Defining the Role of the Apelin Receptor and Mesp in Zebrafish Cardiac Progenitor Development.

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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2016

Abstract

Formation of the vertebrate heart requires a complex series of transcriptional and epigenetic events. The building blocks of this organ, cardiac progenitor cells, must be specified, migrate to the correct embryonic location and remain plastic for a period of time in order to form a number of different cell types. The molecular events responsible for these phenomena remain poorly understood. In this thesis I examine the role of the apelin receptor (aplnr) and the mesp family of transcription factors in cardiac progenitor development. I find evidence that the Aplnr is required to enhance Nodal signaling in order to activate appropriate transcriptional targets at the right time. In the absence of this receptor cardiac progenitor gene expression displays delayed induction kinetics and these cells do not migrate to the correct location in the embryo. I also find that the Aplnr regulates Nodal signaling in a non-cell autonomous manner and may do so by an effect on Nodal ligand cleavage. Finally, I find that a recently discovered ligand for the Apelin Receptor, Apela, may in fact act as an antagonist in this process. When investigating the role of mesp family transcription factors in zebrafish I find that only one of four family members possesses potent cardiogenic activity: mespaa. Surprisingly, the mesp transcription factors are not required for the proper specification of the heart, however they do appear to regulate left-right asymmetry. Finally, using mespaa over-expressing cells to model cardiac progenitor
development, I find that the activation of gata5 (a critical downstream target in cardiac
development) does not occur in the embryo until after the start of gastrulation, challenging
previous views on the timing of this process. Altogether I have discovered a number of novel
insights into the mechanisms of cardiac progenitor development. These have broad ramifications
in the field of developmental and stem cell biology and will be critical in order to harness the
potential of these cells towards treatment of congenital and adult heart disease.
Acknowledgments

Writing an acknowledgements section for what has taken up the bulk of my life for the past six years is somewhat challenging! I would like to start by thanking my parents Mamta and Davendra Deshwar and my two brothers, Amit and Amar, for being a continuing source of support. I would also particularly like to thank my fiancé Shivani Chandrakumar for her encouragement and optimism over the years. She has had to bear the brunt of the highs and lows that come with a career in science and has never once complained! For this and many, many other things I am extremely grateful.

I would next like to thank my supervisor, Dr. Ian Scott. Ian has been an absolutely unbelievable mentor over the past 6 years. He has given me advice and guidance when necessary, but also the complete freedom to explore my own ideas and has really allowed me to become an independent scientist. I cannot say enough good things about Ian as a supervisor, mentor and friend. Thank you for always being excited about any ideas I would bring to the table, good or bad, and for always believing in my work.

It was during the summer in between my second and third year of undergrad that I discovered my true love (SCIENTIFIC love that is), Developmental Biology, and I have been completely enthralled since. Thank you to all of the mentors I had during my undergraduate education who led me down this path and particularly to Dr. William Brook for inspiring me to pursue a PhD and for giving me my first opportunity to explore the field in his lab.

Thank you to Dr. Norm Rosenblum for not only taking me into the MD/PhD program, but for also being a great role model for the career that I hope to one day have. I truly admire the passion and energy that you bring to clinician scientist education, it has allowed me to pursue my dream. I would next like to recognize Dr. Bret Pearson for all of the science, life and terrible music advice that he given me. You have been one of my most important mentors and I want to thank you for always taking time out of your day to chat. I would also like to express my gratitude to my amazing committee, Dr. Freda Miller and Dr. Helen McNeill for all of their helpful suggestions.

I am extremely grateful to all members of the Scott lab, past and present, for what has been an unbelievable ride! I have learned something from each and every one of you and I could not have
had more fun along the way. In particular I would like to thank Dr. Xin Lou for teaching me about how to ask the right scientific questions. I would also like to acknowledge the rest of the “old crew”: Savo Lazic, Jeff Burrows, Sivani Paskaradevan, Bilge Yoruk and Annette Lau. You all helped train me and get me through the most difficult times of my PhD and I cannot thank you enough.

Finally, I would like to express gratitude to the numerous friends and colleagues who have also supported me through the highs and lows of this journey. In particular I would like to mention Rob Vanner and Curtis Woodford. It was a privilege to have two passionate scientific minds to share the long MD/PhD ride with. I would also like to acknowledge Ryan Gaudet and Alex Lin for being two of my main sounding boards for all things science. Finally, while not along for the PhD ride with me, thank you Katrina Piggott, Katie Mullins and Beth Elder, you were always there to listen.

It truly takes a village to have an enjoyable and successful PhD, and for everyone mentioned above and to all those I didn’t have room to name, thank you one more time for everything. I would like to close with my favorite mantra that always keeps me going whenever things get tough:

“Trust the process”

So long!

Ashish
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Chapter 1

1.1 Introduction

1.2 Overview of the Heart and Cardiac Progenitor Cells

The heart is the first functional organ to form in the vertebrate embryo. The adult vertebrate heart is divided into multiple chambers with at least one atrium and one ventricle. On either end of the organ are the inflow and outflow tracts that carry blood into and away from the heart respectively. The heart consists of three different layers: the myocardium, endocardium and epicardium (Xin, Olson, & Bassel-Duby, 2013). The myocardium contains cardiac muscle composed of cardiomyocytes that generate the force for contraction. The endocardium lines the interior of the heart and is composed of endothelial cells while the epicardium forms the outer layer and is primarily made up of connective tissue. There are four main cell types in the adult heart: cardiomyocytes, cardiac fibroblasts, endothelial cells and smooth muscle cells (Pinto et al., 2015). The zebrafish heart can be divided into four chambers: the sinus venosus, atrium, ventricle and bulbus arteriosus (D. Y. R. Stainier & Fishman, 1992).

The genetic control of cardiac development has been an area of intense research over the past several decades (Olson, 2006). A complex milieu of transcriptional and epigenetic events orchestrates the transition of cells from undifferentiated mesodermal progenitors into adult cardiac cell types (Wamstad et al., 2012). Understanding this process is of the utmost importance in order to ultimately address both congenital and adult heart disease. Of particular interest is the formation of cardiac progenitors, the embryonic cells that will give rise to the major cell types of the heart. These cells have been identified in vivo and in vitro and are present at a critical juncture in embryonic development where they have received sufficient instruction to commit to the cardiac lineage, but remain plastic enough to be able to form a number of different cell types (Kattman, Huber, & Keller, 2006; Moretti et al., 2006; Wu et al., 2006). This makes them a particularly unique population which may be ideal for cell based therapy to treat heart disease (Scott, 2012). How these cells are specified, maintained and develop over time remains very poorly understood.
1.3 Early Development of Zebrafish

The unfertilized zebrafish egg consists primarily of yolk with the exception of a small region known as the blastodisc where cleavage takes place. Upon fertilization, cleavage divides the blastodisc into an increasing number of cells until several thousand cells sit on top of the yolk. An important event which happens during this time is the mid-blastula transition (MBT) which takes place at the 10th cell division (Kane & Kimmel, 1993). The MBT is the time at which zygotic transcription begins and up until this point all aspects of development are directed by maternally loaded RNA and proteins. The early zebrafish embryo before the start of gastrulation, known as the blastula, consists of three distinct cell populations; the enveloping layer (EVL), the yolk syncytial layer (YSL) and the deep cells (Kimmel, Warga, & Schilling, 1990). The enveloping layer is formed from the most superficial cells of the embryo which together form a protective epithelium for the embryo. The yolk syncytial layer forms from the fusion of a layer of marginal blastomeres with the underlying yolk to form a syncytium of nuclei (Kimmel & Law, 1985). This layer lies just below the deep cells. The deep cells make up the rest of the blastula and will form the embryo proper.

After 4 hours post fertilization (hpf), the process of epiboly begins. In epiboly, cells in the embryo intercalate together which results in their spread towards the vegetal pole (Warga & Kimmel, 1990). This continues until the yolk is entirely enveloped by cells and it takes 10 hours from the moment of fertilization to complete this process. Shortly after initiation of epiboly, at around 5 hpf formation of the germ layers begins. Cells from the deep layer begin to undergo ingestion and move into the interior of the embryo resulting in the formation of two layers: an internal newly formed hypoblast and superficial (made up of cells that did not undergo ingestion) epiblast. The hypoblast gives rise to the endoderm and mesoderm and the epiblast gives rise to the ectoderm. As gastrulation begins, the dorsal axis of the embryo also becomes apparent. By 6 hpf a well-defined bulge can be observed at the dorsal side of the embryo known as the shield. During this time the embryo also undergoes another process known as convergent extension. Through cell migration and cell re-arrangement, the embryo undergoes convergence (narrowing) along the medial-lateral axis and extension along the anterior posterior axis. At the completion of gastrulation somite formation begins, the central nervous system begins to develop and organogenesis begins in earnest.
Figure 1.1 Zebrafish gastrulation cell movements.

Cellular movements during the three different stages of zebrafish gastrulation: epiboly (2), internalization (3) and convergent extension (4). Reproduced with permission from (Rohde & Heisenberg, 2007).
1.4 Zebrafish as a Model System to Study Cardiovascular Development

The zebrafish is a powerful model to study early embryogenesis and cardiovascular development in particular. Zebrafish embryos undergo extremely rapid morphogenesis which makes them useful given that they can achieve stages in development within hours that can take days in other vertebrate model systems. They are small, durable and easy to house making it feasible for even a small laboratory to maintain thousands of fish. Embryos are easily obtained since zebrafish eggs are externally fertilized and the optically transparent embryos facilitate live imaging. Individual clutches can be as large as several hundred embryos providing abundant sample sizes for high throughput or biochemical studies. The large embryo size allows researchers to not only easily observe the early stages of development but also makes them easily amenable to manipulations such as microinjection and transplantation. Zebrafish first rose to prominence in the early 1990’s due to several large forward genetic screens. Screens carried out in Boston, USA and Tubingen, Germany uncovered mutants in a number of genes critical for early vertebrate development and demonstrated the advantages of using zebrafish as a model system (Mullins, Hammerschmidt, Haffter, & Nusslein-Volhard, 1994; Solnica-Krezel, Schier, & Driever, 1994).

In addition to them being amenable to forward genetic screens, tools are available in zebrafish to easily manipulate gene function in a specific manner. Through the microinjection of mRNA or morpholino antisense oligonucleotides, genes can be over-expressed or knocked down with ease. Morpholino antisense oligonucleotides are 25bp modified RNA molecules that can bind to either the ATG start codon or a splice site of a target transcript (Draper, Morcos, & Kimmel, 2001; Nasevicius & Ekker, 2000). This results in the inhibition of either translation or splicing respectively resulting in a decrease or malformation of the target protein. It should be noted that morpholino phenotypes do not always phenocopy that of a null mutant. Off target effects and p53 mediated apoptosis have both been observed in studies performing morpholino mediated knockdown and in some cases there are extremely poor correlations between morpholino and mutant phenotypes (Kok et al., 2015; Robu et al., 2007). Further complicating this is the fact that a recent study has shown that morpholino mediated knockdown does not always result in the same genetic compensation mechanisms as observed in a genetic null mutant of the same gene.
(Rossi et al., 2015). Taken together this highlights the importance of the creation of a loss of function mutant to confirm any phenotypes observed in morpholino studies.

Generation of stable transgenic insertion lines is a common approach in zebrafish. The Tol2 transposase system can be used to deliver exogenous DNA into the genome albeit with random sites of insertion (Kotani, Nagayoshi, Urasaki, & Kawakami, 2006). It has been found that up to 50 percent of injected embryos will transmit germline insertions to the next generation making it only necessary to raise a small number of injected fish. More direct manipulations of the genome have also quite recently become relatively easy to perform. Both CRISPR-Cas9 or TALEN technology can be used to induce targeted lesions in the genome which can be used to generate loss of function mutations or for the targeted insertion of DNA (knock-in) (Auer, Duroure, Cian, Concordet, & Bene, 2014; Kimura, Hisano, Kawahara, & Higashijima, 2014; Shin, Chen, & Solnica-krezel, 2014). CRIPSR-Cas9 works by generating a guide RNA complementary to the target sequence which recruits the Cas9 enzyme to induce lesions (Hwang et al., 2013). TALENS work in a similar manner in which the TALE nucleases are custom designed to bind to the region of interest to generate breaks in the DNA (Cade et al., 2012). In both cases the induced lesions either facilitate the insertion of exogenous DNA, or their imprecise repair results in disruptions to the locus (insertions or deletions). Together, these tools allow for the extensive manipulation of gene function in zebrafish and facilitate detailed molecular characterization of developmental phenotypes and processes.

Zebrafish also possess several additional advantages which make them particularly useful for studying cardiovascular development. First and foremost is that the heart sits on the ventral body wall of the embryo making it very easy to visualize its formation. The proximity of the heart to the exterior of the embryo makes fluorescent imaging and in situ hybridization easy to perform and image. In addition, the zebrafish embryo is unique in that although circulation begins before the second day of development, the embryo does not require a functional circulatory system until after 7 days post fertilization. Diffusion of oxygen into the body from the water is sufficient up until this time point to adequately serve the needs of embryonic tissues. This is particularly important because embryos with severe cardiovascular defects (eg. a poorly functional or even absent heart) are able to develop for an extended period of time while in other model systems
such as mice, they would die much earlier. This allows for the more complete analysis of a given cardiovascular phenotype.

1.5 Cardiac Morphogenesis in Zebrafish

Cells that will ultimately contribute to the heart can first be traced back to the pre-gastrula embryo at the 512 cell stage where they are located at ventral-lateral positions at the margin (where embryonic cells lie in contact with the yolk) and found between 90 and 270 degrees from the dorsal midline (D. Y. Stainier, Lee, & Fishman, 1993). Several hours later, just prior to the initiation of gastrulation at 40% epiboly, these cells have shifted to more lateral regions between 60 and 140 degrees from dorsal on either side of the embryonic margin (Figure 1.2A) (Keegan, Meyer, & Yelon, 2004). At this point gastrulation begins and these cells involute towards the anterior of the embryo and begin to migrate towards the animal pole. As convergence and extension begins in the embryo, they also begin to move towards the dorsal axis before ultimately arriving at the anterior lateral plate mesoderm (ALPM) at the completion of gastrulation at 10 hours post fertilization (Figure 1.2B). Following gastrulation, cardiac progenitors begin a medial migration towards the midline of the embryo. The two populations of cells meet in the midline and assemble into a structure known as the cardiac cone by the 27 somite stage (Figure 1.2D) (D. Y. Stainier et al., 1993). This cone undergoes rotation and elongation to yield a tube by 24 hours post fertilization. At this point the heart begins to beat. Within the next 24 hours an atrioventricular canal will develop dividing the two chambers and the heart will undergo looping movements in order to establish the proper left-right asymmetry. It is during this time that circulation begins in the embryo and the heart becomes a functional organ. During this time the heart also undergoes the addition of a late differentiating population known as the second heart field. These cells are also derived from the cardiac progenitor pool in the ALPM but instead of contributing to the linear heart tube, remain undifferentiated as the heart cone is formed (Guner-Ataman et al., 2013). Between 24 and 48hpf these cells subsequently incorporate into the heart tube at the venous and arterial poles and differentiate to form myocardium as well as endothelium and smooth muscle of the outflow tract (de Pater et al., 2009; Lazic & Scott, 2011; Zhou et al., 2011). Second heart field addition is critical for proper heart morphogenesis. Cardiac development in other vertebrate systems follows a very similar set of morphogenetic steps: 1) ingression and migration of a bilateral population of progenitors
during gastrulation, 2) migration towards the midline to fuse into a heart tube, 3) second heart field addition to the heart and 4) further morphogenesis including valve formation and looping.

