isoforms (Pitx2a and b) are not expressed in the heart or left LPM (Schweickert et al., 2000). The transcription of Pitx2 is initiated by Nodal under the control of an ASE, but once Nodal expression has ceased Pitx2 expression is maintained by Nkx2.5 (Shiratori et al., 2001). Pitx2 is essential for the proper development of the viscera, including the lungs, heart and gut, and this is reflected both in patients with mutations in PITX2 and in mouse models (Kitamura et al., 1999; Semina et al., 1996). Different organs require different levels of Pitx2c activity, with the lungs needing the highest and the heart needing comparatively lower levels, while the gut requires all three Pitx2 isoforms (Liu et al., 2001). Pitx2 controls multiple aspects of asymmetric gut development, including gut morphogenesis and arterial and lymphatic development, through the upregulation of target genes (Mahadevan et al., 2014; Welsh et al., 2013). Global loss of Pitx2 does not affect heart looping directionality, despite the presence of other cardiac defects (Kitamura et al., 1999). Interestingly, mice specifically lacking the asymmetric expression of Pitx2c in the LPM did not have altered heart looping, embryonic turning or stomach placement, though they did have other laterality defects (Shiratori et al., 2006). This body of evidence suggests that while Pitx2c is essential for establishing the L-R axis of the viscera, it is dispensable for proper leftward cardiac looping, indicating the existence of unknown laterality genes.
Organs can generate L-R asymmetry in three ways: through directional looping as in the heart or gut; through sided differences in branching or size as in the liver; or through the loss of one side of an organ or structure, and this is most common in blood vessels (reviewed in Shiratori and Hamada, 2006). Since these different mechanisms presumably require different biochemical and biomechanical processes to occur normally, different organs must be able to interpret L-R information to give organ specific effects, perhaps through synthesis with dorso-ventral and anterio-posterior information. This review will not investigate global organogenesis further and will subsequently concentrate on cardiogenesis. Since defects in the development of the L-R axis and CHD greatly overlap, they will be discussed together.
An Overview of Cardiogenesis

Cardiac progenitor cells are all ultimately derived from mesodermal cells formed in the anterior region of the primitive streak, though their fate is still plastic at this stage (Tam et al., 1997). The heart and circulatory systems are ultimately derived from the LPM (Gilbert, 2000). The two main contributors of cells to the heart, the first heart field (FHF) and the secondary heart field (SHF), are two distinct cellular populations but have a common origin. By E7.5 these cells migrate anterior-laterally and extend across the midline of the embryo to form two symmetric regions, together termed the cardiac crescent (see Figure 1.4). Cells of the FHF differentiate at the cardiac crescent stage; the SHF contributes cells to the developing heart over a prolonged period of time and SHF derived cells differentiate once they have migrated into the linear heart tube (reviewed in Kelly, 2012). At E8 the FHF cells of the cardiac crescent fuse along the midline to form the linear heart tube. Once this is done, the SHF migrates into the now beating linear heart tube, where it forms a substantial portion of the heart (see Figure 1.5). At E8.5 the heart undergoes rightward looping; the first anatomical evidence of L-R axis asymmetry in the mouse embryo. By E10.5 the chambers of the heart have been firmly defined but the heart remains a tube; septation occurs at E14.5 separating the chambers from each other. This review will not concentrate on cardiogenesis past E10.5 as the Prpf8 homozygous mutant mice die by this point, preventing the analysis of further stages.
Figure 1.4: Early heart development.
Cardiac precursor cells are specified in the anterior mesoderm at E7.0. These contribute to the cardiac crescent and express cardiac marker genes. The cardiac crescent then undergoes morphological changes to form the linear heart tube.
Early Cardiogenic Signalling Molecules

The earliest stages of cardiogenesis are still relatively poorly understood; while the FHF and SHF cardiac lineages were previously thought to come from a separate clonal origin (Meilhac et al., 2004); it has since been found that there is no such common progenitor among Mesp1+ cells (Devine et al., 2014). This indicates that daughter cells of heart field progenitors must assume a FHF or SHF fate rapidly and permanently (Devine et al., 2014). The earliest known cardiac specific transcription factor is MesP1, which is expressed at E6.5-7.0 and is needed for correct heart formation (Saga et al., 2000) and specification of most heart cell types (Saga et al., 2000; Saga et al., 1999). MesP1 expression and cardiac mesoderm specification is induced by Eomes in the presence of low levels of Nodal signalling; Eomes induces definitive endoderm specification in the presence of high levels of Nodal signalling (Costello et al., 2011; van den Ameele et al., 2012). MesP1 can directly promote a cardiovascular cell fate by upregulating cardiogenic genes and downregulating genes promoting alternative cell fates (Bondue et al., 2008). While in vitro cardiac progenitors can undergo multi-lineage differentiation (Bondue et al., 2008; Kattman et al., 2006; Moretti et al., 2006), recent work using an in vivo mouse model contradicts this interpretation. (Lescroart et al., 2014) show that MesP1 expressing cardiac precursors are allocated to either the FHF or SHF in a temporal fashion and have a correspondingly distinct transcriptome. This implies that the cardiac master transcription factor is upstream of MesP1 and is presently unknown, but acts before gastrulation. They go on to show that FHF precursors are unipotent and SHF precursors are bipotent, further contradicting previous work that suggested multipotency.

Many signalling pathways have been found to be involved in early cardiogenesis. Implantation experiments in chicken embryos demonstrated that BMP signalling through Bmp2 can ectopically induce the expression of cardiac myogenesis markers (Andrée et al., 1998; Schultheiss et al., 1997). A large number of mouse lines have since been generated with components of the BMP signalling pathway knocked out; these typically have wide ranging defects including in cardiogenesis (reviewed in Wang et al., 2014). BMP signalling is needed to induce the FHF; the removal of the BMP receptor 1a protein completely prevents maintenance of the cardiac crescent and
primitive ventricle, leaving only a small SHF (Klaus et al., 2007). While the exact function of BMP signalling remains less well characterised in early heart development, its requirement in the formation of the outflow tract (OT) and cardiac cushions is well established. Reduction in BMP signalling either through a hypomorhpic Bmpr2 allele or through NCC specific deletion of Alk2 results in OT septation defects (Délot et al., 2003; Kaartinen et al., 2004). Similarly, myocardial Bmp2 and endocardial Bmpr1a are needed to form the cardiac cushions (Ma et al., 2005). BMP inhibitors such as Noggin are also necessary for cardiomyocyte induction (Yuasa et al., 2005).

It was initially shown in chicken models that canonical Wnt signalling inhibits cardiogenesis to prevent ectopic heart growth (Marvin et al., 2001; Tzahor and Lassar, 2001). While it first appeared that canonical Wnt/β-catenin signalling inhibits cardiogenesis and non-canonical Wnt signalling promotes cardiogenesis, the situation now appears much more complex (for a review see Eisenberg and Eisenberg, 2006). In mice the loss of β-catenin signalling prevents the generation of Isl1+ progenitor cells in the SHF (Klaus et al., 2007). However, in Xenopus, canonical Wnt/β-catenin signalling represses cardiogenesis through inhibition of GATA gene expression (Afouda et al., 2008) and inhibitors of this pathway are needed for cardiogenesis (David et al., 2008). Elegant experiments by (Klaus et al., 2012) reveal that Notch signalling lies upstream of Wnt signalling, which in turn is upstream of Bmp signalling and directly regulates the transcription factors Baf60c, Nsk2.5 and Isl1 as well as Bmp4 signalling. After initial cardiac induction by BMP and Wnt signalling, a host of transcription factors take over, of which this review will explore the two best characterised, GATA4 and Nkx2.5.

GATA4, a zinc finger transcription factor and one of the earliest cardiac markers, is activated by BMP signalling during initial cardiac induction. GATA4 regulates the expression of a number of cardiac structural genes, such as α-myosin heavy chain (Grépin et al., 1994) and injection of GATA4 RNA into Xenopus embryos prematurely activates cardiac genes such as α-myosin heavy chain (Jiang and Evans, 1996). GATA4 is essential for the lateral to ventral folding that generates the linear heart tube and the foregut, but is not essential for cardiomyocyte differentiation (Molkentin et al., 1997). Other GATA transcription factors may be able to compensate for loss of GATA4 and allow cardiomyocyte differentiation. GATA4 and GATA6 double knockout embryos
completely lack the FHF and cannot differentiate cardiac precursor cells into cardiomyocytes; the SHF is still specified but cardiomyocytes do not form (Zhao et al., 2008). Activity of GATA4 is activated by p300 and inhibited by PRC2 through acetylation and methylation of the GATA4 C-terminus respectively (He et al., 2012). GATA4 physically interacts with many other transcription factors such as Nkx2.5 (Durocher et al., 1997), MEF2 (myocyte enhancer factor-2) (Morin et al., 2000) and Tbx5 (T-box 5) (Garg et al., 2003) to activate promoters for cardiac specific genes under their control. Interestingly, the interactions between GATA4 and FOG2 and between GATA4 and Tbx5 are dispensable for cell fate specification, at least in Xenopus (Gallagher et al., 2014). As well as transcription factors, it has been described that GATA4 interacts with CycD2 and CDK4, suggesting a connection between GATA4 activity and the cell cycle (Gallagher et al., 2014; Yamak et al., 2014).

Nkx2.5, the vertebrate homologue to Drosophila tinman, is essential to proper development of both the FHF and SHF. Nkx2.5 is a promoter of many genes involved in cardiogenesis, and physically interacts with transcription factors such as Tbx5 (Hiroi et al., 2001). The importance of Nkx2.5 is highlighted by the great variance in severity of the cardiac defects generated through different mutations in Nkx2.5 (reviewed in Clark et al., 2006). Nkx2.5 is needed for proper development of the myocardium, yolk sac vasculature and proper heart looping, as well as controlling ventricular gene expression (Lyons et al., 1995; Tanaka et al., 1999b). In Nkx2.5 mutant mice cardiac tissue is still present, but heart development is grossly abnormal and stops during looping (Tanaka et al., 1999a). Nkx2.5 is activated by GATA4 in association with Smads 1 and 4, placing Nkx2.5 activation under the control of BMP signalling via Smads (Brown et al., 2004; Liberatore et al., 2002). Nkx2.5 negatively regulates its own expression by repressing Bmp2 expression, inhibiting BMP signalling (Prall et al., 2007). The BMP signalling pathway initiates cardiac specification; its overactivity through loss of Nkx2.5 causes premature specification and a concurrent proliferative failure, leading to CHD.
Cardiac Cell Populations

The FHF is generated from anterior lateral mesoderm and eventually forms the left ventricle and contributes to the atria and inflow tract. *Nkx2.5* and GATA transcription factors activate FHF genes, which ultimately gives rise to the FHF, though both are essential to SHF development as well. *Hand1* (also called *eHand*), is needed for specification of the left ventricle and its expression is dependent on *Nkx2.5* (Yamagishi et al., 2001). *Tbx5* is essential for normal formation of the primitive atria, left ventricle and inflow tract but not right ventricle or outflow tract (Bruneau et al., 2001), suggesting a primarily FHF specific role. SHF specific gene *Isll* is expressed throughout the intraembryonic coelomic lining at E7.5, which contains progenitors of both heart fields (Prall et al., 2007). This is lost by E8.0 in FHF derived structures, though persists in *Nkx2.5* null mutants. Specification of the FHF is poorly understood compared to the SHF, possibly due to its lack of specific genetic markers making FHF specific work difficult. However, the recent discovery of a FHF specific marker, HCN4, will hopefully remedy this situation (Liang et al., 2013).

The SHF is a group of progenitor cells that are derived from pharangeal and splanchnic mesoderm and migrates into the linear heart tube during cardiac looping (Kelly et al., 2001; Waldo et al., 2001). The SHF has come to be defined by its expression of the LIM homeodomain transcription factor *islet-1* (*Isll*); cardiac progenitor cells that express *Isll* are SHF cells, those that do not are FHF cells (Cai et al., 2003). This is a useful definition since without *Isll* structures derived from the SHF are completely absent; these are the entire right ventricle, most of the outflow tract (OT) and a substantial portion of the atria (Cai et al., 2003). However, more recent evidence suggests that this is an oversimplification as *Isll* is also expressed in the NCCs (Engleka et al., 2012) and FHF progenitor cells, albeit at lower levels than in the SHF (Ma et al., 2008). The elongation of the OT is essential for its rotation and is needed for proper cardiac looping (Yelbuz et al., 2002). In the chicken, Shh signalling maintains the SHF by stimulating progenitor cell proliferation, which is needed for OT elongation; a loss of Shh signalling causes arterial pole defects (Dyer and Kirby, 2009). OT elongation is done using similar signalling pathways and transcription factors as used by FHF cells (Waldo et al., 2001). *Isll* expressing cells contribute to many cardiovascular cell lineages including myocyte, endothelial, conduction system and smooth muscle cells.
(Sun et al., 2007). By E9.0 most Isl1 expressing progenitors have migrated onto the linear heart tube and ceased Isl1 expression (Sun et al, 2007).
Figure 1.5: First and secondary heart field contributions.

Schematic showing the contributions of the FHF and SHF to the different regions of the heart. While the left and right ventricles contain mainly derivatives of the FHF and SHF respectively, the atria have an equal proportion of both.
As well as \textit{Isl1}, SHF development requires \textit{Nkx2.5} and \textit{GATA4}. \textit{Mef2c}, needed for the development of SHF derived structures, has both \textit{Isl1} and GATA4 binding sites suggesting it is a downstream effector of \textit{Isl1} (Dodou \textit{et al.}, 2004). \textit{Hand2} (also called \textit{dHand}) is needed for SHF derived cells to populate the right ventricle (Yamagishi \textit{et al.}, 2001). The L-R axis determinants \textit{Foxh1} (Forkhead box h1) and the \textit{Pitx2c} isoform of the \textit{Pitx2} homeobox gene are both essential to SHF formation, which may make the SHF more vulnerable to L-R axis disruptions than the FHF. \textit{Foxh1} associates with \textit{Nkx2.5} to activate transcription of \textit{Mef2c}, under the control of TGF-\beta signalling (von Both \textit{et al.}, 2004). Loss of either \textit{Foxh1} or \textit{Mef2c} completely prevents formation of structures descendant from the SHF (Lin \textit{et al.}, 1997; von Both \textit{et al.}, 2004). Aberrations in \textit{Pitx2c} expression results in a constellation of cardiac defects associated with atrial and ventricular isomerism, suggesting a L-R defect (Campione \textit{et al.}, 2001). Different organs, such as the heart, lungs and gut, require different amounts of \textit{Pitx2c} for correct L-R axis determination (Liu \textit{et al.}, 2001). In the SHF \textit{Pitx2} is needed for OT growth and great vessel development; its loss causes atioventricular misalignment (Ai \textit{et al.}, 2006). \textit{Pitx2} appears to be under the genetic control of both \textit{Nkx2.5} and \textit{Tbx1}, with both genes acting to maintain \textit{Pitx2} expression in the SHF (Nowotschin \textit{et al.}, 2006; Shiratori \textit{et al.}, 2001).

\textbf{Cardiac Looping}

At E8.5 the heart changes from a linear heart tube running down the midline of the organism to a dextrally looped structure; the first physical display of L-R asymmetry (see Figure 1.6). While this is frequently referred to as cardiac ‘looping’, it has come to light that this process is much more complex then the heart tube bending rightwards. Since all of the published material on the biomechanics of heart looping use chicks as a model animal, it will have to be assumed that this section is generally applicable to murine cardiac looping. Heart looping has two phases: the ‘c’ and the ‘s’ phase, which occur in that order (Männer, 2000). In the ‘c’ phase the linear heart tube assumes a ‘c’ shape with the outer curvature pointing to the right of the embryo; this phase involves both ventral bending and dextral torsion. During the ‘s’ phase the atria move so that they are now anterior to the ventricles, following this septation segregates the different chambers; this is very much a simplification of this phase for the sake of clarity.
However, rather than the heart tube simply bending rightwards, the heart tube instead twists along the A-P axis and forms a so-called helical perversion, in which two helices of opposite handedness are connected in the same object, rather than a simple helix (Männer, 2013). Heart looping requires the input of mechanical force from the primitive atria and the splanchnopleure, and altering the strength of these forces can change both the timing and direction of cardiac looping (Taber et al., 2010). Actin polymerisation appears to be needed for the ‘c’ phase of looping, but cytoskeleton contractions are dispensable (Taber, 2006). However, if the splanchnopleure is removed, the heart is able to compensate for the loss of the force it provides through cytoskeletal contraction to stiffen the right side of the myocardium (Nerurkar et al., 2006), demonstrating the existence of compensatory mechanisms. Mechanical models of heart looping suggest that the confined space of the pericardial cavity is important for mechanically compressing the elongating heart tube to form the complex shape of the looped heart (Bayraktar and Männer, 2014). As important the biomechanics of heart looping undoubtedly are (for reviews see Männer, 2000 and Taber, 2006), molecular specification of the L-R axis in the heart must occur immediately prior to looping at the latest for physiological asymmetry to manifest itself. For instance, the model proposed by Taber and colleagues suggests that the left atrium pushes on the heart tube with more force than the right atrium in order to turn the heart dextrally (Taber et al., 2010). This would require the left atrium to identify as ‘left,’ and the greater diameter of the left atrium at this point indicates this asymmetry. Replicating heart looping using a silicone tube which is progressively displaced toward the ‘caudal’ end of the tube supports the hypothesis that the heart tube is biased to loop toward the left before heart looping begins (Bayraktar and Männer, 2014). When the silicone tube is bilaterally symmetrical, there is an even chance of the tube looping to the left as to the right; a small bias to the left or right, however, drives the tube to loop preferentially in that direction (Bayraktar and Männer, 2014).
Figure 1.6: Heart looping.

At E9.0 the ventricle and atrium of the linear heart tube descends and ascends, respectively, producing the dextrally looped heart. By E9.5 the left and right ventricles have specific molecular and genetic identities.
Laterality Defects and Congenital Heart Defects: A Close Connection

Heart development relies on precise spatial and temporal control over gene expression to form a complex 3D shape and as such is sensitive to errors in this process. Mutations in cardiogenic transcription factors can cause a variety of heart defects; confusingly, mutations in the same gene can cause different types of CHD (reviewed in Clark et al., 2005) (see Figure 1.7). Mutations in Nkx2.5, for instance, can cause a variety of types of CHD even in the same family carrying the same mutation (Schott et al., 1998). This heterogeneity in phenotypes contributed greatly to the previous confusion as to the causes of CHD and hints at the great complexity of cardiogenesis. Somatic mutations in genes such as Nkx2.5 and Gata4 do not appear to be associated with CHD (Esposito et al., 2011).

While the role of cilia is well understood in L-R axis establishment, and defects in the L-R axis can cause CHD, it is becoming appreciated that cilia have a direct role in cardiogenesis. Cilia are essential for intercellular signalling, which plays an important role in heart development, and mouse models with mutations in cilia genes often present with forms of CHD not associated with laterality defects (Koefoed et al., 2014; Slough et al., 2008). A forward genetics screen recovered a number of mouse mutant lines with mutations in ciliary genes that presented with CHD but not L-R axis defects (Li et al., 2015). However, CHD is often comorbid with other systemic defects not directly affecting cardiogenic transcription factors, in particular, with laterality defects.
Figure 1.7: CHD and its relationship with cardiogenic transcription factors.

Diagram showing major sites of CHD and linking them to important transcription factors, making it easy to see how mutations in one gene can cause different or multiple defects. The list of transcription factors is illustrative, not exhaustive. ASD: atrial septal defect; AV block: atrioventricular block; AVSD: atrioventricular septal defect; DORV: double outlet right ventricle; PS: pulmonary stenosis; PTA: tricuspid atresia; TOF: tetralogy of Fallot; VSD: ventricular septal defect. Adapted from (Nemer, 2008)
The importance of correct L-R patterning in the process of organogenesis can be seen in instances where it goes wrong. Loss of coherent L-R axis determination can result in *situs inversus*, where the viscera are a mirror image of their correct positioning, termed *situs solitus*. More seriously *situs ambiguus*, or heterotaxy, may arise, this is where only some of the viscera have a mirror image L-R asymmetry to *situs solitus*, or only parts of individual organs have correct L-R asymmetry. These L-R patterning disorders are associated with a number of health problems, and by far the most common of these is CHD (reviewed in Ramsdell, 2005). Disruptions in L-R patterning are associated with a much greater risk of CHD; heterotaxy accounts for an estimated 3% of all CHD cases (Zhu *et al.*, 2006). The mortality rate for heterotaxy is 30%; for left isomerism (two left body sides) and right isomerism (two right body sides) in the first year of life it is 60% and 80% respectively (reviewed in Bowers *et al.*, 1996). CHD may arise from the disruption of the L-R axis patterning causing incorrect looping or chamber specification. However, because transcription factors involved in L-R determination, such as *Foxh1* and *Pitx2*, are also involved in cardiogenesis (Kitamura *et al.*, 1999; von Both *et al.*, 2004) their loss or disruption would not only disrupt L-R patterning but also cardiogenesis even if physically the L-R axis appeared normal. The close relationship between L-R patterning and cardiogenesis can lead to the situation where an isolated case of CHD can be the only presenting symptom of a laterality disturbance (Goldmuntz *et al.*, 2002; Zhu *et al.*, 2006). Indeed, the current incidence rate of laterality defects (1 in 10,000) is considered to be an underestimate as it is not routinely screened for and may pass unnoticed in mild cases (Casey and Hackett, 2000).
Prpf8 and the Spliceosome

The spliceosome is a ribonucleoprotein (RNP) complex that removes the introns in pre-mRNA prior to its export into the cytoplasm as mature mRNA. It contains 6 protein-rich small nuclear RNPs (snRNPs), U1, U2, U4, U5 and U6, which are integral to the spliceosome, as well as many associated proteins (reviewed in Wahl et al., 2009). There are two splicing pathways, the major (U2) pathway (shown in Figure 1.8), which is responsible for over 99% of splicing, and the minor (U12) splicing pathway; only the U5 snRNP is common between them (reviewed in Patel and Steitz, 2003).
Figure 1.8: Diagram illustrating U2 splicing across short exons.

Moving clockwise from top left; the U1 and U2 snRNPs bind to the 5’ splice site (SS) and branch point site (BPS) of the intron, respectively, to form complex A. The U4, U5 and U6 snRNPs are then recruited as a preassembled tri-snRNP, forming complex B, but the whole complex is catalytically inactive. Activation requires the removal of U1 and U4, forming complex B*; this catalyses the first step of splicing, forming complex C. Following this, the second step of splicing occurs, the intron forms a lariat structure bound to the snRNPs and mature mRNA is released. The U2, U5 and U6 snRNPs are then recycled and the lariat is degraded. Adapted from diagram in Grainger and Beggs, 2005
Pre-mRNA Processing Factor 8: Structure and Function

Prpf8 (Prp8 in yeast) is a large (∼256KDa across 42 exons in humans) protein found in both U5 and the U4/U6.U5 tri-snRNP (see Figure 1.8) (Stevens and Abelson, 1999; Stevens et al., 2001). Prpf8 is highly evolutionarily conserved; ClustalW analysis reveals a 61% conservation between human PRPF8 and S. cerevisiae Prp8 and only a 3 amino acid difference between humans and mice. Most experiments exploring its structure and function have been done in yeast, but due to the high conservation it is assumed that these results apply to human PRPF8. Prp8 is the only protein that can be photochemically linked to the 5’ splice site (SS), the branch point site (BPS) and the 3’SS of pre-mRNA and the U5 and U6 snRNAs suggesting that Prp8 interacts directly at these sites (reviewed in Grainger and Beggs, 2005). Experiments in S. cerevisiae reveal Prp8 is essential for splicing pre-mRNA and its depletion halts spliceosome formation at the complex A stage due to the U4/U6.U5 tri-snRNP failing to form and assemble onto the spliceosome (Brown and Beggs, 1992), preventing splicing.

The Role of PRPF8 in Disease

As the causative mutation for the K27 phenotype is most likely in Prpf8, a key component of the spliceosome, our current working hypothesis is that the K27 phenotype is caused by missplicing of certain genes to give rise to the phenotype. Mutations in PRPF8, as well as other spliceosomal genes, are already known to cause retinitis pigmentosa (RP) in humans, a progressive deterioration of the peripheral retina causing night blindness and tunnel vision. All known RP linked PRPF8 mutations are found in the C-terminus, with the vast majority in exon 42 (Maubaret et al., 2011). Patients with RP typically have a delay in spliceosomal assembly and defects in both constitutive and alternative splicing (Tanackovic et al., 2011), showing that splicing is defective. Reproducing these mutations in S. cerevisiae typically prevents U5 snRNP maturation, inhibits the binding of accessory proteins and causes splicing defects (Boon et al., 2007). Other RP mutations modelled in yeast have shown that dysregulated spliceosomal catalytic activity or inhibited protein-protein interactions, rather than build up of immature spliceosomes, can also cause RP (Maeder et al., 2009; Mozaffari-Jovin et al., 2013). Mutations in Prpf3, 8 and 31 have all been seen to specifically affect phagocytosis and adhesion in the retinal pigment epithelium, even in young mice (Farkas et al., 2014). It is not understood how a mutation in a ubiquitously expressed protein could only affect the retina, but it has been suggested that because the retina
requires a higher than normal rate of protein synthesis it could be uniquely vulnerable to
defects in splicing (Tanackovic et al., 2011). Indeed, several components of the
spliceosome, including Prpf8, have upregulated expression in the mouse retina
compared to other tissues (Cao et al., 2011).

Prpf8, Mitosis and Cancer

It is becoming increasingly apparent that components of the spliceosome may play a
direct role in mitosis (reviewed in Hofmann et al., 2010). A slew of recent papers has
connected defects in the spliceosome with defects in mitosis, in particular with
premature sister chromatid separation (reviewed in Valcárcel and Malumbres, 2014).
Regardless as to which spliceosomal component is mutated or depleted (including
PRPF8), the defects in mitosis all appear to largely come from the missplicing of
CDCA5 pre-mRNA leading to depleted levels of sororin protein, which is essential for
stabilising chromosomal pairing in mitosis (Oka et al., 2014; Sundaramoorthy et al.,
2014; van der Lelij et al., 2014; Watrin et al., 2014). PRPF8 may also interact directly
in mitosis as part of a large multi-protein complex in association with topoisomerase
IIa, though its function there remains unknown (De Wever et al., 2012; Lee et al.,
2004). Unsurprisingly, defects in proteins controlling mitotic chromosomal stability are
associated with cancer, though the causal relationship is unclear (reviewed in Losada,
2014). In addition to the role of Prpf8 in mitosis, it is also emerging that Prpf8 plays a
role in the DNA damage response pathway. Disrupting the spliceosome by depleting
levels of PRPF8 increases Trp53 transcription and causes a Trp53 depended cell cycle
arrest, presumably to inhibit tumour formation (Allende-Vega et al., 2013). In the
presence of transcription blocking DNA damage, the protein kinase ATM is activated;
ATM then displaces the spliceosome from damaged DNA, as well as promoting
alternative splicing (Tresini et al., 2015). In light of its role in mitosis and DNA
damage response, it is fitting that components of the spliceosome are emerging as
targets for chemotherapy (reviewed in Bonnal et al., 2012). In keeping with this,
somatic mutations in PRPF8 and other spliceosomal components have been associated
with human cancers, particularly leukaemia (Kurtovic-Kozaric et al., 2015; Makishima
et al., 2012). This is perhaps less surprising when considering their revealed role in
mitosis. These mutations have been confirmed to cause missplicing of specific genes,
such as GATA1, and appear to preferentially affect genes involved in haematopoiesis
and the mitochondrial electron transport chain (Kurtovic-Kozaric et al., 2015). In zebrafish, an ENU derived mutation causing Prpf8 to be prematurely truncated induced missplicing in several genes, including itself and GATA1, as well as embryonic death (Keightley et al., 2013).
The K27 Project

Background

The K27 mutant line was isolated in the ENU mutagenesis screen which investigated mouse chromosome 11 (Hentges et al., 2006; Kile et al., 2003). The mutagenesis screen used balancer chromosomes, which are chromosomes with defined regions of inverted DNA that suppress homologous recombination (Sturtevant, 1921). This allows maximal recovery of lethal alleles within this defined region (see Figure 1.9). The mice used in this study carry the inversion chromosome 11 (Inv(11)8BrdTrp-Wnt3); this inversion was generated by (Zheng et al., 1999) using Cre-loxP chromosome engineering (Ramírez-Solis et al., 1995). The inversion in this chromosome disrupts Wnt3 to give homozygous embryonic lethality (Liu et al., 1999), and has a dominant Agouti marker, which gives balancer carriers yellow coats allowing for convenient identification. By crossing these mice with the mice generated in the ENU mutagenesis screen, mice with the mutation in trans of the balancer chromosome are generated (see Figure 1.10). Crossing this F2 generation with itself gives mice that are either homozygous mutant or mutant/balancer, since homozygous balancer mice are not viable. The simple absence of homozygous mutant mice indicates that a mutation has occurred in an essential gene. This system allows for the rapid identification of lethal mutations within a defined chromosomal region based on their phenotype, which removes any potential bias towards already known genes or phenotypes. Repeated backcrossing and outcrossing to 12S5/SvEvBrd mice was used to remove ENU induced mutations outside of the balancer chromosome region, while retaining mutations in trans of the balancer chromosome. This strategy was used to isolate the I1LJus27 line, which was then divided into two mouse lines, the EHC line which contained a mutation in Myh10, and the K27 line which contained an unknown mutation.
Figure 1.9: A balancer chromosome screen investigating the region of synteny between human chromosome 17 mouse chromosome 11.