**Figure 1.2 Zebrafish heart development**

Location of cardiac progenitors over the course of 48 hpf of zebrafish development. Reproduced with permission from (Bakkers, 2011).
1.6 Molecular Mechanisms of Cardiac Development

Understanding the genetic control of cardiac development has been a major focus of research over the past 30 years. The study of early cardiac development is best divided into two time frames: 1) the development of cardiac progenitors up until the completion of gastrulation (ending at the arrival of progenitors at the ALPM) and 2) once progenitors have arrived at the ALPM and begin to differentiate. The molecular regulation of these two steps will be discussed in turn. For the purpose of my thesis, cardiac progenitors will be defined as the cells that will ultimately contribute to the heart that display fate restriction to this lineage.

Cardiac Progenitors before and during gastrulation

During this time frame these cells undergo a transition from undifferentiated mesoderm into cardiac mesoderm although the exact temporal and molecular nature of this transition remains unclear. Despite this, several genes are known to be required for cardiac development during this time frame and there are numerous signaling pathways that appear to effect the formation of the cardiac progenitor pool. The two genes which have been implicated in heart development during this time frame encode the Apelin receptor (Aplnr) and the transcription factor Mesp1 (Mesp1). The study of these two genes forms the basis for this thesis and thus they will be discussed in detail in a later section.

Several different sets of experiments suggest that during this time frame restricted cardiac progenitors exist. In mouse clonal analysis of cells labelled during early gastrulation identified progenitors that were completely restricted to the cardiac lineage (Devine, Wythe, George, Koshiba-Takeuchi, & Bruneau, 2014). In addition, these cells were further restricted in their contribution to either the first or second heart field. Another recent study has found evidence that the timing of gene expression and the molecular signatures of these two populations has also likely diverged by this time point (Lescroart et al., 2014). In zebrafish, lineage tracing of cells has demonstrated that atrial and ventricular myocardial progenitors are already distinct by the initiation of gastrulation (Keegan et al., 2004; D. Y. Stainier et al., 1993). In addition, these two populations are found to be anatomically segregated in the early embryo. While together these studies provide strong evidence that unique cardiac progenitors exist, the actual molecular events that may or may not be unique to these cells remains unclear.
Wnt, Retinoic acid, Sonic hedgehog and the Nodal signaling pathways all affect cardiac development during gastrulation. Chemical inhibition of the Sonic Hedgehog pathway using cyclopamine was found to dramatically reduce the number of myocardial progenitors in zebrafish (Thomas, Koudijs, van Eeden, Joyner, & Yelon, 2008). Treatment of embryos just before or at the initiation of gastrulation led to a 50% reduction in ventricular myocardial cells with this reduction decreasing as the drug was added at later time points. Furthermore, treatment of mouse embryonic stem cells with a hedgehog pathway agonist increased the number of differentiating cardiac progenitor cells (Birket et al., 2015). While Hedgehog signaling was found to be important for the proper formation of the cardiac progenitor pool, Retinoic Acid (RA) on the other hand was found to play a role in restricting its size. Inhibition of Retinoic Acid signaling prior to gastrulation was found to result in expanded expression of nkx2.5 (one of the first markers of cardiac differentiation, see below) and myl7 (a marker of myocardial differentiation) and an increase in the number of cardiac progenitors at gastrulation (Keegan, Feldman, Begemann, Ingham, & Yelon, 2005). Similar to modulation of Hedgehog, the effect of this inhibition decreased at later time points. The addition of Retinoic Acid inhibitors after the completion of gastrulation had no effect on the size of the myocardial pool. The role of Retinoic Acid signaling in restricting cardiac progenitor formation appears conserved as loss of RA signaling in mice leads to the expansion of the second heart field (Ryckebusch et al., 2008). In contrast to both Hedgehog and Retinoic Acid signaling, the role of Wnt signaling in early cardiac development was found to be considerably more complex. Wnt was found to promote cardiac specification before the initiation of gastrulation but after gastrulation was found to restrict it (Ueno et al., 2007). Over-expression of wnt8 before 6hpf resulted in increased expression of nkx2.5 and myl7 while expression after this time point decreased expression. Providing support to this finding was that over-expression of the Wnt antagonist dkk1 had the opposite response, with expression of nkx2.5 and myl7 expanding when it was over-expressed later in development and shrinking when expressed early. This biphasic response was also conserved when examined in mouse embryonic stem cells with early Wnt stimulation resulting in increased cardiac differentiation of ES cells and late activation reducing it (Ueno et al., 2007). Nodal signaling, unlike the previously discussed signaling pathways, is absolutely essential for the formation of the heart as loss of Nodal signaling or downstream effectors results in the complete absence of cardiac tissue (B Feldman, Dougan, Schier, & Talbot, 2000; Hagos & Dougan, 2007; Kunwar et
A more detailed discussion of Nodal signaling in heart development can be found later in this introduction. Together these results provide strong evidence to suggest that cardiac fate is being influenced just prior to and during gastrulation although the exact nature of this process remains unknown.

**Cardiac Progenitors at the Anterior Lateral Plate Mesoderm (ALPM)**

Once cells have arrived at the anterior lateral plate mesoderm the initiation of a core cardiac transcriptional program begins. This program includes the transcription factors Hand2, Nkx2.5, Gata4/5/6, Tbx5 and Mef2c to initiate the specification and proper differentiation of the heart (Olson, 2006). This core cardiac transcriptional network is extremely well conserved among vertebrates with these components playing critical roles in heart development in chick, mice and the differentiation of human ES cells into cardiac populations (Grépin, Nemer, & Nemer, 1997; Karamboulas et al., 2006; Lyons et al., 1995; Olson, 2006; Srivastava, Cserjesi, & Olson, 1995). Gata5/6 sit at the top of this network in zebrafish and is required to initiate the expression of the rest of the network components (Holtzinger & Evans, 2007; Peterkin, Gibson, & Patient, 2007). Loss of these two transcription factors results in the complete absence of the heart and the expression of nkx2.5, hand2 and tbx5. Gata5/6 seems to be critical not only for the initial expression of these downstream transcription factors but is also required for later differentiation. The position of Gata factors at the top of this network appears to be well conserved as loss of Gata4 and Gata6 in mice results in the complete loss of the heart (Zhao et al., 2008). Hand2 is also a critical regulator of cardiac formation at this time point and is required for proper expansion and differentiation of the cardiac mesoderm (Deborah Yelon et al., 2000). In its absence the cardiac pool does not properly expand following arrival of progenitors at the ALPM and this pool does not properly initiate cmilc2 expression. Nkx2.5, in conjunction with Nkx2.7, plays a very different role and is required to maintain the identity of the ventricle as heart development proceeds in zebrafish (Targoff et al., 2013). In mice Nkx2.5 plays a similar role and is required for the proper initiation of the complete ventricle transcriptional program (Lyons et al., 1995). Finally, the loss of both tbx5 and mef2cb also result in significant cardiac morphogenesis defects underscoring their importance for this process (Garrity, Childs, & Fishman, 2002; Lazic & Scott, 2011; Pi-Roig, Martin-Blanco, & Minguillon, 2014). Altogether, it is this network of transcription factors at this point in development that guides the
differentiation of cardiac progenitors into their respective cell types and drives morphogenesis of the heart.

1.7 The Apelin Receptor

The Apelin receptor (Aplnr, Apj) is G-protein coupled receptor which plays an important role in both early cardiac development and later cardiovascular physiology. It was first identified from a search for genes related to the angiotensin receptor family (O’Dowd et al., 1993). As a G-protein coupled receptor it contains 7 transmembrane domains as well as C- and N-terminal stretches which project into the cytoplasm and extracellular space respectively (O’Carroll, Lolait, Harris, & Pope, 2013).

The roles for the Aplnr is adult physiology are diverse. Aplnr null mice were found to have reduced cardiac contractility in individuals that survived to adulthood (Charo et al., 2009). While this effect was relatively modest, the authors found that under exercise conditions these mice exhibited a striking reduction in exercise capacity. Both sarcomeric shortening and velocity of contraction was found to be reduced in Aplnr null mice when compared to controls. The Aplnr was also found to be expressed in both the hypothalamus and the pituitary gland. Aplnr null mice were found to have a reduction in their ability to elevate the hormones ACTH and corticosterone in the blood in response to different kinds of stress (Newson, Pope, Roberts, Lolait, & O’Carroll, 2013). This suggests a potential role for the Aplnr in hormone release. In addition, Aplnr null mice also exhibit a decrease in drinking behavior and are not able to properly concentrate urine suggesting that it is important in regulating fluid homeostasis (Roberts et al., 2009).

In zebrafish there are two paralogues of the aplnr, aplnra and aplnrb. These two genes show similar but slight differences in their expression patterns during development. aplnra expression is first initiated at the oblong stage (3.7hpf) where it is expressed in the future dorsal part of the embryo (Tucker et al., 2007). At the start of gastrulation expression is found around the margin in the hypoblast, EVL and yolk syncytial layer. At the completion of gastrulation aplnra is strongly expressed in the cephalic mesoderm (which includes the ALPM) as well as the tail of the embryo in the posterior presomitic mesoderm, the somatic epithelium of newly formed somites and adaxial cells. Other areas of expression include the lens primordium and developing otic vesicle. aplnrb expression initiates at the sphere stage (4hpf) where it is expressed in a salt
and pepper pattern in the animal pole of the embryo (Scott et al., 2007). At the initiation of gastrulation its expression becomes similar to *aplnra* where it is found around the margin in the mesendoderm however in contrast, *aplnrb* is not expressed in the dorsal organizer. After gastrulation it also is expressed in the presomitic mesoderm, adaxial cells and the ALPM. Both *aplnra* and *aplnrb* also have documented expression in the developing vasculature.

In zebrafish an *aplnrb* mutant has been described. This mutant, named *grinch*, results in a reduction in the size of and sometimes complete loss of the heart (Scott et al., 2007). Expression analysis at somite stages revealed a decrease in the expression of both *nkx2.5* and *gata5* suggesting a defect in the formation of the anterior lateral plate mesoderm. More detailed analysis revealed that other markers of different cell populations in the ALPM including *fli1* and *tal1* (which mark endothelial progenitors) and *spi1* (myeloid progenitors) were also reduced. This was in contrast to the expression of markers for more posterior mesoderm populations which were unchanged (Paskaradevan & Scott, 2012). Further characterization of *aplnra/b* double morphant embryos revealed an absence of cells in the region of the embryo normally occupied by the anterior lateral plate mesoderm. Furthermore, analysis of the migration of lateral marginal cells during gastrulation revealed a migration delay in which the ingestion of marginal cells and migration towards the anterior of the embryo occurred several hours after when it was observed in WT controls. Marginal cells appeared capable of reaching more proximal regions of the embryo but were not able to reach the ALPM. Together this data suggested that the *aplnr* plays a role in driving migration of cardiac progenitors to the ALPM during gastrulation. In addition to the defects in cardiac progenitor migration, *aplnra/b* morphant embryos were also found to exhibit defects in the formation of the endoderm. A decrease in the expression of *sox17*, *sox32* and *foxa2* was observed in *aplnra/b* morphant embryos suggesting that the Apelin receptor is critical for the formation of both mesodermal and endodermal tissues.

A series of transplantation experiments revealed that the role for the Aplnr in cardiac development in zebrafish is primarily non-cell autonomous (Paskaradevan & Scott, 2012). Margin transplants of WT cells into an Aplnr deficient background were not capable of contributing to the heart at a high frequency while *aplnra/b* morphant cells when transplanted into a WT background were able to contribute at a high frequency. This suggests that the Aplnr
is required in the environment around the cardiac progenitors during their development. How the Aplnr functions in a non-cell autonomous manner however remains unclear.

The role of the Aplnr in the proper formation of the cardiovascular system appears to be conserved in vertebrates. In mice Aplnr is expressed in both the gastrulating mesoderm and the second heart field and Aplnr null mice exhibit severe defects in cardiovascular development (D’Aniello et al., 2013). More than 50 percent of Aplnr null embryos die in utero with a further 20% dying just after birth (Kang et al., 2013). Null embryos exhibited an insufficient maturation of the yolk sac vasculature and severe defects or lack of the anterior cardinal veins and dorsal aorta. Capillary density was also reduced in these embryos and developing blood vessels were deficient in vascular smooth muscle. Aplnr null hearts displayed a thin myocardium, improper looping and ventricular septation defects. The Apelin receptor has also been implicated in the development of the cardiovascular system in Xenopus laevis. Injection of morpholinos against the aplnr resulted in the disruption or absence of the intersegmental vessels (Cox, D’Agostino, Miller, Heimark, & Krieg, 2006). A different group also reported that in addition to the vasculature defects a profound effect on the heart was observed. They found that injection of aplnr MO into Xenopus embryos also resulted in a small tubular heart with reduced heartbeat and blood flow (Inui, Fukui, Ito, & Asashima, 2006).

Finally, the Apelin receptor has also been demonstrated to be important for the proper differentiation of cardiomyocytes from embryonic stem cells in culture. Over-expression of Aplnr in mouse embryonic stem cells results in enhanced cardiac differentiation of embryoid bodies, while Aplnr inhibition leads to impaired cardiac differentiation (D'Aniello et al., 2013; D'Aniello et al., 2009). Cells in which Aplnr is knocked down exhibited a delay in the initiation of both Brachyury and Mesp1 expression suggesting a delay in mesoderm formation and differentiation. In addition to this delay the total number of MF-20 (a marker of cardiomyocyte differentiation) positive cells was reduced suggesting that the ability of these cells to form cardiomyocytes was decreased. Further evidence to suggest that Aplnr is critical in early cardiac progenitor development was found by over-expressing Aplnr in Cripto null cells. Cripto is an essential co-factor of the Nodal signaling pathway (discussed in greater detail later) and Cripto null cells were found to generate neuronal tissues instead of cardiomyocytes in culture (D’Aniello et al., 2009). Over-expression of Aplnr was remarkably found to re-direct Cripto null
mouse embryonic stem cells from a neuronal to cardiac fate suggesting a critical role in this process. Despite a wealth of evidence suggesting a role for the Aplnr in heart development, how it is important at a molecular level remains unclear.

1.8 Ligands for the Apelin Receptor

Two endogenous ligands for the Aplnr have been discovered. Apelin was the first ligand identified while Apela was identified more recently by two groups and initially named Ela and Toddler respectively (Chng, Ho, Tian, & Reversade, 2013; Pauli et al., 2014; Tatemoto et al., 1998).

**Apelin**

The *Apelin* gene codes for a 77 amino acid protein which possesses an N-terminal secretory signal sequence which gets cleaved to form a mature protein (Tatemoto et al., 1998). Apelin proteins exist as dimers endogenously and the protein can be cleaved at several different points to yield proteins of 36, 17 and 13 amino acids. Apelin-13 is sometimes pyro-glutamylated at the N-terminal glutamine residue.

*Apelin* is not expressed until the end of gastrulation in zebrafish where it is found in the axial mesoderm and subsequently the developing notochord (Scott et al., 2007). By 18hpf its expression shifts to only the posterior part of the notochord and it begins to be expressed in the developing heart and by 24hpf expression also spreads throughout the head (Zeng, Wilm, Sepich, & Solnica-Krezel, 2007). *apelin* over-expression in zebrafish was found to result in embryos completely lacking a heart. Both *cmlc2* and *nkx2.5* expression were completely absent in these embryos and they also exhibited a delay in epiboly. While the effect of *apelin* over-expression on cardiovascular development was striking, *apelin* knockdown via morpholino antisense oligonucleotides did not result in a strong effect on heart development. *apelin* MO embryos were only found to exhibit a mild decrease in *cmlc2* expression unlike the phenotypes observed in *aplnra/b* loss of function.

Several lines of evidence suggest that Apelin does not act on the Aplnr to regulate cardiovascular development in zebrafish. First and foremost is the lack of expression during gastrulation which is the time point at which the Apelin receptor appears to be required. Second, mice mutant for
**Apelin** and zebrafish embryos injected with *apelin* morpholinos have no or only very mild cardiovascular defects (Kuba et al., 2007; Zeng et al., 2007). Together these pieces of evidence suggested that either the Aplnr is capable of activity in the absence of a ligand, or the ligand remained to be identified.

**Apela**

Apela was first identified in zebrafish in a search for un-annotated protein coding sequences. Ribosome profiling data was paired with RNA-seq datasets to identify RNAs undergoing translation that had not previously been linked to a protein coding gene (Pauli et al., 2014). *apela* was previously thought to code for a long non-coding RNA but actually codes for a 54 amino acid protein. This protein has an N-terminal signal secretory sequence of 22 amino acids which gets cleaved to result in the mature protein (Chng et al., 2013). It was confirmed to result in a secreted protein as an Apela-GFP fusion was observed in the extracellular space in zebrafish and Apela protein was detected in the supernatant from *Xenopus* embryos over-expressing the zebrafish protein. Unlike *apelin*, expression of *apela* first begins around 4hpf where it is expressed ubiquitously in the blastoderm of the embryo throughout gastrulation. At the completion of gastrulation it is expressed in the notochord, lateral plate mesoderm and endoderm.

The functional consequences of loss of *apela* were evaluated through the generation of several different null mutants made using TALENS. *apela* homozygous mutant embryos exhibited a similar loss of function phenotype as the *grinch* mutants and *aplnra/b* morphants with a small or absent heart and disrupted endoderm formation (Chng et al., 2013). More detailed analysis revealed a delay in the ingression of lateral margin cells during gastrulation similar to what our group observed in *aplnra/b* morphants. These cells did not ingress until more than 30 minutes after their WT counterparts and once cells did begin to move, they did so at a reduced rate and accumulated at the margin (Pauli et al., 2014). Epiboly also appeared to be delayed as a result. Interestingly, the over-expression of *apela* was found to yield phenotypes similar to the loss of function phenotype with embryos exhibiting defects in cardiac formation.

The similarity in phenotypes in *aplnr* and *apela* loss of function suggested perhaps that Apela could be the endogenous ligand for the Aplnr during gastrulation. To more directly address this
question *apela* was over-expressed as a fusion with alkaline phosphatase in 293T cells and then applied to cells over-expressing the *aplnr*. Cells over-expressing either *aplnra/b* or the human *APLNR* gene were capable of binding the fusion protein while mutant *aplnrb* and other G-protein coupled receptors were not (Chng et al., 2013). Furthermore, over-expression of *apela* was found to induce the internalization of GFP tagged Aplnra and Aplnrb while again, it did not induce a similar response in other GPCRs (Pauli et al., 2014). These lines of evidence together suggest that Apela acts as a ligand of the Apelin receptor.

While much of the initial reports of Apela’s function in development have focused on early gastrulation and heart formation, it appears to possess additional functions in development. Recent work has demonstrated that together with Apelin, Apela is required for the proper midline migration of angioblasts during vasculogenesis in zebrafish (Helker et al., 2015). Furthermore, and perhaps more surprisingly, Apela seems to also play a role in the development of embryonic stem cells. *APELA* was found to be highly expressed in human embryonic stem cells and subsequently downregulated upon differentiation (Miura et al., 2004). Interestingly, loss of *APELA* resulted in a significant reduction in growth and the loss of pluripotency in human embryonic stem cells (L. Ho et al., 2015). This indicates a critical role for APELA in embryonic stem cell maintenance and development. Surprisingly, *APLNR* is not expressed in these cells and *APLNR* knockdown has no effect on pluripotency suggesting that APELA acts through a different receptor in this context. In addition, a recent study has found that the *Apela* RNA plays a non-coding role in regulating p53 mediated apoptosis in mouse embryonic stem cells (Li et al., 2015). Altogether the function of Apela seems to be extremely diverse and further investigation is needed to fully appreciate its function.

### 1.9 Apelin Receptor Signaling

How the Aplnr signals to initiate a cellular response has remained a complex and relatively poorly understood process. As a G-protein coupled receptor, Aplnr activity results in the exchange of a GDP for a GTP in an associated Galpha subunit of a heterotrimeric G protein complex. Depending on the G-protein that is affected this can then result in the activation of different intracellular signaling cascades. The Aplnr has been implicated in signaling through a number of different G-proteins including Gi/o, Ga11, Ga12, Gq/11 and Ga13 (O’Carroll et al.,
It should be noted however that most of these associations relate to Apelin-Aplnr signaling and not Apela-Aplnr signaling given that Apela was discovered just recently.

Two pathways which have been demonstrated to be activated by Aplnr-Apelin signaling are the MAPK/ERK pathway and PI3K/AKT pathway. Each of these pathways has been implicated in the downstream effects of Apelin-Aplnr signaling in multiple different contexts including cardiac progenitor formation, endothelial cell function and cancer (D’Aniello et al., 2009; Masri, Morin, Cornu, Knibiehler, & Audigier, 2004; Masri, Morin, Pedebernade, Knibiehler, & Audigier, 2006; Picault et al., 2014). Interestingly both pathways in some cases appear to be converging on the activation of the serine/threonine kinase P70S6K. In addition to these pathways Apelin-Aplnr signaling has also been shown to result in the inhibition of adenylate cyclase and subsequently prevent the formation of cAMP (Masri et al., 2006; Scott et al., 2007). A summary of some of the relevant intracellular signaling cascades downstream of the Aplnr is provided below. It should be noted that a recent study has shown both ERK phosphorylation and cAMP inhibition as a result of Apela-Aplnr signaling suggested that these pathways may be shared by both ligands (Deng, Chen, Yang, Feng, & Hsueh, 2015).

Figure 1.3 Aplnr signaling pathways
Summary of some of the different signaling pathways that the Aplnr has been shown to act through. Reproduced with permission from (Chapman, Dupré, & Rainey, 2014).

An additional signaling mechanism of Aplnr that was identified in endothelial cell development is the ability to induce the translocation of the histone deacetylase HDAC4/5 out of the nucleus into the cytoplasm in order to promote gene expression (Kang et al., 2013). Finally, a ligand independent signaling role for the Aplnr has also been discovered. Scimia and colleagues (2012) found that the Aplnr can act as a stretch receptor in order to mediate cardiac hypertrophy. Cardiomyocytes in which the Aplnr was knocked down when subjected to stretch showed a reduced Frank Starling gain (a measure of force that can be recruited by stretch) when compared to control. Furthermore, the stretch response mediated by the Aplnr was found to result in an increase in the expression of genes associated with hypertrophy and increased cell size. Interestingly, this response was found to be mediated by the binding of B-arrestins as opposed to the more traditional binding of G-proteins. Stretch was found to increase B-arrestin recruitment to the Aplnr and knockdown of B-arrestins led to a loss of the increase in hypertrophic markers in response to stretch. Instead of acting through a G-protein coupled pathway, stretch was actually found to reduce G-protein activation. Together these results demonstrate the Aplnr is capable of signaling through multiple pathways and performing a variety of functions to regulate both development and adult homeostasis. In the context of early heart development, the nature of Aplnr signaling remains unknown.

1.10 The Nodal Signaling Pathway

Nodal ligands are members of the TGFβ superfamily of proteins and are secreted morphogens that diffuse through the extracellular space and enact concentration dependent responses in target tissues (Y. Chen & Schier, 2001). Nodals are essential proteins which play a highly conserved role in axis determination, germ layer specification and patterning in the early vertebrate embryo.

Nodal ligands are secreted as pre-proteins into the extracellular space where they exist as homodimers. The enzymes Furin and Pace4 cleave the pro-domain off the ligand which leads to the formation of the mature protein (Beck et al., 2002). Un-cleaved immature ligands also
possess signaling activity albeit less than the activated form of the protein. The immature and mature ligands also exhibit different levels of stability in the embryo. The immature protein has been found to be much more stable while the mature form gets quickly degraded (Good et al., 2005). Nodal signal reception is performed by type 1 (Acvr1b) and type 2 (Acvr2a/b) serine threonine kinase receptors that interact with the co-factor Cripto (One eyed pinhead). Cripto is an EGF-CFC type protein which is essential for Nodal signaling and its loss has the same phenotype as mutants for the Nodal ligands (Gritsman et al., 1999). After the ligand is bound to the receptor complex, this results in the phosphorylation of the type 1 receptor and the subsequent phosphorylation of Smad2 or Smad3. Phosphorylated Smad2/3 forms a complex with Smad4 and together they enter the nucleus to enact a transcriptional response.

The Nodal signaling pathway is antagonized at several different points along the pathway. Secreted Lefty proteins acts as antagonists by binding the Nodal ligands and preventing their interaction with the receptor complex and by binding to Cripto and preventing its participation in Nodal ligand reception (C. Chen & Shen, 2004). The Cerberus proteins are another family of secreted proteins which are capable of binding the Nodal ligands and acting as antagonists (Piccolo et al., 1999). Several different genes also act to antagonize the Nodal pathway by regulating the presence of the type 1 and type II receptors. Dapper2 and Rock2a both act to promote the degradation of Nodal receptors to reduce the levels of Nodal signaling (L. Zhang et al., 2004; Y. Zhang et al., 2009). Finally, the kinase Araf has been shown to negatively regulate Nodal signaling by phosphorylating Smad2 to promote its degradation (Liu et al., 2013).

Nodal ligands behave as morphogens which means that the response of a receiving cell to a Nodal signal depends on the dose received (Hagos & Dougan, 2007). Cells will express different genes depending on the threshold of Nodal signaling achieved and as such there are high and low threshold Nodal targets (Y. Chen & Schier, 2001). Achieving a given Nodal threshold can also be a function of time, if a cell stays in contact with the Nodal signal for long enough a given threshold can be attained even from a relatively weak signal (reference).
Figure 1.4 Nodal signaling pathway

Schematic outlining the key features of the Nodal signaling pathway. Reproduced with permission from (Shen, 2007).

1.11 Nodal Signaling in Early Zebrafish Development

There are three Nodal ligands in zebrafish: Squint, Cyclops and Southpaw. *squint* is maternally contributed and becomes zygotically expressed around the margin before the start of gastrulation (Rebagliati, Toyama, Fricke, Haffter, & Dawid, 1998). By the shield stage expression at the
margin is absent and is limited to faint expression at the dorsal part of the embryo. *cyclops* expression starts at the dome stage where it is also found around the margin, and subsequently becomes enriched and eventually restricted to the dorsal part of the embryo during gastrulation. *southpaw* is not expressed until after gastrulation and along with *squint* and *cyclops*, plays a role in left-right asymmetry during this time frame. Expression of *cyc* and *sqt* at the margin is required to induce both mesoderm and endoderm formation in zebrafish. Both ligands are expressed in the marginal blastomeres themselves as well as the underlying yolk syncytial layer and have overlapping functions in early development and patterning. Squint and Cyclops differ in their ability to act at a distance. While Squint is capable of inducing target genes at long distances from its site of secretion, Cyclops does not have this same ability (Y. Chen & Schier, 2001). Double mutant *squint* and *cyclops* embryos completely lack the endoderm and most mesoderm including the notochord, anterior somites, heart, pronephros and blood (B Feldman, Gates, & Egan, 1998). Marginal blastomeres fail to involute during gastrulation in these embryos and the entire hypoblast (later beneath the epiblast consisting of the mesoderm and endoderm) is absent. Depletion of the two ligands specifically in the yolk syncytial layer alone results in the loss of the endoderm and the dorsal shield suggesting that YSL expression of these ligands is critical for development (Fan et al., 2007).

### 1.12 Mesp Genes in Cardiac Development

The *mesp* family of genes codes for transcription factors that possess a basic helix-loop-helix (bHLH) DNA binding and dimerization domain. These genes have been demonstrated to be important for cardiac progenitor formation and migration in multiple model systems (Kitajima, Takagi, Inoue, & Saga, 2000; Satou, Imai, & Satoh, 2004).

Mice possess two Mesp genes, *Mesp1* and *Mesp2*. *Mesp1* was first discovered in a screen for genes preferentially expressed in the allantois and its expression is first observed at the onset of gastrulation where it is expressed in the primitive streak (Y Saga et al., 1996). Expression is found throughout the developing mesoderm (both embryonic and extraembryonic) as cells ingress however it is not expressed in either the ectoderm or the endoderm. Following gastrulation *Mesp1* is down-regulated in most of the newly formed mesoderm but maintains expression in the allantois and the lateral margin of the primitive streak. Later in development *Mesp1* is expressed is the presomitic mesoderm. *Mesp1* null mice were found to exhibit varying
degrees of cardia bifida and randomized looping (Y Saga et al., 1999). Despite these morphological defects, the myocardium appeared well developed and both the cardiac jelly and cardiac trabeculation appeared normal. These defects were attributed to a migration delay of cardiac progenitors during gastrulation as Mesp1 expressing cells remained in the primitive streak longer than controls.

Mesp2, in contrast to Mesp1, is not highly expressed during gastrulation (Yumiko Saga, Hata, Koseki, & Taketo, 1997). Mesp2 was found to exhibit very low levels of expression during early gastrulation in a pattern similar to Mesp1 and its expression was extremely transient (Kitajima et al., 2000). Strong Mesp2 expression is not observed until somitogenesis where, like Mesp1, it is also expressed in the presomitic mesoderm (Yumiko Saga et al., 1997). Loss of Mesp2 results in somitogenesis defects and homozygous null embryos die shortly after birth. Interestingly, it was found that Mesp2 expression was upregulated and persisted in Mesp1 null mutants suggesting that it may be compensating for Mesp1 function (Sawada et al., 2000). Double Mesp1/Mesp2 knockout embryos display severe defects in development including a lack of a heart, somites and gut (Kitajima et al., 2000). Embryos appear to lack the entire mesoderm and detailed analysis of embryos during gastrulation revealed that mesoderm cells were not able to ingress at all through the primitive streak. In order to determine if Mesp1/2 is required in the mesoderm itself for proper migration or in the surrounding environment chimeric embryos were generated in which double mutant embryos were fused with WT embryos. Interestingly, double mutant cells were not able to contribute to the heart or the anterior head mesenchyme but yet contributed extensively to other tissues from the lateral plate mesoderm, notochord and paraxial mesoderm. This suggests that Mesp1/2 are required cell autonomously for cardiac development.

Study of the mesp family of transcription factors in other model systems has also revealed a conserved role in heart development. Cs-mesp, the sole mesp orthologue present in the basal chordate C. savignyi is expressed in the cells that give rise to the heart (Satou et al., 2004). Knockdown of Cs-mesp using morpholinos resulted in the complete absence of the juvenile heart. Furthermore, cardiac progenitors displayed a reduction in the expression of the Ciona orthologues of Nkx2.5 and Hand2. Consistent with Mesp playing a conserved role in cardiac development is the finding that morpholino injections against the Xenopus Mesp1 orthologues
mespaa and mespab results in a decrease in the expression of the cardiac progenitor markers dkk1, tbx5 and tbx20 (Kriegmair et al., 2013).