More than half of human chromosome 17 is conserved with mouse chromosome 11 (purple region). Regions lacking synteny are in black. The 24cM inversion between $Wnt3$ and $Trp53$ (orange region) is within this conserved region. Taken and adapted from Kile et al, 2003 and (Hentges and Justice, 2004)
Figure 1.10: Schematic showing creation of l11Jus27 mouse line.

C57BL/6J males treated with ENU (black mice) are mated with females carrying the balancer region between Wnt3 and Trp53 (brown mice). The inversion contains the K14-Agouti mutation giving light ears and tails, marking balancer carriers. The resulting G1 generation is mated with mice carrying the balancer and the Rex mutation (curly haired mice, shown with broken outline), which marks non-mutated chromosomes. G2 mice carrying the balancer and mutation (yellow with a normal coat) are mated with each other to produce homozygous mutants in the G3 offspring. Homozygous balancer carriers are Wnt3 deficient and die. The type of chromosome is shown beneath the diagrams (blue with orange: balancer; blue with red star: mutated; black: Rex mutation carrier.). Adapted from Hentges and Justice, 2004.
Next generation sequencing on the K27 mutant line has revealed a number of mutations; however only 1 is in an exon, making it our strongest candidate (Tenin, G. pers. comm.). This mutation is a missense mutation in the gene *Prpf8*, which codes for a component of the spliceosome that is essential in both the major and minor splicing pathways (Patel and Steitz, 2003). The mutation is a point mutation causing an A to G transition that substitutes a serine residue for an asparagine residue at amino acid 1531 on exon 28. Bioinformatic analysis reveals that this residue is conserved between mice and yeast and is immediately upstream of both a potential phosphokinase C phosphorylation target and a conserved coiled coil domain (Grainger and Beggs, 2005). Protein modelling experiments suggest that the interaction between Prpf8 and its binding partner, Aar2, may be altered in our mutant protein. From this point the K27 line will be referred to as the *Prpf8<sup>N1531S</sup>* line. Approximately half of *Prpf8<sup>N1531S</sup>* homozygous mutant embryos display reversed cardiac looping along with profound heart dysmorphology. They also display global developmental delay compared to littermates and yolk sac defects, though the phenotype can be highly varied in severity.
Aims and Objectives

The aim of this PhD project is to characterise the phenotype of the Prpf8<sup>N1531S</sup> homozygotes, determining what developmental processes are affected and finding the cause of these defects. The overarching hypothesis throughout this project is that the mouse line has defects in cardiogenesis and laterality establishment, which are caused by the Prpf8<sup>N1531S</sup> mutation. This hypothesis was interrogated in two ways. First; the phenotype was investigated to determine the nature and cause of the morphological defects we observe. Second, the role of Prpf8 in embryogenesis and the functional consequences of the N1531S mutation were investigated to determine whether a defect in Prpf8 could cause our phenotype.

To characterise the phenotype, in situ hybridisation was used to visualise the expression pattern of genes important in cardiogenesis and L-R axis establishment. We also investigated the node of Prpf8<sup>N1531S</sup> homozygotes using immunofluorescent staining, electron microscopy and videomicroscopy to determine whether there were defects present at the node.

In order to confirm that Prpf8 is needed in embryogenesis and that the N1531S mutation affects Prpf8 function, we used other model organisms. We determined the effect that malfunctional Prpf8 has on embryogenesis by knocking down Prpf8 in zebrafish embryos with morpholinos. The specific N1531S base change was investigated using yeast models, to provide biochemical data on the consequences that this mutation has on splicing.
Chapter 2: Methods and Materials
Methods and Materials:

General lab reagents were supplied by Sigma. Primer sequences can be found in Appendix 1 and were bought from Eurogentec (http://www.eurogentec.com/lifescience.html).

2.1: Frequently used solutions

10x Phosphate buffer solution (PBS)
11.5g Na2HPO4
2g K2HPO4
80g NaCl
2g KCl
To 1l with H2O

PTW
PBS
0.1% Tween-20

4% Paraformaldehyde (PFA)
8g PFA
Make up to 200ml with PBS, heat and stir until dissolved.

10X TAE buffer
242g Tris base
57.1ml acetic acid
100ml 0.5M EDTA pH 8.0
To 1l with H2O

TE buffer
1ml 1M Tris-HCl pH 7.5
200µl 0.5M EDTA pH 8.0
To 100ml with DEPC treated H2O

Hybridisation mix recipe (total 50ml):
50% Formamide (Sigma F7503)
1.3x SSC pH 7.5
5mM EDTA pH8.0
50g/ml Yeast tRNA (Invitrogen 15401-011)
0.5% CHAPS (Sigma 26680, dissolved in H2O)
100µg/ml Heparin (Sigma H9399),
0.2% Tween-20 (Sigma)

5x MAB pH7.5 recipe:
11.6g maleic acid (Sigma M0375)
8.7g NaCl
Adjust pH to 7.5 with NaOH and make upto 200ml with H2O. Autoclave
5x MABT pH 7.5
To 50ml of MAB add 0.5ml 10% Tween-20.

NTMT:
1ml 5M NaCl
2.5ml 2M Tris-HCl pH 9.5
2.5ml 1M MgCl₂
5ml 10% Tween-20
39ml H₂O.

Zebrafish embryo water:
1ml of 4% Instant Ocean aquarium sea salt (Spectrum Brands)
2ml of 0.1% methylene blue (Sigma MB1)
Make up to 1l with H₂O

MS222 solution:
2g MS222 (Sigma E10521)
10.5ml 1M Tris pH9
To 500ml with ddH₂O
2.2: Genotyping

Yolk sacs were lysed in 50-100μl of 50mM NaOH at 95°C for at least 30 minutes. 2μl of this is used in a PCR reaction using primers complimentary for MIT327 and MIT35 (sequences in Appendix 1). If tissue for genotyping had previously been incubated in media containing serum, the tissue was washed in 200ml PBS and then centrifuged before lysing in NaOH.

2.3: PCR

PCR reaction mixture composition: 1μl primer mix, 3μl MyTaq Red Mix (Bioline 25044), 1-2μl DNA/cDNA made up to 10μl with H2O. Samples were initially denatured at 94°C for 5 minutes before undergoing 40 cycles for denaturing at 94°C for 1 minute, annealing at 60°C for 1 minute and extending at 72°C for 1 minute 30 seconds before undergoing a final extension for 5 minutes at 72°C.

2.4: Cycle sequencing PCR

2μl BigDye (ABI) mix, 2μl primer, 5μl sequencing buffer, Xμl DNA/cDNA made up to 20μl with H2O. The reaction took place in a thermocycler on the following program:

1: Heat lid
2: 96°C for 1 minute
3: 96°C for 30 seconds
4: 50°C for 15 seconds
5: 60°C for 4 minutes
Repeat the last three steps 24 times
6: Store at 4°C

2.5: Sample precipitation for cycle sequencing

PCR products were pipetted into an Eppendorf tube to which 16μl H2O and 64μl 95% ethanol was added. The samples were vortexed and left to stand at room temperature for 15 minutes before being centrifuged at 13000RPM for 20 minutes. The supernatant was discarded and 250μl 70% ethanol was added to tubes, which were vortexed. The samples were then centrifuged at 13000RPM for 10 minutes and the supernatant
discarded. The pellets were then dried and stored at -20°C before being sent for sequencing. Sequences uploaded to the sequencing server were visualised on 4Peaks software (Nucleobytes).

2.6: qPCR reactions in triplicate

Reaction mix for Prpf8 TaqMan probe (Applied Biosystems Cat: 4331182) for each biological sample: 14µl 10x buffer, 14µl 50mM MgCl₂, 5.6µl dNTP, 1.4µl HGS Diamond Taq polymerase (Eurogentec TAQ-1011-1000+), 91µl H₂O, 7µl cDNA.

This was divided into 66.5µl aliquots, to which either 1.2µl GAPDH primer and 1.8µl Sybr green 1 or 3µl Prpf8 TaqMan probe was added. 23µl of the reaction mixture was added to each well.

Reaction mix for Trp53 primers for each biological sample: 21µl cDNA (25ng/µl), 84µl GoTaq qPCR master mix (Promega A6002), 111µl H₂O. This was divided into 72µl aliquots to which 6µl primer was added. 24µl of the reaction was added to each well.

Reaction mixtures were ran on a DNA Engine Opticon 2 continuous fluorescence detector (BioRad). Samples were initialised at 95°C for 10 minutes before undergoing 40 cycles of denaturing at 95°C for 10 seconds and annealing at 60°C for 1 minute and then being read. The melting curve extending from 40°C to 100°C was generated, with samples held for 1 minute each °C and read every 1°C. The raw data were processed on Opticon monitor 3 (BioRad) software and subsequently analysed using Excel (Microsoft). Samples were normalised against expression levels of GAPDH, a housekeeping gene.

2.7: Glass wool purification of DNA from agarose gel

The gel bands were cut out and placed over silane trated glass wool in a small Eppendorf tube, which has a small hole in the base. The small tube was placed in 1.5ml Eppendorf tube and spun at 5000RPM for 5 minutes. An equal volume as the flowthrough of isopropanol and 1/10 the volume of the flowthrough of 3M NaAc was added to the flow through, which was mixed thoroughly and stored overnight at -20°C.
The DNA was then spun at 13000RMP for 10 minutes, aspirated and resuspended in 20µl H₂O.

2.8: DNA purification using Bioline kit (Cat no:52059)

The Bioline ISOLATE II PCR and gel kit was used for routine DNA purification from agarose gel fragments or enzymatic reaction mixtures, following manufacturer’s instructions. Heated elution buffer was always used to recover DNA.

2.9: Generating linear template for DIG labelled RNA probes

Two methods were used to generate RNA probes, the PCR method and the plasmid method.

In the PCR method, primers designed to amplify a ~600bp region of the gene of interest was designed with T3 or T7 promoter sites 5’ of the genetic sequence on the sense primer. These were used to generate a linear template via PCR amplification, which was purified and used to generate antisense RNA.

In the plasmid method, plasmid containing templates for antisense RNA synthesis were isolated by miniprep and linearised by an appropriate restriction enzyme. To the linear plasmid reaction mixture was added 0.5µl 10mg/ml proteinase K, 25µl 1% SDS and 4.5µl H₂O; this was incubated at 45°C for 40 minutes. This reaction mixture was then purified with the Bioline ISOLATE II kit following the PCR clean up protocol. DNA was eluted in 2 passes of 30µl of preheated elution buffer. The DNA was then precipitated by adding 60µl isoproponol and 6µl 3M NaAc pH 5.2 and incubating the reaction mixture at -20°C overnight. DNA was centrifuged and washed twice in 75% ethanol, then dried and resuspended in 20µl of DEPC treated H₂O.
2.10: Synthesising DIG labelled RNA probe

To an appropriate amount of linear template 2µl 10x transcription buffer (Roche); 2µl DIG RNA labelling mix (Roche); 2µl of the appropriate RNA polymerase (Roche); 0.5µl RNase inhibitor (Promega) was added, and volume made up to 20µl with DEPC treated H2O. The reaction mixture was incubated at 37°C for 2 hours. After incubation 1µl RNase-free DNase was added and the mixture incubated at 37°C for 15 minutes. Afterwards 100µl TE (pH 7.5-8.0), 10µl 4M LiCl and 300µl isoproponol were added and the mixture stored overnight at -20°C. The reaction mixture was then centrifuged at 13000RPM for 20 minutes, the supernatant removed and the pellet was washed in 75% ethanol in DEPC treated H2O. The pellet was centrifuged at 13000 rpm for 5 minutes, the supernatant was removed and the pellet was dried and resuspended in 20µl TE buffer (pH 7.5).

2.11: RNA extraction:

Tissue samples stored at -20°C in RNA later (Ambion) or Trizol (Invitrogen) were placed in 100-200µl of TRI Reagent (Sigma-Aldrich, T9424) or Trizol, homogenised, and left to stand for at least 5 minutes. 0.2ml of chloroform was added per 1ml of TRI Reagent, the samples were vortexed for 15 seconds and left to stand for 15 minutes at room temperature. Samples were then centrifuged at 12,000RPM for 15 minutes at 4°C. The top aqueous phase was transferred to a fresh tube. 0.5ml of isoproponol per 1ml of TRI Reagent was added, the samples vortexed and then left to stand for 10 minutes at room temperature. The samples were then centrifuged at 12,000 RPM for 10 minutes at 4°C to pellet the RNA. The supernatant was discarded and the pellet washed in 1ml of 75% ethanol per 1ml of TRI Reagent used. The pellet was dried and resuspended in 30µl DEPC treated H2O. Yield was found by measuring absorbance at 260nm in a spectrophotometer or by Nanodrop. RNA was stored at -80°C.

2.12: Reverse transcription

RNA was first cleared of DNA with a DNase reaction. 5µg of RNA, 2µl 5x reaction buffer (Bioline) and 1µl RNase-free DNase 1 (Promega M6101) were made up to 10µl with DEPC treated H2O and incubated at 37°C for 30 minutes. 1µl 25mM EDTA was
added and the sample was incubated at 70°C for 15 minutes. Following this, the Tetro cDNA synthesis kit (Bioline 65042) was used to generate cDNA. 1µl of random hexamer primers, 1µl 10mM dNTP mix, 2µl 5x reverse transcriptase buffer, 1µl RiboSafe RNase inhibitor and 1µl Tetro reverse transcriptase were all added to the reaction mixture, which was made up to 20µl with H2O and incubated at 42°C for 1 hour. cDNA was stored at -20°C or, for long term storage, -80°C.
2.13: Embryo dissection for whole mount *in situ* hybridisation

Pregnant mice were allowed to progress until embryos were the required age. They were then euthanized using a schedule 1 method. Embryos were then dissected out into 1% phosphate buffered solution (PBS), extraembryonic membranes were removed and the yolk sac saved for genotyping. If the headfold had closed, a hole was made in the back of the head to prevent trapping of in situ staining. Embryos were fixed in 4% paraformaldehyde (PFA) in 1% PBS for 1 hour at room temperature or overnight at 4°C. After fixation, embryos were washed twice in 1% PBS, then twice in 50% MeOH/PTW (1%PBS, 0.1% Tween 20) and then stored at -20°C in 100% MeOH.

2.14: Whole mount *in situ* hybridisation

Embryos were rehydrated and washed twice in PTW. They were then digested with 10µg/ml of Proteinase K in PTW at room temperature for 5 minutes at E6.5-7.5 and an extra 5 minutes per day up until E10.5. Embryos were then refixed for 20 minutes in 4% PFA with 0.1% glutaraldehyde in PTW. Embryos were then washed in PTW twice, then washed in 1:1 PTW/hybridisation mix, then washed again in pure hybridisation mix before incubating embryos in fresh hybridisation mix at 65°C for 1.5 hours. 1ml of prewarmed hybridisation mix containing roughly 1µg/ml DIG labelled RNA probe was added and the embryos were returned to 65°C. The embryos were incubated for between 1 and 4 days.

Post hybridisation the embryos were washed twice with prewarmed hybridisation mix before excess probe and mRNA was digested with 20/ml RNase A (Roche 109169) in hybridisation mix at 37°C. The embryos were then washed in hybridisation mix twice at 65°C, then once in 1:1 hybridisation mix/MABT at 65°C. Embryos were then rinsed and washed in MABT for 15 minutes and then incubated for 1hr at room temperature in 1x MABT, 2% blocking reagent in H2O (1.5ml total volume) on a rocker. Embryos were then incubated for 1.5hrs in 1x MABT, 2% blocking reagent, 20% sheep serum (Sigma) in H2O (1.5ml total volume) at room temperature on a rocker. Finally, embryos were incubated overnight at 4°C in 1x MABT, 2% blocking reagent, 20% sheep serum and 1/1000 dilution of Ant-Digoxigenin-AP (Roche 1093274) on a rocker.

Post antibody incubation the embryos were rinsed 3 times in MABT and transferred to a glass scintillation vial. They were then washed 3x 1hr in 3-4ml MABT on a rocker,
then washed 2x 10 mins in 3-4ml of NTMT before being incubated in 3ml NTMT with 3µl NBT-BCIP (Roche 11681451001) or 30µl 5-Bromo-6-chloro-3-indoyl phosphate p-toluidine salt (Acros Organics 337290500, dissolved in dimethylformamide (Sigma D4551) at 50mg/ml). Embryos were allowed to develop in the dark on a roller at room temperature until colour was strong enough (typically 2-5 days). Embryos were then washed 3x in PTW and stored in PTW at 4°C.
2.15: Bacterial transformation

For purchased competent cells the manufacturer’s instructions were followed. For homemade competent cells the following protocol was used. 50µl of chemically competent cells had 2-6µl of circular plasmid DNA added to them and were left on ice for 1 hour. The cells were then heat shocked for 1 minute at 42°C, returned to ice for 30 seconds before adding 250µl of LB broth and incubating on a shaker for 1-2 hours at 37°C. 75-100µl of this broth was then plated on LB agar plates containing 100µg/ml ampicillin. The plates are incubated over night at 37°C.

2.16: Bacterial miniprep

Kits from Qiagen (Cat no. 12123) or Bioline (Cat no. 52056) were used, a general protocol is provided here. A 5ml culture of a single bacterial colony containing the desired plasmid was grown up in LB broth overnight at 37°C. 4ml in total of bacterial broth was spun down in a centrifuge in each 2ml centrifuge tube. The bacterial pellet was resuspended in 250µl buffer 1 containing RNase A. 250µl of buffer 2 was then added and the tube was inverted several times to ensure mixing; the lysis reaction proceeded for 4 minutes. 350µl of chilled buffer 3 was then added to neutralise the lysis buffer; tubes were inverted several times and incubated on ice for 5 minutes. Following this, the tubes were centrifuged at 13000 rpm for 10 minutes to pellet the precipitate. The supernatant was transferred to a brand specific spin column and the columns were centrifuged for 30 seconds at 8000 rpm. The appropriate wash buffers were then added to columns, which were then centrifuged for 30 seconds. Flow through was discarded and columns were centrifuged for 2 minutes to remove residual buffer. Columns were then placed in fresh Eppendorf tubes and 50µl elution buffer, which had been heated to 70°C, was added to the centre of each column and left to stand for 2 minutes. Columns were then centrifuged for 5 minutes and elutant was collected in and stored at -20°C.

2.17: PRPF8 bacterial maxiprep

A kit from Qiagen (Cat no 12162) was used and the protocol for low-copy plasmids using the QIAGEN-tip 500 provided by the manufacturer was followed. Colonies
containing the desired plasmid were picked and grown up in 5ml of LB broth with 100µg/ml ampicillin for 4 hours at 37°C. 2.5ml of these starter cultures were transferred into 500ml of LB broth with 100µg/ml ampicillin and grown overnight at 37°C. Cells were centrifuged and resuspended in 10ml buffer P1. 10ml of buffer P2 was then added, the reaction mixture was inverted several times and the lysis reaction proceeded for 4 minutes. 10ml of chilled buffer P3 was then added and the mixture was mixed thoroughly and incubated on ice for 20 minutes. The sample was then centrifuged at 20000 rpm for 30 minutes at 4°C. To remove precipitate that would not pellet, the supernatant was passed through filter paper. The supernatant was then passed through an equilibrated QIAGEN-tip 500, which was then washed with 2x30ml buffer QC. Plasmid was eluted into a 50ml Falcon tube with 15ml buffer QF, which was prewarmed to 70°C. 10.5ml isopropanol was added to precipitate DNA, and the solution was centrifuged at 18000 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed with 5ml of 70% ethanol and centrifuged at 18000 rpm for 10 minutes. The supernatant was removed and the pellet air-dried before being resuspended in 100µl of elution buffer P (Bioline). Quantity and quality of the plasmid was assessed via Nanodrop and gel electrophoresis. 160µg of each plasmid and a glycerol stock of each plasmid were shipped on dry ice to Colin Johnson, St James’ Hospital, Leeds.
2.18: Scanning electron microscopy

Pregnant mice were allowed to progress until embryos were the required age. They were then euthanized using a schedule 1 method. Embryos were then dissected out into 1% phosphate buffered solution (PBS), extraembryonic membranes were removed and the yolk sac saved for genotyping, but the amniotic membrane was kept intact. Embryos were fixed in 2.5% glutaraldehyde and 4% PFA in 0.1M HEPES (pH 7.4) at least overnight. Embryos were stored up to a month in fixative. When ready for processing, the embryos were washed 5x5 minutes in ddH2O, fixed for 1hr in 1% OsO4 in 0.1M HEPES (pH 7.4) at room temperature. There were then washed for another 5x5 minutes in ddH2O and subjected to an EtOH dehydration series (25%, 50%, 70% EtOH for 20 minutes each) and embryos were stored in fresh 70% EtOH at 4°C. Embryos were then put through 1x90% and 3x100% EtOH washes at 20 minutes each before critical point drying. Embryos were washed 3x30 minutes in liquid CO2 at X pressure before the temperature was raised to X°C. Embryos were then mounted on EM stubs with graphite sticky tape (company) and epoxy (?) using forceps. Embryos were imaged at high vacuum at 10KV on a Quanta FEG 250 electron microscope (FEI).

2.19: Transmission electron microscopy

Pregnant mice were allowed to progress until embryos were the required age. They were then euthanized using a schedule 1 method. Embryos were then dissected out into 1% phosphate buffered solution (PBS), extraembryonic membranes were removed and the yolk sac saved for genotyping, but the amniotic membrane was kept intact. Embryos were fixed in 2.5% glutaraldehyde and 4% PFA in 0.1M HEPES (pH 7.4) at least overnight. Embryos were stored up to a month in fixative. Embryos were then processed, stained and sectioned by the EM facility and imaged on a Tecnai 12 Biotwin transmission electron microscope (FEI).
2.20: Fish morpholino injections

Splice blocking Morpholinos were designed to exons 7 and 24 of Zebrafish Prpf8. Concentrations and volumes required to give a penetrant phenotype without excessive morpholino toxicity were empirically determined. Injection volume was confirmed using a graticule. Morpholinos (GeneTools) were diluted in 1% PBS and 0.5% Phenol red and injected using a microinjector and borosilicate glass needles (Havard Apparatus 30-0038). Zebrafish embryos were injected at the 1-2 cell stage into the yolk cell and were arranged for injection on 3% agarose plates created using casting moulds. 3% agarose was made using zebrafish embryo water. These create trenches in the agarose into which embryos can be securely placed for injecting. After injecting, embryos were transferred to plates of zebrafish embryo water. Embryos were allowed to develop until 72 hours post fertilisation in an incubator at 37°C before the chorion was removed using forceps and embryos were immobilised using MS-222 solution. Heart looping and kyphosis was then assessed before embryos were either destroyed or stored in Trizol at -20°C prior to RNA extraction.

Morpholino sequences can be found in Appendix 1

Control: 0.52nl of 0.5mM control morpholino (4.4ng)
P53 morpholino: 0.52nl of 0.3mM Trp53 morpholino (1.22ng)
Exon 7 morpholino: 0.52nl of 0.3mM exon 7 morpholino (1.33ng)
Exon 24 morpholino: 2nl of 0.7mM exon 24 morpholino (11.8ng)

2.21: Detection of morpholino induced splice variants

Zebrafish morphant embryos that had been stored in Trizol had RNA extracted and cDNA synthesised as described previously. Only embryos with the morphant phenotype were examined. Exons flanking the morpholinos target sequence were amplified via PCR and then visualised on a 2% agarose gel. Aberrant sized transcripts were cut out, purified and sequenced via cycle sequencing as described above.
2.22: Chamber slide preparation

Squares of PVC tape was applied to non-adhesive microscope slides and a chamber in the middle of the square was cut out using a scalpel. The resulting chamber was washed and any attached adhesive and other detritus was removed. Only 1 layer of tape is needed if the embryo was flat or trimmed close to the node. If the embryo was quite rounded, more layers were needed to prevent the embryo from folding over or the node from becoming compressed against the coverslip.

2.23: Embryo dissection for immunofluorescence

Pregnant mice were allowed to progress until embryos were the required age. They were then euthanized using a schedule 1 method. Embryos were then dissected out into 1% phosphate buffered solution (PBS), extraembryonic membranes are removed and the yolk sac saved for genotyping. Embryos were fixed in 4% paraformaldehyde (PFA) in 1% PBS for 2 hour at 4°C. After fixation, embryos were washed twice in 1% PBS, then taken through a MeOH/PBS dehydration series (25%, 50%, 75%, 100%) then stored at 4°C in fresh 100% MeOH between 1 and 5 days. Embryos to be stained for ZO-1 or Prpf8 were not fixed in PFA prior to the MeOH dehydration series.

2.24: Immunofluorescence Staining

Embryos prepared for immunofluorescent staining were rehydrated through a methanol series and then washed 3x20 minutes in PBS before a 90 minutes incubation in 5% horse serum, 0.5% Triton X-100 in PBS at room temperature. Embryos were then incubated overnight at 4°C on a rocker in 5% horse serum, 0.5% Triton X and stained against acetylated tubulin (1:500, Sigma T7451) and gamma tubulin (1:500, Sigma T5192) or acetylated tubulin and ZO.1 (1:100, Life Technologies 40-2300). Embryos were then washed 3x20 minutes in PBS. Immunocomplexes were then incubated with goat anti-mouse (1:500, Alexa fluor 488) and goat anti-rabbit (1:500, VectaStain BA 1000) antibodies in PBS for 2 hours at room temperature. Embryos were washed 3x10 minutes in PBS and then incubated with 1:500 streptavidin-Cy3 (GE PA43001) in PBS for 30 minutes. Embryos were then rinsed and washed 3x20 minutes in PBS. If stained with DAPI, embryos were incubated with 50ng of DAPI in PBS for 10 minutes and then
washed 1x10 minutes in cold PBS. The node region of the embryo was dissected out and arranged ventral side up and then mounted in Prolong Gold Antifade (Life Sciences, P36934) in a home-made chamber slide, which was cured for at least 2 days before imaging at room temperature. Images were taken on a Leica TCS SP5 AOBS upright confocal using a 63x/1.40 HCX PL Apo objective and 1.0-2.0x digital zoom using sequential imaging at format 2048 x 2048. Images were collected using the following detection mirror settings: DAPI 389-505 nm; Alexa Fluor 488 544-572 nm; Cy3 563-724 nm, using the 405 nm (25%), 488 nm (20%) and 543 nm (100%) laser lines respectively.

When staining using rabbit anti-Prpf8 antibodies, the above protocol was followed, but instead of 1:500 streptavidin Cy3, 1:250 Cy5 (GE PA45001) was used to detect anti-Prpf8 antibodies. Images were collected using the following detection mirror settings: DAPI 389-505 nm; Alexa Fluor 488 544-572 nm; Cy5 639-800 nm, using the 405 nm (25%), 488 nm (20%) and 633 nm (100%) laser lines respectively. Anti-Prpf8 from Abcam were used at a 1:100 dilution; antibodies provided by Colin Johnson were used at a 1:50 dilution.
2.25: Nodal flow videomicroscopy

Embryos were cultured in high glucose DMEM modified with HEPES without glutamine and sodium pyruvate (Sigma D6171), supplemented with 10% heat inactivated FBS (Gibco 10500064). This will be subsequently referred to as node media. 50ml aliquots were stored at -20°C before use.

Overnight preperation:

Frozen node media was thawed at 4°C in a refrigerator. Gel packs normally used for keeping deliverables cold were incubated at 37°C overnight. Homemade chamber slides were prepared, washed and stored under a lid to protect from dust. The camera (Leica MC170 HD) was mounted onto the microscope (Leica DMRB) in advance to save time.

Node videomicroscopy

PBS and thawed node media were warmed in a 37°C water bath. For visualising nodal flow, a 1:100 dilution of latex microbeads (Sigma L1398) in node media in an Eppendorf tube was warmed at 37°C. A pregnant female was sacrificed to obtain embryos at E8.5 and the intact uterine horn was placed in the warmed PBS, to prevent embryos from becoming cold. The warmed node media, PBS with the uterus and 1:100 dilution of media with microbeads were all placed in a polystyrene box, with the warmed gel packs used to keep all the reagents warm. Embryos were dissected out from the uterus one at a time in warmed node media, with only embryos with 1-3 somites used for flow visualisation. Yolk sacs were retained for genotyping. Embryos were mounted on the homemade chamber slides, excess media was removed, and the 1:100 media/latex beads dilution was added. Embryos were then positioned ventral side up, and a coverslip was added. If necessary, the coverslip was gently pushed onto the slide to ensure surface tension kept the coverslip in place. Embryos were then incubated at 37°C for at least 10 minutes before being imaged on the Leica DMRB at 40X using a Leica MC170 HD microscope camera. Videos lasting 5 minutes were taken with a video grabber program (VGA2USB, Epiphan Video) using an MPEG4 decompressor at maximum quality. Video analysis was performed using the Manual Tracker plugin (Cordelieres, F.) from Fiji (Schindelin et al., 2012). Movies were
converted to .tiff stacks containing 150 frames from the first minute of filming and the path of 5 beads was tracked.