In vitro, the over-expression of Mesp1 has been used to effectively generate populations of cardiac progenitors. Transient expression of Mesp1 in mouse embryonic stem cells was found to dramatically increase the differentiation of these cells into cardiomyocytes and also accelerated the rate of this process (Bondue et al., 2008). These cells formed not only cardiomyocytes, but also smooth muscle cells and vascular endothelial cells, together making up the three main cell types in the heart. Mesp1 over-expressing cells upregulated the expression of the core cardiac transcriptional network including Nkx2.5, Gata4/6, Mef2c and Hand2 among others while not showing any increase in the expression of ectoderm or endodermal genes. Interestingly, Mesp1 was found to repress the expression of pluripotency genes, genes involved in mesoderm formation and also some endoderm genes suggesting perhaps a critical role for Mesp1 in the differentiation of cardiac mesoderm by repressing earlier/alternative fates.

1.13 Potential of Mesp Expressing Cells

While Mesp is undoubtedly an important regulator of cardiac development, numerous lines of evidence have emerged which suggest that it may in fact be a more general regulator of mesoderm development and may govern the formation of many different cell types. The first of these lines of evidence is that Mesp expressing cells contribute to a variety of different tissues. Lineage tracing using a Mesp1-CRE revealed extensive contributions of Mesp1 expressing cells to the yolk sac vascular endothelium and hematopoietic progenitors in the blood islands (Chan et al., 2013). Furthermore, Mesp1 expressing cells give rise to satellite cells (the precursors to skeletal muscle cells) in the craniofacial skeletal muscles and the diaphragm.

In vitro, it has been demonstrated that depending on the timing and culture conditions, Mesp1 over-expression can be used to drive the formation of other mesodermal cells types. Early expression of Mesp1 before it is normally induced for the generation of cardiomyocytes resulted in the generation of hematopoietic progenitors instead (Chan et al., 2013). In contrast, when Mesp1 was induced in cells cultured in serum free conditions, these cells differentiated into paraxial mesoderm. The timing of Mesp expression may not only just regulate the decision between cardiac and non-cardiac fates but may also regulate the formation of different cardiac
populations as well. Recently, it has been found that a difference in the timing of \textit{Mesp1} expression during gastrulation is found between first and second heart field progenitors (Lescroart et al., 2014). Cells expressing \textit{Mesp1} early in gastrulation (E6.25-6.75) went on to contribute to first heart field derived structures while cells expressing \textit{Mesp1} slightly later (E7.25) predominantly contributed to second heart field structures.

1.14 Zebrafish Mesp Genes

Zebrafish possess four \textit{mesp} family members: \textit{mespaa}, \textit{mespab}, \textit{mespba} and \textit{mespbb}. \textit{mespaa} and \textit{mespab} are orthologues of \textit{Mesp1} in mice and \textit{mespba} and \textit{mespbb} are orthologues of \textit{Mesp2}. Both \textit{mespaa} and \textit{mespab} are first expressed at 40\% epiboly around the margin with the exception of the dorsal part of the embryo (Cutty, Fior, Henriques, Saúde, & Wardle, 2012; Sawada et al., 2000). Margin expression persists throughout gastrulation where both \textit{mespaa} and \textit{mespab} are expressed in the developing mesoderm. After gastrulation, expression of both members is restricted to the pre-somatic mesoderm. In contrast, \textit{mespba} and \textit{mespbb} do not show any expression during gastrulation. Expression of these two family members is initiated after gastrulation where they are also expressed in the presomitic mesoderm in the developing somites. Knockdown of the \textit{mesp} genes in zebrafish has not yet been reported although the over-expression of \textit{mespba} was found to disrupt somite formation (Sawada et al., 2000). Whether or not Mesps play a role in cardiac progenitor development in zebrafish remains unknown.

1.15 Thesis Rationale and Summary

Despite several decades of focused research, the molecular mechanisms that underlie the initial specification and formation of cardiac progenitors have remained elusive. While multiple signaling pathways have been implicated in this process, the exact molecular events remain unknown. Two genes, \textit{aplnr} and \textit{mesp}, have been directly tied to this process although how exactly they regulate cardiac progenitor development is unclear. Through the study of the Aplnr and Mesps numerous lessons can be learned. How a progenitor pool is specified, migrates and is maintained during early development are questions that have large ramifications in our understanding of developmental biology in general, but also in our ability to generate and maintain stem cells populations in vitro for regenerative therapies.
The Aplnr has also been found to be important in a wide range of both developmental processes and adult physiology and its mechanisms of signaling are extremely diverse and unfortunately, poorly understood. Understanding how the Aplnr regulates the formation of the heart in zebrafish will provide insight into not only the process of early cardiac progenitor formation, but will also have ramifications in all other fields in which the Aplnr is important including hormone regulation and cardiac contractility and function. While we have attained some understanding of how the Aplnr regulates heart formation at a cellular level, by regulating the migration of cardiac progenitors to the ALPM, we need to understand how it regulates this process at a molecular level to truly make gains into how the Aplnr functions in development and disease.

The mesp family of transcription factors are well studied regulators of cardiac progenitor formation. The ability of the mesp genes to direct cardiac differentiation and the requirement for them in heart development in a variety of model systems is clear. Despite this understanding, how mesp genes actually regulate heart formation in vivo remains poorly understood. Downstream targets of Mesp in vivo, the timing of mesp induction for cardiac progenitor specification and the potential of mesp expressing cells remain important questions to be addressed in order to fully understand their function.

In this thesis I describe significant progress towards addressing some of the key questions in early cardiac progenitor development. I describe a novel mechanism of Aplnr action in which it regulates Nodal signaling to ensure the proper timing of migration and gene expression in cardiac progenitors. I find that it modulates Nodal signaling in a non-cell autonomous fashion perhaps by regulating the extra-cellular processing of the Nodal ligand. More surprisingly, I find that the newly discovered ligand for the Apelin receptor, Apela, may act as an antagonist in this context. Finally, I find that while the Mesp family of transcription factors is seemingly not essential for cardiac progenitor development in zebrafish, Mespaa retains an extremely potent ability to drive cardiac progenitor formation. I also discover a new role for the Mesp family of transcription factors in establishing the proper left-right asymmetry of the heart. This work both clarifies and expands on the molecular events underlying the complex process of cardiac progenitor development and provides a number of novel insights with broad implications in developmental and stem cell biology.
Chapter 2

2.1 Materials and Methods

2.2 Zebrafish Mutants, Lines and Imaging

Zebrafish embryos were raised at 28 degrees Celsius according to standard techniques (Westerfield, 1993). \textit{Tg(myl7:EGFP)}^{twu34}, \textit{Tg(gata5:EGFP)}, \textit{Tg(kdrl:EGFP)}^{s843}, \textit{Tg(acta:EGFP)}^{f13}, \textit{aplrb}^{608} (grinch, p.W90L) and \textit{ela}^{hr13} zebrafish lines have been previously described (Beis et al., 2005; Chng et al., 2013; Higashijima, Okamoto, Ueno, Hotta, & Eguchi, 1997; Huang, Tu, Hsiao, Hsieh, & Tsai, 2003; K. Kikuchi et al., 2011; Scott et al., 2007).

\textit{aplrb}^{hu4145} (p.W54X) fish were a gift from Stephan Schulte-Merker. The \textit{aplrb}^{ins} mutant allele was purchased from Znomics, Inc. \textit{aplrb}^{ZM00177433Tg} has a (c.886_887insTg(ZM)) retroviral insertion that disrupts the ORF of the single coding exon. The \textit{mespaa}^{hse11} mutant line was generated using the CRISPR/Cas system. The oligos TAGGACGTCCGGTTCTCTTCGG and AAACCCGAAGAGAACCGGACGT were annealed and ligated into the pT7-gRNA vector digested with BsmBI to create the guide RNA via in vitro transcription (Jao, Wente, & Chen, 2013). Imaging was performed using a Leica DFC320 camera on a Leica M205FA stereomicroscope and a Nikon A1R confocal microscope.

2.3 Microarrays

An Agilent zebrafish microarray (V3: 026437) was used to compare the gene expression profile of WT vs \textit{aplrb} morphant embryos. 4 replicates were performed and for each experiment 20 embryos were collected at 50% epiboly (5.25 hpf) and total RNA was prepared using the Ambion RNAqueous kit. Microarray results were analyzed using Genespring v11.0.1 (Agilent Technologies, Inc.). As recommended by the manufacturer, the data was normalized using Agilent’s Spatial Detrending Lowess normalization. All data analysis was performed on log2-transformed data. Standard single factor t-tests were used followed by ranking with fold changes. After normalization and averaging the four chips, the data was filtered to remove the probes that showed no signal in order to avoid confounding effects on subsequent analysis (probes below the 20\textsuperscript{th} percentile of the distribution of intensities were removed). MIAME-compliant microarray data was submitted to GEO (accession #GSE58683).
2.4 Morpholino and RNA injection

Embryos were injected at the one cell stage according to standard procedures. Translation blocking MOs against *aplnra* (5' – cgtgattcggccgtgc - 3') and *aplnrb* (5' – agagaagtttcatgtgc - 3') have been previously described (Scott et al., 2007). *aplnra/b* morphant embryos were co-injected with 0.5 ng of *aplnrb* MOs and 1 ng of *aplnra* MOs. YSL injections were performed by injecting into the yolk at the 1,000-cell stage. The translation blocking MOs against *lefty1* (5' - cgccgactgacttttcaag - 3') has been previously described (Benjamin Feldman et al., 2002). *lefty1* morphant embryos were injected with 6 ng of MOs per embryo. A translation blocking morpholino against *mespba* (5' - tgttggggtgggtgggtc - 3') has been previously described (Kawamura et al., 2005). Translation blocking morpholinos for *mespab* (5' - catggttcagtgggtggttc - 3') and *mespbh* (5' - tgtggttcattgtgggtggtc - 3') were ordered from Gene Tools (Oregon, USA) and the following doses were injected per embryo for the respective *mesp* MOs: *mespab* MO 1 ng, *mespba* MO 0.75 ng and *mespbh* MO 1 ng. Full-length coding sequences of zebrafish *mespaa*, *mespab*, *mespba*, *mespbh* and *msgn1* were PCR amplified from cDNA templates and cloned into pCS2+. *In vitro* transcribed RNA was prepared using the mMessage Machine Kit (Ambion) and purified using the MegaClear kit (Ambion). 150 pg of *msgn1*, *mespaa*, *mespab*, *mespba* and *mespbh* were injected per donor embryo. 250 pg of *mespaaΔnls-ecr* was injected per donor embryo. 0.5 pg of taram-a* RNA was injected per donor embryo (Renucci, Lemarchand, & Rosa, 1996).

2.5 RNA *in situ* hybridization

RNA ISH was carried out using DIG labelled antisense probes as previously described (Thisse & Thisse, 2008). Double ISH was performed against a fluorescein-labelled *gfp S65C* probe using previously established protocols (Zhou et al., 2011). Fluorescein-labelled probes were detected using INT/BCIP and DIG labelled probes with NBT/BCIP. Probes for *mespaa*, *mespab*, *sqt* and *cyc* were prepared from templates containing full length coding sequences. Probes for *myl7*, *nkx2.5*, *gsc*, *ntl*, *sox17*, *sox32*, *flh*, *gata5*, *lefty1* and *lefty2* have been previously described (Alexander, Rothenberg, Henry, & Stainier, 1999; Bisgrove, Essner, & Yost, 1999; J. N. Chen & Fishman, 1996; Y Kikuchi et al., 2001; Reiter et al., 1999; Schulte-Merker et al., 1994; Talbot et al., 1995; D Yelon, Horne, & Stainier, 1999). ImageJ analysis software was used to document *sox17* and *sox32* cell numbers and spread.
2.6 Transplantation

Transplantation was performed as previously described (Scott et al., 2007). Donor embryos were injected with 5% tetramethylrhodamine dextran (10,000 MW, Molecular Probes) as a lineage tracer. Transplants were performed by placing 20-40 cells into the margin or animal cap of a host embryo at the sphere stage (4 hpf). For cyc/sqt animal cap transplants 200pg of gfp S65C RNA was co-injected into donor embryos. Double ISH was performed to visualize donor cells. 200 pg of sqt-GFP RNA was injected into donor embryos used for gradient experiment transplants. The pro-protein convertase site mutant Sqt-GFP construct has been previously described (Tessadori et al., 2015). mesp co-transplant experiments were performed by aspirating cells from the separate donors into the needle together before being placed in the animal cap of a host. mespab over-expressing donor embryos were injected with a AlexaFluor647 conjugated Dextran (Thermofisher).

2.7 Western Blot

200 pg of sqt-GFP RNA was injected per embryo. The sqt-GFP construct has been previously described (Jing et al., 2006). 50 embryos per condition were lysed and protein amounts were equalized before loading. Biorad Mini-PROTEAN Precast 4-15% gradient gels were used. Blots were first treated with Rabbit anti-GFP (Torrey Pines Scientific) at a dilution of 1:1000 in 5% non-fat milk. Goat anti Rabbit HRP (Biorad) was then used at 1:5000. After developing blots were stripped and re-probed with mouse anti-Gapdh (Molecular Probes) diluted 1:1000 which was then detected using the same secondary as above. Western blots were imaged using a Chemidoc-MP gel imager (BioRad) and band intensity was quantified using Image Lab software (BioRad).

2.8 Drug treatments

Nodal inhibition was performed by treating embryos with SB505124 (10 uM) (Sigma) in egg water/0.1% DMSO. The APLNR agonist ML233 was obtained from Glixx Laboratories (Southborough, MA, USA). Embryos were treated with 2.5 μM ML233 in egg water/1% DMSO from the sphere stage onwards. For mespaaΔnls-ecr transplants 20uM tebufenozide was dissolved in egg water/0.1% DMSO.
2.9 Zebrafish embryo dissociation

De-chorionated embryos were transferred to a 1.5mL Eppendorf tube when they reached the desired stage. After incubation in 200ul calcium-free Ringer solution (116 mM NaCl, 2.6mM KCl, 5mM HEPE, pH 7.0) for 10 min, embryos were transferred into a 24-well plate filled with 500ul TrypLE solution (GIBOCO, TrypLE Express Enzyme, cat #: 12604-013) at room temperature. Embryos were gently homogenized every 5min with P1000 tips. Dissociation was monitored under a dissection scope until most cells were in a single-cell suspension. The cell suspension was transferred into 200ul ice-cold FBS to stop the reaction. Cells were centrifuged at 300g for 3min at 4 degrees Celsius and washed with 500ul cold DMEM with 10% FBS before re-suspension in 500ul cold DMEM with 1% FBS. Right before the flow cytometry analysis, cells were filtered through a 40um strainer and DAPI was added at a concentration of 5ug/ml to exclude any dead cells. 85-100 transplanted embryos were collected and dissociated for each sample.

2.10 Flow cytometry analysis

The single-cell suspension was analyzed using a Becton Dickinson LSR II flow cytometry analyzer (BRVU). Information on 180,000 to 250,000 events was acquired for each sample. The same acquisition and analysis gate settings were used for both control and mespaa-overexpressing cells from the same stages. Analysis was carried out using FlowJo software. Cell-doublets and dead cells were excluded first based on forward scatter, side scatter and the DAPI channel. Gates for discriminating GFP positive and negative cells were set based on GFP negative control cells of the same stages and applied on the Alex647+ population that represented the transplanted cells.

2.11 Primers and Genotyping of Mutants

In the aplnrb<sub>hd4145</sub> mutant allele a premature stop codon has been induced into the coding sequence at amino acid position 54. Primers used for genotyping are used to amplify a 215 bp product which when cut with AciI yields fragments of 140 bp, 52 bp and 25 bp, the mutant allele will not be cut with AciI. Forward primer: 5’-catcttcacctggagctcactg-3’ Reverse primer: 5’-agcaccacatagctgctgatctt-3’. For genotyping the allele of aplnrb<sub>grinch</sub> the same primers were used.
as for the *hu4145* allele, but the resultant PCR product was instead cut with EaeI, generating a 141 bp of the 215 bp product in the mutant allele.

Genotyping the *aplnra* allele was performed using the following primers to detect the presence of the insertion: Forward primer: 5’-acctggaacatgtggttc-3’;
Reverse primer: 5’-aaccggattgaggcagctgtgac-3’. To determine the presence of the WT *aplnra* allele the following forward primer is used instead: Forward primer: 5’-ctcggtttcttgcctttc-3’.

### 2.12 Mesp Mutant Constructs

All *mespaa* mutant constructs were generated by site directed mutagenesis. Primers used to generate the dimerization mutant (p.F113P) were as follows: Forward: 5’-acagacgcagcagcagctgtggttc-3’ and Reverse: 5’-catcactcaggacccccctcgctgtcgtg-3’. The HLH binding domain mutant (p.KLR97_99EDE) was constructed using primers: Forward: 5’- gagcttgtgtagatcctcatctcatctctcctccgcgcgcgtgcagtcgctgc-3’ and Reverse: 5’-ggcagactgcaagcagcggaggatgagttgctcacaagagct-3’. Overlapping amplicons were generated using flanking primers, followed by full-length amplification of *mespaa* coding sequence and subcloning into pCS2+. *mespaa*Δnls-*ecr* was generated by first creating a *mespaa-* *ecr* construct. The *ecr* sequence was ligated upstream of the *mespaa* coding sequence in PCS2+ and then the nls mutant was created using the following primers: Forward: 5’-gacagatgtccagcgggcaaccgcagctcacaagttc-3’ and Reverse: 5’-aggaactctgatgtggggtgccgctcagctgccagatctgtc-3’. To create the N-terminal and C-terminal *mespaa-mespab* swaps the unique PstGI and BclI restriction enzyme sites that flank the HLH domain were used.
Chapter 3

3.1 The Apelin Receptor enhances Nodal signaling for proper cardiac progenitor development.

3.2 Introduction

During gastrulation, complex cell movements occur which results in the localization of progenitor populations to discrete embryonic regions for subsequent organogenesis. Loss of Apelin receptor (Aplnr) function in zebrafish, as demonstrated in the grinch (aplnrβ) mutant, results in an absence or decrease in cardiogenesis, and affects expression of the earliest known cardiac mesoderm markers (Scott et al., 2007; Zeng et al., 2007). In zebrafish, the Apelin receptor has been implicated in the movement of cardiac progenitors during gastrulation to the anterior lateral plate mesoderm (ALPM), the site of heart development. Aplnrα/b-depleted cells show a delay in anterior migration during epiboly (Paskaradevan & Scott, 2012). The role of Aplnr in the proper formation of the heart appears to be conserved in vertebrates. In mice, Aplnr (also known as Apj) is expressed in the gastrulating mesoderm, with Aplnr mutant mice exhibiting incompletely penetrant cardiovascular malformations including thinning of the myocardium, ventricular septation defects, an enlarged right ventricle and improper heart looping (Kang et al., 2013). In vitro, over-expression of Aplnr in mouse embryonic stem cells results in enhanced cardiac differentiation of embryoid bodies, while Aplnr inhibition leads to impaired cardiac differentiation (D’Aniello et al., 2009, 2013).

While a role for Aplnr signaling in the earliest events of cardiac development is evident, how Aplnr functions in this context remains unclear. Numerous studies have described roles for the classical Apelin / Aplnr hormone GPCR (G-protein Coupled Receptor) signaling pair in adult physiology, however in the context of early heart development Apelin does not appear to be the correct Aplnr ligand (Chng et al., 2013; Paskaradevan & Scott, 2012; Szokodi et al., 2002). Further, the requirement for Aplnr in cardiac development appears to be primarily non-cell autonomous, which is to say that Aplnr is not required in the cells that will form the heart per se but rather in surrounding cells (Paskaradevan & Scott, 2012).
Recently, an evolutionary conserved 32-amino acid secreted peptide named Apela was discovered to be the long sought after early ligand for Aplnr (Chng et al., 2013; Pauli et al., 2014). Apela, also known as Ela/Toddler parallels the spatiotemporal transcription of \textit{aplnra/b} in zebrafish, and null \textit{apela} mutant embryos have a similar cardiac phenotype to that of the \textit{aplnrb}^{\textit{grinch}} mutant. Analysis of \textit{apela} mutant embryos also revealed a migration defect during early gastrulation, in which lateral mesendodermal cells showed a delay in ingression from the margin, similar to what has been observed in embryos deficient for Aplnr function.

In this thesis I find that the Apela/Aplnr signaling axis modulates Nodal/TGFbeta signaling during gastrulation, a key pathway essential for mesendoderm induction and migration (Carmany-Rampey & Schier, 2001; Dougan, Warga, Kane, Schier, & Talbot, 2003). Loss of function of Aplnr leads to a reduction in Nodal target gene expression, whereas activation of Aplnr signaling increases the expression of these same targets. By elevating Nodal levels in \textit{aplnra/b} mutant/morphant embryos, we are able to fully restore cardiac differentiation. I also find that loss of Aplnr attenuates the activity of a point source of the Nodal ligands Squint and Cyclops, suggestive of a direct effect on the Nodal signal itself. Consistently, I provide evidence that the Aplnr cascade may in part regulate Nodal ligand processing to achieve optimal mesendodermal differentiation. Most unexpectedly, I find that the loss of Ela may actually result in the elevation of nodal signaling and that there are subtle phenotypic differences between Aplnr and Ela loss of function. This points to the possibility that Ela may work as an antagonistic ligand to Aplnr, suggesting a model in which the antagonism between Ela and its receptor Aplnr fine-tunes Nodal activity during the onset of gastrulation to initiate the migration of lateral margin cells and proper heart formation. I propose that the Elabela-Aplnr signaling cascade may therefore act as a rheostat for the Nodal/TGF\(\beta\) pathway during the earliest stages of cardiogenesis.

3.3 Results

3.3.1 \textit{aplnra/b} double mutants phenocopy \textit{aplnra/b} morpholino injected embryos.

Previous analysis of Aplnr function in zebrafish was performed using either mutants for \textit{aplnrb} (\textit{grinch}) or through the injection of morpholino anti-sense oligonucleotides against both \textit{aplnra/b}
Given the discrepancies that have been observed between mutant and morpholino phenotypes in other contexts, I wished to pursue mutant analysis of \textit{aplnra} and \textit{aplnra/b} together. An allele containing a large viral insertion (5 kb) into the region coding for the seventh transmembrane domain in the \textit{aplnra} locus (\textit{aplnra}^{ins}) was obtained from Znomics and subsequently characterized in detail (Figure 3.1A). Homozygous \textit{aplnra}^{ins} embryos recapitulated the phenotype of \textit{aplnrb}^{grinch} with similar pericardial edema (Figure 3.1C), reduced \textit{nkh2.5}-positive cells at 16 hours post fertilization (hpf) (Figure 3.1F-H) and reduced \textit{myl7}-positive cardiomyocytes at 2 days post fertilization (dpf) (Figure 3.1J-K). In addition, the number and spread of \textit{sox17}-positive cells was significantly reduced in homozygous \textit{aplnra}^{ins} when compared to wildtype (WT) at 8 hpf (Figure 3.1M-N, P-Q). \textit{sox17} expression marks endoderm progenitors at this stage. These phenotypes are highly similar to those observed in mutants for \textit{aplnrb} suggesting common function.

Double \textit{aplnrb}^{hu4145}, \textit{aplnra}^{ins} mutants were then generated to evaluate functional redundancy for these two paralogues in early development. Double mutant embryos exhibited normal morphology at 2 dpf with pericardial edema (Figure 3.1D). In less than 5% (n=21) of double mutant embryos extreme phenotypes were also observed including cyclopia and tail bifurcations (Figure 3.1E). In contrast to \textit{aplnra} or \textit{aplnrb} single mutants, which usually possess significant cardiac tissue at 2 dpf, double mutant embryos exhibited either complete absence of or an extremely small heart (Figure 3.1L). In addition, double \textit{aplnra}; \textit{aplnrb} mutant embryos exhibited a further reduction in both the spread and number of \textit{sox17} expressing cells when compared to the \textit{aplnra}^{ins} single mutant (Figure 3.1M-Q). \textit{nkh2.5} expression was negligible in double mutants suggesting a complete absence of early cardiac progenitors (Figure 3.1I). It should be noted that the double mutant phenotype faithfully phenocopies that seen with the injection of \textit{aplnra/b} morpholinos (MOs) both at the morphological level and the expression of these three diagnostic genes (\textit{sox17}, \textit{nkh2.5} and \textit{myl7}). Taken together, these data suggests that Aplnra is required for both proper endoderm differentiation and cardiac development and that Aplnra and Aplnrb have redundant roles in these processes.
3.3.2 Aplnr deficient embryos show a reduction in Nodal signaling.

To gain insight into how Aplnr signaling regulates early cardiac development, I pursued a gene expression profiling approach. Comparative microarray analysis at 5.25 hpf (50% epiboly) of cDNA from wildtype (WT) and aplnra/b morphant (injected with morpholinos against aplnra and aplnrb) embryos revealed a reduction in a set of genes known to be downstream of the Nodal signaling pathway. Previously published work has identified 72 Nodal regulated genes in zebrafish at 6 hpf (Bennett et al., 2007). Strikingly, 23 of these 72 genes were down-regulated in aplnra/b morphants, suggesting a decrease in Nodal signaling in the absence of Apelin receptor function (Figure 3.2A). Given the known role of Nodal signaling in induction and migration of the mesendoderm (Carmany-Rampey & Schier, 2001; B Feldman et al., 2000; Gritsman et al., 1999), I wondered if the Aplnr might work upstream or in parallel to the Nodal pathway. I next sought to confirm attenuated Nodal signaling following aplnra/b knockdown by means of chemical inhibition. Embryos were incubated from the sphere stage (4hpf) onwards with increasing concentrations of SB505124, which acts as a dedicated Alk4/5/7 antagonist. While WT embryos treated with a suboptimal concentration of the drug and aplnra/b MO embryos alone exhibited normal morphology at 24hpf, aplnra/b MO embryos treated with the same concentration of the drug exhibited complete cyclopia, a hallmark of reduced Nodal signaling in zebrafish (Figure 3.2B-D).
Figure 3.2 – *aplnra/b* MO injected embryos have a reduction in Nodal target gene expression and are sensitive to Nodal chemical inhibition

(A) List and Venn diagram of 23 Nodal target genes found to be down-regulated in a microarray of *aplnra/b* morphant embryos compared to WT at 5.25 hpf (50% epiboly). (B-D) Eye and whole body morphology in WT embryos treated with SB505124 (B), *aplnra/b* MOs (C) and *aplnra/b* MOs with SB505124 (D).

I next used RNA whole mount *in situ* hybridization to look at the expression of several direct downstream targets of Nodal in *aplnra/b* morphant and *aplnra/b* double mutant embryos. The canonical Nodal target genes *floating head* (*flh*), *goosecoid* (*gsc*) and *sox32* all showed reduced expression in both morphant and mutant embryos at 8 hpf relative to WT embryos (Figure 3.3A-C,E-G,I-K). Analysis of *sox32* expression, which marks endodermal precursors, revealed both a reduced number of endoderm cells and a decreased extent of endodermal migration (quantified
in Figure 3.3M-N), consistent with previous analysis of *aplnrb* and *apela* mutants (Chng et al., 2013; Pauli et al., 2014). Further analysis of additional Nodal target genes *lefty1* and *lefty2* also revealed a decrease in expression before and at the beginning of gastrulation (Figure 3.3O-T).

I next examined if the ectopic activation of Aplnr could suffice to increase the expression of Nodal target genes. Over-expression of both known ligands of Aplnr, *apelin* (*apln*) and *apela* each result in phenotypes similar to Aplnr loss-of-function, possibly as a consequence of ligand-mediated receptor internalization and signal desensitization (Chng et al., 2013; Paskaradevan & Scott, 2012; Pauli et al., 2014; Scott et al., 2007; Zeng et al., 2007). To bypass this limitation, I made use instead of ML233, a non-peptide small molecule agonist of APLNR (Khan et al., 2011). Treating embryos injected with 150 pg of *aplnrb* RNA with 2.5 μM of ML233 resulted in a significant increase in the expression of the three Nodal targets *gsc*, *flh* and *sox32* relative to WT (Figure 3.3D,H,L). A greater number of endoderm cells were evident that migrated to a more anterior position following this treatment (Figure 3.3M-N). Over-expression of *aplnrb* or ML233 treatment alone resulted in increased *flh* and *gsc* expression, whereas *sox32* expression was largely unaffected by ML233 or *aplnrb* alone (Figure 3.4A-I). ML233 had no effect on *gsc*, *flh* or *sox32* expression in *aplnra/b* morphants, indicating that the action of ML233 is Aplnr-dependent (Figure 3.5A-F). To confirm that the effect of Aplnr activity on *gsc*, *flh* and *sox32* was mediated by its effect on the nodal signaling pathway, overexpression of *aplnrb* and treatment of embryos with ML233 in *oep* morpholino injected embryos was performed. These embryos did not display any expression of *gsc*, *flh* or *sox32*, arguing against a scenario where Aplnr signaling is acting in parallel to Nodal signaling (Figure 3.5G-L).
Figure 3.3 - Aplnr Deficient Embryos Exhibit a Reduction in Nodal Signaling

(A-N) Visualization of the expression of the canonical nodal target genes gsc, flh and sox32 in WT (A,E,I), aplnra/b MOs injected embryos (B,F,J), aplnra \textsuperscript{ins}; aplnrb \textsuperscript{mut445} double mutant embryos (C,G,K) and aplnrb RNA injected embryos treated with the agonist ML233 (D,H,L).
Embryos are viewed from the dorsal side. Quantification of the number and spread of *sox32* expressing cells (M,N). Data are represented as means ± SEM. * p<0.05. *** p<0.001 unpaired two-tailed t-test. (O-T) *In situ* hybridization showing expression of *lefty1* at 4 hpf (O, R) and 5.5 hpf (P, S) and *lefty2* at 5.5 hpf (Q, T) in *aplnra/b* morphant embryos when compared to WT from an animal pole (top of embryo) view.

![Embryos view](image)

**Figure 3. 4 – Effect of *aplnrb* over-expression or ML233 treatment alone on Nodal target gene expression.**

(A-I) Expression of the canonical Nodal target genes *gsc, flh* and *sox32* in WT (A-C), *aplnrb* RNA injected embryos (D-F) and embryos treated with the agonist ML233 (G-I). Embryos are viewed from the dorsal side with anterior to the top.
Figure 3.5 – ML233 acts to boost Nodal target gene expression through the Aplnr and Aplnr signaling is not in parallel to Nodal.

(A-F) Expression of the canonical Nodal target genes gsc, flh and sox32 in aplna/b morphant embryos with (A-C) or without ML233 (D-F). Embryos are viewed from the dorsal side with anterior to the top. (G-L) Expression of the canonical Nodal target genes gsc, flh and sox32 in oep morphant embryos with (G-I) or without the injection of aplnb RNA and ML233 addition (J-L). Embryos are viewed from the dorsal side with anterior to the top.
3.3.3 Elevated levels of Nodal can rescue the Aplnr Loss of Function Heart phenotype.