2.26: Nodal cilia videomicroscopy

Overnight preperation:

Frozen node media was thawed at 4°C in a refrigerator. Gel packs normally used for keeping deliverables cold were incubated at 37°C overnight. Homemade chamber slides were prepared, washed and stored under a lid to protect from dust.

Node videomicroscopy

PBS and thawed node media were warmed in a 37°C water bath and the 37°C temperature controlled incubator on the microscope was turned on. A pregnant female was sacrificed to obtain embryos at E8.5 and the intact uterine horn was placed in the warmed PBS, to prevent embryos from becoming cold. The warmed node media, PBS with the uterus and 1:100 dilution of media with microbeads were all placed in a polystyrene box, with the warmed gel packs used to keep all the reagents warm. Embryos were dissected out from the uterus one at a time in warmed node media, with only embryos with 1-3 somites used for flow visualisation. Yolk sacs were retained for genotyping. Embryos were mounted on the homemade chamber slides ventral side up, and a coverslip was added. If necessary, the coverslip was gently pushed onto the slide to ensure surface tension kept the coverslip in place. Embryos were imaged on a Nikon TE2000 PFS microscope using a 100x/1.49 Apo TIRF objective. Videos lasting 20 seconds were collected on a FASTCAM SA3 (Photron) at 125 frames per second and analysed using FIJI.
2.27: Site directed mutagenesis

The Q5 Site Directed Mutagenesis kit (NEB) was used to induce site directed mutations following kit instructions and using kit components.

For creation of true wild type human *PRPF8* from IMAGE clone (which contained a missense mutation (a759g) and is likely to be highly deleterious to protein function) 24.72ng of plasmid was used. The primers used to substitute the incorrect nucleotide for the correct nucleotide created a new restriction site for Eco47III, which was used to screen for bacterial colonies containing the correct clone prior to sequencing.

Primers used:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>hcDNAPrpf8NEBSDM For</td>
<td>5’- ACTTACCAGCgCTGGCAGTTTC -3’</td>
</tr>
<tr>
<td>hcDNAPrpf8NEBSDM Rev</td>
<td>5’- GGAGCCATTTACATACCTCCTG -3’</td>
</tr>
</tbody>
</table>

Primers were annealed at 65°C.

For creation of the K27 mutation (N1531S) from true wild type *PRPF8* 130.18ng of plasmid was used. Rather than creating the exact K27 mutation (aat to agt), it was decided to create a synonymous mutation (aat to agc). This created a BseYI restriction site that was used to screen for colonies containing the correct clone prior to sequencing.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>K27hprpf8NEBSDM For</td>
<td>5’- CAGATTCACAgcCGTAGATTCACC -3’</td>
</tr>
<tr>
<td>K27hprpf8NEBSDM Rev</td>
<td>5’- GTTCAGGCCTGATCGCTG -3’</td>
</tr>
</tbody>
</table>

Primers were annealed at 59°C.
2.28: FLAG tag cloning

Oligonucleotides containing a FLAG tag sequence, a start codon, a Kozak sequence and a Cla1 restriction site were cloned into the true wild type and the K27 mutation plasmids at the AgeI restriction site. Oligonucleotide sequences are provided in Appendix 1.

Oligonucleotide annealing:

2μl of sense and antisense oligonucleotides were annealed to each other in reaction mixtures containing 2μl 10x ligation buffer (Roche) and 14μl H2O. Annealing took place in a thermocycler on the following program:

1: Heat lid
2: 95°C for 5 minutes
3: Ramp to 50°C at 0.1°C/second
4: 50°C for 10 minutes
5: Chill to 4°C at 1°C/second

Plasmid digest:

2μg of plasmid containing PRPF8 were digested with AgeI for 1 hour at 37°C. 0.5μl of CIP (NEB) was added and the reaction mixture was incubated at 37°C for 1 hour. The reaction mixture was purified using the NEB clean up kit following the protocol for PCR clean up. The plasmid was eluted in 30μl of elution buffer heated to 70°C and concentration was found by Nanodrop.

Plasmid ligation:

50ng of plasmid was ligated to 52ng of annealed oligonucleotides in a reaction mixture containing 4μl 5x buffer and 0.5μl T4 ligase (Roche), in 20μl total reaction volume. Ligation occurred in a thermocycler on the following program:

1: Heat lid
2: 37°C for 20 minutes
3: 16°C for 15 minutes
Repeat last two steps twice
4: 37°C for 10 minutes
5: Store at 4°C
Plasmid transformation:

The reaction mixture was then adjusted to a 1:5 dilution and 2µl of the diluted reaction was used to transform Top10 OneShot chemically competent cells (Invitrogen). As a control, cut but unligated plasmid was also transformed into Top10 OneShot cells. 100µl of transformed cells were spread on LB agarose plates containing 100µg/ml ampicillin; colonies were observed on the plate which received cell transformed with ligated plasmid only. Colonies were picked for miniprep via standard protocol, with some bacterial broth of each picked colony used to create glycerol stocks. Plasmids containing the FLAG insert were detected by ClaI digestion and gel electrophoresis, and the orientation of the insert was found by cycle sequencing. Glycerol stocks of plasmids containing successful ligations were retained.
2.29: CRISPR cloning

Targets for CRISPR/Cas9 editing were identified using a web tool (Zhang, 2015, [http://crispr.mit.edu](http://crispr.mit.edu)). Two G residues, to allow for T7 polymerase directed transcription, and BsmBI restriction sites are appended to the 5’ of the each sense oligonucleotides ordered; only a BsmBI restriction site is added to the antisense oligonucleotides. Complimentary pairs of oligonucleotides were annealed as previously described for FLAG tag cloning. Oligonucleotide sequences are provided Appendix 1.

Ligation of oligonucleotides in gRNA vector was performed using a protocol from (Jao et al., 2013). 1µl annealed oligonucleotides, 400ng gRNA vector, 1µl 10x NEBuffer 3, 1µl 10x T4 ligase buffer (Roche), 0.5µl BsmBI, 0.3µl BglII, 0.3µl Sall, 0.5µl T4 DNA ligase were combined and made up to 10µl with H2O. The ligation reaction was performed in a thermocycler on the following program:

1: Heat lid
2: 37°C for 20 minutes
3: 16°C for 15 minutes
Repeat steps 2-3 twice
4: 37°C for 10 minute
5: 55°C for 15 minutes
6: 80°C for 15 minutes
7: Store at 4°C

2µl of the ligation product was used to transform Top10 OneShot cells, 100µl of the transformed cells was plated onto LB agarose plates containing 100µg/ml ampicillin.

Successful ligation was determined by cycle sequencing using M13F primers and suitable clones were stored as glycerol stocks.
2.30: Animal husbandry

The PrpfsN1531S strain is derived from the llJus27 strain (Kile et al., 2003) and has been extensively backcrossed to the 12S5/SvEvBrd line carrying the chromosomal inversion Inv(11)8BrdTrp53-Wnt3. Mice were maintained in the Biological Service Facility of the University of Manchester, UK, according to Home Office requirements and with local ethical approval. Personal licence number 40/10317; Home Office procedure project licence number 40/3406. Mice were euthanised using a Schedule 1 method according using Home Office standards.

Zebrafish embryos were obtained using timed matings, with fertilised eggs collected and immediately used for morpholino injections. The Flk-GFP-Casper (Flk-GFP+/+, roy/r-, nacre/r-) strain was exclusively used as this strain expresses GFP in endothelial cells and has no pigmentation. Zebrafish were housed in the Biological Service Facility of the University of Manchester, UK, according to Home Office requirements and with local ethical approval, under procedure project license number 70/8132. Zebrafish embryos were destroyed 3 days post fertilisation, which is before falling under Home Office protection (6 days post fertilisation). As such, no personal licence is required.

2.31: Statistical Analysis

Statistical analysis and the generation of graphs from data was performed on GraphPad Prism 6.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. A paired t-test (two tailed) with Welch’s correction was used to investigate the difference in somite numbers between E8.5 embryos. Differences in nodal cilia lengths and numbers were investigated using a Mann-Whitney test. Fishers exact test (two-tailed) was used to analyse the zebrafish morpholino data. The comparative CT method was used to investigate the qPCR data (Schmittgen and Livak, 2008), with the results subjected to an unpaired t-test with equal standard deviation.
Chapter 3: Characterising the $Prpf^N1531S/N1531S$ Phenotype
3.0 Introduction

Investigating the phenotype of mutant animals is a cornerstone of both developmental biology and genetics. By finding which processes are defective in the embryogenesis of mutant embryos and identifying the causative mutation, the developmental function of a gene can be elucidated. Mutagenesis studies can reveal phenotypes caused by disruptions in the function of genes; mutagenesis is often a key step to finding the function of an unknown gene. Mutagenesis screens in animal models are particularly important in investigating human birth defects, as the functions of many genes are conserved between humans and model organisms. Human birth defects can frequently be replicated in model organisms such as mice.

In particular, we have employed a mutagenesis screen in mice to isolate mutants with cardiac developmental defects in order to identify genes that may contribute to human congenital heart defects. One such mutant, the \( Prpf8^{N1531S} \) mouse, was selected for further study in this thesis research due to the apparent cardiac morphological defects in mutant embryos. While heterozygotes appear normal, \( Prpf8^{N1531S} \) homozygous embryos usually begin to die by E10.5, but typically look similar to E9.5 heterozygous embryos and have a number of defects in cardiac morphogenesis, laterality establishment and yolk sac remodelling, as well as developmental delay. This chapter will describe all these phenotypes except the L-R axis defects, which will be discussed in chapter 4.

3.1 Allantois and Yolk Sac Development

The correct formation of the yolk sac, placenta and umbilical vessels is essential for embryos to be carried to term; defects in human placental development affect about a third of pregnancies, which is very high when compared to other animals (reviewed in Jauniaux et al., 2006). While there are differences between mouse and human placental development and structure, the different cell types in the placenta are comparable between the two species, and genes required for murine placental development are expressed in a similar manner in humans (reviewed in Rossant and Cross, 2001). In both humans and mice the allantois is essential for umbilical cord formation and the yolk sac is an essential source of haematopoiesis, both of which are required for
successful embryogenesis (reviewed in Freyer and Renfree, 2009). This introduction will be referring to mouse development unless otherwise stated.

The allantois and yolk sac are extraembryonic membranes that receive major contributions from epiblast cells that have passed through the primitive streak from E7.0 until E7.5 (Kinder et al., 1999). During this period yolk sac undergoes vasculogenesis to form the primitive capillary plexus (reviewed in Garcia and Larina, 2014). At E8.0 the allantois elongates until it connects with the chorion, to which the allantois attaches during chorioallantoic fusion; the allantois also undergoes vasculogenesis at this point but this is not dependent upon chorioallantoic fusion (Downs et al., 1998). Between E8.5 and E9.5 the yolk sac undergoes vascular remodelling through angiogenesis to create a mature network of blood vessels able to convey blood flow throughout the yolk sac; this is dependent upon sufficient cardiac output and blood viscosity (Lucitti et al., 2007). From E9.5 to E10.5 the chorionic surface begins to develop villi into which blood vessels from the allantois spread, this will form the future labyrinth, analogous to the human chorionic villi; the rest of the chorion will form the other placental structures (reviewed in Watson and Cross, 2005). Prpf8^{N153S} homozygous embryos do not develop past this stage, precluding further analysis.

Genetic Control of Allantoic and Yolk Sac Development

A number of genes have been described as being essential for proper allantoic development and chorioallantoic fusion, though many have incompletely penetrant phenotypes (reviewed in Inman and Downs, 2007; Watson and Cross 2005). Mutations in genes coding for the cell adhesion molecules Vcam1 and its receptor α4 integrin prevent chorioallantoic fusion in a large subset of embryos, presumably due to weakened intercellular attachment (Inman and Downs, 2007). Bmp signalling appears to be important for allantoic development, with mutant Bmp2, Bmp4 and Bmp5/Bmp7 double mutant mice all reported to have defects in allantoic development and chorioallantoic fusion (Lawson et al., 1999; Solloway and Robertson, 1999; Ying and Zhao, 2001). However, many genes reported to be essential for proper allantoic
development or chorioallantoic fusion have no obvious specific role in either of these processes, underscoring the incomplete understanding of these processes.

The genetic control of yolk sac formation and maturation is better understood than the control of allantoic development. The initial formation of the primitive capillary plexus through vasculogenesis is reliant upon VEGF signalling mediated by the receptor Flk1 to generate both capillaries and haematopoietic cells (reviewed in Garcia and Larina, 2014). The tyrosine receptor kinases Tie-1 and Tie-2 and their ligands, angiopoietins 1 and 2, play key roles in yolk sac remodelling as controllers of angiogenesis. Tie-2 appears to have a pivotal role in driving angiogenesis, while Tie-1 is essential for maintaining blood vessel integrity (Sato et al., 1995). Angiopoietins 1 and 2 act as an agonist and antagonist of Tie-2 activity and angiogenesis, respectively (Davis et al., 1996; Maisonpierre et al., 1997; Suri et al., 1996). Angiopoietins 3 and 4 exist in mice and humans respectively and bind to Tie-2 in vitro, though their function in vivo is unknown (Valenzuela et al., 1999).

**Cardiogenesis**

As heart development has already been covered in depth in Chapter 1, this section will simply serve as a reminder of events in early cardiogenesis that are relevant to the phenotype of Prpf8N1531S homozygous embryos, as well as discussing the two genes that were investigated in this project, *Isl1* and *Irx4*.

Cardiac progenitor cells originate from mesoderm that has passed through the primitive streak during gastrulation (Tam et al., 1997). Both the heart and circulatory system come from the LPM, with the FHF and SHF making the majority contribution to the heart itself (Gilbert, 2000). At E8.0 both the FHF and SHF are found in the cardiac crescent below the headfold, which reaches across the embryonic midline. Atrial and ventricular identity is specified at the cardiac crescent stage, prior to the formation of these structures (Bruneau et al., 2000). The FHF forms the linear heart tube at E8.0 by migrating across the midline of the embryo, fusing the cardiac crescent. After this has occurred, the SHF migrates into the heart tube to form much of the heart; this migration
is also essential for proper heart looping. The FHF forms the left ventricle and contributes to the atria and inflow tract while the SHF forms the right ventricle, OT and contributes to the atria. Heart looping begins at E8.5 and is complete by E9.5; at this point the left and right ventricles have acquired distinct molecular identities. At E10.5 the chambers of the heart have been specified but septation has not yet occurred; K27 embryos die at this stage.

**Genes controlling cardiogenesis**

A great many genes have been described as affecting some aspect of cardiogenesis (reviewed in Clowes *et al.*, 2014), so this introduction will only discuss the most pertinent to this thesis research project. *Nkx2.5* and *Gata4* are regulators of many genes needed for specification and function of future cardiac cells (Hiroi *et al.*, 2001; Jiang and Evans, 1996). *Isl1* is essential for the SHF to contribute to the heart tube and its expression is often used to define the SHF (Cai *et al.*, 2003). The genes *Hand1* and 2 are essential for ventricular morphogenesis, and are preferentially expressed in the left and right ventricles, respectively (Srivastava *et al.*, 1997). Despite their apparent compartmentalisation, *Hand2* can compensate somewhat for the loss of *Hand1*, though CHD are still observed (McFadden *et al.*, 2005).

**Isl1**

The LIM homeodomain transcription factor *islet-1* (*Isl1*) is expressed in cells that contribute to the future SHF (Cai *et al.*, 2003). These SHF progenitor cells go on to form the entire right ventricle, most of the outflow tract (OT) and a substantial portion of the atria (Cai *et al.*, 2003). Other cardiac progenitor cells, such as the NCCs and FHF progenitor cells do express *Isl1*, though at lower levels than SHF progenitors (Engleka *et al.*, 2012; Ma *et al.*, 2008). *Isl1* expressing cells contribute to many cardiovascular cell lineages including myocyte, endothelial, conduction system and smooth muscle cells (Sun *et al.*, 2007). By E9.0 most *Isl1* expressing progenitors have migrated into the linear heart tube and ceased *Isl1* expression (Sun *et al.*, 2007). *Isl1* and GATA4 bind to recognition sites on *Mef2c*, which directs the development of the SHF (Dodou *et al.*, 2004). *Isl1* itself appears to be under the control of one or more Forkhead transcription factors, as a number of evolutionarily conserved Forkhead binding sites have been
found in an enhancer for *Isl1* expression in the *Isl1* locus (Kang et al., 2009). *Isl1* expression in cardiac progenitors also appears to be directly upregulated by β-catenin signalling (Lin et al., 2007).

**Irx4**

*Iroquois homeobox gene 4* (*Irx4*) is a highly conserved gene that plays an important role in ventricle specification and the maintenance of ventricle specific genes (Bao et al., 1999; Bruneau et al., 2000; Bruneau et al., 2001). *Irx4* expression is restricted to the cardiac ventricle, as well as the hindbrain. Ventricle expression of *Irx4* appears to be dependent upon both *Hand2* and *Nkx2.5* expression; mice with non-functional *Nkx2.5* do not express *Irx4*, while mice missing *Hand2* initiate but do not maintain *Irx4* expression (Bruneau et al, 2000). Interestingly, mice null for *Irx4* survive into adulthood, but eventually develop cardiomyopathy caused by impaired ventricular function; embryonic expression of *Hand1* was lower in these mice than in controls (Bruneau et al, 2001). Mutations in *IRX4* have been linked to ventricular septal defects in humans, possibly due to defects in protein-protein interactions between IRX4 and its binding partners (Cheng et al., 2011).

**Summary**

The development of the yolk sac and chorioallantoic fusion, as well as proper heart development, are required for successful embryogenesis. Defects in all of these processes can result in birth defects or spontaneous abortion. *Prpf8*<sup>N1531S</sup> homozygous embryos fail to undergo proper yolk sac and heart formation, as well as fail to undergo chorioallantoic fusion, which we have investigated to characterise the *Prpf8*<sup>N1531S</sup> homozygous phenotype.
3.2 Results

3.2.1 Morphological analysis of Prpf8\textsuperscript{N1531S/N1531S} embryos

While heterozygotes appear normal, homozygous embryos suffer from a wide range of morphological defects. At E8.5 there are few obvious differences between Prpf8\textsuperscript{N1531S} homozygous and heterozygous embryos, though developmental delay is already noticeable in Prpf8\textsuperscript{N1531S} homozygotes by this stage (Figure 3.1). At E9.5 the Prpf8\textsuperscript{N1531S} homozygous embryos may have a grossly distended heart tube, with atrial, ventricle and OT dysmorphia (Figure 3.1). These embryos frequently fail to undergo embryonic turning with the neural tube kinking and the body wall and anterior neural tube failing to close. The yolk sac fails to remodel, a process that requires sufficient cardiac output to occur (Lucitti et al, 2007) and chorioallantoic fusion often does not happen; this may be the cause of embryonic death (Figure 3.2). The Prpf8\textsuperscript{N1531S} phenotype is typically more pronounced with increasing developmental age. However, the phenotype is highly variable in its severity, and in some instances only a relatively subtle cardiac defect is evident, along with the developmental delay. Similarly, while the yolk sacs of Prpf8\textsuperscript{N1531S} homozygous embryos never achieve the complex vascularisation seen in heterozygotes, sometimes large vessels with very few branches are seen in Prpf8\textsuperscript{N1531S} homozygotes. At E10.5 the developmental delay, heart defects and lack of yolk sac remodelling are frequently profound in Prpf8\textsuperscript{N1531S} homozygous embryos (Figures 3.3 and 3.4). At E10.5 some Prpf8\textsuperscript{N1531S/N1531S} embryos appear dead, the rest die the following day. One of the most striking hallmarks of the Prpf8\textsuperscript{N1531S} homozygous mutants is that a large proportion of the mutants have reversed heart looping; indicating the presence of a L-R axis defect, though due to the early death of the embryos abnormal cardiac looping is the only morphological evidence of L-R axis defects (Figure 3.3). No evidence of laterality abnormalities has been found in adult heterozygous mice (n = 8)
Figure 3.1: Morphological analysis of E8.5 and E9.5 embryos.

Figure: Embryos at E8.5 and E9.5 from two dissections prior to fixation; extraembryonic membranes have been removed. A: Heterozygous embryo showing headfold, somites, neural tube and node (the node is outlined). B: Prpf8^{N1531S/N1531S} embryo showing the headfold, somites, neural tube and node (the node is outlined). No obvious defects are apparent. C: Heterozygous embryo displaying normal development, the heart is indicated with an arrow. D: Prpf8^{N1531S/N1531S} homozygous embryo, which has failed to turn, displaying open neural tube (grey arrow) and a distended and reversed heart tube (white arrow). Scale bars = 0.5mm.
Figure 3.2: Morphological analysis of E9.5 yolk sac vasculature.

E9.5 littermate embryos with yolk sacs, prior to fixation. A, C: Heterozygous embryo showing prominent yolk sac and umbilical blood vessels, respectively (arrows). B, D: *Prpf8<sup>N1531S/N1531S</sup>* embryo completely lacking both yolk sac and umbilical vessels, though chorioallantoic fusion appears to have occurred in this instance. The primitive capillary plexus has formed correctly (indicated by the arrow in B) but the umbilical vessels have not (indicated by the arrow in D). Scale bars = 0.5mm.
Figure 3.3: Morphological analysis of E10.5 embryos.

Series of E10.5 littermate embryos prior to fixation highlighting the variability of the $Prpf8^{N1531S/N1531S}$ phenotype.  A: Heterozygous embryo showing no abnormalities.  B: $Prpf8^{N1531S}$ homozygous embryo showing a mild phenotype; heart looping is reversed and the heart tube and embryo itself is less developed than expected for this developmental stage, but no other defects are apparent.  C: $Prpf8^{N1531S}$ homozygous embryo showing severe developmental delay.  The embryo has not undergone turning, chorioallantoic fusion has not occurred, the heart tube is very underdeveloped and the gut and neural tube have not closed. Scale bars = 0.5mm.
Figure 3.4: Morphological analysis of E10.5 yolk sac vasculature.

E10.5 heterozygous and homozygous embryos prior to fixation.  

A: Heterozygous embryo inside intact yolk sac showing clear evidence of yolk sac remodelling with prominent large and small blood vessels  

B: Prpf8\textsuperscript{N1531S/N1531S} embryo in yolk sac with no evidence of yolk sac remodelling and no blood vessels at all. Scale bars = 0.5mm.
Figure 3.5: Morphological analysis of E10.5 chorioallantoic fusion.

E10.5 heterozygous and homozygous embryos prior to fixation. A: Heterozygous embryo with successful chorioallantoic fusion and a prominent and blood filled umbilical artery and vein. B: Prpf8<sup>N1531S</sup> homozygous embryo with chorioallantoic fusion, but the attachment to the chorionic plate appears tenuous and the umbilical vessels are dysmorphic and presumably nonfunctional. C: Prpf8<sup>N1531S</sup> homozygous embryo in which chorioallantoic fusion has not occurred, precluding the formation of umbilical vessels. The severity of the Prpf8<sup>N1531S/N1531S</sup> phenotype between B and C appears similar, despite the differences in chorioallantoic fusion. Scale bars = 0.5mm.
Due to the incomplete penetrance of the different aspects of the $Prpf8^{N1531S/N1531S}$ phenotype, the frequency of stereotypical aspects of the $Prpf8^{N1531S/N1531S}$ phenotype was found. A total of 20 dissections taken at E10.5, comprising of 92 heterozygous and 53 homozygous embryos, were investigated (Figure 3.6 A). While no heterozygous embryos had abnormalities in cardiac looping, 38% of $Prpf8^{N1531S}$ homozygous embryos had reversed heart looping, indicating a highly penetrant laterality defect. Yolk sac defects were also highly penetrant, with 68% of embryos showing no sign of vascularisation, the remainder showed very primitive yolk sac remodelling compared to littermates. Approximately 49% of $Prpf8^{N1531S}$ homozygous embryos had not undergone chorioallantoic fusion; the allantois was sometimes seen to be full of blood. Yolk sac vascularisation was rare when chorioallantoic fusion defects were also present (Figure 3.6 B). About 30% of $Prpf8^{N1531S}$ homozygous embryos still had open anterior neural tubes, which could suggest either a patterning defect or embryonic delay; this may be somewhat more common in embryos with avascular yolk sacs (Figure 3.6 C). About half (53%) of $Prpf8^{N1531S}$ homozygous embryos had some form of oedema suggestive of cardiac insufficiency; this was the only aberrant phenotype that was occasionally (3% of embryos) observed in heterozygous embryos. As well as incorrect heart looping, other heart defects were observed in $Prpf8^{N1531S/N1531S}$ embryos such as inflated inflow tracts, misaligned atria or ventricles in relation to the rest of the body or grossly inflated and distended heart tubes; collectively these were noted as miscellaneous CHD and were seen in 81% of homozygous mutant embryos. This did not appear to be any more or less likely to occur with with any of the other defects observed, possibly because CHD is so common in $Prpf8^{N1531S}$ homozygous embryos. In addition, the hearts of $Prpf8^{N1531S/N1531S}$ embryos at E10.5 had a level of chamber maturation similar to that of E9.5 embryos or earlier.
Figure 3.6: Penetrance of different aspects of the Prpf8<sup>N1531S</sup> phenotype in E10.5 embryos.

A: Graph showing the percentage of embryos displaying various phenotypes seen in Prpf8<sup>N1531S/N1531S</sup> embryos. These abnormal phenotypes are largely absent from heterozygotes. B: Graph showing co-occurrence of yolk sac remodelling defects with chorioallantoic fusion defects. Yolk sac vascularisation may be less likely to occur in the presence of chorioallantoic defects. C: Graph showing colocalisation of yolk sac vascularisation with neural tube (NT) defects. Failure to close the neural tube appears to be somewhat more common in embryos with avascular yolk sacs. Data collected from 20 different dissections containing 92 Prpf8<sup>+/N1531S</sup> embryos and 53 Prpf8<sup>N1531S/N1531S</sup> embryos.
**Shh expression analysis**

The Prpf8<sup>N1531S</sup> homozygotes frequently present with an open anterior neural tube, which is a common feature in embryos with increased Shh pathway activation; mutants with decreased Shh expression exhibit holoprosencephaly (reviewed in Li *et al.*, 2011; Murdoch and Copp, 2010). Shh is expressed in the notochord and the floor plate of the neural tube (Echelard *et al.*, 1993) and is frequently used as a midline marker (see Figure 3.7). In addition, Shh is heavily expressed in the node, and can be used to assess whether the node is present in embryos. Mice lacking Shh have L-R axis defects including bilateral Lefty2 and Ptx2 expression (Meyers and Martin, 1999); this is due to the role of Shh in establishing the midline, rather than Shh having a L-R axis specific function (reviewed in Shiratori and Hamada, 2006). Previous lab members had investigated the expression of Shh and its ligands, Gli1, 2 and 3; they found that all the genes investigated were downregulated, except for Gli3, which was upregulated (Stephen, L. 2013). Gli1 functions only as a transcriptional activator, while Gli2 is mainly an activator of transcription it can also be processed into a transcriptional repressor; Gli3 is mainly a repressor of transcription but can be processed into a transcriptional activator (reviewed in Li *et al.*, 2011). These alterations in the expression levels of the three Gli genes could suggest that Shh signalling is impaired. The expression pattern at E8.5 was not previously investigated; however experiments at this timepoint would help determine if midline specification is altered in Prpf8<sup>N1531S</sup> homozygous embryos.

It was found that in Prpf8<sup>N1531S</sup> heterozygous embryos at E8.5 there were no observable deviations from the published expression pattern of Shh (*n*=4, see Figure 3.6). In Prpf8<sup>N1531S</sup> homozygous embryos there was strong staining in the node, confirming that the node was present in these embryos (*n*=6/6). There was also staining in the neural tube, but this appeared somewhat patchy in some embryos, with sections of the neural tube remaining unstained (*n*=3/6). In addition, staining appeared somewhat fainter in Prpf8<sup>N1531S/N1531S</sup> embryos (*n*=5/6), supporting the results of previous lab members (Stephen, L. 2013). This also shows that the neural tube defects observed are not caused by increased Shh expression.
Figure 3.7: In situ hybridisation with RNA probe detecting Shh mRNA.