As Nodal signaling is reduced in aplnr mutant embryos, I reasoned that increasing Nodal may ameliorate or rescue cardiogenesis in the absence of Aplnr function. To test this hypothesis, I took two complementary approaches. I first elevated the levels of endogenous Nodal signaling by injecting a MO against lefty1, a direct negative feedback Nodal antagonist (Benjamin Feldman et al., 2002). lefty1 MO was injected into embryos bearing two different aplnrb mutant alleles, s608 (p.W90L) and hu4145 (p.W54X), which exhibit a small heart (Figure 3.6A-C). While the penetrance of the heart phenotype varied within each clutch, lefty1 MO treatment was capable of rescuing both mutant lines, with nearly all embryos showing rescue of the small heart phenotype (Figure 3.6D, note that 25% of embryos in a given cross would be homozygous null mutants). One caveat of this approach is that aplnra/b gene expression is regulated by Nodal signaling (Pauli et al., 2014). It is therefore conceivable that elevating Nodal levels in aplnrb single mutants provides rescue simply by elevating aplnra gene expression. To address this issue, lefty1 MO was injected into embryos generated from an in-cross of aplnra; aplnrb heterozygous parents. Embryos were evaluated for heart formation by WISH for myl7 gene expression and subsequently genotyped. Strikingly, over 60% of aplnra/b double mutants exhibited proper cardiac formation when injected with lefty1 MOs, which was not observed in un-injected mutant siblings (Figure 3.6E-H). This suggests that elevated Nodal signaling is capable of rescuing the Aplnr cardiac phenotype, even in the complete absence of Aplnr function.

As a complementary approach, I attempted to specifically elevate the levels of Nodal signaling within lateral margin cells and see if this rescued cardiac contribution. To perform this experiment, donor cells from myl7:EGFP transgenic embryos either injected or uninjected with taram-a* RNA encoding a hyper-activated Nodal receptor (Renucci et al., 1996) were transplanted to the margin of aplnra/b morphant hosts (Figure 3.6I). As our lab has previously shown (Paskaradevan & Scott, 2012), WT cells placed at the margin of aplnra/b morphant hosts contributed to the myocardium at an appreciably reduced frequency (7.8%, N=3, n=157) as compared to when WT hosts were used (Figure 3.6J). In contrast, taram-a* overexpressing cells, when transplanted to the margin of aplnra/b morphant embryos, contributed to the myocardium
at a much higher frequency (20.7%, N=3, n=164) with no significant difference when compared
to transplantation of WT cells into WT hosts (22.5%, N=4, n=234), suggesting a near complete
rescue (Figure 3.6J-N”). It should be noted that transplantation of WT taram-a* expressing cells
into the margin of WT embryos did not increase the contribution of donor cells to the heart
(22%, N=4, n=213). These experiments further argue that the heart defects observed in aplnr
deficient embryos are suppressed if Nodal signaling is increased.

Figure 3.6 - Elevation of Nodal Signaling in aplnr Mutant/Morphant Embryos Rescues
Cardiogenesis.
(A-C) myl7 ISH showing a representative heart phenotype at 48 hpf in a WT embryo (A) and two different aplnr mutant alleles; hu4145 (B) and grinch<sup>608</sup> (C). Anterior is oriented towards the left. (D) Quantification of the number of embryos with a small heart from individual clutches in which half were injected with lefty<sup>1</sup> MO. Clutches were obtained from crosses of aplnr heterozygous mutants. (E-H) Classification of heart phenotype in aplnra<sup>ins</sup>; aplnr<sup>hu4145</sup> double mutant embryos when injected with lefty<sup>1</sup> MO as compared to un-injected embryos. Severity of cardiac phenotypes was scored based on myl ISH (H). (I) Schematic displaying the transplantation of injected donor cells into the margin of host embryos. Contribute of transplanted cells to the heart is scored based on expression of the myl7:EGFP transgene in donor cells. (J-N") Margin transplants of WT or taram-a<sup>*</sup> overexpressing myl7:EGFP cells into WT or aplnra/b morphant embryos. Arrow indicates the heart. Embryos are displayed from a lateral view with the anterior of the embryo towards the right. Data are represented as means ± SEM. * p < 0.05, n.s. = not significant, Tukey’s Multiple Comparison test following significant (p < 0.05) one way ANOVA. (O-R) Gross morphology and myl7 expression at 24 hpf in WT (O), embryos injected with a sub-optimal dose of oep MOs (P), aplnra/b morphant embryos (Q) and aplnra/b/oep morphant embryos (R).

3.3.4 Loss of the Aplnr leads to a delay in Mesp expression.

At this point my results led me to speculate as to how a reduction in Nodal signaling may lead to a delay in mesendodermal ingression during gastrulation. Previous work has shown that Nodal target genes are activated depending on the dose and/or associated time of exposure to the Nodal ligand (Hagos and Dougan, 2007). I hypothesized that Aplnr may be required to boost the Nodal signal in order to activate the expression of genes required for ingression at the right time in development. A particularly interesting category of genes which were down-regulated in the aplnra/b MO microarray was the mesp family of transcription factors. Mesp genes have been shown to regulate the migration of mesoderm through the primitive streak during gastrulation in mice and are essential for cardiac formation (Kitajima et al., 2000). All mesp family members for which probes were present on the microarray showed reduced expression (Table 3.1). By RNA in situ hybridization, I confirmed that both mesp<sub>a</sub>a and mesp<sub>b</sub> are expressed around the margin during gastrulation and their expression is dramatically decreased in aplnra/b MO embryos when
compared to wild type embryos at 50% epiboly (5.25 hpf, Figure 3.7E-F, I-J). To determine if mesp genes are Nodal targets, embryos were treated with the Nodal inhibitor SB505124 from 4.5-5.5 hpf and found that this completely abrogated mespaa and mespab expression (Figure 3.7A-B). In addition, animal cap transplants of squint over-expressing cells induced mespaa/mespab expression (Figure 3.7C-D). Taken together, this suggests that Nodal is necessary and sufficient for mesp gene expression. In order to distinguish whether Aplnra/b is essential for mesp transcription in general, or simply for the proper temporal activation of mesp expression, I performed RNA in situ hybridization for both mesp family members at a later time point. Strikingly, at shield stage (6 hpf, 45 minutes after 50% epiboly) in aplnra/b MO embryos expression of both mespaa and mespab appeared to largely recover (Figure 3.7G-H, K-L). This indicates that the loss of Apelin receptor can result in the delay of Nodal target gene expression and is required for the proper temporal activation of mesp genes.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_15_P116279</td>
<td>mespaa</td>
<td>-3.24</td>
</tr>
<tr>
<td>A_15_P627536</td>
<td>mespaa</td>
<td>-3.04</td>
</tr>
<tr>
<td>A_15_P630646</td>
<td>mespba</td>
<td>-2.08</td>
</tr>
<tr>
<td>A_15_P108949</td>
<td>mespba</td>
<td>-2.04</td>
</tr>
</tbody>
</table>

Table 3.1 - Microarray Data for mesp Family Genes on aplnra/b morphant Array.

Fold change for all mesp family probes present on the microarray. Fold change is comparing WT to aplnra/b morphant embryos from normalized data from four biological replicates.
Figure 3.7 – Loss of Aplnr Results in a Delay in mesp Gene Expression

(A-B) Animal view of expression of mespaa and mespab at 50% epiboly (5.25 hpf) in embryos treated with 10 μM of SB505124 from 4-5.25 hpf. Animal cap view with dorsal to the bottom. (C-D) Animal view of expression of mespaa and mespab at 50% epiboly (5.25 hpf) in embryos in which cells expressing 4 pg of sqt RNA were transplanted into the animal cap. Animal cap view with dorsal to the bottom. (E-L) Expression of mespaa and mespab at 50% epiboly (5.25 hpf) (E,F,I,J) and the shield stage (6 hpf) (G,H,K,L) in WT and aplnr/a/b morphant embryos when examined by in situ hybridization. Embryos are viewed from the animal pole with dorsal at the bottom.
3.3.5 Loss of Aplnr reduces the activity of Nodal ligands in a non-cell autonomous manner.

To investigate the link between Aplnr and Nodal, we next examined whether the decrease in Nodal gene targets observed in the absence of Aplnr function was the consequence of decreased expression of the Nodal ligands. Surprisingly, at the sphere stage, both Nodal ligands exhibited an increase in expression, possibly the result of the embryo trying to compensate for a lack of Nodal signaling (Figure 3.8A-B,E-F). Later in development, at 30 percent epiboly (4.7hpf), a now slight decrease in both ligands was observed, consistent with the fact that the nodal ligands regulate their own expression in a positive feedback loop (Figure 3.8C-D,G-H). By 50 percent epiboly (5.25hpf) no difference was observed in the expression of the two Nodal ligands (Figure 3.8I-J,K-L). Altogether this suggests that the defect in Nodal signaling observed in Aplnr loss of function is not the consequence of a reduction in ligand expression.
Figure 3.8 – Nodal Ligand Expression in aplnra/b Morphant Embryos.

(A-L) Lateral view of expression of the Nodal ligands sqt (A-D, I-J) and cyc (E-H, K-L) at sphere (4 hpf), 30% epiboly (4.7 hpf) and 50% epiboly (5.25 hpf) in WT and aplnra/b morphant embryos.

I next sought to determine if Aplnr might directly act on the Nodal signaling pathway. To address this question, I took advantage of a previously developed zebrafish Nodal point source assay (Y. Chen & Schier, 2001). Nodal over-expressing cells were transplanted into the animal cap of host embryos and the ability to induce target genes was subsequently evaluated (Figure 3.9A). While in WT (WT host and donor) embryos 4 pg of squint RNA was found to be sufficient to induce gsc expression, in MO (host and donor) embryos gsc induction was never observed with the same dose (Figure 3.9B,F,J). To evaluate if this was a complete loss of Squint activity or simply a reduction, we looked at the ability to induce the low threshold Nodal target gene ntl (Y. Chen & Schier, 2001). In contrast to gsc, ntl expression was induced at a high frequency in both WT and MO embryos, demonstrating that the loss of Aplnr resulted in an attenuation of Squint activity (Figure 3.9C,G,J). We performed similar experiments with Cyclops and found that cyc RNA was less effective at inducing both gsc and ntl in aplnra/b morphant embryos, suggesting that Aplnra/b regulates the activity of both Nodal ligands (Figure 3.9D-E,H-J).

To understand how Aplnr could affect the Nodal signaling pathway I needed to consider a number of different potential mechanisms of action including effects on Nodal ligand generation/secretion, movement-processing or signal reception/transduction. To begin to distinguish between these different possibilities I wanted to determine whether Aplnr is required in Nodal secreting cells, Nodal ligand receiving cells, or in the environment in between signal generation and reception. I started by performing further transplantation experiments to determine whether Aplnra/b is required in the Nodal secreting or surrounding cells. At a higher dose of squint RNA (40 pg) a ring of ntl expression was induced around the transplanted donor cells (Figure 3.9K). While WT into WT and MO into WT transplants consistently resulted in a strong ring of ntl expression, WT into MO and MO into MO transplants resulted in a smaller
expression domain (Figure 3.9L-N). This suggests that Aplnra/b are not required in Nodal secreting cells per se, but instead required in the surrounding environment.

Figure 3.9 – The Aplnr Attenuates the Activity of Sqt and Cyc in a non-cell autonomous manner.

(A) Animal cap transplant of GFP expressing control cells detected by ISH. (B-J) Animal cap transplants of sqt or cyc overexpressing cells into WT (B-E) or aplnra/b morphant embryos (F-I). gsc and ntl expression is displayed in blue and gfp expressing donor cells are marked in brown. Both donor cells and hosts are of the same background (WT into WT or morphant into morphant). Embryos are viewed from the animal pole with dorsal at the bottom. Data are
represented as means ± SEM. (K-N) Animal cap transplants of cells expressing high levels of sqt RNA. ntl expression is visualized in blue and gfp expressing donor cells are brown. Four different combinations of donor/host cells were examined, WT into WT (donor into host) (K), aplnra/b morphant into WT (L), WT into aplnra/b morphant (M) and aplnra/b morphant into morphant (N). When a large ring of expression was induced the donor cells are circled in white. Embryos are viewed from the animal pole with dorsal at the bottom.

To investigate the requirement for Aplnr in Nodal signal reception, I took advantage of the unique properties of the yolk syncytial layer (YSL). squint and cyclops are both expressed in the YSL during early gastrulation and are required for proper endoderm induction as their loss specifically in the YSL results in the complete absence of sox17 expression (Fan et al., 2007). While the Nodal ligands are expressed in the YSL and are important for development, the Nodal co-receptor oep is not expressed in the YSL and thus this tissue should not respond to them (Fan et al., 2007). This provides a useful developmental scenario where Nodal is being expressed by the YSL and received by endoderm progenitors. Endogenous aplnra/b expression in the YSL has also been documented (Tucker et al., 2007). If the Aplnr is simply required in Nodal signal reception/transduction, depletion of Aplnr in the YSL should not have an effect on the formation of the endoderm. When aplnra/b MOs were targeted to the YSL (via injection into the yolk at the 1,000 cell stage) (Fan et al., 2007), endoderm formation was perturbed, as shown by a dramatic reduction in the number of sox17-expressing cells (Figure 3.10C-F, I-J). Consequently, a high percentage of embryos exhibited cardia bifida (88/154 = 57%) (Figure 3.10G,K). Cardia bifida is the formation of two separate hearts resulting from the failure of cardiac progenitors to converge towards the midline after gastrulation and is typically the consequence of aberrant endoderm formation (Yutaka Kikuchi et al., 2001; Ye & Lin, 2013). Consistently, cardiac specification appeared normal in these embryos following YSL injection of aplnra/b MO as indicated by strong nkx2.5 expression (Figure 3.10H,L). This result provides evidence that the Aplnr is required non-cell autonomously for endoderm formation as loss of Aplnr function outside of the signal receiving cells (endoderm) results in a defect. This complements previously published experiments suggesting that Aplnr acts non-cell autonomously for cardiac progenitor development (Paskaradevan and Scott, 2012). Together these pieces of evidence suggest a role for Aplnr in neither the Nodal secreting cells (shown by transplants performed above) nor the
ligand receiving cells (endoderm and cardiac progenitors) but rather in the surrounding environment.

Figure 3.10 – The Aplnr is required non-cell autonomously for endoderm formation.

(A-B) Visualization of rhodamine dextran co-injected with MOs when specifically injected into the YSL. (C-D) Quantification of the number and spread of sox17-positive cells following YSL injection of aplnra/b MOs. (E-L) In situ hybridization displaying the expression of sox17 (E-F and I-J) and nkd2.5 (H,L) in WT and YSL aplnra/b MOs injected embryos (E, I - dorsal view with anterior to the top, F, J - lateral view with dorsal to the left). (G, K) A comparison between control myl7:EGFP transgenic embryos (G) and those injected with aplnra/b MOs into the YSL (K). Embryos are viewed from the ventral side with the anterior at the top.
3.3.6 The Aplnr is required for proper Nodal ligand processing

Given that the effect of the Aplnr on Nodal signaling did not appear to be either on the Nodal secreting cells or the Nodal receiving cells, I wanted to investigate if it may be important for proper gradient formation. For example, it could be important for movement of the Nodal ligands through the extracellular space. To address this question, transplants were performed in which cells from donor embryos injected with sqt-GFP RNA were placed in the animal cap of embryos to generate an ectopic Sqt-GFP gradient. Sqt-GFP has previously been demonstrated to have similar signaling activity as WT Sqt and has been used to monitor Nodal ligand movement and stability (Jing, Zhou, Wang, & Chen, 2006; Müller et al., 2012). I observed no striking differences in Sqt-GFP gradients in aplnra/b morphants when compared to WT, suggesting that gradient formation is not the primary defect in the absence of Aplnr activity (Figure 3.11A-H). Sqt-GFP was observed in the extracellular space at some distance from donor cells with no discernable difference from WT. I additionally examined the levels of Sqt-GFP protein in WT, Aplnr deficient and apela mutant embryos via western blot using an anti-GFP antibody. In this experiment two Sqt species can be detected following injection of sqt-GFP, a larger unprocessed immature form of Sqt and a smaller mature form with more potent signaling activity (Beck et al., 2002). Intriguingly, the formation of the mature Sqt ligand showed a delay in aplnra/b morphant embryos when compared to WT, and consequently the immature ligand took a longer period of time to clear from the embryo (Figure 3.11-J). I next examined the dynamics of Sqt-GFP processing in an apela (ela$^{hr13}$) mutant background. Strikingly, apela mutant embryos displayed a higher amount of both unprocessed and processed Sqt-GFP when compared to WT at 4-6 hpf (Figure 3.11-I). Together, this data suggests that Nodal ligand protein dynamics are affected in both Aplnr and Apela loss-of-function, each showing different effects on Nodal protein levels and processing.

A recent study has indicated that Sqt-GFP lacking the pro-protein convertase cleavage site is not effectively secreted from the cell (Tessadori et al., 2015). They concluded that processing actually occurs inside the cell as opposed to in the extracellular space as indicated by previous work (Beck et al., 2002). This would contradict my proposed model given that I find defects in Nodal processing and altogether have found that this effect is likely happening in the extracellular environment. To more directly address this question I performed animal cap
transplants of donor cells expressing the cleavage mutant used in their study and examined hosts two hours after transplantation. Abundant GFP protein was visualized in the extracellular space indicating the ability of the cleavage mutant Sqt-GFP to be secreted at high levels (Figure 3.11K-N). This contradicts findings of the previous study and suggests that Nodal ligand processing may in fact occur in the extracellular space.
Figure 3.11 - Nodal Protein Dynamics are Affected in Aplnr and Ela Loss-of-function Embryos.

(A-H) Animal pole view of animal cap transplants in which Sqt-GFP expressing cells marked with rhodamine dextran were transplanted into WT (A-D) and aplnra/b morphant (E-H) embryos (both donor and host cells were either WT or morphant). (I) Western blot probed with anti-GFP antibody displaying the presence of unprocessed immature Sqt-GFP protein (pre) and mature processed protein (mat) at 4 hpf, 6 hpf and 8 hpf in WT, aplnra/b morphant and ela\textsuperscript{br13} mutant embryos. Gapdh protein was subsequently detected as a loading control. (J) Quantification of the ratio of WT to apln morphant and ela mutant unprocessed and processed Sqt-GFP. Levels were normalized to Gapdh loading controls in all cases. (K-N) Dorsal view of animal cap transplants in which cells expressing Sqt-GFP with a mutated pro-protein convertase cleavage site marked with rhodamine dextran were transplanted into WT embryos.

3.3.7 Apela may act as an antagonist of the Apelin receptor

Given that in apela mutant embryos I observed an increase in the amount of both un-processed and processed Squint ligand, I wondered if perhaps Apela does not act as a simple agonist for the Aplnr. While phenotypically both Aplnr and Apela deficient embryos exhibit similar features, I noticed a difference in the staining of sox17 when performing in situ hybridization experiments. When WT, aplnra/b morphant and apela (ela\textsuperscript{br13}) homozygous mutant embryos were compared for intensity of staining of sox17 at an equal time point, apela mutants did not phenocopy aplnra/b loss of function. apela mutant embryos appeared to have a less substantial decrease in the number of sox17 expressing cells when compared to WT but more surprisingly, appeared to stain at a higher intensity than both WT and aplnra/b morphant embryos (Figure 3.12A-F). This was in contrast to the aplnra/b morphant embryos where sox17 positive cells stained less intensely when compared to WT. Given these discrepancies and the previous difference in Nodal ligand processing, I wondered if perhaps apela actually behaves as an antagonist for Aplnr. I then reasoned that if Apela acts to limit Aplnr signaling, an excess of Aplnr in an apela null background should exacerbate the phenotype. Strikingly, injection of aplnrb RNA into embryos from ela\textsuperscript{br13} heterozygote in-crosses consistently increased the penetrance of the small heart
phenotype (Figure 3.12G) suggesting that 1) Aplnr remains biologically active even in the absence of its endogenous ligand and 2) an excess of active Aplnr may be responsible for the apela loss of function phenotype.

Figure 3.12 – Aplnr and Ela display differences in sox17 expression and aplnr over-expression exacerbates the ela mutant phenotype.
(A-F) Dorsal and lateral views of *sox17* expression in WT, *aplnra/b* morphant and *ela* mutant embryos at 70 percent epiboly (8 hpf). (G) Quantification of the number of embryos with a small/absent heart from 4 individual clutches of *ela*br13 heterozygous in-crosses with or without the injection of 150 pg of *aplnrb* RNA. Note that 25% of embryos would on average be expected to be *ela* mutants.

### 3.4 Discussion

#### 3.4.1 The Aplnr enhances Nodal signaling for proper cardiac progenitor development.

Altogether, I have found that the endoderm and cardiac defects in fish lacking *aplnr* can be likely attributed to decreased Nodal signaling. I propose a model where Aplnr activity enhances the effect of Nodal signaling that allows for a Nodal threshold to be met at the right time in order to induce the expression of genes required for ingression of lateral marginal cells and proper heart formation. In the absence of *aplnr* function, a longer time frame is required for cells to reach this threshold of Nodal signaling, resulting in a delay in internalization (Figure 3.13A-B). These results are consistent with previous observations on both the non-cell autonomous and temporal roles of Aplnr signaling in cardiac specification (Paskaradevan & Scott, 2012; Scott et al., 2007).
Figure 3.13 – Aplnr is Required to Enhance Nodal Signaling for Proper Cardiac Development.
(A-C) Model for the Nodal-mediated nature of the Aplnr phenotype. In WT embryos (A), Aplnr is required for the appropriate Nodal threshold to be reached in order to initiate the expression of the downstream program to drive the ingresson of cardiac progenitors and their migration towards the anterior of the embryo. In aplnr and apela mutant embryos it takes a longer period of time in order for this threshold to be reached and results in a delay in gene expression and ingresson of the mesendoderm. In Aplnr deficient embryos the levels of Nodal do not accumulate until later in development (B) while in apela mutants (C) Nodal levels may peak before cells are competent to respond. In both cases cardiac progenitors are unable to migrate all the way to the heart forming regions.

3.4.2 Aplnr and Nodal signaling

The link between the aplnr mutant phenotype and Nodal signaling fits well into the context of previously published literature. Nodal signaling establishes the mesendoderm and a loss of Nodal signaling or its downstream transcriptional effectors results in a heartless phenotype (Benjamin Feldman et al., 2002; Kunwar et al., 2003). Furthermore, cells lacking the functional Nodal co-receptor Oep (Cripto) are unable to internalize during gastrulation and cannot contribute to the mesoderm or endoderm (Carmany-Rampey & Schier, 2001). These cells stay at the margin and continue to move towards the vegetal pole. Likewise, aplnra/b morphant cells display delayed ingresson kinetics that do not support proper cardiac development (Paskaradevan & Scott, 2012). However, unlike the loss of Oep, loss of Aplnr results in a partial and not total loss of Nodal activity, and mesendodermal ingresson is evident, albeit at a later time. This also provides a plausible explanation for the incomplete penetrance of the cardiac phenotype observed in aplnra, aplnrb and ela mutant embryos (Chng et al., 2013; Scott et al., 2007). In mouse embryonic stem cells, graded Nodal signaling over 18 hours regulates differentiation to mesendodermal fates, with very subtle (2-fold) changes in levels of phospho-Smads having profound effects (Lee et al., 2011). As gastrulation proceeds far more quickly in zebrafish than it does in mice, this may also explain why profound cardiogenesis defects are not frequently seen in Aplnr/Apj mutant mice (Kang et al., 2013).
My data using the viral insertion allele into zebrafish *aplhra* supports the notion that both paralogues fulfill a common role during cardiogenesis. In contrast to previous work with *aplhr* MOs (Nornes, Tucker, & Lardelli, 2009), I find that *aplhra* mutants do not have epiboly defects, but rather share features of the *aplhrb*<sup>grinch/hur4145</sup> cardiac and endoderm phenotypes. This finding is supported by the fact that a different loss of function allele, *aplhr*<sup>max</sup> generated by our collaborator Dr. Bruno Reversade displays an identical phenotype to our insertional allele (Deshwar at al., submitted). As loss of either *aplhra* or *aplhrb* can have effects on cardiac progenitor specification, I hypothesize that both act in concert to modulate Nodal signaling, with loss of either potentially resulting in a sufficient decrease to impinge on cardiogenesis.

It has been previously demonstrated that distinct Nodal target genes require different Nodal activity thresholds for activation (Y. Chen & Schier, 2001). The level of Nodal signal that a cell perceives depends on both the concentration and duration of the signal (Dubrulle et al., 2015; Hagos & Dougan, 2007). YSL injection of *aplhr* MOs had a more subtle effect on development, with reduced endodermal cell number but no apparent effect on endoderm migration or cardiac progenitor specification, likely reflecting a more moderate effect on Nodal signaling levels. In *aplhr* mutants, given the reduction in Nodal signaling, marginal cells likely require a longer exposure to Nodal ligands before a certain threshold is reached to induce migration and *mesp* expression. This may explain why endodermal progenitors do eventually migrate and why *mesp* expression recovers in *aplhr*<sub>a/b</sub> morphant embryos. In support of this hypothesis, *mesp* expression in *aplhr* morphant embryos at 50% epiboly (5.25 hpf) appears to be retained in the most marginal blastomeres, consistent with the fact that these blastomeres are closest to the source of Nodal ligand. The basic helix-loop-helix Mesp transcription factor family has been shown to regulate the migration of mesoderm through the primitive streak in mice, and play key roles in cardiac development in several contexts (Bondue et al., 2008; Kitajima et al., 2000; Saga et al., 1999; Satou et al., 2004). This provides a molecular mechanism for how reduced levels of Nodal may translate into a delay of cell movement during gastrulation. However, I do not believe that defects in *mesp* expression can fully account for the *aplhr* cardiac phenotype. *mespaa* overexpression was not sufficient to rescue cardiogenesis in *aplhra/b* morphants or mutant. However, finer temporal expression of *mesp* expression may be required for proper cardiac specification.
3.4.3 Aplnr autonomy and mechanism of action

The cellular autonomy of Aplnr function in cardiac progenitor development has been an area of confusion, notably as both cell autonomous (Scott et al., 2007; Zeng et al., 2007) and non-autonomous (Paskaradevan and Scott, 2012) roles have been documented. My results clearly show that Aplnr is not absolutely required in cells expressing the Nodal ligands for ligand secretion or activity. Furthermore, I have also bolstered previous data suggesting that the Aplnr is not strictly required in Nodal receiving cells either with my experiments in which I knocked down Aplnra/b in the YSL. Altogether this suggests that the Aplnr may be required to modulate Nodal signaling in the extracellular space through perhaps acting on Nodal ligand stability and/or processing in aplnr morphant embryos.

In both aplnr and ela deficient embryos I find aberrations in the levels of both unprocessed and processed exogenous squint ligand although my findings are not wholly consistent as aplnr loss of function appears to demonstrate a defect in processing while ela loss of function indicates an effect on stability. These two phenomena are intimately linked which may explain the discrepancy (Good et al., 2005). My analysis at this point has been limited to the over-expression of a GFP fusion that undoubtedly does not mimic the endogenous dynamics of the Nodal ligands. Through the generation of specific antibodies to the Nodal ligands I may be able to see a clearer picture of the effect of aplnr loss on their dynamics and this is an interesting avenue for future study.

A recent report has suggested that in zebrafish embryos unprocessed Nodal ligands are not secreted which brought into question whether or not the Aplnr could be acting in a non-cell autonomous manner to regulate Nodal processing (Tessadori et al., 2015). In my hands, using a sensitive Nodal point source assay, I found that a cleavage site mutant form of Sqt is secreted. All previous work in mouse also suggests that Nodal ligands are processed extracellularly, following secretion (Beck et al., 2002). I have attempted to rescue the Aplnr phenotype with injection of Furins/Pace4 that catalyze Nodal cleavage but was not successful (results not shown). It may be that subtle changes in the kinetics of Nodal ligand processing that occur in aplnr mutants are instrumental for the cardiac progenitor defects.
It is also possible that rather than playing a strictly cell autonomous (in cardiac progenitors) or non-autonomous (in Nodal signal sending cells) role, a threshold level of Aplnr activity is required to ensure that proper levels of Nodal signaling occur. The interpretation of the transplantation experiments may therefore be confounded by the size of donor tissue. The role of a “community effect” in amplifying the Nodal signal to drive collective epithelial-to-mesenchymal transition during gastrulation has recently been described (Voiculescu, Bodenstein, Lau, & Stern, 2014). As Aplnr is both activated by Nodal (at the level of gene expression) and in turn potentiates Nodal signaling, this may provide a feed-forward mechanism to help achieve maximal Nodal signaling for proper gastrulation in a timely manner. Mechanistically, how Aplnr activity impinges on the Nodal pathway (and perhaps Nodal ligand processing) remains to be determined. Signaling cascades downstream of Aplnr, both G protein-dependent and -independent, have been described. Which of these are required for Aplnr function in cardiac development, or if a new pathway is involved, remains to be elucidated.

An unconventional but intriguing possibility involves the ability of Aplnr to be activated by stretch, as previously documented in adult cardiomyocytes (Scimia et al., 2012). Mechanical stretch has recently been implicated as a cue for cell fate determination and pre-stress of the ECM has been shown to affect TGF activity (Klingberg et al., 2014). In particular, mechanical strain experienced by invaginating mesendodermal precursors is crucial for expression of ntl and mesodermal specification in zebrafish (Brunet et al., 2013). One conceivable scenario is that Aplnr is activated by strain during epibolic movements, leading to a potentiation of the Nodal pathway. Future work is necessary to investigate whether the Aplnr acts as a stretch receptor in the context of early gastrulation and how this may mediate Nodal signaling.

### 3.4.4 Apela antagonism of the Aplnr

In this study I find several pieces of evidence that suggest a difference in the phenotypes of Aplnr and Apela loss of function. This data complements that found by our collaborators Dr. Bruno Reversade and his lab members Dr. Serene Chng and Dr. Leno Ho. They have made a number of interesting findings which directly relate to my dataset and shall be summarized. They have found that apela mutant embryos are actually less sensitive to Nodal chemical inhibition, that they display a slight increase in ARE-luciferase activity (a reporter of Nodal signaling levels) when compared to WT and that increasing Nodal levels in the embryo using lefty1/2
morpholinos actually leads to an exacerbation of the apela mutant phenotype. Furthermore, they find that by treating apela mutant embryos with a chemical antagonist of the Aplnr ML221 they are actually able to rescue the cardiac phenotype to a certain extent suggesting that Apela does in fact act to antagonize the Aplnr for proper cardiac development. Taken all together I believe that there is strong evidence that Apela acts as an antagonist for the Aplnr although this leads to questions around how this fits into my current model.

An important question is how the loss of the receptor and its antagonist can both result in a delay in migration during gastrulation. Both Aplnr and Apela loss-of-function result in the delay in the ingress of lateral marginal cells and a delay in the expression of the pro-cardiac transcription factor mespaa/ab (data for Apela not shown) and yet both show an opposite effect on Nodal signaling in the embryo. It is well documented that the timing and level of Nodal signaling is crucial for the proper specification and migration of the mesendoderm (Hagos and Dougan, 2007). I hypothesize that in Ela loss-of-function embryos Nodal signaling attains its peak too early in development, before mesendodermal progenitors are properly competent to respond (Figure 3.13C). Providing support to this hypothesis is a recent study that demonstrated that some Nodal target genes cannot be induced until proper competency has been achieved by the cell (Dubrulle et al., 2015). If Nodal levels were to peak too early in development, by the time cells became competent to perceive the signal, overall Nodal levels would be lower than required to push cells to the proper threshold in a timely fashion and a delay in migration/target gene expression would result. Further study and a better method to visualize Nodal dynamics in time and space in the embryo will be required to better apprehend this phenomenon.