E8.5 embryos stained for Shh expression. A: Heterozygote showing normal uninterrupted Shh expression along the midline of the embryo, with intense staining at the node (black arrow; n=4). B: Prpf8^{N1531S/N1531S} embryo showing similar expression pattern as in the heterozygote embryo, with similar intense staining at the node. However, midline staining appears to be interrupted at several points (white arrows; n=5/6). Scale bars = 0.5mm.
While it had been noted that the $Prpf8^{N1531S/N1531S}$ embryos were clearly developmentally delayed by E9.5, it was less clear whether the delay was noticeable by E8.5. Anecdotally, it had been noticed that $Prpf8^{N1531S}$ homozygous embryos tend to have fewer somites than their littermates at E8.5. To confirm that delay had begun by E8.5, 10 dissections, taken between 12:00 and 13:00, were examined and the average number of somites was counted for $Prpf8^{N1531S}$ heterozygous and homozygous embryos, in litters containing at least 2 homozygotes embryos (Figure 3.8). By taking the average somite number and ensuring multiple $Prpf8^{N1531S/N1531S}$ embryos were present in the dissections analysed, the effect of potential outliers on the distribution of the data was minimised.
Figure 3.8: Analysis of somite number in E8.5 embryos.

The average number of somites in Prpf8\textsuperscript{N1531S} homozygous embryos is markedly fewer than in littermate heterozygous embryos (p > 0.0001; two-tailed paired t-test; 10 pairs). The scatterplot shows the median somite number (±SD), revealing almost no overlap in the distribution of average somite numbers between heterozygote and Prpf8\textsuperscript{N1531S/N1531S} embryos. The paired scatterplot shows the average somite number for pairs of genotypes in each dissection. All dissections contained at least 2 Prpf8\textsuperscript{N1531S/N1531S} embryos and were taken between 12:00 and 13:00 at E8.5.
From Figure 3.8, it can be seen that at E8.5 it is clear that $Prpf8^{N1531S}$ homozygous embryos experience developmental delay compared to their heterozygous littermates. That there is no overlap in the standard deviation between the two genotypes suggests the difference between the two is highly significant; this is supported by a paired t-test ($p>0.0001$). The mean number of somites for heterozygotes is 6.34 while $Prpf8^{N1531S/N1531S}$ embryos have a mean number of 3.74; at this stage of development a pair of somites is formed every hour (Tam, 1981), indicating that $Prpf8^{N1531S}$ homozygous embryos are delayed in development by several hours with respect to their heterozygous littermates. This delay becomes more pronounced with age. Directly comparing the average somite number of the two genotypes in each pair shows that the number of somites in $Prpf8^{N1531S}$ homozygous embryos may not be correlated with the average number of somites in heterozygotes. Indeed, the litters with the two highest numbers of average somites in heterozygote embryos (8.0 and 6.3 somites) have the joint second lowest average number of somites for $Prpf8^{N1531S/N1531S}$ embryos (2.7 somites). Pearson’s correlation coefficient ($r=0.325$) does suggest a weak direct correlation, but this was not found to be significant ($p=0.359$). This could indicate that developmental delay may affect different $Prpf8^{N1531S/N1531S}$ embryos to a different degree, causing some $Prpf8^{N1531S/N1531S}$ embryos to have far fewer somites than their heterozygous littermates, while other $Prpf8^{N1531S}$ homozygotes are less affected. However, the difference between the average number of somites in $Prpf8^{N1531S}$ heterozygous and homozygous embryos is only somewhat variable between litters (SD is ±1.3 somites). This indicates that the severity of developmental delay in $Prpf8^{N1531S/N1531S}$ embryos varies little between different litters at E8.5. Taken together, this analysis demonstrates that the developmental delay observed in $Prpf8^{N1531S}$ homozygous embryos starts at or before the beginning of somitogenesis.
**Dll-1 expression analysis**

Defects and delays in somitogenesis could be caused by expression defects in genes essential for somite specification, which have not previously been investigated in $Prpf8^{N1531S/N1531S}$ embryos. Notch signalling is essential for somitogenesis and the formation of a segmented body axis (Ferjentsik et al., 2009). Dll-1 is a Notch ligand that is essential for proper somitogenesis and is expressed in the presomitic mesoderm and in the posterior half of the somites; mouse embryos with mutated Dll-1 display defects in somite morphology (Hrabě de Angelis et al., 1997). Oscillations in the expression of genes of the Notch, FGF and Wnt pathways, including Dll-1, coordinate somitogenesis (reviewed in Gibb et al., 2010). Altering the synchronicity of these oscillations by extending protein half-life of Hes7 results in fused somites after the fourth somite (Hirata et al., 2004). Interestingly, accelerating the expression of Hes7 by removing introns also abolished oscillatory Hes7 expression, resulting in fused somites, highlighting the role of introns and splicing in controlling gene expression (Takashima et al., 2011). In addition, Notch signalling and Dll-1 specifically are required for establishing the L-R axis (Kitajima et al., 2013; Krebs et al., 2003; Przemeck et al., 2003). Krebs and colleagues reported that Dll-1 knockout mice showed delays in forming mesodermal somites, but did not further elaborate (Krebs et al., 2003). We therefore investigated the expression pattern of Dll-1 to determine whether defects in Dll-1 expression could account for the delay in somitogenesis or the L-R axis defects we observe.

At E9.5 in both $Prpf8^{+/N1531S}$ and $Prpf8^{N1531S/N1531S}$ embryos, Dll-1 was expressed in a pattern comparable to the published expression pattern ($n=3$ for both genotypes, see Figure 3.9). The somites and the presomitic mesoderm appear to be specified and formed correctly in $Prpf8^{N1531S/N1531S}$ embryos, with no sign of fused somites. Preliminary analysis of $Prpf8^{N1531S/N1531S}$ embryos at E8.5 also did not reveal any differences, but not enough embryos were analysed for this to be conclusive. Nevertheless, it appears that Dll-1 is normally expressed in $Prpf8^{N1531S/N1531S}$ embryos.
Figure 3.9: In situ hybridisation with RNA probe detecting Dll-1 mRNA.

E9.5 embryos stained for Dll-1 expression. A, B: Prpf8^{N1531S} heterozygote and homozygote respectively, showing similar expression patterns (n of both groups = 3). C, D: The tails of the embryos shown in A and B, respectively. Dll-1 is correctly expressed at the posterior of each somite, as well as in the presomitic mesoderm in both cases. Scale bars = 0.5mm.
3.2.2 Cardiac Gene Expression Analysis

To determine if the distended heart tubes observed in Prpf8 mutants resulted from inappropriate cardiac gene expression, the expression of genes involved in cardiogenesis was examined by in situ hybridisation. Previous lab members have investigated the genes Nsk2.5, Myl4 and dHand, which did not reveal any profound changes in cardiac gene expression in homozygous mutant embryos (Mitchell, K. 2009; Stephen, L. 2013). Because of the developmental delay of approximately one day, Prpf8$^{N1531S}$ homozygotes were compared with similarly staged wild type controls as well as heterozygous littermates (which show a more advanced developmental morphology). Although the homozygous embryos are developmentally delayed their age will still be referred to by days post-conception rather than by their physical appearance. This delay becomes progressively more pronounced; at E8.5 there is a subtle difference between mutants and heterozygotes while at E10.5 mutants appear a full day younger by morphological landmarks.

The expression of the gene Irx4 was investigated as it is essential for ventricle formation and provides a useful ventricle specific marker (Bruneau et al, 2000), which is valuable when mutants can have greatly distended hearts making the division between the atria and ventricles difficult or impossible to identify. In situ results (Figure 3.10) show Irx4 is expressed in Prpf8$^{N1531S}$ homozygotes at E10.5, indicating the ventricles are specified properly ($n = 3$), though the regions of expression can appear more or less normal depending on how malformed the heart is (compare Figure 3.9 B and C). Nevertheless, correct cardiac chamber specification appears to progress normally. Irx4 expression also marks the future hindbrain (Bruneau et al, 2000); this region of the brain is also correctly specified in Prpf8$^{N1531S/N1531S}$ embryos.
Figure 3.10: In situ hybridisation with probe detecting ventricle marker *Irxi* mRNA.

A: E9.5 heterozygous embryo showing normal ventricular and hindbrain expression of *Irxi*. B and C: E10.5 *Prpf8<sup>Nov3315</sup>* homozygous embryos also showing normal ventricular and hindbrain expression of *Irxi*, indicating these structures are correctly specified, despite the dysmorphology of the heart (n=3). Scale bars = 0.5mm.
The expression of SHF marker *Isl1* was investigated via *in situ* to see whether the SHF was specified properly in *Prpf8*<sup>N1531S</sup> homozygotes, because *Isl1* marks SHF progenitors (Cai *et al.*, 2003). It was found that *Isl1* was expressed and that its expression pattern was reduced in area, though not intensity, in mutants (n = 4, see figure 3.11). However, since mutants experience developmental delay it is mostly likely this change in expression pattern reflects this delay rather than a genuinely aberrant expression pattern and that if the embryos were allowed to develop further *Isl1* would be expressed normally. In support of this view, *Fgf8*, another marker for the SHF, was found to be normally expressed (Stephen, L. 2013). SHF derived structures, such as the right ventricle and OT, appear to be present in *Prpf8*<sup>N1531S</sup> homozygotes from morphological observations, despite often being obviously malformed. However, it is unknown whether they express genes specific to the right ventricle and OT correctly.
Figure 3.11: In situ hybridisation with RNA probe detecting *Isll* mRNA.

E9.5 embryos stained for *Isll* expression. A: Heterozygote showing normal *Isll* expression in the foregut, tail, splanchnic mesoderm (arrow), neural tube and outflow tract (*n* = 3). B: *Prpf8^E1531S/N1531S* embryo showing similar expression pattern as in the heterozygote embryo; neural tube expression is very faint and head expression is absent, possibly due to developmental delay (*n* = 4). Scale bars = 0.5mm.
3.3 Discussion:

This analysis of the phenotype of Prpf8<sup>N1531S</sup> homozygotes builds upon work carried out by previous lab members. It was previously shown by histological analyses that mutants have a thinned and more disorganised myocardium as well as a lack of remodelling of the brain vasculature. Ultrasound investigation of mutants in the womb revealed a somewhat less frequent heartbeat than heterozygous littermates (Stephen, L. 2013). Interestingly, culturing the mutant hearts <i>ex vivo</i> allows them to survive far past when the whole embryo would have died (Tenin, G. pers comm.). The Prpf8<sup>N1531S/N1531S</sup> homozygous mutant hearts still contract when divided into pieces, with the left ventricle, where contraction initiates, having the same contraction rate as the whole heterozygote heart, indicating that the pacemaker cells are present and functional (Tenin, G. pers. comm). This suggests that the heart cells themselves are fine, but the heart as a whole structure is faulty. It is also possible that the culturing process itself allows the cells to survive, by providing growth factors missing in the Prpf8<sup>N1531S</sup> homozygous mutant embryos as they develop, for example.

Due to the frequent failure of the neural tube to close, previous lab members investigated the possibility of cranial patterning defects, by detecting the expression of Engrailed and Fgf8, which were normally expressed (Mitchell, K. 2009; Stephen, L. 2013). However, Shh and its effectors, the Gli transcription family, did show changes in expression levels, with Shh, Gli1 and Gli2 expression reduced in Prpf8<sup>N1531S/N1531S</sup> embryos, while Gli3 expression was increased (Stephen, L. 2013). We have also reported a reduction in Shh expression at E8.5, along with evidence of interruptions in Shh along the midline. The consequences of this are unclear, as in depth analysis of the neural tube has not been performed and Prpf8<sup>N1531S/N1531S</sup> embryos die before many Shh dependant structures, such as digits, form. Previous lab members had also carried out expression analysis of cardiac genes, including Nkx2.5, Nppa, Actc1 and Myl4. Most of these genes, as well as Irx4 and Isl1 shown here, showed no overt changes in expression that correlated with the morphological defects observed. Actc1 and Myl4 were downregulated, suggesting a defect in myogenesis (Mitchell, K. 2009); interestingly downregulation of Actc1 is associated with increased apoptosis in human cardiomyocytes (Jiang <i>et al.</i>, 2010). Both the heart and head appear to be specified correctly in Prpf8<sup>N1531S/N1531S</sup> embryos, indicating that defects occur in processes in
parallel with or downstream of tissue specification. Another possibility is that the problem occurs before heart specification and in a global process, such as cell migration or proliferation, but manifests itself as a heart defect.

Highly specific phenotypes have been previously reported in knockout mice that are null for genes which have a global function. Mice null for the genes coding for components of the mTORC2 complex, which helps control cell growth and survival, such as rictor and mLST8, die at midgestation with yolk sacs that have not remodelled (Guertin et al., 2006). In a similar manner, Akt1−/−/Akt3+/− mice die at midgestation with cardiovascular and nervous system defects; Akt1 and 3 code for protein kinase Bα and γ respectively (Yang et al., 2005). Therefore it is not unprecedented that mutations in a gene coding for a protein with as broad a function as Prpf8 could cause as specific a constellation of defects as seen in Prpf8N1531S homozygous embryos. Defects in genes with a global function have previously been shown to cause delayed embryonic development. Loss of the protein IKAP causes embryonic delay in mice, as well as abnormal brain and cardiovascular development, and death by E10.5 (Dietrich et al., 2011). Mice null for Blm, which codes for a helicase important in suppressing homologous recombination (reviewed in Wu and Hickson, 2006) die midway through gestation with embryonic delay and increased apoptosis (Chester et al., 1998).

Quantification of the various aspects of the phenotype seen in Prpf8N1531S homozygotes revealed the variability in the penetrance of aspects of this phenotype. While most of the phenotypes occurred in more than a third of mutant embryos, unlooped (mesocardia) hearts where no directionality could be determined, were seen only twice. This finding suggests that mesocardia is not a bona fide phenotype, but instead that these hearts were so grossly malformed as to preclude meaningful interpretation of looping directionality. The rate of heart looping reversal we observe in our embryos is in agreement with the rate seen in other published mouse mutants, which tends to vary between 30 and 50%. The heart defects we observe suggest that our mutants also have defects in cardiogenesis, as well as defects in heart looping caused by laterality defects. The origin of the laterality defects has been investigated in detail and will be the subject of the next chapter of this thesis. The CHD we see in the Prpf8N1531S homozygous embryos is dissimilar to the CHD seen in strict models of laterality disturbance; in these
models the CHD is more similar to that seen in humans and includes atrial and ventricular isomerism. In the Prpf8<sup>N1531S/N1531S</sup> embryos the heart tube is often malrotated with respect to the embryo, may be dilated and never forms mature chambers. These defects would be fully expected to cause cardiac insufficiency, as indicated by the less frequent heart beat observed (Steven, L. 2013.), and this is the likely cause of the oedema which was frequently seen. Cardiac insufficiency may also be responsible for the failure in yolk sac remodelling (Lucitti et al, 2007). Due to the almost complete penetrance of cardiac defects, and the universal incidence of yolk sac defects, and the fact these defects occur simultaneously, it is not possible to establish causality. It is possible that cardiac defects cause yolk defects, or vice versa, or that both these defects occur independently. The origin of the CHD is currently unknown, but it does not appear to be caused by a failure of cardiac cell specification, as all cardiac markers so far investigated appear to be expressed normally. It may be that a specific, as yet unidentified, gene is missexpressed or misspliced in the Prpf8<sup>N1531S</sup> homozygotes, leading to CHD. Alternatively it is possible that the heart is highly sensitive to global defects in cell migration or survival, for instance, and so is the organ most severely affected. If the second hypothesis were true, this would mean that the Prpf8<sup>N1531S</sup> mutation is not cardiac specific. A third, if unlikely, hypothesis is that Prpf8 has a cardiac specific function.

It is difficult to speculate whether the failure of yolk sac remodelling is due to cardiac insufficiency or failure of chorioallantoic fusion. While occasionally large, primitive vessels are seen in the yolk sacs of Prpf8<sup>N1531S</sup> homozygous embryos at E10.5, remodelling never approaches the level seen in littermate heterozygous embryos. Frequently yolk sac vessels are not seen at all in Prpf8<sup>N1531S</sup> homozygotes. Failure of chorioallantoic fusion occurs in 49% of embryos; this incomplete penetrance is consistent with other reported mutants with defects in chorioallantoic fusion (reviewed in Inman and Downs, 2007). The formation of the vitelline vessels appears unaffected. The incomplete penetrance of the chorioallantoic fusion defects compared to the universally seen defects in yolk sac remodelling may suggest that either heart defects or an intrinsic yolk sac defect is the primary cause of the lack of yolk sac remodelling. However, yolk sac vascularisation seems to be much less likely to occur if chorioallantoic fusion has failed, which could suggest that yolk sac vascularisation is dependent upon chorioallantoic fusion. There are many examples of embryos that have
no sign of yolk sac vascularisation despite successful chorioallantoic fusion, suggesting that while necessary, chorioallantoic fusion is not sufficient for yolk sac remodelling to occur. Considering that cardiac output is needed for yolk sac remodelling (Lucitti et al., 2007), the loss of this connection between the heart and the yolk sac may cause or exacerbate yolk sac defects. Interestingly only angiogenesis is negatively affected; yolk sac vasculogenesis seems unaltered as the primitive capillary plexus is formed and appears normal. While yolk sac remodelling has been conclusively shown to be reliant upon cardiac output (Lucitti et al., 2007), no studies have been done that suggest that chorioallantoic fusion is reliant upon cardiac output. This supports the hypothesis that Prpf8\textsuperscript{N1531S} homozygotes have defects specific to the chorion and/or allantois that inhibit their proper function. Even when fusion does occur, this connection is tenuous and the umbilical vessels do not form correctly. The failure of the extraembryonic membranes to form properly precludes the development of Prpf8\textsuperscript{N1531S} homozygotes to term.

As well as the defects described above, Prpf8\textsuperscript{N1531S} homozygous embryos also experience developmental delay. Prpf8\textsuperscript{N1531S} homozygous littermates have fewer somites compared to heterozygotes, however, there appears to be no disruption in somitogenesis as measured by Dll-1 expression and the somites do not appear malformed. Defects in genes that compose the segmentation oscillation clock typically result in gross defects in somites, which we do not observe in Prpf8\textsuperscript{N1531S} homozygous embryos, arguing against defects in the oscillation clock. Even alterations in the rates of gene expression or the half-life of proteins such as Hes7 result in malformed somites (Hirata et al., 2004; Takashima et al., 2011). However, it is possible that if the splicing of a member of the oscillation clock was slowed by the Prpf8\textsuperscript{N1531S} mutation, somitogenesis could be slowed without the gross defects in somite morphology by extending the periodicity of the oscillations, as long as the oscillations themselves were maintained. Alternatively, developmental delay could be caused by the defects in the extraembryonic membranes reducing the supply of essential nutrients or growth factors, accounting for the severe delay seen at E10.5, with Prpf8\textsuperscript{N1531S} homozygous embryos appearing one or two days younger than heterozygous littermates. However, this delay is seen at E8.5, before the defects in extraembryonic membranes are seen, and could feasibly have started earlier still. This result suggests another cause for the embryonic delay, at least prior to the failure of yolk sac remodelling. This cause could be a general
defect in cellular proliferation, survival, differentiation or movement in either the whole embryo or a critical region, such as the primitive streak, slowing embryonic development. This developmental delay may be the ultimate origin of the failure of the neural tube to close, since the head region appears to be correctly specified (Mitchell, K. 2009; Stephen, L, 2013).

3.4 Conclusion

*Prpf8*\textsuperscript{N1531S/N1531S} embryos have a diverse phenotype, with many aspects of embryonic development affected. The heart, L-R axis and yolk sac are all abnormal, and there is embryonic delay starting by the onset of somitogenesis. This mutant phenotype could be caused by defects in the progenitor cells of different tissues that are affected simultaneously, creating defects in the descendant tissues. Alternatively, some or all of the aspects of the *Prpf8*\textsuperscript{N1531S/N1531S} phenotype could be caused by a global defect in, for example, cell survival. Whether the phenotype is directly caused by defects in Prpf8 itself or whether disturbance of Prpf8 functionality causes changes in splicing or function of other genes to give rise to our phenotype is, at present, unknown. However, it appears that the specification of cardiac cells and specific cardiac regions, such as the ventricles, is unaffected.
Chapter 4: Investigating the laterality defect in $Prpf8^{N2531S/N1531S}$ embryos
4.1 Introduction

Left-Right Establishment: a concise summary

The earliest known events in murine laterality establishment start at E7.5 and the L-R axis is determined by E8.5 (Collignon et al., 1996). Whether the L-R axis is determined earlier than this in mice, as is the case in other model organisms (reviewed in Vandenberg and Levin, 2013), is unknown. The organ of L-R asymmetry in mice is the embryonic node (Figure 1.2), which is the homologue to the Spemann-Mangold organiser and Henson’s node in Xenopus and chickens respectively. At the node, motile cilia drive a leftward flow of fluid (Nonaka et al., 2002) and it is this flow which determines laterality. While there are two models as to how this results in L-R axis establishment, the most well supported suggests that mechanosensory immotile cilia on the node crown cells detect the force of fluid and trigger a release of Ca\(^{2+}\) (McGrath et al., 2003; Takao et al., 2013). This results in preferentially left sided Nodal expression around the node, which then induces Nodal expression in the left LPM (Brennan et al., 2002). Cerl2, which encodes an inhibitor of Nodal, is preferentially expressed on the right of the node, where it acts to prevent ectopic Nodal signalling in the right LPM (Marques et al, 2004). Nodal then induces the expression of its additional inhibitors Lefty 1 and 2 in the midline and left LPM, respectively (Saijoh et al., 2003). These act to prevent ectopic expression in the right LPM, which would result in laterality defects. Nodal in the LPM induces the expression of Pitx2c (Shiratori et al., 2001), which is thought to control the asymmetry of the viscera.

Research performed during this thesis project has revealed that the Prpfs\(^{N1531S/N1531S}\) homozygous embryos have defects in both node morphogenesis and ciliogenesis; this introduction will now cover both of these subjects in greater depth.

Genetic control of embryonic node formation

Since the node is a product of the anterior primitive streak, genes that are needed for primitive streak morphogenesis, such as T, which codes for brachyury (King et al., 1998), and FoxH1 (Yamamoto et al., 2001) are also needed for proper node formation
and/or function. Genes that are largely specific to node formation are less well understood. *Noto* is one of the more well characterised genes required for node morphogenesis and is essential to proper node formation (Alten et al., 2012a). Loss of Noto function results in an abnormal node with no delineation between the pit and crown cells, as well as impaired ciliogenesis (Beckers et al., 2007). Other genes identified as essential for node morphogenesis have very disparate functions. *Epb4.115* is needed for correct integration of the rosettes of node precursor cells into the visceral endoderm; loss of Ebp4115 results in multiple nodes possibly due to malfunctioning MET (Lee et al., 2010). *Rac1* and *Zic3* are important in cell migration; loss of function of *Rac1* results in the formation of flat, multiple ‘nodes,’ (Migeotte et al., 2011) while loss of *Zic3* prevents the overlying endoderm from being removed (Sutherland et al., 2013). Proper control of the cell cycle also appears to be important in node morphogenesis; knocking out *Acvr1* in the epiblast, which controls BMP dependent inhibition of the cell cycle, results in a flattened node morphology and cilia defects (Komatsu et al., 2011). As important as proper node formation is, nodal cilia must also be correctly formed for the L-R axis to be correctly specified.

**Genetic control of nodal ciliogenesis**

Due to the absolute requirement for cilia in the node for left-right axis specification, malfunction in genes required for formation or function of cilia are likely to cause defects in laterality establishment. Some examples include genes required for cilia assembly and disassembly (Kinzel et al., 2010) as well as motor proteins (Nonaka et al., 1998; Supp et al., 1999). Genes required for the creation of nodal cilia specifically presumably exist because nodal cilia are the only known motile 9+0 cilia, unfortunately these genes have not yet been found. In *Xenopus* embryos ectopic expression of *Foxj1* can induce the formation of motile cilia that resemble those found in the gastrocoel roof plate on non-ciliated cells (Stubbs et al., 2008). *Foxj1* induces the expression of a suite of ciliogenesis promoting genes such as *Rfx3* and *Tektin* and is in turn under the control of *Noto* (Alten et al., 2012a; Yu et al., 2008). Loss of functional *Foxj1* completely prevents the formation of 9+2 motile cilia but allows the formation of shorter, though still non-functional, nodal cilia; primary cilia remain unscathed (Brody et al., 2000; Chen et al., 1998). As well as forming correctly, cilia must be localised to the posterior of the node pit cells to create a directional flow. Models of the nodal flow show that
cilia angled perpendicularly to the nodal pit will not produce a directional flow, but cilia tilted posteriorly will (Cartwright et al., 2004). This was subsequently confirmed in vivo (Nonaka et al., 2005). Noto and Foxj1 control the position of cilia and the loss of either gene perturbs correct cilia localisation (Alten et al., 2012a). Information from the planar cell polarity (PCP) pathway is essential for cilia localisation; loss of function in genes such as Vangl1 and 2 and Dvl1, 2 and 3 causes L-R axis defects by interfering in proper flow directionality (Hashimoto et al., 2010; Song et al., 2010). The requirement of the PCP pathway in controlling cilia localisation appears to be evolutionarily conserved across vertebrates, and in all species examined the cilia need to be localised to the posterior of node pit cells (Antic et al., 2010; Borovina et al., 2010).

Mouse models of cilia motility defects display L-R axis defects

Many different mouse lines have been established with mutations in genes essential for cilia motility that display L-R axis defects. These typically fall into two categories, either cilia are absent, as in the case in lines with mutations in Kif3a, Kif3b and Ifi88 (Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 1998), or cilia motility is reduced, as in mutants for Dpcd, Rfx3 and inversin (Okada et al., 1999; Shinohara et al., 2012). Mouse with defective left-right dynein appear to be unusual in that the nodal cilia are completely immotile with no detectable movement, as opposed to beating slowly compared to control embryos as seen in Rfx3 and other mutants. Nevertheless, mice which either lack cilia or have cilia motility defects exhibit a high penetrance of laterality defects including situs inversus and heterotaxy, demonstrating that L-R axis determination has been randomised. The exception to this is the inv mouse line (inversion of turning) which carries a partial deletion in inversin (Morgan et al., 1998). These mice are extremely unusual in that they consistently display situs inversus rather than a randomisation of the L-R axis, and concurrently display reversed Nodal expression (Lowe et al., 1996; Yokoyama et al., 1993), which would suggest that the nodal flow is reversed. However, the nodal flow in inv/inv mice proceeds to the left, albeit slowly (Okada et al., 1999), even though the cilia themselves beat at speeds comparable to wild-type embryos (Watanabe et al., 2003). It is possible that the slow nodal flow is due to the aberrations in node morphology that are also present in inv/inv embryos (Okada et al., 1999). While inversin is found in nodal motile cilia, it is not found in the putative mechanosensory cilia, which suggests it is not needed to detect the
nodal flow; indeed it is possible to restore normal laterality by artificially inducing a leftward nodal flow (Watanabe et al., 2003), which could not occur if flow detection was impaired. Why the sluggish leftward flow seen in inv mice consistently results in situs inversus while an artificially imposed leftward flow restores laterality establishment is unknown. The inv mouse line remains a challenge to explain using current models of murine laterality establishment, which suggest that the leftward nodal flow is the ultimate arbiter of laterality. It may yet be seen that mice, like other model organisms, establish laterality before the formation of the embryonic organiser and inversin may be required in this undiscovered process.
4.2 Results

4.2.1: Cilia Immunofluorescence

In order to determine whether defects at the node could be the cause of the laterality defects in the Prpf8$^{N1531S}$ homozygotes, we performed immunofluorescence staining to label nodal cilia (Figures 4.1 and 4.2). Cilia were labelled with anti-acetylated tubulin antibodies, while the centrosomes were labelled with anti-γ-tubulin antibodies. We found that cilia were present in both genotypes, and that centrosomes were correctly localised to the base of the cilia. It also appeared that cilia may be somewhat shorter, but as anti-acetylated tubulin labels structures other than cilia, the length and number of cilia could not be determined with confidence. When confocal Z-stacks were resliced to examine the nodes from the side, it became apparent that the node of Prpf8$^{N1531S/N1531S}$ embryos was much shallower than the node of Prpf8$^{+/N1531S}$ embryos. While this could have been an artefact of the sample mounting process causing the tissue to be crushed, this flattening was never seen in stage-matched heterozygous embryos, suggesting that this is a real morphological difference between the two genotypes.
Figure 4.1: Immunofluorescent labelling of cilia at the node

Whole mount staining of the nodes of embryos taken at E8.5 confirms that cilia are present at the node. Cilia were labelled with anti-acetylated tubulin antibodies (green), while the centrosomes were labelled with anti-γ-tubulin antibodies (red). Images are max projections of confocal Z-stacks. A: Prpf8\textsuperscript{N1531S} heterozygous node (n=8). B: Prpf8\textsuperscript{N1531S} homozygous node (n=10). There are no apparent differences in the staining pattern between genotypes, with centrosomes localising to the base of cilia. C: Reslice of A showing a side-on view, revealing the pit shape of the node. D: Reslice of B, showing that the Prpf8\textsuperscript{N1531S} homozygous node is curiously flattened. The boxed area has been blown up in Figure 4.2. Scale bars = 10\(\mu\)m.
Figure 4.2: Enlarged portion of the node with immunofluorescently labelled cilia.

The boxed areas in Figure 4.1 have been enlarged to better show colocalisation of cilia (green) and centrosomes (red), examples are indicated with arrows. A: Prpfs$_{N1531S}^{N1531S}$ heterozygous node (n=8). B: Prpfs$_{N1531S}^{N1531S}$ homozygous node (n=10). There are no apparent differences in the distribution of acetylated-tubulin or $\gamma$-tubulin between genotypes, with centrosomes localising to the base of cilia. C: Reslice of A showing a side-on view of the node. D: Reslice of B, showing that the Prpfs$_{N1531S}^{N1531S}$ homozygous node is flat compared to the node of heterozygotes. Scale bars = 5$\mu$m.
4:2:2 Electron microscopy

The immunofluorescence experiments suggested that there were defects in cilia and node morphology in Prpf8N1531S homozygous embryos. To identify node structural defects in greater detail, scanning electron microscopy (SEM) was used to resolve the node and nodal cilia in order to examine both for signs of dysmorphology, while avoiding potential artefacts from mounting the tissue for immunofluorescence imaging.

The SEM experiments revealed clear differences in node morphology between Prpf8N1531S heterozygous and homozygous embryos (Figure 4.3). At the late headfold (LHF) stage all Prpf8N1531S heterozygous embryos have highly ciliated nodes and most nodes are recessed with respect to the visceral endoderm (n=5). However, stage matched Prpf8N1531S homozygous embryos never have pit shaped nodes (n=5). At 2-3 somites Prpf8+/N1531S embryos clearly have a pit shaped node which is deeply recessed with respect to the visceral endoderm, as seen in published literature. Stage matched Prpf8N1531S/N1531S embryos, however, frequently have flat nodes (n=6/8 embryos). This strongly suggests that the flattened nodes seen in homozygous embryos are not due to delayed growth but due to an intrinsic defect in node morphogenesis. Due to the awkward positioning of one of the Prpf8N1531S/N1531S embryos at the 2-3 somite stage, cilia could not be counted or measured reliably, as the node was partially obscured.

The nodal cilia of Prpf8N1531S/N1531S embryos are clearly present and do not have any gross morphological changes that are immediately apparent (Figure 4.4). However, when the mean lengths of the nodal cilia of Prpf8 heterozygotes (n= 672) and homozygotes (n= 556) at 2-3 somites are compared, it can be seen that the cilia of homozygotes are shorter (p<0.0001) by 0.45μm, a loss of about 19% of the total length compared to controls (Figure 4.5, for a Tukey box-plot representation of the data see Appendix 7). It is not unreasonable that this alteration in cilia length could result in a reduction in the force generated by the ciliary beat, and so in the strength of the nodal flow, though to what extent is unclear. Unexpectedly, the cilia of Prpf8N1531S homozygotes (n= 299) are longer than that of heterozygote controls (n= 438) at the LHF stage (p<0.05), with the mean of the Prpf8N1531S homozygotes 0.043μm longer than that
of the heterozygotes. This may reflect the difficulty in accurately stage matching embryos at the LHF stage, when anatomical markers are not forthcoming, and could indicate the presence of slightly older embryos in the \( Prpf8^{N1531S/N1531S} \) results. Alternatively, this discrepancy may be a genuine finding and, though the biological consequences of marginally longer cilia are probably minor, it could be a reflection of disorganised ciliogenesis. Though there appears to be trend towards fewer cilia in \( Prpf8^{N1531S/N1531S} \) embryos, this is not significant; due to the wide range of datapoints in the control genotype it is possible that a difference in the distributions of average cilia number between control and homozygous embryos would be confirmed if more embryos are examined (Figure 4.6).

Transmission electron microscopy (TEM) was used to determine whether the nodal cilia of \( Prpf8^{N1531S} \) heterozygotes and homozygotes had any differences in their ultrastructure which could be predicted to impair cilia function (Figure 4.7). Shortened nodal cilia are frequently associated with defects in ciliary ultrastructure (Alten et al., 2012a) and could be expected to be found in the cilia of \( Prpf8^{N1531S/N1531S} \) embryos. However, there were no perceptible differences between the nodal cilia of \( Prpf8^{N1531S/N1531S} \) heterozygotes and homozygotes by TEM (\( n \) of embryos for both genotypes = 3). The presence of dynein arms and the 9+0 arrangement of microtubules can be seen in all genotypes. This suggests that cilia formation is not grossly disturbed, and that the nodal cilia phenotype is relatively subtle compared to the phenotype seen in other published mutants with nodal cilia defects.
Figure 4.3: SEM images showing the node and nodal cilia.

Top row: Whole mouse embryos taken at LHF and 2-3 somite stages. At LHF stage n=5 for both genotypes; at 2-3 somite stage n=7 for heterozygotes and n=8 for homozygotes. The pit shape of the node is immediately obvious in heterozygous embryos but the node appears flat in homozygous embryos (arrow). Scale bars are 100µm. Bottom row: Magnified images of the node from the panel immediately above. The node (outlined in yellow) is clearly highly ciliated both in Prpf8<sup>N1531S</sup> heterozygotes and homozygotes. Scale bars are 10µm.
**Figure 4.4**: Nodal cilia of Prpf8^N1531^ heterozygotes and homozygotes.

Top row: Magnified SEM images showing the node and nodal cilia of LHF stage embryos. Bottom row: Magnified SEM images displaying the node and nodal cilia of embryos at the 2-3 somite stage. Scale bars are 5µm; the anterior-posterior axis for all images is displayed in the top left image. No gross morphological differences between Prpf8^N1531^ heterozygotes and homozygotes at either stage are immediately obvious.
Figure 4.5: Comparison of the lengths of nodal cilia between Prpf8\textsuperscript{+/N1531S} and Prpf8\textsuperscript{N1531S/N1531S} embryos.

The mean length of nodal cilia was found for Prpf8\textsuperscript{N1531S} heterozygotes and homozygotes at the LHF and 2-3 somite stage. This is graphically represented above in the bar graph (error bars are SEM). The stark difference between the mean length of the cilia in Prpf8\textsuperscript{N1531S} heterozygotes and homozygotes at the 2-3 stage is clear when comparing the data.
Figure 4.6: Comparison of the numbers of nodal cilia between $Prpf8^{+/N1531S}$ and $Prpf8^{N1531S/N1531S}$ embryos.

The number of nodal cilia was found for $Prpf8^{+/N1531S}$ heterozygotes and homozygotes at the LHF ($n=5$ embryos for both genotypes) and 2-3 somite stages ($n=7$ for both genotypes) and shown in the scatterplot above. The median and interquartile ranges are shown. There is a clear trend towards fewer cilia in $Prpf8^{N1531S}$ homozygotes, but this is not significant.
TEM cross sections of nodal cilia were examined for defects in ciliary ultrastructure. \( Prpf8^{+/+} \), \( Prpf8^{+/N1531S} \) and \( Prpf8^{N1531S/N1531S} \) nodal cilia all had dynein arms and the expected 9+0 configuration of microtubules. Shrinkage of the plasma membrane in the \( Prpf8^{+/N1531S} \) and \( Prpf8^{N1531S/N1531S} \) nodal cilia is likely a processing or fixing artefact (\( n=3 \) embryos for all genotypes). Scale bars are 200nm.

**Figure 4.7: TEM of nodal cilia**
4.2.3 Node videomicroscopy

The SEM images showed that the node is misshapen and nodal cilia are significantly shorter. However, it remained unclear whether fluid movement at the node was dysfunctional. However, it was also plausible that defects in other processes, such as the detection of the nodal flow by immotile cilia, or the release of Ca$^{2+}$, could cause our phenotype without requiring a fault in the nodal flow. We therefore visualised the nodal flow itself by comparing the movement of microbeads in the nodes of $Prpfs^{N1531S}$ heterozygous and homozygous embryos at 2-4 somites (Figure 4.8).

All but 1 of the $Prpfs^{+/N1531S}$ embryos investigated had the expected leftward movement of microbeads in the node indicative of a functional node and nodal cilia ($n=5/6$). In 5/7 $Prpfs^{N1531S/N1531S}$ embryos there was no evidence of microbead motion in any direction; instead beads moved at random via Brownian motion. In one of $Prpfs^{N1531S/N1531S}$ embryos beads appeared to become trapped in vortices presumably created by motile cilia, but no leftward flow was established. Finally, in one homozygous mutant embryo the leftward nodal flow appeared to be established normally, indicating that a small subset of embryos may establish leftward nodal flow. Taken together, these results show that the vast majority (6/7) of $Prpfs^{N1531S/N1531S}$ embryos do not generate a coherent nodal flow. The analysed video are included in the attached DVD and described in Appendix 5.
Figure 4.8: Stills from videos investigating microbead movement in the nodes of Prpf8^{+/N1531S} and Prpf8^{N1531S/N1531S} embryos.

Five individual beads in each embryo were manually tracked and their paths visualised. The dot at the end of each path corresponds to the final position of the bead in the video. The anterior of the node is at the top of the image, and the left of the node is at the right of the image. Left: Prpf8^{+/N1531S} embryo showing the concerted movement of beads from the right to the left of the node as described in previously published experiments (n=5). Right: Prpf8^{N1531S/N1531S} embryos showing the absence of the directional movement of the microbeads, indicating a lack of nodal flow (n=6).
Based on the results of the nodal flow microbead experiments, we hypothesised that cilia motility was compromised in $Prpf8^{N1531S/N1531S}$ embryos. We performed direct visualisation of nodal cilia using DIC microscopy and a camera recording at 125 frames per second to test this hypothesis. While $Prpf8^{N1531S}$ heterozygous embryos had many cilia rotating rapidly ($n=3$), in the $Prpf8^{N1531S/N1531S}$ embryos most cilia were immotile ($n=2$). In the first $Prpf8^{N1531S/N1531S}$ embryo the two cilia within the mutant node that did show definite motor driven movement moved slowly and only one moved in a rotational fashion, the other simply moved back and forth. A few other cilia appeared to move around a very narrow axis of rotation; this may have simply been Brownian movement and not motor driven, or they could have been caught in the movement of fluid from the few motile cilia. The motile cilia in the $Prpf8^{+/N1531S}$ embryos were not observable down the eyepiece of the microscope and could only be seen in the recorded video files; this is typical since normally nodal cilia move too quickly to be seen directly by the human eye. However, the motile cilia in the $Prpf8^{N1531S/N1531S}$ embryo were slow enough to be directly observed, highlighting their motility defect. In the second $Prpf8^{N1531S/N1531S}$ embryo only two motile cilia could be observed at the node, these rotated somewhat more slowly than in $Prpf8^{N1531S}$ heterozygotes; all other cilia were immotile. If these $Prpf8^{N1531S}$ homozygous embryos are representative of the $Prpf8^{N1531S/N1531S}$ phenotype, it provides direct confirmation that nodal cilia motility is impaired in $Prpf8^{N1531S/N1531S}$ homozygotes and explains the lack of nodal flow and the origin of the laterality defects observed. These videos are included in the attached DVD and described further in Appendix 5.
4.2.4 Laterality gene *in situ* hybridisation

In light of the defects at the node, the expression patterns of several genes essential to L-R axis establishment were investigated via whole mount *in situ* hybridisation. These genes were *Cerl2, Nodal, Lefty 1, Lefty 2* and *Pitx2* (Figure 4.9). Defects in cilia driven nodal flow causes defects in the expression patterns of all of these genes and aberrations in the expression patterns of all of these genes have previously been shown to induce laterality defects (reviewed in Shiratori and Hamada, 2006). By visualising the expression pattern of these genes it can be confirmed whether the L-R axis pathway is disrupted downstream of the defects observed at the node of *Prpf8*N1531S homozygous embryos.
Top row: The expression pattern of key laterality genes in Prpf8\(^{+/N1531S}\) heterozygotes.

Bottom row: The expression of the same genes in Prpf8\(^{N1531S/N1531S}\) embryos. Arrows indicate regions of interest. Embryos were stage matched and processed simultaneously with heterozygous embryos. The expression patterns of Cerl2 \((n=12)\), Nodal \((n=21)\), Lefty 1 and 2 \((n=10)\) and Pitx2 \((n=38)\) in Prpf8\(^{N1531S/N1531S}\) embryos were all found to be aberrant. Scale bars are 0.5mm.
As can be seen in Figure 4.9, there were significant alterations in the expression of all the laterality genes examined in Prpf8<sup>N1531S</sup> homozygotes compared to heterozygotes, which showed a normal expression pattern. In the case of Cerl2, Nodal and Pitx2, the expression patterns were quantified and have been presented graphically in Figure 4.9

Cerl2, an important inhibitor of Nodal expression, was found to have randomised or absent expression in Prpf8<sup>N1531S</sup> homozygotes instead of the stereotypical right sided perinodal expression usually seen (n=12). In addition it was seen that most homozygous embryos had far lower levels of expression than in controls.

Nodal was also seen to have randomised or absent perinodal expression, instead of the normal preferentially left sided expression (n=21). As well as randomised expression, the intensity of Nodal expression around the node was often reduced in Prpf8<sup>N1531S/N1531S</sup> embryos compared to Prpf8<sup>+/N1531S</sup> controls. Intriguingly, Prpf8<sup>N1531S</sup> homozygous embryos never showed the expected pattern of expression of Nodal in the LPM. 19/21 embryos showed no sign of LPM expression at all; the remaining two embryos only had Nodal expression in the more posterior portion of the LPM, posterior to the cardiac precursor cells.

Lefty1 and Lefty2 expression was detected using a probe complimentary to both genes. While control embryos showed normal expression, demonstrating the effectiveness of the probe, Prpf8<sup>N1531S</sup> homozygotes did not show any sign of expression of either gene (n=10).

Pitx2 expression was, like Nodal and Cerl2 expression, completely randomised or absent (n=38). Importantly, in embryos with missing LPM expression, Pitx2 headfold expression remained unaffected. When embryos in which both heart looping directionality and preferential sided expression of Pitx2 could be confidently determined were examined more closely, it was seen that there was sometimes a discrepancy between Pitx2 expression and the direction of cardiac looping (Figure 4.10). Out of the 21 embryos where Pitx2 expression had either left or right sided
expression, 6 had *Pitx2* expression *in trans* of heart looping (29%). An example can be seen in the bottom far right panel of Figure 4.9.

![Graphical representation of the in situ results of Nodal, Pitx2 and Cerl2 in *Prpf8*^{N1531S/N1531S} embryos.](image)

**Figure 4.10:** Graphical representation of the *in situ* results of *Nodal, Pitx2* and *Cerl2* in *Prpf8*^{N1531S/N1531S} embryos.

Top left, middle and right: Parts-of-whole graph showing the frequency of left, right, bilateral or absent expression of *Nodal, Pitx2* and *Cerl2*, respectively. Bottom: Bar chart showing the results of scoring individual embryos for both the direction of heart looping and the expression pattern of *Pitx2*. Bars representing *Pitx2* expression *in trans* of heart looping are indicated with arrows.
4.3 Discussion

At the beginning of the PhD project it had been appreciated that the Prpf8\textsuperscript{N1531S/N1531S} embryos experienced laterality defects, but the precise nature and origin of these defects was unknown. The results presented here clearly show that these embryos fail to establish the L-R axis very early in development, well before anatomical abnormalities are apparent. The randomisation of the expression patterns of cardinal laterality genes such as Nodal, Cerl2 and Pitx2 are consistent with the defects in node architecture and nodal flow that we observe in Prpf8\textsuperscript{N1531S/N1531S} embryos. Interestingly, we report that in a subset of Prpf8\textsuperscript{N1531S/N1531S} embryos the directionality of heart looping is opposite to the sidedness of Pitx2 expression. While mice null for Pitx2 loop correctly, implying that Pitx2 is dispensable for correct looping directionality (Kitamura et al., 1999), no literature describing the uncoupling of the sidedness of Pitx2 expression and heart looping directionality could be found. In the chicken, however, several examples exist of reversed heart looping in the presence of normally sided Pitx2 expression (Linask et al., 2003; Patel et al., 1999; Schlange et al., 2001), demonstrating that Pitx2 expression can be uncoupled from heart looping directionality.

The most parsimonious hypothesis for the defects seen in the L-R axis would be that defects in node morphology and/or node cilia inhibit the formation of the nodal flow, and this causes the downstream defects in the spatial expression of L-R axis genes. It is presently impossible to know whether it is the changes in the node shape or the length of the nodal cilia that is most responsible for the failure of the nodal flow to form. There is some evidence to suggest that the cilia themselves have motility defects, with almost all nodal cilia observed being immotile, and the three cilia that did move beat slowly; this would be expected to compromise the nodal flow. Cilia motility defects could be caused by defects in ciliary motor proteins, such as Kif3a or left-right dynein, which would impede cilia motility and cause L-R axis defects (Marszalek et al., 1999; Supp et al., 1999). It is also possible that the Ca\textsuperscript{2+}-release is perturbed, but this cannot be determined by the data presented here. Another possibility is that PCP defects are present at the node, which can prevent the cilia from being correctly positioned at the posterior of the nodal pit cells (Song et al., 2010; Hashimoto et al., 2010). The SEM images do not show overt defects in cilia positioning in the node of Prpf8\textsuperscript{N1531S/N1531S}.
embryos, but more in depth analysis is needed to confirm this. It may be that cells from the visceral endoderm do not correctly flank the pit of the node in Prpf8<sup>N1531S</sup> homozygotes, as seen in Rac1 mutants, which have midline defects (Migeotte et al., 2011). There may be subtle interruptions in Shh expression in Prpf8<sup>N1531S</sup> homozygotes (see Figure 3.6), which could indicate the presence of subtle midline defects in Prpf8<sup>N1531S/N1531S</sup> embryos.

The results of the in situ expression analysis in the Prpf8<sup>N1531S/N1531S</sup> embryos are difficult to reconcile completely with published literature. The consistent lack of Nodal expression in the LPM is at odds with the expression pattern seen in iv (inversus viscerum) mice, which like Prpf8<sup>N1531S</sup> homozygotes lack a nodal flow but possess nodal cilia (Okada et al., 1999). In iv/iv embryos Nodal expression in the LPM is randomised or bilateral, but absent expression in the LPM is uncommon; seen in only 9 of 32 iv/iv embryos reported (Lowe et al., 1996). The causative mutation in this mouse line was found to be in the gene coding for left-right dynein; subsequent targeted mutations confirmed that this gene is required for nodal cilia motility (Supp et al., 1999; Supp et al., 1997). Mice with non-functional left-right dynein have immotile nodal cilia and L-R axis defects, but no other defects were noted and homozygous mice survived until weaning (Supp et al., 1997). Importantly, male mice were fertile and tracheal cilia beat normally in mice with a targeted mutation to left-right dynein, possibly suggesting that left-right dynein is only important in nodal cilia functionality (Supp et al., 1997). Prpf8<sup>N1531S</sup> homozygotes also have immotile nodal cilia, but in addition display many other defects and are embryonic lethal; this is presumably because many organ systems are affected by mutating such a critical splicing protein as Prpf8. In embryos missing nodal cilia entirely, the Nodal-Lefty2-Pitx2 pathway appears to be bilaterally activated in the majority of embryos (Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 1998), which we do not observe in Prpf8<sup>N1531S/N1531S</sup> embryos (Figure 4.9). The mutants described in these papers also display other defects, including embryonic lethality and neural tube defects, possibly reflecting the consequences of removing cilia globally. These defects are also seen in Prpf8<sup>N1531S</sup> homozygotes even though nodal cilia are present in homozygous mutant embryos.
Embryos with defective nodal Ca\(^{2+}\) signalling appear to have the phenotype most closely resembling Prpf\(^{N1531S/N1531S}\) embryos. Ca\(^{2+}\) signalling in the node is thought to be mediated by the Pkd2-Pkd1l1 protein complex in the cilia of nodal crown cells, which detects the nodal flow and triggers a release of Ca\(^{2+}\), initiating L-R axis establishment (reviewed in Yoshiba and Hamada, 2014). Embryos missing Pkd2 (Pkd2\(^{-/-}\)) or homozygous for a point mutation in Pkd1l1 (Pkd1l1\(^{rks}\)), lack expression of Nodal in the LPM and are missing Lefty 1 and 2 expression entirely (Field et al., 2011; Pennekamp et al., 2002), similar to the Prpf\(^{N1531S/N1531S}\) embryos. However, there are significant differences between the Pkd2\(^{-/-}\) and Pkd1l1\(^{rks}\) embryos and Prpf\(^{N1531S/N1531S}\) embryos. Unlike the Prpf\(^{N1531S}\) heterozygotes, Pkd2\(^{-/-}\) embryos have a normal leftward nodal flow and are simply unable to sense this flow (Yoshiba et al., 2012). In both Pkd2\(^{-/-}\) and Pkd1l1\(^{rks}\) embryos, both Nodal and Cerl2 were reported as having consistently symmetrical perinodal expression, though the number of embryos examined was not shown for Pkd2\(^{-/-}\) embryos. While Pitx2 expression was randomised in Pkd2 null embryos (Pennekamp et al., 2002), embryos with a point mutation in Pkd2 (Pkd2\(^{lrm4}\)) consistently lack expression of Pitx2 in the LPM (Field et al, 2011), possibly reflecting differences in genetic background or severity of the defects in Pkd2 function between the two mutants. In Prpf\(^{N1531S}\) homozygotes, while there are certainly some embryos with symmetric perinodal expression of Cerl2 and Nodal and missing LPM expression of Pitx2, these are only a subset of the expression patterns observed (Figure 4.2). In Prpf\(^{N1531S/N1531S}\) embryos, we frequently observe a reduction in both Cerl2 and Nodal expression compared to controls, as seen by in situ staining (Figure 4.9), which was not reported in Pkd2\(^{-/-}\), Pkd2\(^{lrm4}\) or Pkd1l1\(^{rks}\) mutants, but was seen in embryos where Ca\(^{2+}\) levels were reduced pharmacologically (Takao et al., 2013).

The shorter cilia in the Prpf\(^{N1531S/N1531S}\) embryos is reminiscent of defects seen in Rfx3\(^{-/-}\) mutant embryos, which exhibit a delay in nodal cilia growth leading to shorter nodal cilia in Rfx3 deficient embryos (Bonnafe et al., 2004). Rfx3 deficient embryos have laterality gene expression patterns similar to that seen in Prpf\(^{N1531S/N1531S}\) embryos; Rfx3\(^{-/-}\) embryos also have defects in cilia motility (Shinohara et al., 2012). Embryos deficient in Noto and Foxj1 also have shortened and largely immotile cilia (Alten et al., 2012a; Beckers et al., 2007). However, in each of these cases, the defect in cilia growth is more severe than seen in Prpf\(^{N1531S/N1531S}\) embryos. This suggests that Prpf\(^{N1531S}\) homozygotes have a relatively mild cilia growth defect which may or may not play a
major role in the lack of nodal flow observed. Consistent with this, *Noto* and *Foxj1* mutant nodal cilia have defects apparent via TEM (Alten et al., 2012a), which we have not detected *Prpf8*<sup>N1531S/N1531S</sup> embryos. To our knowledge there is no published material linking Prpf8 to *Noto, Foxj1* or *Rfx3*. However, knocking down *PRPF8* in human cells does cause a reduction in cilia number, which is consistent with Prpf8 playing a role in ciliogenesis (Wheway et al., 2015).

*Prpf8*<sup>N1531S</sup> homozygous embryos frequently (6/8) have nodes which appear correctly shaped, with little evidence of disorganisation, but are completely flat compared to control embryos (Figure 4.3). Very few mutant mouse lines have been described with node morphology specific defects, as opposed to midline defects which also affect the node. Embryos with *Acvr1* specifically knocked out in the epiblast display a flattened node which appears similar to the *Prpf8*<sup>N1531S/N1531S</sup> phenotype (Komatsu et al., 2011). These *Acvr1* mutants also display shortened cilia and nodal flow defects. However, in these embryos, *Nodal* and *Lefty2* are consistently present, if misslocalised, in the LPM, while in *Prpf8*<sup>N1531S/N1531S</sup> embryos the expression of both *Nodal* and *Lefty2* is consistently absent in the LPM. *Acvr1* is needed for BMP induced cell cycle arrest in the node, which is essential for ciliogenesis (Komatsu et al., 2011), potentially nodal cell cycle arrest could be disrupted in *Prpf8*<sup>N1531S/N1531S</sup> embryos. Embryos with no functional *Noto* are described as having flattened nodes, but these are frequently irregularly shaped and express both *Nodal* and *Cerl2* in the pit of the node (Beckers et al., 2007); *Prpf8*<sup>N1531S/N1531S</sup> embryos have both these genes correctly localised to the crown of the node. Embryos which lack *Rac1* specifically in the epiblast also display flattened nodes, but the node is irregularly shaped and the border between the node pit and the surrounding visceral endoderm is abolished (Migeotte et al., 2011).

After combing the available literature, it appears that the node dysmorphology seen in the *Prpf8*<sup>N1531S/N1531S</sup> phenotype is unique, making it challenging to predict how these node morphological defects affect node function. There does not appear to be a deregulation of node formation, as seen in *Noto* mutants, instead only the process of forming the pit shape of the node is affected. Clearly the node is correctly segregated into pit and crown cells, judging by the shape of node and the correct localisation of *Nodal* and *Cerl2* to the node crown. It is reasonable to propose that the pit shape of the...
node is needed for proper fluid dynamics, as in vivo the leftward flow occurs in the middle of the node cavity, while both at the top and bottom of the node a rightward return flow is found travelling along Reichert’s membrane and the node pit, respectively (reviewed in Cartwright et al., 2008). A flat node would greatly reduce the distance between these three streams of fluid, possibly resulting in less of a coordinated movement of fluid and more of a cacophony, even if the cilia functioned normally. Alternatively, Reichert’s membrane, which covers the embryo and node in vivo, could simply be crushing the cilia and impairing their movement. Neither of these hypotheses satisfactorily explain the lack of a nodal flow after Reichert’s membrane has been removed, however, arguing in favour of cilia motility defects as the cause of the nodal flow defects. Nevertheless, either of the aforementioned hypotheses could exacerbate the impaired nodal flow caused by weakly motile cilia, as seen in the preliminary videomicroscopy. Shinohara and colleagues report that as few as two motile cilia are needed to confer correct L-R axis asymmetry, and that only a weak localised leftward flow is sufficient for correct Cdr1 and Pitx2 expression (Shinohara et al, 2012). However, the mutants described by Shinohara and colleagues have cilia that moved at a similar speed to controls, there were simply fewer moving cilia (Shinohara et al, 2012). It is unclear whether only a few, slowly rotating and shortened cilia, as seen in Prpf8N1531S homozygotes, could initiate laterality establishment. Recent models on the nodal flow show that Reichert’s membrane slows the movement of fluid compared to the rate of movement seen in embryos with the membrane removed (Smith et al., 2011). This could make the cilia motility defects in the Prpf8N1531S homozygotes more pronounced than the videomicroscopy would suggest. The cilia motility defect we observe does not appear to come from gross defects in the ciliary dynein arms or axonemes as these structures appear intact, though their functionality has not been tested and the cilia are correctly localised to the basal body.

While we show that Prpf8N1531S homozygous embryos have defective node formation and nodal cilia motility, it is possible that Prpf8 may have roles upstream of the nodal flow in L-R axis establishment. The presence of node morphogenesis defects suggests that embryonic defects may be present at earlier embryonic stages than those investigated here. The events in the L-R axis pathway upstream of the nodal flow are completely unknown in mice, however in other model organisms such as zebrafish, Xenopus and chickens it is known that the asymmetric distribution of ion transporters is
needed for L-R axis establishment early in embryogenesis (Kawakami et al., 2005; Levin et al., 2002). In Xenopus it has been shown that protein trafficking mediated by Rab GTPases is essential for this asymmetric distribution (Vandenberg et al., 2013). If a similar process occurs in mouse embryos, this could also be disrupted in Prpf8\textsuperscript{N1531S} homozygotes, compounding the defects caused by defective nodal flow. Alternatively, the Prpf8\textsuperscript{N1531S} mutation may simply be disturbing gross morphological processes upstream of node formation, which then results in abnormal node morphogenesis, causing laterality defects.

4.4 Conclusion

In conclusion, while the Prpf8\textsuperscript{N1531S} heterozygotes establish proper laterality and have no apparent node cilia defects, Prpf8\textsuperscript{N1531S} homozygotes exhibit an array of L-R axis defects. The nodes of Prpf8\textsuperscript{N1531S} homozygotes are flat compared to controls and have shortened and largely immotile cilia. These defects, either singly or in concert, lead to a lack of nodal leftward fluid flow. This flow defect results in downstream abnormalities in the expression patterns of genes essential for L-R axis establishment and consequentially defects in heart looping.
Chapter 5: Investigating Prpf8 in multiple model organisms
5.1 Introduction to Prpf8

As discussed in the introduction (Chapter 1.3), Prpf8 is an extremely highly conserved component of the eukaryotic spliceosome (reviewed in Granger and Beggs, 2005). It is essential for splicing both the major and minor pathways and directly interacts with the 5′SS, BPS and 3′SS of the pre-mRNA (reviewed in Grainger and Beggs, 2005), highlighting its importance. Mutations in both human PRPF8 and yeast Prp8 can cause splicing and spliceosomal defects; this includes inducing mutations in Prp8 that are analogous to disease causing mutations in PRPF8 (Boon et al., 2007; Brown and Beggs, 1992; Maeder et al., 2009; Mozaffari-Jovin et al., 2013; Tanackovic et al., 2011). While defects in spliceosomal proteins including PRPF3, PRPF8 and PRPF31 have long been known to cause RP (reviewed in Mordes et al., 2006), it has recently become appreciated that mutating or depleting spliceosomal genes, including PRP8, causes defects in mitosis (reviewed in Valcárcel and Malumbres, 2014) and is associated with cancer (Kurtovic-Kozaric et al., 2015; Makishima et al., 2012). There is some, limited, evidence to suggest that PRPF8 may play a direct role in mitosis (De Wever et al., 2012).

5.1.2 The Effect of mutations in Prp8 on splicing

In splicing, the 5′SS (or splice donor) undergoes a transesterification reaction and is bound to the BPS to form the lariat intermediate; this is the first step of splicing. Following this, the 5′ exon attacks the 3′SS in a transesterification reaction to ligate the two exons together and release the lariat intermediate; this is the second step of splicing (reviewed in Wahl et al., 2009). Between the two steps the spliceosome must undergo rearrangement of its active site (Figure 5.1) (Liu et al., 2007; Schellenberg et al., 2013). Introducing mutations into critical nucleotides of the 5′SS and BPS can inhibit both the first and second steps while mutations in the 3′SS only inhibit the second step; all cause an increase in the abundance of the lariat intermediate (Liu et al., 2007). Specific mutations in spliceosomal machinery, including Prp8, can confer the ability to overcome splice site mutations, allowing splicing to proceed even if the splice site is mutated, and are described as suppressors of splice site defects (Grainger and Beggs, 2005). Splicing in the presence of splice site defects appears to be accomplished by
altering the equilibrium between the two arrangements of the active site of the spliceosome; favouring one step at the expense of the other (Query and Konarska, 2004). For instance, intronic 3’SS mutations inhibit the second step of splicing, but in the presence of a mutant that suppresses second step splicing defects the second conformation of the spliceosome is favoured, compensating for inhibition of the second splicing step reaction. Suppressors of first step splicing defects display increased lariat accumulation at the expense of mature transcript, while suppressors of second step defects have reduced lariat intermediate and increased mature transcript (Liu et al., 2007). Both types of suppressor mutation have been described in different alleles of Prp8, and both types of mutation are largely found affecting residues on the surface of the cavity that forms the active site of the spliceosome (Galej et al., 2013; Liu et al., 2007).
Figure 5.1: Schematic showing the two splicing steps in the active site of the spliceosome.

The spliceosome undergoes dynamic rearrangements to reconfigure the active site for the two steps of splicing. In the first step, the branch point (orange circle) attacks the 5’ splice site of the intron, creating the lariat intermediate. Following this, the active site undergoes rearrangement. Then, the 5’ exon attacks the 3’ splice site, ligating the 5’ and 3’ exons together (green and red boxes respectively). The spliced mRNA and lariat are then released (not shown). Splice site suppressor mutations alter the kinetics of active site rearrangement, to promote either the first or second step arrangement of the spliceosomal active site.

Adapted from Query and Konarska, 2004
5.1.3 Structure of Prp8

Prp8 is a highly modular protein with specific domains performing different functions, and some of these domains are more essential to the ability of Prp8 to splice pre-mRNA than others. For instance, there are many examples of RP patients with mutations in PRPF8 at the C-terminus, but no examples of patients with mutations elsewhere in PRPF8. This could suggest that mutations elsewhere are either embryonic lethal or harmless in humans, however, due to the high degree of sequence conservation and the essential role PRPF8 has in splicing it seems likely that these mutations are embryonic lethal. Many yeast Prp8 mutants exist with mutations outside the C-terminus of Prp8, and these show defective splicing (reviewed in Grainger and Beggs, 2005). Moreover, structural and biochemical investigations suggest the C-terminus is largely involved in protein-protein interactions, while the large central domain forms the active site (Boon et al., 2006; Galej et al., 2013). Therefore, it can be proposed that the patient mutations in the C-terminus still leave some PRPF8 functionality, while mutations outside the C-terminus severely or totally abrogate PRPF8 function by incapacitating the spliceosomal active site, resulting in embryonic lethality. It is therefore important to place the Prpf8N153S mutation in context with known protein domains and mutations that affect domain functionality in order to predict the effect of the Prpf8N153S mutation on Prpf8 functionality. Since yeast is highly amenable to biochemical investigation, most structural and functional analyses have been done in Prp8 rather than PRPF8; due to the high degree of conservation between the two proteins it is assumed that results in Prp8 apply to PRPF8, as well as mouse Prpf8.

Biochemical studies of Prp8 have revealed much about the organisation, structure and function of the protein domains found in Prp8. Prp8 can be dissected into four functional domains (Figure 5.2) consisting of residues 1-394; 443-770; 771-2170 and 2179-2413 (Boon et al., 2006). Close to the N-terminus is a nuclear localisation signal needed for both U5 snRNP nuclear accumulation and for U4/U6.U5 tri-snRNP formation (Boon et al., 2007). The C-terminal domain of Prp8 contains an MPN (Mpr1/Pad1 N-terminal) domain that does not coordinate Zn2+ ions and so likely does not have catalytic activity; instead it allows the C-terminus to interact with other spliceosomal proteins (Pena et al., 2007; Zhang et al., 2007). It is in this domain that RP linked mutations are found in humans.
The most pertinent domain of Prp8 to this project is the large central domain spanning residues 771-2170. Interestingly, this central domain of Prp8 is thought to have evolved from prokaryotic group II intron reverse transcriptases, with the N-terminal and C-terminal domains acquired to facilitate protein-protein interactions (Dlakić and Mushegian, 2011; Galej et al., 2013). This evolutionary history probably accounts for the lack of homology that Prp8 has to other eukaryotic proteins. The crystal structure of a large fragment of Prp8 consisting of residues 885-2413 has shed much light on this central domain (Galej et al, 2013). Galej and colleagues demonstrated that residues 885-1824 form a novel protein domain that is subdivided into a polymerase-like domain (residues 885-1375), a linker domain (residues 1376-1649) and a type II restriction endonuclease-like fold (residues 1653-1824) (Galej et al, 2013). A conserved RNA recognition motif had been proposed between residues 1058-1151 (Grainger and Beggs, 2005), but the resolved structure of this region argues against this prediction (Galej et al, 2013). A modified RNAse H fold spanning residues 1839-2092 is present that appears to bind and position RNA for splicing rather than degrade it (Galej et al., 2013; Pena et al., 2008; Ritchie et al., 2008). This RNAse H domain switches between open and closed conformations and only the former contains a Mg$^{2+}$ ion (Schellenberg et al., 2013). The closed and open conformations are also shown to correspond to the first and second steps of splicing, respectively (Schellenberg et al, 2013). It has been suggested that the domain responsible for splicing cofactor activity lies within residues 1503 and 1673; transposon inserts into this region are lethal in S. cerevisiae (Turner et al., 2006). This region is highly conserved (Grainger and Beggs, 2005) and contains a number of 3’SS suppressor mutations (Umen and Guthrie, 1996); it is now known that this region includes part of the linker and endonuclease domains (Galej et al, 2013). In addition, there is a point of contact somewhere between residues 1585 and 1598 and the BP, indicating that some or all of the region between residues 1503-1673 form part of the active site of Prp8 (Galej et al, 2013). It is within this region that our mouse mutation lies, at residue 1531 in mouse Prpf8, which corresponds to residue 1603 in yeast Prp8 (Figure 5.2).
Figure 5.2: Schematic of the domain architecture of Prp8.

A: Prp8 contains a number of protein domains, the functions of which are beginning to be understood. The structure of residues prior to the RT domain is unknown. The N1603S substitution is towards the C-terminal end of the linker region (red arrow). NLS: nuclear localisation signal; RT: reverse transcriptase. Adapted from Galej et al, 2013 with data from Boon et al, 2007. B: Structure of mouse Prp8 and Aar2 generated from publicly available data (Galej et al, 2013). Residue 1531 (purple) of Prp8 is at the interface between Prp8 and Aar2 (Simon Lovell, pers. comm.).
5.14 Binding Partners of Prp8

Prp8 has a number of protein binding partners, which are essential to its function or assembly. The most relevant to this project are the snRNP Brr2 and the protein Aar2. Aar2 and Brr2 bind competitively to the C-terminus of Prp8; Aar2 binds to Prp8 in the cytoplasm and, upon phosphorylation, is replaced by Brr2 in the nucleus (Figure 5.3) (Boon et al., 2007; Weber et al., 2011; Weber et al., 2013). Only Brr2 is found in mature spliceosomes; it has been suggested Aar2 functions to inhibit precocious Brr2 activity (Weber et al., 2011). X-ray crystallography of Prp8 (residues 885-2413) in complex with Aar2 revealed that the C-terminus of Aar2 is essential for stabilising Prp8 and interacts with multiple protein domains (Galej et al., 2013). This stabilisation sequesters the Jab1/MPN domain of Prp8 which is the binding partner for Brr2, preventing Brr2 from binding to Prp8 (Nguyen et al., 2013). Aar2 may also have a role in preventing undesirable RNA binding, ensuring that the U4/U6 di-snRNA binds to Prp8 after it has been unwound by Brr2 (Weber et al., 2013). Aar2 is also essential for spliceosomal recycling and depleting Aar2 protein levels causes an accumulation of unspliced transcripts (Gottschalk et al., 2001).

Brr2 is an RNA helicase required to unwind the U4/U6 di-snRNA and enable proper spliceosomal catalytic function (Laggerbauer et al., 1998; Raghunathan and Guthrie, 1998), creating the activated complex B* (see figure 1.8). Only the N-terminus of Brr2 interacts with RNA (Hahn et al., 2012; Kim and Rossi, 1999); the C-terminus has no catalytic activity but is involved in protein-protein interactions (Cordin et al., 2014); the C-terminus of human BRR2 binds to the C-terminus of PRPF8 (Liu et al., 2006). It has since been found that the N-terminus of yeast Brr2 interacts with the Jab1/MPN domain of Prpf8 (Nguyen et al., 2013). The interaction between Prp8 and Brr2 seems to be quite complicated, as Prp8 appears to both enhance (Maeder et al., 2009) and reversibly inhibit Brr2 activity (Mozaffari-Jovin et al., 2013). Brr2 may be needed to remodel the catalytic centre of the spliceosome in order to proceed from step 1 to step 2 of splicing (Hahn et al., 2012).
Summary

Prp8 is integral to the assembly of the spliceosome, and also forms a large part of its active site. It directly interacts with the 5’SS, BPS and 3’SS of the spliced intron, and many mutations have been described in Prp8 that can interfere with the kinetics of the splicing. While Prp8 must have many more protein partners, the interaction between Prp8, Aar2 and Brr2 is essential for spliceosomal assembly and activation. Aar2 binds to Prp8 in the cytoplasm and the Prp8-Aar2 protein complex is imported into the nucleus. There, Aar2 is phosphorylated and removed from Prp8, allowing Brr2 and the U4/U6 di-snRNA to bind to Prp8, activating the spliceosome.

Previously published mouse mutants of Prpf8 have focussed on mutations known to cause RP in humans, to better understand how defects in splicing contribute to the RP phenotype (Farkas et al., 2014; Graziotto et al., 2011). Deramaudt et al, 2005 report that mouse embryos with both copies of Prpf8 knocked out are not viable, but the phenotype was not reported. A zebrafish ENU mutagenesis screen did generate a zebrafish line with severely truncated Prpf8. Impaired heamatopoises and myeloid differentiation were identified in these fish (Keightley et al, 2013). To our knowledge the Prpf8\textsuperscript{N1531S} mutation is the first reported animal model with a mutation in the catalytic centre of Prpf8, and the first investigation of the function of Prpf8 in embryogenesis.
Figure 5.3: Diagram showing interaction of Prp8 with binding partners.

1: Prp8 binds to Aar2 in the cytoplasm and is imported into the cell nucleus through the nuclear pore complex. 2: Aar2 prevents both Brr2 and the U4/U6 di-snRNA from binding Prp8; Aar2 is phosphorylated and removed. 3: Brr2 binds to Prp8; Aar2 is dephosphorylated. 4: The U4/U6 di-snRNA binds to Prp8; Aar2 is exported to the cytoplasm for reuse. Adapted from Weber et al., 2013.
5.2: Results

5.2.1: Isolating the causative mutation in the \textit{I1Jus27} mouse line

Due to the random nature of ENU mutagenesis the causative mutation in the \textit{I1Jus27} mouse line was initially unknown (see Figure 1.11). However, previous lab members had narrowed the region of interest on chromosome 11 to a 6Mb region (Figure 5.4 A). Subsequently next generation sequencing was performed which revealed a missense mutation in Prpf8 which resulted in an asparagine residue being replaced by a serine residue at position 1531, which is highly conserved (Tenin, G pers. comm.). Both asparagine and serine are small, polar amino acids, which can play important roles in protein active sites. However, the absolute conservation of N1531 throughout the eukaryote lineage (Figure 5.4 B) suggests that this asparagine residue is essential for protein function. This is supported by bioinformatic analysis using PROVEAN (Choi \textit{et al.}, 2012) suggesting that the N1531S mutation should be highly deleterious. This mutation is not normally found in either the 129S5 or C57BL/6 mouse lines (Figure 5.4 C), demonstrating the mutation is a result of the ENU treatment. However, in order to obtain additional support for our hypothesis that the N1531S substitution directly causes the \textit{I1Jus27} phenotype we decided to examine Prpf8 mutations in other model organisms.
Figure 5.4: Genetic analysis of l11Jus27 mice.

A: Schematic showing recombinant mapping of l11Jus27 phenotype to a region of chromosome 11 containing Prpf8. The mutation is carried on the chromosome with the C57BL/6 genotype. B: Clustal analysis of Prpf8 orthologues in mouse, zebrafish, Drosophila melanogaster and Saccharomyces cerevisiae showing that residue 1531 is absolutely conserved across eukaryotes. C: Sanger sequencing of different mouse genotypes confirming that N1531S substitution is present in heterozygous and homozygous mice carrying the l11Jus27 phenotype, but not in wild type mice from either a 129S5 or C57BL/6 background.
5.2.2: Zebrafish morpholino analysis

In order to determine whether a reduction in Prpf8 activity could lead to embryonic defects, we took advantage of the fact that Prpf8 is extremely highly conserved and investigated loss of Prpf8 in a zebrafish model. Zebrafish have several advantages over mice for studying embryonic development: they develop externally and generate many embryos, allowing high numbers of offspring to be generated rapidly and economically. Zebrafish embryos are also transparent until pigment cells develop, making them ideal for studying early embryogenesis. The casper zebrafish line is truly transparent; this line can be crossed with transgenic lines which label specific tissues with GFP to aid analysis of tissue morphogenesis. Finally, it is possible to knockdown a gene of interest using targeted morpholinos, which can interfere with either the initiation of translation or with splicing of target splice sites; either method results in a reduction of mature protein. However, translation inhibiting morpholinos require Western blots for confirmation, while splice site blockers only require PCR analysis for confirmation. Since morpholinos are resistant to degradation (Corey and Abrams, 2001), their effects are persistent throughout early embryogenesis until they are diluted through embryonic growth. By comparing the effects of morpholinos targeted against Prpf8 splice sites to the effect of a published control morpholino in injected (morphant) embryos, it can be determined whether a reduction in Prpf8 affects embryogenesis.

We designed splice-blocking morpholinos directed against exon 7 (Figure 5.5) and exon 24 of zebrafish Prpf8. These exons were chosen because their size is not a multiple of three, which means that if they were cleanly removed then the downstream transcript would be in a different frame, crippling Prpf8 functionality. Injecting zebrafish embryos with these morpholinos resulted in comparable defects in morphogenesis. Transgenic zebrafish with GFP expressed from a Flk1 promoter, to label blood vessel endothelium, on a Casper background were used (a kind gift from Adam Hurlstone); this allowed convenient visualisation of the heart and vasculature. Injecting 1 cell stage embryos with 0.52nl of 0.3mM exon 7 or 2nl of 0.7mM exon 24 morpholino was sufficient to generate a large number of morphant embryos; injecting 0.5nl of 0.5mM control morpholino had no observable effect on embryogenesis. These were the lowest concentrations of morpholino that would reliably generate a morphant phenotype; stronger concentrations were not used to avoid off target effects since mRNA rescue
may not be possible, due to the large size of the Prpf8 transcript. We observed few signs of morpholino toxicity, with the prevalence of the ‘monster’ phenotype (Bedell et al., 2011) being low (example in Figure 5.5 G). However, we did observe darkening of the CNS in embryos injected with morpholinos against exon 7. This phenomenon is associated with off target upregulation of Trp53 (Robu et al., 2007); simultaneously knocking down Trp53 ameliorated this without affecting other aspects of the phenotype. These embryos were analysed at 72 hours post fertilisation (hpf) by which time heart looping had concluded. It was frequently observed that morphant embryos had spinal curvature and tail defects. These defects varied in severity from minor notches or kinks at the end of the tail to gross dysmophlogy along the A-P body axis. Morphant embryos also appeared to have a shorter A-P body axis, though this was not measured. This phenotype is consistent with the reported phenotype of a zebrafish mutant with a premature truncation in Prpf8, resulting the loss of most of the mature protein (Keightley et al., 2013). However, we report that morphant embryos have an increased rate of heart looping defects. Morphants did not show an increased prevalence of heart looping reversal; instead the atria and ventricles failed to ascend and descend, respectively, resulting in a more linear, primitive heart tube (Figure 5.6). Defects in the aortic arches were also observed, with fewer arches being present in morphants. It is unclear whether Keightley et al (2013) investigated heart and aortic morphology in their mutant line, but they did not report defects in these structures.
Figure 5.5: Investigation of the Prpf8 morphant phenotype at 72 hours post fertilisation.

A: Zebrafish embryos that received control morpholino injections at the one cell stage have no apparent phenotype. B: Zebrafish embryos that received injections of a morpholino against exon 7 of Prp8 at the one cell stage frequently show a variety of defects, including spinal and tail curvature (arrows). C-G: Fixed embryos treated with morpholino against exon 7 of Prpf8 showing the range of morphant phenotypes. From least to most severe: C: normal/slight; D: tail kink/notch; E: medium; F: severe; G: monster. These embryos have been stained with DAB to show erythrocytes (red). As the phenotype becomes more severe staining in the heart becomes less pronounced and spinal, tail and heart deformities become more severe (arrows). Figures C to G are to scale with each other. All scale bars = 0.5mm.
Figure 5.6: Diagram showing heart looping defects in transgenic zebrafish injected with morpholinos directed against *Prpf8*

Zebrafish expressing GFP under a Flk1 promoter were treated with morpholinos targeting exon 7 of *Prpf8*. The hearts (outlined in white) and aortic arches (arrow) of control embryos have developed normally, while embryos treated with morpholinos against *Prpf8* have defects in heart looping and aortic arch morphogenesis. Images of zebrafish are to scale with each other. *Prpf8* \textsuperscript{N1531S} homozygotes frequently have heart looping defects even in the absence of laterality defects. All scale bars = 0.1mm.
While the rate of heart looping reversal (Figure 5.7 A and B) was no different between Prpf8 knockdown embryos and controls, the incidence of linear or unlooped hearts was much greater in knockdowns compared to all other outcomes (Figure 5.7 C and D, p<0.0001). Importantly, there was no difference in the incidence rate of unlooped hearts between embryos treated with morpholinos against exon 7 and exon 24 of Prpf7, or between embryos which were treated with morpholinos against both Trp53 and exon 7 of Prpf8 and embryos which received only morpholinos against exon 7 of Prpf8. This suggests that this is a bona fide result of a reduction in Prpf8 protein levels. It is possible that the cardiac phenotype observed in morphant embryos is not cardiac cell autonomous and is simply a read out of defects in global morphogenetic process, which could also be the case in Prpf8N1531S homozygotes. While rates of spinal defects are much higher in all groups of Prpf8 morphant embryos (Figure 5.7 E and F), the rate of the tail notch/kink phenotype is much lower in embryos which received morpholinos against exon 24 instead of exon 7, however, the reason for this finding is unclear.

Confirmation that the splice blocking morpholinos were indeed inhibiting the proper splicing of Prpf8 was done by PCR analysis to find evidence of missplicing. It was seen that the exon 7 morpholino resulted in the retention of most, but not all, of intron 7, which was not seen in control embryos (Figure 5.8 A, B), which introduces a premature stop codon. The morpholino targeted against exon 24 resulted in the exclusion of much, but not all, of exon 24 in morphant embryos (Figure 5.8 C, D), with presumably deleterious effects on protein functionality. With both morpholinos there was mature wild type transcript present, consistent with a knockdown rather than a knockout effect. Though the knockdown effect appears modest, this is probably because cDNA was generated after the morphant phenotype was scored, at 72hpf. By this timepoint much of the morpholino could be diluted or sequestered, but any permanent defects caused in early embryogenesis would persist. The intron-exon structure of Prpf8 is remarkably well conserved between mice and zebrafish, so it is possible to directly compare the structure of Prpf8 between the two species (see Appendix 6 for a diagram).
Figure 5.7: Graphical representation of zebrafish morpholino data.

A, B: Rates of different types of observed heart defects in injected zebrafish embryos were compared. While rates of heart looping reversal and gross absence of moving heart tissue appeared similar in all groups, hearts were more likely to fail to loop at all in embryos which received morpholinos against Prpf8 compared to those which received a control morpholino. C, D: The failure rate of heart looping was significantly higher and at the expense of the total of the other possible outcomes of heart looping (designated remainder) in Prpf8 morphant embryos compared to controls. There was no difference between the two different Prpf8 morpholinos (C), or between embryos which received morpholinos against exon 7 of Prpf8 and Trp53 (p53) and those which only received a Prpf8 morpholino (D). E, F: rates of spinal deformities as displayed in Figure 5.5 in control and morphant embryos. Control embryos showed low levels of all type of deformities, while morphant embryos showed a variety of defects. Embryos which received the morpholino against exon 24 of Prpf8 had a much lower rate of the tail notch/kink phenotype and a concurrent rise in the normal/slight phenotype than embryos that received a morpholino against exon 7; the rates of other outcomes appears comparable (E).
Figure 5.8: Diagram showing that the splice blocking morpholinos do inhibit target splice sites.

A: Agarose gel of PCR analysis showing that primers against exon 6 and intron 7, and exon 6 and exon 8, generate a product corresponding to a partial retention of intron 7 in cDNA from morphant but not control embryos. B: Results of Sanger sequencing (black) were analysed by BLAT and compared to the genomic Prpf8 sequence (blue) and confirmed that most of intron 7 was retained. C: Agarose gel of PCR analysis showing that the amplicon of primers against exon 23 and exon 26 show an additional band in morphant embryos lower than the expected amplicon size. D: Results of Sanger sequencing of the additional band were analysed by BLAT and compared to the genomic Prpf8 sequence, which confirmed that most of exon 24 is lost.
5.2.3: Yeast Prp8 model

While the zebrafish morpholino experiments strongly suggest that the presence of non-wild type Prp8 transcript can cause broad defects in embryogenesis, it remained unclear what effect the N1531S substitution would have on Prp8 functionality. To address this question, we used a yeast (S. cerevisiae) model, in which a multitude of biochemical tools can be used. In addition to this, several investigations of Prp8 function have previously been performed in yeast, which means our results are comparable to published data. The yeast Prp8 data presented here was generated in collaboration with Ray O’Keefe.

Prp8 was cloned into the shuttle vector pRS413, to allow for easy manipulation. In order to determine the effect that the N1603S mutation (analogous to the N1531S mutation in mouse) had on yeast growth, genomic Prp8 was knocked out in haploid yeast, which had wild type Prp8 on a plasmid with a URA3 selectable marker to maintain viability, as Prp8 is an essential gene. These colonies were then transformed with pRS413 containing Prp8 and grown on media containing 5-fluoroorotic acid, which selectively kills yeast containing URA3. This ensures that only yeast colonies with Prp8 on the pRS413 vector alone would be viable. To confirm this, knockout out yeast colonies which were transformed with the empty pRS413 vector were plated at 30°C; these transformants did not form colonies (Figure 5.9 A), confirming that these colonies contained no copies of Prp8. In contrast, knockout yeast which received the pRS413-PRP8 construct did form colonies. Interestingly, knockout yeast transformed with pRS413-PRP8N1603S also formed colonies, suggesting that the Prp8N1603 allele retains at least some functionality, as cells with this allele as their sole source of Prp8 were viable. Yeast pRS413-PRP8N1603S transformants remained viable when grown at 16°C and 37°C.

Although yeast colonies using only Prp8N1603S were viable, it remained unknown whether splicing was affected. To investigate this, the ACT1-CUP1 splicing reporter construct was introduced into haploid yeast with the endogenous copy of CUP1 disabled. The ACT1-CUP1 construct contains the 5’ exon and intron of ACT1 fused to the copper resistance gene CUP1 (Lesser and Guthrie, 1993). In order for the yeast cell
to synthesise mature CUP1 protein, acquire copper resistance and be able to grow on copper enriched media, the ACT1 intron must be successfully spliced. The efficiency of splicing can be gauged by finding the highest concentration of copper that the yeast can tolerate; if splicing is more efficient then more CUP1 is produced and therefore colonies tolerate higher copper concentrations. The spliceosome can be challenged by inducing 5’SS, BPS or 3’SS mutations in the ACT1 intron of the ACT1-CUP1 fusion gene. By comparing the maximum tolerance of yeast using only wild type Prp8 or Prp8\textsuperscript{N1603} in the pRS413 vector, the splicing efficiencies between the two Prp8 alleles when confronted with intronic splice site mutations can be compared; wild type splice sequences would not give a phenotype, as \textit{Prp8}\textsuperscript{N1603S} is viable. Figure 5.9 B shows that the Prp8\textsuperscript{N1603S} allele is much less efficient at splicing the ACT1-CUP1 construct when challenged with 5’SS, BPS or 3’SS mutations, compared to wild type Prp8. This suggests that that the Prp8\textsuperscript{N1603S} allele does not increase splice site fidelity. Primer extension analysis from Prp8\textsuperscript{N1603} colony extracts shows that when challenged with a BPS mutation (A259C) more lariat intermediate is accumulated, while when confronted with a 3’SS mutation (A302U) less mature mRNA is present, compared to wild type Prp8 (Figure 5.9 C). This shows that the Prp8\textsuperscript{N1603} allele promotes the first step of splicing at the expense of the second step of splicing, and so is a first step allele of Prp8.
Figure 5.9: Investigation of N1603S substitution phenotype in yeast.

A: Yeast colonies growing using Prp8 in a pRS413 vector with genomic yeast Prp8 knocked out. Left: Yeast colonies using pRS413 without Prp8 fail to grow, confirming genomic Prp8 is non-functional; middle and right: yeast colonies are viable using pRS413 containing either wild type or mutated (N1603S) Prp8.

B: Comparison of splicing efficiencies using an ACT1-CUP1 splicing reporter. This contains an artificial intron which must be spliced for copper resistance to be conferred; more efficient splicing correlates with increased copper resistance and therefore growth at higher concentrations of copper sulphate (CuSO₄). Yeast using wild type and N1603S Prp8 were challenged with mutations at the 5'SS (G5A), BPS (C256A and A259C) and the 3'SS (A302G and A302U) at increasing concentrations of CuSO₄; in each case the N1603S allele of Prp8 was less efficient at splicing the reporter as growth (the bars on the graph) was inhibited at lower concentrations of CuSO₄.

C: Primer extension analysis in Prp8 and Prp8ₐN1603S colonies. Top row shows lariat accumulation in Prp8ₐN1603S colonies when challenged by the BPS mutation A259C. Bottom row shows depletion of mature mRNA product when challenged by 3’SS mutation A302U in yeast with Prp8ₐN1603S. These results are consistent with a first step allele of Prp8. Ray O’Keefe, pers. comms.
5.2.4: Prpf8 Expression analysis

The expression domain of a gene is often essential to understanding its role in embryogenesis, as its location determines what genes it can interact with and highlights tissues in which the gene of interest is likely to be important. To our knowledge the expression pattern of Prpf8 has not been found in any model organism, making it difficult to predict processes that could be affected in Prpf8<sup>N1531S/N1531S</sup> embryos. We therefore designed three probes for <i>in situ</i> hybridisation against different regions of Prpf8. Probe 1 did not generate good quality PCR amplification product and was discarded. Probe 2 hybridises to the 5’ untranslated region and exon 1, while probe 3 hybridises to exons 37-40, but not intronic sequence (Figure 5.10). Sequence analysis with BLAT confirmed that these probe sequences were unique and should not hybridise to other genes.

![Figure 5.10: Schematic of in situ probes against Prpf8 alongside genomic Prpf8.](image)

Images show high and low magnification (top and bottom of A and B) of <i>in situ</i> probe sequence (black) against genomic Prpf8 (blue). A: Probe 2 hybridises against the 5’ UTR and exon 1 of Prpf8. B: Probe 3 hybridises against exons 37-40 of Prpf8 mRNA, but not pre-mRNA.
We then investigated the expression pattern of *Prpf8* in *Prpf8*+/N1531S E10.5 embryos and compared the patterns of probe 2 and 3 to each other. The staining pattern of the two antisense probes mirrored each other, strongly suggesting that they were detecting the same gene with no off target effects. The embryos stained using probe 3 appear lighter than those stained with probe 2, this may be an artefact of the destaining treatment used to enhance contrast (Figure 5.11). Control embryos stained using the sense sequences of each probe showed no signs of signal development, confirming that staining using the antisense probes was not due to background (Figure 5.11).

*Prpf8* was expressed widely throughout the embryo at E10.5, as expected for a gene involved in splicing, which occurs in all tissues. Staining appeared to be particularly intense in the limb bud, pharyngeal arches and branchial arch arteries, as well as regions of the head including the eye, olfactory pit and future oral cavity. There also appeared to be salt-and-pepper staining along the trunk of embryos stained with probe 2 (Figure 5.12). Both probes showed some evidence of intersomite staining; this was more prominent with probe 3, possibly due to the destaining treatment. Unexpectedly, staining in the heart was consistently absent using either probe.

At E8.5, when the the laterality genes *Nodal, Lefty 1, Lefty 2* and *Pitx2* are expressed in highly specific areas including the node and LPM, *Prpf8* expression remained global (Figure 5.13). Staining did appear particularly intense in regions of the headfold, the anterior neural tube and and possibly in the lateral plate mesoderm. At the 5 somite stage, staining becomes either more prominent in the somites or downregulated in tissue surrounding the somites. Staining was either low or undetectable in the posterior neural tube and node. Consistent with the staining pattern in E10.5 embryos, the cardiac crescent remained unstained.

We investigated the expression pattern of *Prpf8* as measured by *in situ* hybridisation in *Prpf8* N1531S homozygous embryos. Unfortunately, in the batch labelled with probe 3 the closest stage matched heterozygous embryos failed to develop signal in the assay; as a result the available controls are 3 or 4 somites younger than the *Prpf8* N1531S homozygotes (Figure 5.12 C, D). Nevertheless, there appears to be no discernible
difference in the expression pattern of *Prpf8* in *Prpf8^{N1531S}* heterozygotes and homozygotes.
Figure 5.11: *In situ* hybridisation with probes detecting *Prpf8* at E10.5.

A, D: Sense probes show no colour reaction or non-specific background (*n* = 6 for probe 2; *n* = 4 for probe 3). B, C: Antisense probe 2 shows staining in regions of the head including the future oral cavity and eye (blue arrow) as well as in the pharyngeal arches (yellow arrow), limb bud (white arrow) and the walls of the pharyngeal arch arteries (green arrow), as well as salt-and-pepper staining in the trunk (red arrow) and between somites (orange arrow) (*n* = 3). The heart shows no staining (black arrow). E, F: Antisense probe 3 shows a similar pattern to antisense probe 2 (*n* = 4). Scale bars = 0.5mm.
Figure 5.12: Magnified areas of *in situ* hybridisation with probes detecting *Prpf8* at E10.5.

A: Sense probes show no colour reaction or non-specific background. B: Antisense probe 2 show staining in the walls of the pharyngeal arch arteries, as well as salt-and-pepper staining in the trunk (black arrow). C: Antisense probe 3 shows a similar, though less prevalent, salt-and-pepper staining pattern along the tail (black arrow).
Figure 5.13: In situ hybridisation with probes detecting Prpf8 at E8.5.

A and B: Staining pattern of Prpf8 probe 2 on Prpf8<sup>N1531S</sup> heterozygotes (n = 6) and homozygotes (n = 4), respectively. C and D: Staining pattern of Prpf8 probe 3 on Prpf8<sup>N1531S</sup> heterozygotes (n = 10) and homozygotes (n = 12), respectively, showing a similar expression pattern to A and B. Expression appears to be almost global, though expression appears reduced in the neural tube of all embryos, and between the somites of older embryos (A and D). Scale bars = 0.5mm.
5.2.5: Immunofluorescence of Prpf8

Despite the satisfactory resolution of the embryonic expression domains of Prpf8, it remained unclear as to how defects in Prpf8 could cause the cilia and L-R axis defects seen in the Prpf8N1531S homozygotes when Prpf8 expression is almost global. Therefore, we investigated the protein localisation of Prpf8. We detected Prpf8 with an anti-Prpf8 antibody, and found that in both heterozygotes and homozygotes Prpf8 was localised to the nucleus (Figure 5.14). Control experiments in which the Prpf8 antibodies were omitted showed only background staining. During the course of this project, Wheway and colleagues, reported that knocking down Prpf8 in both mouse inner medullary collecting duct and human retinal pigmented epithelial cell lines caused ciliogenesis defects (Wheway et al., 2015). They also found that Prpf8 localised to the basal body of both primary cilia and the highly modified cilia in the retina, suggesting Prpf8 may have functions outside of splicing. Therefore, it is possible that Prpf8 has a direct function in cilia and that the N1531S mutation could be causing a cilia specific defect.

Using antibodies raised against Prpf8 (a kind gift from Colin Johnson), we investigated the localisation of Prpf8 in the mouse node at E8.5, when the cilia driven flow should be operating (Figure 5.15 and 5.16). Cilia were detected using an anti-acetylated tubulin antibody. We found that Prpf8 appeared to be localised to the basal body of node cilia, possibly with faint signal along the cilium itself. The anti-Prpf8 antibody also appeared to label the plasma membrane, which may be due to background non-specific staining, but not the nucleus. Omitting the Prpf8 primary antibody in control experiments showed only low levels of background staining. In both Prpf8N1531S heterozygotes and homozygotes, these anti-Prpf8 antibodies appeared to preferentially label cilia basal bodies, with no differences between the two genotypes. Interestingly we were unable to detect basal body localised Prpf8 using a different antibody raised against Prpf8 purchased from a commercial supplier (Abcam ab79237). This commercially available antibody detected labelling in the nuclear speckles, in which the spliceosome is found. The difference in staining patterns could be due to the different conditions in the nucleus compared to the cytoplasm, which could affect antibody binding. Alternatively, the antibodies may recognise different epitopes on Prpf8 which may be obscured due to
the presence of proteins bound to Prpf8; for instance, the Abcam antibody may recognise an epitope that is obscured by basal body proteins. Again, there appeared to be no difference between Prpf8\textsuperscript{N1531S} heterozygous and homozygous embryos, showing that Prpf8 localisation is unaffected.
Figure 5.14: Localisation of Prpf8 in the nucleus.

The staining pattern of anti-Prpf8 (red) in the nucleus was investigated via confocal microscopy; cilia were labelled with anti-acetylated tubulin (green) and nuclei were stained with DAPI (blue). In both heterozygotes (A) and homozygous (B) embryos Prpf8 was localised to the nuclear speckle with these antibodies. Due to difficulties with sample processing, the images for the homozygous embryonic node are of very poor quality; thus this image demonstrates Prpf8 localisation in the neural tube. A: *Prpf8^+/N1531S* embryonic node showing cilia and Prpf8, which is localised to the nuclear speckle. B: *Prpf8^N1531S/N1531S* embryonic headfold, labelled as in A. The staining pattern of Prpf8 is indistinguishable to A. Scale bars are 10µm. A’, B’: Regions of A and B, respectively, cropped and blown up. The region blown up is indicated by a white box in A and B. Scale bars are 5µm. n= 3 for both genotypes.
Figure 5.15: Localisation of Prp8 in the node.

The staining pattern of anti-Prp8 (red) in the embryonic mouse node was investigated via confocal microscopy; cilia were labelled with anti-acetylated tubulin (green) and nuclei were stained with DAPI (blue). There was no evidence of nuclear localisation of Prp8 using these anti-Prp8 antibodies. However, Prp8 and cilia did appear to co-localise, with more Prp8 signal at the presumptive basal body of the cilia. All images are maximum intensity projections of confocal z-stacks. A, B and C: Staining pattern of indicated antibodies in Prp8+/N1531S embryos (n = 5). D, E and F: Re-slice of A, B and C, respectively, showing side view of the node. G, H and I: Staining pattern of indicated antibodies on Prp8N1531SN1531S embryos (n = 7). J, K and L: Re-slice of G, H and I, respectively, showing side view of the node, which is noticeably flatter compared to Prp8+/N1531S embryos. No difference in the staining pattern of either antibody can be discerned. The region within the white box has been blown up in Figure 5.16. Scale bars = 10µm.
Figure 5.16: Enlarged image showing Prpf8 localised at the node.

Blown up regions of Figure 5.15; cilia are labelled green, Prpf8 in red and nuclei are in blue. Prpf8 and cilia appear to co-localise, with more Prpf8 signal at the presumptive basal body of the cilia. An example is indicated with an arrow. A, B and C: Staining pattern of indicated antibodies in Prpf8+/N1531S embryos (n= 5). D, E and F: Re-slice of A, B and C, respectively, showing side view of the node. G, H and I: Staining pattern of indicated antibodies on Prpf8N1531S/N1531S embryos (n= 7). J, K and L: Re-slice of G, H and I, respectively, showing side view of the node, which is noticeably flatter compared to Prpf8+/N1531S embryos. No difference in the staining pattern of either antibody can be discerned, though background is higher in the Prpf8N1531S/N1531S embryo. Scale bars = 5µm.
5.2.6 qPCR analysis of Prpf8 and Trp53

While *in situ* hybridisation is excellent for resolving expression domains, it can only offer a semiquantitative result when investigating changes in gene expression levels. In order to resolve whether the level of Prpf8 expression is different between Prpf8$^{+/N1531S}$ and Prpf8$^{N1531/N1531S}$ embryos, qPCR was used. As depleting Prpf8 has previously been shown to result in an increase in Trp53 expression (Allende-Vega et al., 2013), Trp53 expression levels were also investigated. In both genes there appeared to be no changes in the fold expression between Prpf8$^{N1531S}$ heterozygotes and homozygotes as measured by qPCR (Figure 5.17).
Figure 5.17: Quantification of expression levels of target genes by qPCR.

The fold change in expression between Prpf8\textsuperscript{N1531S} heterozygotes and homozygotes in whole E10.5 embryos was investigated by qPCR. The average expression levels of three biological replicates, performed in triplicate, are shown here. Both Trp53 (p53) and Prpf8 itself showed no changes in expression levels between genotypes. Expression levels were normalised to GAPDH expression.
5.2.7: Biochemical analysis of Prpf8

The initial bioinformatic analysis of the N1531S mutation, using publicly available data (Galej et al., 2013), suggested that replacing the asparagine residue with serine would strengthen the protein-protein interaction with Aar2. Since Aar2 must be removed to allow Brr2 to bind to Prpf8 and activate the spliceosomal catalytic site (Weber et al., 2011), difficulty in removing Aar2 could plausibly interfere in Prpf8 and spliceosomal function. We collaborated with the Johnson lab to investigate whether the N1531S mutation alters the binding affinity of Prpf8 for target proteins, including Aar2 and Brr2, via mass spectrometry.

Since at the time there was no full length cDNA sequence available for mouse Prpf8, human PRPF8 in a pCMV-Sport6 vector was used. The IMAGE clone plasmid initially contained a mutation at nucleotide 759 (a759g), resulting in an arginine residue being replaced by a histidine residue, with possible deleterious consequences due to the biochemical differences between the two residues. This mutation was corrected by site directed mutagenesis to give a true wild type PRPF8 cDNA sequence (Figure 5.18).

Following this, the same site directed mutagenesis method was used to generate the N1531S mutation in the human wild type sequence. The mouse Prpf8N1531S mutation is aat to agt while the mutation induced by site directed mutagenesis is aat to agc; this creates a new BseYI restriction site with which to screen successful mutant plasmids while retaining the N1531S residue change. A Kozak sequence and FLAG tag was then cloned into the two PRPF8 sequences, PRPF8 and PRPF8N1531S at a unique AgeI restriction site, upstream of and in frame with the PRPF8 start codon (Figure 5.19). This subcloning generated a FLAG tagged PRPF8 protein, with some translated vector sequence between the FLAG tag and PRPF8 itself. The orientation of the FLAG tag was confirmed by Sanger sequencing (Figure 5.20). A maxiprep of these two constructs was then provided to the Johnson lab to perform transfections, protein purification and then mass spectrometry.
Figure 5.18: Site directed mutagenesis of human *PRPF8* cDNA clone.

A site directed mutagenesis strategy was used to both correct the sequencing error in the IMAGE cDNA clone and induce the N1531S substitution. Sanger sequencing was used to confirm this. Targeted nucleotides are highlighted.
Figure 5.19: Schematic showing the insertion of a FLAG tag into a construct containing human Prpf8.

Oligonucleotides were designed to clone into an AgeI restriction site upstream of the Prpf8 start codon (at the 3’ end). A ClaI restriction site was included to screen colonies which contained the insert, then Sanger sequencing was used to determine the orientation of the insert.

Figure 5.20: Confirmation of FLAG tag insertion

Sanger sequencing was used to find the direction of the oligonucleotide insertion (highlighted in yellow). In the top the AgeI site has been highlighted.
5:3 Discussion

The discovery that a point mutation in *Prpf8* is the causative mutation in the *Il1Jus27* mouse line has been somewhat challenging to reconcile with the available literature. Quite how the *Prpf8*N1531S mutation results in cardiac and laterality phenotypes is unknown. Immunofluorescence shows that *Prpf8*N1531S is correctly localised to the nuclear speckle, confirming that protein is made and localised in the spliceosome properly. The yeast data strongly suggests that the N1531S mutation should impair splicing in mouse embryos. This could result in lower levels of mature mRNA or improper splicing of target genes, leading to the inclusion of intron and exclusion of exons. In other models of spliceosomal defects, including *Prpf8*, some authors have reported that only a small subset of genes have splicing errors and it is thought that it is defects in these target genes that leads to the reported phenotype (Keightley *et al.*, 2013; Kurtovic-Kozaric *et al.*, 2015). However, disease causing mutations in PRPF8 alter alternative splicing, affecting the inclusion of about 20% of human exons (Korir *et al.*, 2014). In either case, in the *Prpf8*N1531S homozygotes the missplicing of genes needed for cardiac, L-R axis and yolk sac morphogenesis may be what leads to the phenotype observed. The sheer number of genes involved in these processes necessitates the use of next generation sequencing technology to find misspliced transcripts, which may be comparatively rare. An alternative hypothesis, that Prpf8 has a previously unknown function in cilia, is supported by observations reported here and elsewhere (Wheway *et al.*, 2015) that Prpf8 is localised to the cilium and is required for ciliogenesis. Mutations in *Prpf8* and other spliceosomal genes can cause retinitis pigmentosa, a ciliopathy, which could also suggest a cilia specific function for spliceosomal genes (Tanackovic *et al.*, 2011). However, some of the retinitis pigmentosa mutations in PRPF8 have been shown to generate global splicing defects (Korir *et al.*, 2014), making it impossible to test these hypotheses separately. It is important to note that the retinitis pigmentosa mutations are all located very far from the N1531S mutation in the Prpf8 protein (Maubaret *et al.*, 2011), so it is possible that they interfere with separate functions of Prpf8. Finally, it is possible that an unknown function of Prpf8 in the cilium causes the L-R axis defects, but the missplicing of target genes causes the cardiac and yolk sac defects.
When this project started no information about the embryonic processes affected by Prpf8 was available. We therefore confirmed that perturbing Prpf8 function in zebrafish embryos by morpholino injection can cause embryonic defects, and due to the high degree of conservation this data should be applicable to mouse models as well. While off target effects are always a concern with morpholinos (reviewed in (Bedell et al., 2011), the morphant phenotype we recovered closely resembled that of a zebrafish mutant line published after the completion of our morpholino experiments, confirming specificity (Keightley et al., 2013). In addition, we still recovered the morphant phenotype even when Trp53 was also knocked down, confirming the Prpf8 morphant phenotype was not due to non-specific upregulation of p53. Bioinformatic investigation using BLAT suggests that Prpf8 does not appear to have undergone genome duplication in zebrafish, removing the risk that a divergent copy of Prpf8 that would not be targeted by the morpholinos could compensate for the knockdown of Prpf8. Similarly, we confirmed using BLAT that the morpholino injection created aberrant transcript sequences were specific to Prpf8. However, we could not use mRNA injection to rescue the Prpf8 knockdown phenotype as the coding sequence of Prpf8 is too long for in vitro mRNA synthesis. Therefore, we relied on comparisons between the two morpholino phenotypes with each other and with a published mutant to determine specificity. While we were able to recover a cardiac phenotype in the zebrafish morphants, laterality defects were not observed. This could be because the zebrafish and mouse L-R axis establishment pathways have diverged, making Prpf8 essential for cardiac laterality only in the mouse. Alternatively, it is possible that the Prpf8\textsuperscript{N1531S} mutation results in a gain-of-function phenotype in Prpf8\textsuperscript{N1531S} homozygotes, which would be expected to give a different phenotype to knocking down Prpf8 expression. This could account for the lack of a laterality phenotype in Prpf8 morphant zebrafish. The spinal defects we observed in the morphant embryos may be analogous to the failure of embryonic turning we frequently see in Prpf8\textsuperscript{N1531S} homozygotes. We had generated guide RNA constructs for use with Crispr/Cas9 gene editing technology (See Appendix 4) in order to insert our specific base change into zebrafish Prpf8, but these experiments were not pursued due to the limited time remaining in the studentship.

The expression pattern of Prpf8 as resolved by our antisense riboprobes is broadly consistent with its presumably global role in splicing. Certain tissues, particularly the
future oral cavity and limb bud, showed stronger staining than other regions; this could indicate a requirement for higher levels of pre-mRNA splicing in these regions. This is purely speculation as we could not find any papers describing rates or levels of splicing in mouse embryos. As Prpf8 is increasingly thought to be essential for cell division (reviewed in Valcúrcel and Malumbres, 2014), high Prpf8 expression could also indicate sites of comparatively higher cellular proliferation. Interestingly, the salt-and-pepper staining observed on the trunk, between the somites and in the limb bud was reminiscent of phosphohistone-H3 labelling, which marks dividing cells. The consistent labelling of the pharyngeal arch arteries with the Prpf8 probes, along with the disruption of aortic arch development in zebrafish morphants, suggests that these analogous structures are sensitive to Prpf8 disruption. Defects in pharyngeal arch development are a common cause of CHD in humans. Surprisingly, the heart itself was not labelled by either probe against Prpf8, and this was highly consistent. It is almost inconceivable that the heart does not require Prpf8 for splicing, given its importance in the spliceosome. It is also unlikely that the heart was unstained for technical reasons, as the *Irx4 in situ* probe was consistently successful using the same protocol (see Figure 3.7). It is more likely that cardiac levels of Prpf8 mRNA are too low to be detected by *in situ* hybridisation, indeed, we were able to generate an amplicon using primers specific to Prpf8 mRNA by PCR from E10.5 heart cDNA (see Appendix 3). The posterior neural tube and node were similarly unlabelled, even though Prpf8 protein was detected by immunofluorescence; this again suggests that levels in some embryonic regions are too low to be detected by *in situ* hybridisation. The Prpf8 sense probes generated little to no background suggesting that the staining with the antisense probes represents an accurate readout of the Prpf8 embryonic expression pattern. Similarly, it is unlikely that the antisense staining is a result of trapped probe generating background, as this is more often associated with structures containing cavities such as the hindbrain and heart.

The N1531S mutation is not expected to alter levels of Prpf8 transcript and this was confirmed by qPCR. While knocking down Prpf8 using siRNA has previously been shown to increase Trp53 expression in cell lines (Allende-Vega *et al.*, 2013), we did not see a similar result in the Prpf8^{N1531S} homozygotes by qPCR. This could indicate that splicing defects caused by the N1531S mutation are too subtle to be detected by the
pathway that upregulates Trp53 expression in response to spliceosomal defects. Alternatively, it could suggest that splicing is not affected in the \( Prpf8^{N1531S} \) homozygotes and instead a presently unknown function for Prp8 in the cilium or elsewhere is negatively affected. Both hypotheses are at odds with the position of the mutation in the spliceosomal active site and the yeast data, which suggests that the N1531S mutation should severely affect splicing.

In order to investigate the effect of the N1531S mutation biochemically we moved to a yeast model in collaboration with Ray O’Keefe. We showed that the analogous mutation, N1603S, is viable in yeast across a range of temperatures, but the efficiency of splicing is reduced without increasing the fidelity of any step. Using primer extension analysis, it is clear that the \( Prp8^{N1603S} \) allele is a first step allele. Interestingly, is has been reported that in yeast \( Prp8^{W1609R} \) is a second step allele with increased 3’S5 fidelity (Umen and Guthrie, 1996); that mutations in residues so close together can have such disparate phenotypes underlines the complexity of the spliceosome. In addition, we have generated FLAG tagged copies of the human \( PRPF8 \) cDNA clone with and without the N1531S mutation. By using mass spectrometry to compare what proteins and in what ratios the two alleles of PRPF8 bind to, we can further elucidate the potential mechanisms behind the phenotype seen in the \( Prpf8^{N1531S} \) homozygotes. The protein biochemistry experiments, however, are beyond the scope of this thesis and are being performed by another collaborator, Colin Johnson.

5.4 Conclusion

In summary, we have established that the causative mutation in the \( llJus27 \) mouse line is a point mutation in the spliceosomal gene, Prpf8. The N1531S substitution is predicted to decrease the efficiency of splicing, based on work in yeast models. The presence of aberrant copies of \( Prpf8 \) mRNA causes defects in zebrafish embryogenesis, including cardiac defects consistent with those in the \( Prpf8^{N1531S} \) homozygotes. However, the expression pattern of \( Prpf8 \) in mouse embryos at E8.5 and E10.5 shows much lower levels of \( Prpf8 \) transcript in both the node and the heart, which is difficult to reconcile with the phenotype we describe. Nevertheless, we do show by
immunofluorescence that Prpf8 protein is localised to the basal body of nodal cilia at E8.5, which could suggest a direct role for Prpf8 in cilia formation, maintenance and/or function. There appears to be no difference between \( Prpf8^{+/N1531S} \) embryos and \( Prpf8^{N1531S/N1531S} \) embryos in either Prpf8 expression pattern or localisation to cilia, suggesting that malfunctional, rather than absent, Prpf8 is the cause of the \( Prpf8^{N1531S} \) phenotype. Finally, we have generated FLAG tagged human \( PRPF8 \) mRNA clones for use in future protein biochemistry experiments by a collaborator, which will shed further light on the \( Prpf8^{N1531S} \) phenotype.
Chapter 6: Final discussion:
6.1.1: Phenotypical analysis of \textit{Prpf8}^{N1531S}

The role of mouse Prpf8 in embryogenesis has not previously been investigated in published literature. We show that the phenotype of the \textit{Prpf8}^{N1531S} homozygous embryos affects many different developmental processes, including the heart, L-R axis and yolk sac; heterozygotes are unaffected. We build upon the work of previous lab members (Mitchell, K. 2009; Stephen, L. 2013) who characterised the cardiac phenotype and provide additional evidence that the heart tube is correctly specified, despite its gross dysmorphology, with the expression pattern of the cardiac marker genes \textit{Irx4} and \textit{Isl1} apparently normal. These genes label the ventricles and SHF, respectively. This suggests that Prpf8 does not have a role in cardiac specification, but is still required for proper cardiogenesis. We also quantify the prevalence of various aspects of the \textit{Prpf8}^{N1531S} phenotype and show that, as well as fully penetrant yolk sac remodelling defects, chorioallantoic fusion frequently fails. The origin of the cardiac and the yolk sac and chorioallantoic defects remains obscure and will require further analysis. However, mutations in cilia are known to cause CHD (Li et al., 2015) Prpf8 is known to be required for ciliogenesis (Wheway et al., 2015), and we observe defects in nodal cilia in \textit{Prpf8}^{N1531S} homozygotes, making it possible that the CHD we observe in the \textit{Prpf8}^{N1531S} homozygotes is also due to a ciliopathy. We also report that the neural tube floor plate and the somites of the embryo are specified correctly, as measured by \textit{Shh} and \textit{Dll1} expression respectively. However, there may be subtle defects in neural tube or notochord formation, as \textit{Shh} expression appeared interrupted in a manner reminiscent of the pattern of \textit{Shh} expression in \textit{α5} integrin null embryos (Goh et al., 1997). Interestingly, \textit{α5} integrin is expressed in the allantois (Girós et al., 2011) and integrins are essential for chorioallantoic fusion (reviewed in Inman and Downs 2007). More in depth analysis of neural tube formation, Shh pathway activity and integrin activity may reveal further insights into the \textit{Prpf8}^{N1531S} phenotype. While a loss of Shh signalling is classically associated with holoprosencephaly and excessive Shh signalling is associated with exencephaly, many mutant mouse lines with ciliary defects and a reduction of Shh signalling have been reported to display exencephaly, as seen in \textit{Prpf8}^{N1531S} homozygotes (reviewed in Li et al., 2011).
6.1.2: \(Prpf8^{N1531S}\) embryos have profound laterality defects.

While it had previously been observed that \(Prpf8^{N1531S}\) homozygotes frequently showed reversed cardiac looping, only preliminary investigations into the origin of this defect had been done. Here, we show that the \(Prpf8^{N1531S}\) homozygotes have profound and wide-ranging laterality defects, demonstrating that Prpf8 is required for L-R axis establishment in a manner consistent with its emerging role in ciliogenesis (Wheway et al., 2015). Immunofluorescence and SEM images of the node show that cilia are present at the node in \(Prpf8^{N1531S}\) homozygotes; but the cilia are significantly shorter. In addition, there is a trend towards fewer cilia in \(Prpf8^{N1531S}\) homozygotes, but this result is not statistically significant over the number of embryos examined. These results are consistent with Prpf8 being required for ciliogenesis, as reported elsewhere (Wheway et al., 2015). TEM images of nodal cilia did not reveal any overt changes in the ultrastructure of from \(Prpf8^{N1531S/N1531S}\) embryos, with the axonemes and dynein arms appearing normal. Preliminary videomicroscopy of nodal cilia shows that the nodal cilia are either immotile or move very slowly, demonstrating that \(Prpf8^{N1531S}\) homozygotes have a defect in cilia motility. The microbeads introduced into the nodes of \(Prpf8^{N1531S}\) homozygous embryos do not move, which is consistent with defects in generating fluid flow in the node. The leftward movement of fluid in the node is essential for proper L-R axis establishment, and a lack of movement causes L-R axis defects (Nonaka et al., 2002). It is unknown whether there are defects specific to the release of \(Ca^{2+}\), which is essential for L-R axis establishment (McGrath et al., 2003; Takao et al., 2013).

As well as cilia defects, we observed that the node of \(Prpf8^{N1531S}\) homozygotes is very flat compared to \(Prpf8^{N1531S}\) heterozygotes, indicating that the node itself has not formed properly. This is particularly interesting as processes which govern node formation are largely unknown. However, proper control over cellular proliferation is needed for the node to form properly (Komatsu et al., 2011) and Prpf8 plays an essential role in cell cycle progression (reviewed in Valcárcel and Malumbres, 2014). Defects in the cell cycle could be the cause of the node morphogenesis defects observed in \(Prpf8^{N1531S}\) homozygous embryos. However, no proliferation defects have been
reported in the hearts of Prpf8<sup>N1531S</sup> homozygous embryos (Stephen, L. 2013), suggesting that any proliferation defects could be specific to the node.

As a result of these cilia and node defects, nodal cilia in Prpf8<sup>N1531S</sup> homozygotes do not generate a directional flow as measured by the movement of microbeads in cavity of the node. In agreement with the lack of a nodal flow, in situ analysis reveals that all the L-R axis pathway genes analysed were missexpressed. The expression patterns of Nodal, Cerl2 and Pitx2 were completely randomised while Lefty1 and Lefty2 were both consistently not expressed. Shh was found to be expressed in the midline, suggesting the lack of Lefty1 expression is not due to a failure in midline specification. We also show that the expression of Pitx2 can occur in trans of heart looping directionality. This suggests that the direction of heart looping is decided upstream of Pitx2 or the embryo synthesises information from Pitx2 and other transcription factors to determine the direction of cardiac looping. Aberrant expression of all of these genes has been shown to cause L-R axis defects in mice (reviewed in Shiratori and Hamada, 2006). Therefore, missexpression of these laterality genes is sufficient to cause the cardiac looping reversal that we see in Prpf8<sup>N1531S</sup> homozygotes. Interestingly, the aberrant expression patterns of all these genes in the Prpf8<sup>N1531S</sup> homozygotes most closely resembled embryos in which Ca<sup>2+</sup> levels were reduced pharmacologically (Takao et al., 2013). To our knowledge the Prpf8<sup>N1531S</sup> mutation is the first demonstration of a mutation in a spliceosomal protein generating a cilia motility defect or L-R axis defect.
6.1.3: Confirmation that Prpf8 is the causative gene in Prpf8N1531S homozygotes.

Previous lab members had performed mapping experiments confirming that the causative mutation lay in a region of chromosome 11 that contained Prpf8. Subsequent next generation sequencing revealed that the only exonic mutation was the Prpf8N1531S mutation. Here, we show that in zebrafish, the accumulation of aberrant Prpf8 transcript can cause morphological defects, including cardiac defects, reminiscent of defects seen in Prpf8N1531S homozygous embryos, showing that Prpf8 is essential for proper embryogenesis. Importantly, the phenotype recovered from the morpholino experiments is very similar to that seen in a zebrafish Prpf8 mutant (Keightley et al., 2013). Using a yeast model, it was shown that the analogous mutation, N1603S, reduces the efficiency of splicing and is a first step splice allele (O’Keefe, R., pers. comm.). This shows that mutating asparagine to serine at residue 1531 is highly likely to interfere with Prpf8 and splicingomous function in mice. Without RNA-Seq analysis it is not possible to know whether aberrant mRNA transcripts are present in abundance in the Prpf8N1531S homozygotes. Culturing human cells containing mutations in PRPF8 known to cause RP show only a small subset of transcripts are misspliced (Tanackovic et al., 2011). It is probable that this is the case in the Prpf8N1531S homozygotes as well, as a complete inability to splice transcripts would be expected to cause very early lethality, which is presumably what occurs in embryos with other splice site components globally knocked out (Ding et al., 2004; Isono et al., 2005; Xu et al., 2005). An alternative hypothesis is the alternative splicing is altered in Prpf8N1531S homozygotes resulting in protein isoforms occurring in improper tissues, as seen in cardiac specific knockouts of ASF/SF2 (Xu et al., 2005). If these transcripts are identified in Prpf8N1531S homozygotes, this could reveal novel protein isoforms.

We also reveal for the first time the embryonic expression pattern of Prpf8, which appears to be preferentially expressed in the head, pharyngeal arches, limb bud, between somites and possibly in dividing cells; expression was not detected in the heart. It is possible that if embryonic development proceeded past E10.5, defects would appear in these tissues as well. It is difficult to speculate what other defects Prpf8N1531S homozygotes would display if they lived longer due to the diverse array of defects
linked to mutations in spliceosomal proteins. As well as RP, human diseases associated with mutations in spliceosomal proteins include myeloid malignancies (Kurtovic-Kozaric et al., 2015) and autosomal-dominant hypotrichosis simplex (Pasternack et al., 2013). A number of mouse lines with mutations in spliceosomal proteins have been generated, but none display a phenotype similar to the Prpf8\textsuperscript{N1531S} homozygotes. Cardiac specific ablation of the spliceosomal proteins SC35 and ASF/SF2 both generated mice with postnatal cardiac defects, but not CHD (Ding et al., 2004; Xu et al., 2005). Mice heterozygous for a knockout allele of the splicing factor Sf3b1 displayed rib and vertebral defects due to improper Hox gene expression (Isono et al., 2005). In addition, Prpf8 has a major role in cell cycle progression (reviewed in Valcárcel and Malumbres, 2014) and faulty Prpf8 could be expected to interfere with mitosis, which could influence the Prpf8\textsuperscript{N1531S} phenotype in concert with splicing defects. Taken together, the available evidence suggests that that when mutating spliceosomal proteins the phenotype recovered is highly specific to the spliceosomal protein, and in the case of large proteins, to the protein domain mutated.

Wheway and colleagues show that Prpf8 is localised to the basal body of the primary cilium in both human and mouse cultured cells (Wheway et al., 2015). Here, we show that Prpf8 is also localised to the basal bodies of nodal cilia, as well as the nucleus, and that the localisation of Prpf8 is not disrupted in Prpf8\textsuperscript{N1531S} homozygotes. These localisation experiments suggest that Prpf8 may have a cilia specific function, which is supported by the cilia defects we observe in the Prpf8\textsuperscript{N1531S} homozygotes. It is not known if Prpf8 localises to 9+2 motile cilia. Whether Prpf8\textsuperscript{N1531S/N1531S} embryos have defective primary cilia functionality is unknown, but is thought to be unlikely. The alterations in Gli2 and Gli3 expression seen in Prpf8\textsuperscript{N1531S} homozygotes (Stephen, L. 2013) could be caused by defective Shh signalling, which is cilium dependent (reviewed in Jacob and Briscoe, 2003), but the Prpf8\textsuperscript{N1531S} phenotype does not resemble the common phenotype of embryos with aberrant Shh signalling due to primary cilium defects (Huangfu et al., 2003), though a decrease in Shh signalling can cause exencephaly (reviewed in Li et al., 2011). In addition, primary cilia are essential for cardiogenesis (reviewed in Koefoed et al, 2014) and mutant embryos lacking primary cilia do have a thin myocardium (Slough et al, 2008), which is seen in Prpf8\textsuperscript{N1531S/N1531S}
embryos (Stephen, L., 2013). Therefore, primary cilia defects could contribute to the cardiac defects seen in the $Prpf8^{N1531S/N1531S}$ embryos and should be investigated further.

It is still entirely possible that the function of Prpf8 at the cilium is undisturbed in $Prpf8^{N1531S}$ homozygotes and it is the missplicing of genes needed for cilia function that causes the $Prpf8^{N1531S}$ phenotype. Another, if unlikely, possibility is that the ciliogenesis defects are caused directly by mutated Prpf8, but the nodal cilia motility defects are caused by the missplicing of other genes, or vice versa. This missplicing could be caused by changes in the binding affinity of Prpf8 for spliceosomal proteins. Similarly, changes in the binding affinity of Prpf8 for ciliary proteins could result in cilia defects. To investigate this we have generated FLAG tagged human PRPF8 for ongoing biochemical experiments investigating the possibility of alterations in the binding affinity of PRPF8 for protein partners, particularly AAR2 and BRR2.

6.1.4: Final conclusions

ENU mutagenesis is a powerful tool for investigating genetics because it is an unbiased method of investigating gene function. Typically, screens are performed to identify mutant lines displaying an interesting phenotype and the causative mutation is then found (reviewed in Cordes, 2005). Because ENU induces point mutations ENU models closely resemble disease causing mutations found in nature. While classical gene knockout studies are valuable in suggesting functions for unknown genes, the phenotype of these mutations may be severe. Embryonic lethality may occur at the earliest timepoint the gene of interest is required at, preventing analysis of later functions the gene may have. Missense mutations can generate gain-of-function and hypomorphic alleles, both of which can have different phenotypes to the knockout allele (reviewed in Justice et al., 1999). Hypomorphic alleles still retain some functionality in their protein product and embryogenesis may proceed further, allowing the other, later functions of the gene to be studied. ENU is a potent mutagen, and has been used to create multiple mutations in the same gene, an allelic series (reviewed in Cordes, 2005). Allelic series allow the function of different protein domains and isomorphs to be
investigated; one allele may have a very different phenotype from another, shedding light on the multiple functions of a single gene product (reviewed in Justice et al, 1999).

We have characterised an ENU derived mouse line with a causative point mutation in Prpf8 which has profound defects in embryogenesis. Homozygous Prpf8 knockout embryos are reported to be nonviable, but no further analysis was reported (Deramaudt et al., 2005), precluding comparison with the Prpf8^{N153S} homozygotes. We present novel data showing that Prpf8 is essential for L-R axis establishment and provide evidence supporting the hypothesis that Prpf8 has a role in cilia function and motility, as well as splicing.
Chapter 6.2: Limitations

There are a number of limitations with the work presented in this thesis. The use of animal models, while necessary, does not always translate to human disease. The lack of human patients with mutations outside of the C-terminus of Prpf8 makes it impossible to determine whether the N1531S mutation would exhibit a similar phenotype in humans as it does in mice. It is possible that there the developmental processes affected by the N1531S mutation have diverged between humans and mice, altering or removing the function of Prpf8 in those processes, even though Prpf8 itself remains highly conserved and essential to the spliceosome. There are some differences between human and mouse yolk sac development and placentation (reviewed in Freyer and Renfree, 2009; Jauniaux et al., 2006; Rossant and Cross, 2001) and these processes could be affected in a species specific fashion by mutant Prpf8. The L-R axis pathway has diverged between model organisms (reviewed in Vandenberg and Levin, 2013) and obvious ethical considerations make investigating human embryonic L-R axis establishment impossible. This evolutionary divergence may or may not mean that the role of Prpf8 in murine L-R axis is conserved in humans and other animals. However, knocking down PRPF8 in both mouse and human cells does impede ciliogenesis (Wheway et al., 2015), suggesting at least some conservation of Prpf8 function at the cilia.

We have been unable to determine whether or not splicing is affected in the Prpf8$^{N1531S/N1531S}$ homozygotes. It therefore remains unclear as to how the N1531S mutation generates the phenotype we observe, which impedes interpretation of the phenotype. If a subset of transcripts specific to each of the affected tissues is misspliced, then the phenotype could be caused entirely by splicing defects. Until RNA-Seq is performed, which detects all transcripts presents in a tissue sample, the effect of the N1531S mutation on the transcriptome cannot be determined. In addition, the presence of misspliced transcripts could identify functions for novel genes, or novel functions for well characterised genes. If misspliced transcripts are present, it may be difficult to dissect defects caused by faulty Prpf8 activity at the cilium from defects caused by other, missspliced, proteins.
Experiments in animal models are inherently more expensive and time consuming than \textit{in vitro} experiments; this is especially pronounced in mouse models. Tissue and cell culture models are amenable to experiments involving the addition of pharmacological agents. In addition, these models are easy to scale up as a large number of plates can be generated with which to test many different reagents, which can be imaged throughout culturing. This principle also holds true for animal models which develop externally, such as birds and fish, which have been widely used in pharmacological investigations. Mice, however, are placental mammals and culturing them \textit{ex vivo} requires specialised equipment, reagents and expertise that our lab does not have access to (reviewed in Piliszek \textit{et al.}, 2011). Testing a battery of pharmacological inhibitors of splicing, for example, on cultured mouse embryos in order to recover a phenotype similar to the \textit{Prpf8}^{N1531S} phenotype in mice is simply not feasible. It would be even more challenging to specifically target a region, such as the node, with a pharmacological inhibitor as the entire embryo would be immersed in culturing media. Likewise, genetic experiments which would be possible in yeast are challenging in mice due to the cost and difficulty in generating transgenic mouse embryos, so creating a screen of Prpf8 alleles in mice would be prohibitively expensive, if potentially informative. There is also the possibility that the genetic background of the strain of mouse that carries the \textit{Prpf8}^{N1531S} mutation may influence the resulting phenotype, which has previously been noted in genetically engineered mice (reviewed by Doetschman, 2009). If the \textit{Prpf8}^{N1531S} phenotype is a result of the missplicing of a subset of genes, splice site single nucleotide polymorphisms may alter which genes are missspliced, or the ratio of missspliced to properly spliced mRNA, giving a different phenotype.
Chapter 6.3: Future Directions

6.3.1 General phenotype analysis future directions.

This thesis research and previous projects have made some progress in investigating the other aspects of the Prpf8<sup>N1531S/N1531S</sup> phenotype, particularly the cardiac defects. However, there is a great deal that remains unknown. The origin of the cardiac defects remains obscure, but there does not appear to be a defect in specifying the structures of the heart, as the inflow tract, atria, ventricles and outflow tract are all present. In situ analysis of other genes required for cardiogenesis may reveal changes in the expression pattern of these genes. It had previously been reported that Myl4 and Actc1 were downregulated in Prpf8<sup>N1531S/N1531S</sup> embryos (Mitchell, K. 2009); other genes that control myogenesis, such as MyoD1 (Sartorelli et al., 1990), could also be downregulated.

The defects in yolk sac remodelling and chorioallantoic fusion seen in Prpf8<sup>N1531S/N1531S</sup> embryos remain relatively unexplored, though as before, in situ analysis of genes important in these two processes may shed more light on the matter. The failure of the allantois to fuse to the chorion could be because the allantois is too short to reach the chorion; this could be caused by defects in cellular proliferation or migration. Labelling proliferating cells with anti-pHH3 antibodies would enable quantitative differences between Prpf8<sup>N1531S</sup> heterozygous and homozygous allantoides to be found. If disaggregated allantoides can be cultured, then a scratch wound assay would detect differences in cell migration. Alternatively, chorioallantoic fusion may fail because molecules required for cell adhesion, such as VCAM-1 and α-4-integrin (reviewed in Inman and Downs, 2007) are absent or misslocalised. Again, in situ analysis and antibody staining would determine whether these proteins are expressed and localised correctly, but this should be performed on both the chorion and allantois.

There are other aspects of the Prpf8<sup>N1531S</sup> homozygous phenotype that remain obscure. The LHF stage (E8.0) is the earliest embryonic stage examined in detail. At this stage
heterozygous embryos appeared to be forming a pit shaped node, while homozygous embryos were not. In addition, Prpf8<sup>N1531S</sup> homozygous embryos already show developmental delay at this stage as compared to heterozygous littermates. Reduced cellular proliferation could explain both the developmental delay and the flattened node, if forming a pit shaped node is dependent upon cellular proliferation, which is unknown. Whole mount pHH3 staining at E7.0 would reveal any changes in the number of proliferating cells prior to the onset of the Prpf8<sup>N1531S</sup> homozygous phenotype. Similarly, whole mount staining for activated Caspase-3 would determine whether the rate of cell death is increased, which would also interfere with embryonic development, particularly if a specific tissue such as the heart or node were affected. If the number of dying cells was found to be higher, this could be due to defects in the DNA damage response pathway (Tresini et al., 2015). Antibodies against phosphorylated ATM could detect activation of the DNA damage response pathway. Alternatively, the defects in node morphogenesis could be a sign of defects in the midline, as indicated by the patchy Shh expression pattern at E8.5 and the changes in the expression pattern of Shh pathway genes (Stephen, L. 2013). The expression patterns of notochord specific genes such as T or Noto should be investigated to determine whether the alterations in Shh expression are due to defects specific to Shh expression or due to defects in the structures of the midline.

In addition to investigating the Prpf8<sup>N1531S</sup> homozygous phenotype, it is unknown whether there are any age related diseases in the Prpf8<sup>N1531S</sup> heterozygotes. While histological analysis of the eyes Prpf8<sup>N1531S</sup> heterozygotes at 18 months did not reveal any overt changes (Tenin, G. pers. comm.), it is possible more detailed analysis could reveal defects consistent with RP. While RP is often isolated, it can occur alongside renal defects in Senior-Loken syndrome, and renal defects are associated with other ciliopathies (reviewed in Ware et al., 2011). While there has been no indication that Prpf8<sup>N1531S</sup> heterozygotes have renal defects, a more detailed examination may reveal subtle changes in kidney morphology.
6.3.2: Further investigation into the L-R axis phenotype

We have made a great deal of progress in understanding the origin of the laterality defects seen in Prpf8\textsuperscript{N1531S} homozygotes and have shown that these defects arise in the seat of L-R axis establishment, the node. Finishing the preliminary videomicroscopy would reveal whether nodal cilia have impaired motility, as would seem to be the case. These experiments are ongoing. However, there are a few other avenues to pursue as well. It is possible that the motor proteins of the nodal cilia in Prpf8\textsuperscript{N1531S} homozygotes are missing or misslocalised; immunofluorescent staining would determine this. The expression patterns of Nodal, Cerl2 and Pitx2 may indicate that there is a defect in Ca\textsuperscript{2+}-release or signalling as well. Ca\textsuperscript{2+} is released in the nodal crown cells preferentially on the left side of the node (McGrath et al., 2003); Ca\textsuperscript{2+} localisation could be detected with a Ca\textsuperscript{2+} sensitive fluorescent dye to detect defects in Ca\textsuperscript{2+} release. However, because Ca\textsuperscript{2+} is released in response to nodal flow, defects in nodal flow also cause aberrations in Ca\textsuperscript{2+} release even in the absence of defects in the Ca\textsuperscript{2+} release mechanism itself. Artificially rescuing nodal flow in Prpf8\textsuperscript{N1531S} homozygotes would be expected to rescue L-R axis defects, if nodal flow detection and Ca\textsuperscript{2+}-release and response mechanisms are intact (Nonaka et al., 2002). This is, however, a technically challenging experiment to perform and requires specialised equipment and embryo culturing techniques with which we do not have experience.

Other genes not investigated here are also important for L-R axis determination. In particular Wnt3a and components of the Notch pathway lie upstream and directly control Nodal and Cerl2 expression (Kitajima et al., 2013; Krebs et al., 2003; Nakaya et al., 2005; Przemeck et al., 2003). As Notch signalling is upstream and independent of nodal flow (Kitajima et al., 2013), defects in Notch pathway genes would indicate that L-R axis defects occur upstream of nodal flow. Preliminary in situ analysis of Dll1 expression did not reveal any overt changes in gene expression, but other genes could be affected. Defects in the Notch pathway could compound the effects of the observed cilia and node defects, but would not be expected to cause these defects (Krebs et al., 2003). Wnt3a may cause defects in mechanosensory cilia (Nakaya et al., 2005) but it is unknown whether these cilia are affected in Prpf8\textsuperscript{N1531S} homozygous mutants; these defects would be downstream of the observed nodal flow defects in any case.
The consistent defects in node morphology are one of the most interesting aspects of the L-R axis phenotype in Prpf8\textsuperscript{N1531S} homozygous embryos because node morphogenesis is very poorly understood. Unfortunately this lack of prior knowledge makes analysis of the flat node phenotype challenging as very few genes are known to control this process. Furthermore, the phenotypes generated by the loss of function of these genes are dissimilar to the node phenotype we report. Nevertheless, in situ analysis of Noto, which specifies the node, and genes that are essential for proper ciliogenesis, such as Foxj1 and Dnahc11, could shed more light on the Prpf8\textsuperscript{N1531S} phenotype (Alten et al., 2012a). However, it seems likely that the flattened node phenotype is not a result of defective node specification but defective morphogenesis. Analysis of the basic morphological processes that are needed for node morphogenesis, such as EMT, MET and cell migration, proliferation and adherence could be a more profitable avenue of exploration, but would probably require a great deal of time to pursue.

Defects in PCP at the node result in the nodal cilia not being correctly localised to the posterior of nodal pit cells (Antic et al., 2010; Nonaka et al., 2005). This is normally visible by SEM (Mahaffey et al., 2013) but preliminary analysis of SEM images of the nodes from Prpf8\textsuperscript{N1531S/N1531S} embryos suggests that cilia seemed to be properly localised. Staining Prpf8\textsuperscript{N1531S/N1531S} embryonic nodes with antibodies against ZO1 and γ-tubulin, to label cell membranes and ciliary basal bodies respectively, would resolve this question. These data, combined with data generated by data from the finished videomicroscopy, could be used to build a mathematical model describing the alterations in the force generated by the nodal cilia of Prpf8\textsuperscript{N1531S/N1531S} embryos.
6.3.3: Future directions investigating the effect of the N1531S mutation on Prpf8

We have presented data showing that it is a point mutation in Prpf8 that causes the Prpf8N1531S homozygous phenotype. The yeast data strongly suggests that splicing should be impaired in Prpf8N1531S homozygous, but PCR analysis of target genes has not detected any evidence of exon inclusion or intron retention. RNA-Seq analysis would capture all transcripts present in the embryo, including any misspliced pre-mRNA. This experiment would greatly advance the search for misspliced transcripts and would be particularly helpful in finding evidence of missplicing in genes that are not obviously connected to the Prpf8N1531S homozygous phenotype. This analysis could reveal novel functions for previously annotated genes and help identify the function of completely uncharacterised genes, as well as confirming that the spliceosome is negatively affected in Prpf8N1531S homozygous mice.

In order to find the biochemical consequences of the N1531S mutation, mass spectrometry is currently being performed on FLAG tagged human wild type PRPF8 and PRPF8N1531S. This will reveal if PRPF8N1531S has an altered binding affinity for ciliary proteins that could explain the cilia defects we observe. This will also reveal if PRPF8 has altered affinity for AAR2 and Brr2, though it cannot quantitatively find changes in the binding strength between two proteins. Surface plasmon resonance analysis can quantitatively find the binding constant, and therefore the affinity, that PRPF8 has for AAR2. This would confirm whether the strength of the bond between PRPF8 and AAR2 is increased, as suggested by protein models.

It would be very interesting to determine the tissue specific consequences of the N1531S mutation. This would determine whether the phenotypes we observe are due to a global defect in embryogenesis or are caused by specific defects in multiple tissues. It is presently unknown whether the cardiac phenotype we observe is due to a cardiac specific function of Prpf8 or if defects in tissues providing signalling molecules, for example, cause the heart defects seen. Similarly, if a node specific function of Prpf8 could be established, this would greatly support the hypothesis that Prpf8 is important in
node morphogenesis and/or cilia function. This could be done using pharmacological inhibitors of splicing, but not without the possibility of non-specific effects and toxicity. It would also be difficult to target a specific tissue in cultured whole embryos. Knocking out Prpf8 in specific tissues could be accomplished using Cre/loxP technology. Targeted deletion of an essential exon of Prpf8 in specific tissues can be done in mice expressing Cre under a tissue specific promoter; the node, for instance, can be targeted using the promoter for Noto, though this also targets the notochord (Alten et al., 2012b). However, a tissue specific homozygous knockout phenotype may be different or more severe than that seen in the Prpf8\textsuperscript{N1531S} homozygous embryos, as Prpf8\textsuperscript{-/-} embryos are not viable (Deramaudt et al., 2005). Embryos that carry a Cre/loxP site in wild type Prpf8 on one chromosome and the Prpf8\textsuperscript{N1531S} allele on the other chromosome would overcome this problem if Cre expression is driven by a tissue specific promoter. This would generate the Prpf8\textsuperscript{N1531S} phenotype in target tissues while the rest of the embryo would retain a functional copy of Prpf8. In this way the effects of the Prpf8\textsuperscript{N1531S} mutation could be investigated in separate tissues. This approach may enable the segregation of the different aspects of the Prpf8\textsuperscript{N1531S} homozygous phenotype, and possibly allow the embryos to develop further. If this were the case, this would allow the effects of malfunctional Prpf8 to be determined in organs that develop after the Prpf8\textsuperscript{N1531S} homozygotes die. It would be particularly interesting to determine if a cardiac specific Prpf8\textsuperscript{N1531S} mouse develops postnatal defects similar to mice with cardiac specific knockouts of other spliceosomal genes (Ding et al., 2004; Xu et al., 2005). These genetic manipulations would take a great deal of time and money to perform and analyse but could be very informative.

While mutations in spliceosomal proteins can cause RP, and mutations in ciliary proteins can cause retinal defects and laterality defects in the same individual (reviewed in Ware et al, 2011) there is no information linking spliceosomal defects and laterality defects. Examining patient data to determine whether patients with RP are more likely to display heterotaxia could find other spliceosomal genes with a phenotype similar to the Prpf8\textsuperscript{N1531S} phenotype without resorting to creating transgenic mouse lines.
Bibliography


factor RFX3 directs nodal cillum development and left-right asymmetry specification. Mol Cell Biol 24: 4417-4427.


Bowers PN, Brucekner M, Yost HJ. 1996. The genetics of left-right development and heterotaxia. Semin Perinatol 20: 577-588.


Jiang HK, Qiu GR, Li-Ling J, Xin N, Sun KL. 2010. Reduced ACTC1 expression might play a role in the onset of congenital heart disease by inducing cardiomyocyte apoptosis. Circ J 74: 2410-2418.


Stephen L. 2013. Identification and characterisation of cardiac defects in mouse models isolated from a random chemical mutagenesis screen. In: Faculty of Life Sciences. Manchester, United Kingdom: University of Manchester. p 280.


Appendices
Appendix 1: Primer sequences

All primers shown are in a 5’ to 3’ orientation. Extension temperatures are 60°C unless specified otherwise.

Table 1: Primers for whole mount in situ probe synthesis:

T7 promoter sequences are in bold, T3 promoter sequences are underlined

<table>
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<td>Shh</td>
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<tr>
<td>Dll-1</td>
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<td>Prpf8 2</td>
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Table 2: Primes for sequencing human PRPF8 IMAGE clone:

Primers cover the entire inserted cDNA clone and are ordered from 5’ to 3’ of the inserted sequence.

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<td>5</td>
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Sequence for finding nucleotide change causing the N1531S point mutation in mice:

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<th>Direction</th>
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</thead>
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</table>
Table 3: Primers for cloning sequences into plasmids:

**FLAG tag insertion primers:**

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Table 4: CRISPR gRNA target sequence primers:

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<tr>
<td>1 (genomic sense)</td>
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<td>Reverse</td>
</tr>
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<td>2 (genomic antisense)</td>
<td>TAGGCATAGACTCTTCAAAACCAC</td>
<td>Forward</td>
</tr>
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<td>2 (genomic antisense)</td>
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Table 5: Genotyping primers:

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Table 6: qPCR primers:

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Table 7: Zebrafish morpholino confirmation primers against Prpf8:

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<td>Exon 6</td>
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<td>Intron 7</td>
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<tr>
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</table>
Appendix 2: Investigating gaps in next generation sequencing

Table 1: Primers for investigating gaps in next generation sequencing:

Only primers which generated amplicons that were successfully sequenced are presented here. No changes were found that could affect gene function in Prpfs11531S homozygous genomic DNA.

<table>
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</tr>
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</tr>
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Table 2: Gaps in next generation sequencing:

Green = Confirmed, no changes; Yellow = under investigation

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Appendix 3: Prpf8 expression in heart and yolk sac

Due to the apparent absence of Prpf8 expression in the heart via in situ hybridisation, primers covering exons 23-25 were used to find the presence of Prpf8 mRNA to determine if Prpf8 is actively transcribed. cDNA generated from the trunk, heart and yolk sac of E10.5 embryos was investigated. As intron 23 is very large (~2.5 kilobases), contaminating genomic DNA cannot generate an amplicon. The PCR reaction generated the same product in all three pools of cDNA, confirming that Prpf8 transcript is found in all three tissues.

<table>
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<td>25</td>
<td>GCTCTCCATGGCTGTATGT</td>
<td>Reverse</td>
</tr>
</tbody>
</table>
Prpfl8 is expressed in the heart and yolk sac.

Primers specific to Prpfl8 mRNA generated amplicons using cDNA generated from the trunk, heart and yolk sac of Prpfl8^{N153S} heterozygous embryos.
Appendix 4: CRISPR/Cas9 gRNA cloning

Confirmation that the target sequences were successfully cloned into the gRNA vector was achieved using cycle sequencing. Inserted sequences are highlighted.

Appendix 5: Attached DVD

The attached DVD contains the movie files of both the direct cilia videomicroscopy and the detection of nodal flow using microbeads. $Prp8^{N153S}$ heterozygotes and homozygotes are in their respective folders labelled ‘het’ and ‘hom’ respectively. Videos are .avi files saved using JPEG compression and run at 10 frames per second. Only illustrative portions of the videos are included due to the large file size of the original data. In the nodal flow videos, the left of the node is always to the right of the video. In the cilia videomicroscopy videos, arrows indicate examples of motile cilia.
Appendix 6: Architecture of Prpf8 in mouse and zebrafish

A: Comparing the amino acid sequences of mouse (green-blue, top) and zebrafish (dark blue, bottom) using BLAT shows that the intron-exon structure of Prpf8 is almost identical between the two species. The Prpf8$^{N1531S}$ mutation (red arrow), exon 24 (orange arrow) and exon 7 (black arrow) are indicated. B: Comparing the translated aberrant transcript generated by the exon 7 morpholino (green-blue) to mouse Prpf8 (dark blue) shows that the structure of exon 7 is conserved between mouse and zebrafish. C: Comparing the translated aberrant transcript generated by the exon 24 morpholino (green-blue) reveals that most of exon 24 is still missing when compared to mouse Prpf8 (dark blue).
Appendix 7: Comparison of the lengths of nodal cilia between $Prpf8^{+/N1531S}$ and $Prpf8^{N1531S/N1531S}$ embryos.

The median length of nodal cilia was found for $Prpf8^{N1531S}$ heterozygotes and homozygotes at the LHF and 2-3 somite stage. This is graphically represented above in the Tukey boxplot, which shows the median (the bar) and the interquartile range as a box (stretching between the 25th to the 75th percentiles), and the ‘whiskers,’ which extend to 1.5x the interquartile range of the data. Individual outliers outside the whiskers are shown as symbols. The stark difference between the length of cilia in $Prpf8^{N1531S}$ heterozygotes and homozygotes at the 2-3 somite stage is clear when comparing the median of the two boxplots.