### 3.4.5 Aplnr and its effect on the lateral plate mesoderm

An interesting question is why the ALPM is particularly affected by dampened levels of Nodal in Aplnr loss of function. Recent work suggests that cardiac progenitors, which arise from the ALPM, are specified very early during gastrulation, and as such these cells may be exquisitely sensitive to Nodal signaling levels and timing (Devine et al., 2014; Lescroart et al., 2014). Furthermore, these cells are among the first to ingress during gastrulation and travel the greatest distance to the far animal pole to reach their target destination. It is possible that a delay in their migration makes it impossible for them to travel this large distance in time. Alternatively, while subsequent migration from the lateral margin does occur, it may be that these cells require an
earlier specification event to occur to properly migrate to the ALPM and form cardiac mesoderm. Teasing apart the migration versus differentiation phenotypes, which are intimately linked, will require more detailed analysis of gene expression signatures in cardiac progenitors. As to why lateral populations are specifically affected and not dorsal ones where Nodal signaling is particularly prevalent, we speculate that the levels of Nodal modulated by Aplnr will not have as large of consequence in a high Nodal signaling environment like the shield/dorsal aspect of the embryo. Furthermore, only *aplnra* and not *aplnrb* is expressed in the dorsal part of the embryo (Tucker et al., 2007).

### 3.5 Summary

In conclusion, I find that Aplnr is required to enhance Nodal signaling in order to activate genes required for proper cell movement and consequently cardiac development at the right time. This work opens several lines of future investigation on the early events required for the movement of the mesendoderm during gastrulation and early cardiac progenitor development. The levels and timing of key signaling pathways such as Nodal/TGFβ are absolutely essential for…. and my work demonstrates how subtle changes in this pathway can have a dramatic impact on development. In addition, the non-autonomous effect on Nodal ligand dynamics opens up an intriguing site of pathway regulation that at this point has not been explored. Similar mechanisms to those described here for Aplnr signaling may also therefore remain to be discovered for other major developmental pathways. Further, given that Aplnr signaling has been shown to regulate multiple aspects of adult physiology, the role of this novel signaling mechanism and the potential functions of Apelin and Apela in the context of physiological homeostasis and disease are areas of great interest.
Chapter 4

4.1 Mespaa has a potent and conserved pro-cardiac function in zebrafish

4.2 Introduction

Cardiogenesis is first apparent in the vertebrate embryo based on the expression of genes in the cardiac mesoderm (*Nkx2.5* and *Isl1* being key examples) that mark cells that will contribute to the heart (Cai et al., 2003; Guner-Ataman et al., 2013). Expression of these markers is orchestrated by a network of core cardiac transcription factors (including Gata4/5/6, Mef2c and Hand1/2) that is highly conserved (Lazic & Scott, 2011; Peterkin et al., 2007; Deborah Yelon et al., 2000; Zhao et al., 2008). Definitive markers of restricted cardiac progenitors prior to *Nkx2.5/Isl1* expression are not known however using both traditional cell tracing and genetic strategies these cells have been identified to be present as early as the onset of gastrulation (Devine et al., 2014; D. Y. Stainier et al., 1993). In zebrafish, cells that contribute exclusively to atrial or ventricular myocardium can be identified at 5 hours post-fertilization (hpf) (Keegan et al., 2004). Similarly, clonal analysis in mouse has shown that the first and second heart field lineages of cardiac progenitors diverge at early gastrula stages (Devine et al., 2014; Lescroart et al., 2014).

How and when cardiac progenitors are first specified, the transcriptional program guiding this process and the migration cues responsible for the movement of these cells during gastrulation remains unknown. One set of genes that has been implicated in all of these processes is the *Mesp* family, which encode basic helix-loop-helix (bHLH) transcription factors. In mouse, *Mesp1* and *Mesp2* are both expressed in the gastrulating mesoderm as it moves through the primitive streak (Kitajima et al., 2000; Saga et al., 1996). Loss of both *Mesp1* and *Mesp2* in mice leads to the impaired migration of mesodermal cells during gastrulation, resulting in their accumulation at the primitive streak and a complete absence of cardiac mesoderm (Kitajima et al., 2000; Saga et al., 1996). The role of *Mesp1/2* as an important regulator of cardiac development may be widely conserved as the loss of *Cs-Mesp* in the sea squirt *Ciona savigni* results in the complete absence of the heart (Satou et al., 2004). However, morpholino-mediated knockdown of *Mesp*
orthologues in *Xenopus* only resulted in minor cardiac defects (Kriegmair et al., 2013).

Comprehensive loss of function data in other model systems is lacking with respect to Mesp function in cardiogenesis.

Over-expression of *Mesp1* in embryonic stem (ES) cells leads to the up-regulation of the core cardiac transcription factors and promotes the formation of cardiac cells (Bondue et al., 2008). While once postulated as a master regulator of cardiac development given its ability to direct cells to form cardiac cells *in vitro*, Mesp1 appears to play an important role in the generation of a broad range of mesodermal tissues (Bondue et al., 2008; Chan et al., 2013). Depending on the time of induction and culture conditions, *Mesp1* over-expression is able to direct mouse ES cells into hematopoietic, cardiac or skeletal myogenic cell populations. Furthermore, lineage tracing of *Mesp1*-expressing cells in mice revealed contributions to the yolk sac, facial muscle and haematopoietic lineages (Chan et al., 2013; Yoshida et al., 2008). These observations contrast those made in other model systems and raise questions about the important nature of the *Mesp* genes in cardiac development across species.

While the exact timing and mechanism of early cardiac progenitor specification remains unclear, recent evidence has suggested that this may occur early in development. Single *Mesp1*-expressing cells marked during early gastrulation in mice were found to either contribute to cardiac or non-cardiac tissues exclusively, with no clones contributing to both populations (Devine et al., 2014). Furthermore, the timing of *Mesp1* expression appears to distinguish the first from second heart field progenitors in the early gastrula embryo, with each population possessing a unique molecular signature suggesting an early divergence of these two lineages (Lescroart et al., 2014). Subsequent to Mesp1, Gata family members are key initiators of the cardiogenic program, with mouse *Gata4/6* mutants being acardiac (lacking a heart) (Zhao et al., 2008). In zebrafish the cardiac transcriptional network is initiated by *Gata5/6* (Holtzinger and Evans, 2007; Lou et al., 2011; Peterkin et al., 2007). Embryos lacking these two genes completely lack a heart and early cardiac gene expression including *nkx2.5*, *hand2* and *tbx5*. Expression of *gata5* is evident with mesendoderm formation in zebrafish, well before the onset of *nkx2.5* expression (Reiter et al., 1999). When Gata5/6 activity is first required during cardiac development, and its relationship to Mesp activity, remain key questions to be addressed.
In this study I investigated the role of the *mesp* family of transcription factors in zebrafish cardiovascular development. Through gain-of-function approaches I found that Mespaa is the only Mesp family member capable of making cells competent to form cardiac tissues at a high frequency. I further found that *mesp* family members are capable of promoting a variety of mesodermal fates including vascular endothelium and facial muscle. Strikingly, *mespaa* null mutants displayed no defects in cardiovascular specification and development, with further depletion of other *mesp* family members having no effect. However, a large percentage of *mespaa* null embryos displayed defects in cardiac laterality. Using *mespaa* over-expressing cells as an *in vivo* model for cardiac progenitor development, I found that while *mespaa* expression is required early in gastrulation for pro-cardiac activity, initiation of *gata5* expression and unique cellular behaviors in these cells were not apparent until the completion of gastrulation. This suggests an extended time period in which *mesp* activity is required to poise cardiac progenitors for the initiation of the core cardiac transcriptional network. Taken together, my work suggests that while retaining its ability to promote cardiac progenitor formation, *mesp* gene function may be dispensable for the cardiac regulatory network in zebrafish.

### 4.3 Results

#### 4.3.1 *mespaa* is sufficient to direct cells to a myocardial fate

Zebrafish possess four *mesp* family genes, *mespaa/ab* (homologues of *Mesp1*) and *mespba/bb* (homologues of *Mesp2*) (Cutty et al., 2012; Sawada et al., 2000). In order to evaluate a role for these genes in cardiac development, animal cap transplants were performed in which *mesp* over-expressing cells from *myl7:EGFP* transgenic embryos were placed in the animal cap of host embryos and the ability of these cells to contribute to the myocardial lineage (based on GFP expression) was evaluated at 48hpf (Figure 4.1A). As a control, the closely related bHLH family member *msgn1* (*mespo*) was also examined. Donor embryos were injected with a rhodamine conjugated dextran that allows donor cells to be traced in the host embryo. Wild-type (WT) cells placed in this region of the host embryo do not contribute to cardiac tissues (Lou et al., 2011), providing a robust readout of potential pro-cardiac activity. Strikingly, when *mespaa* over-expressing cells were transplanted into the animal cap, at 48hpf they were found to contribute to
an appreciable portion of the myocardium. This was evident from the large number of GFP +’ve/rhodamine +’ve cells that could be observed throughout the heart in host embryos (Figure 4.1F-H). mespaa over-expressing cells contributed to the heart at a very high frequency, with 81% of host embryos containing GFP +’ve myocardial cells (Figure 4.1B). This was much greater than what has been observed for gata5 (25%) or gata5/smarcd3b (50%) over-expressing cells in an identical assay (Lou et al., 2011). Surprisingly, none of the other three mesp family members were able to efficiently promote a myocardial fate, with transplantation of mespab, mespba and msgn1 over-expressing cells all failing to result in observable myocardial contribution (Figure 4.1B and I-K). While mespbb did demonstrate pro-cardiac activity, this was only evident in a far lower (18%) number of host embryos (Figure 4.1B). I do not believe these results reflect improper expression of some mesps, as 1) both higher and lower concentrations of RNA were attempted for all five genes with no significant differences observed (data not shown); and 2) mesp family members were as active as mespaa in this assay when other cell fates were analyzed (see Figure 4.2). Together these results indicated that despite the extreme redundancy of this gene family, mespaa is the sole cardiogenic mesp in zebrafish.
Figure 4.1 – Mespaa is the only potent cardiogenic Mesp in zebrafish.
Given the ability of Mespaa but not Mespab to promote myocardial fate, I next sought to identify motifs or regions of Mespaa critical for this activity. All 4 zebrafish Mesps have high sequence conservation in the bHLH DNA-binding and dimerization domain, with little similarity evident in regions N- and C-terminal to this (Figure 4.2A). To identify cardiogenic motifs I created chimeric Mesps in which I exchanged the N- or C-terminal portions of Mespaa and Mespab and evaluated pro-cardiac activity. Interestingly, the addition of either the N- or C-terminal domain of Mespaa to Mespbb was able to confer some pro-cardiac activity (Figure 4.2B, fusions 2 and 3). In contrast, the addition of either the N- or C-terminal domain of Mespab to Mespaa had little to no effect on its ability to drive myocardial contribution (Figure 4.2B, fusions 1 and 4). Given that there are no conserved motifs between the N- and C-terminal regions of Mespaa, this likely suggests that the potent ability of Mespaa to drive cardiac fates is the result of the combinatorial activities of several motifs. To further investigate the functional properties of Mespaa two additional mutants were generated, one with mutations in key residues (KLR to EDE) in the basic motif that prevent DNA-binding activity in other bHLH factors, and the other in the dimerization domain (F113P) that prevents dimerization of many bHLH factors (Schindler et al., 2014). I pursued these studies as previous work on the cardiogenic bHLH transcription factor Hand2 surprisingly found that its ability to promote cardiomyocyte differentiation was independent of its DNA-binding activity (Schindler et al., 2014). When over-expressed both Mespaa mutants were unable to drive myocardial contribution (Figure 4.2B), suggesting that Mespaa promotes cardiogenesis as a DNA-bound transcription factor dimerized with other (b)HLH factors.
Figure 4.2 – Pro-cardiac activity of Mespaa maps to both N- and C-terminal portions of the protein.

(A) Schematic comparing the protein sequence of all four zebrafish Mesp family members. (B) Schematic of various Mesp fusion and mutant constructs and the ability of these constructs when RNA was over-expressed to confer pro-cardiac activity to transplanted cells in the animal cap.
4.3.2 *mespaa* over-expressing cells are capable of forming multiple mesodermal lineages

While *Mesp1* has been previously postulated as a master regulator of heart development, recent evidence suggests that it is critical for the development of multiple mesodermal lineages (Chan et al., 2013; Devine et al., 2014). In order to evaluate the potential of *mesp* expressing cells, animal cap transplants were performed using transgenic lines indicative of various cell fates as donors. I first wanted to determine whether *Mespaa* and other Mesps are capable of driving cells to other cardiovascular lineages: the vascular endothelium and endocardium. Cells from *kdrl:EGFP* (expressed in all vascular endothelium, including endocardium) transgenic donor embryos either injected or not injected with *mesp* RNA were placed in the animal cap of host embryos, with transplants examined at 48hpf. As expected, in controls (no *mesp* RNA) WT cells placed in the animal cap never contributed to the endocardial lineage (Figure 4.3A-C, G). In contrast, *mespaa* over-expressing cells contributed with a high propensity to the endocardium, as demonstrated by GFP positive cells found throughout the heart (Figure 4.3D-F). This occurred at a very high frequency (85%, Figure 4.3G) and in many cases *mespaa* over-expressing cells were found to make up what appears to be the entire endocardium. Intriguingly, GFP +`ve cells were not restricted to the heart but instead were found throughout the vasculature, suggesting that *mespaa* over-expressing cells can form vascular endothelium as well (Figure 4.3D-F). While *mespaa* was the only zebrafish *mesp* capable of making cells competent to form myocardium at a high frequency, I wondered if the other *mesp* family members might be able to make cells differentiate into vascular endothelium and/or endocardium. Interestingly, all three additional *mesp* family members were capable of making cells competent to form endothelial cells (Figure 4.3G). Of these, only *mespbb* was able to promote endocardial fate at a high frequency (52%). This provides further evidence that *Mespaa* is the most potent cardiogenic (cardiomyocyte and endocardium) *Mesp* in zebrafish, with *Mespbb* as the other *Mesp* family member possessing pro-cardiac activity.

Recent experiments have shown that cardiac progenitors of the second heart field share a common progenitor with craniofacial muscle (Lescroart et al., 2010; Tirosh-Finkel, Elhanany, Rinon, & Tzahor, 2006). To further evaluate the potential of *mesp* over-expressing cells, an *acta:EGFP* transgenic line (expressed in skeletal muscle) was used in transplantation
experiments (Higashijima et al., 1997). Control (un-injected) donor cells were not observed to contribute to facial muscle (Figure 4.3H-J). In contrast, over-expression of \textit{mespaa}, \textit{mespba} and \textit{mespbb} were all sufficient to promote facial muscle fate at a high frequency, whereas \textit{mespab} and \textit{msgn1} were not competent to do so (Figure 4.3K-M, N). This provides further evidence that Mesps convey a more general mesodermal potential, as opposed to one that is cardiac-restricted.

As my over-expression experiments were carried out in the artificial context of the animal cap, where \textit{mesp} gene expression and mesendodermal specification do not normally occur, I next addressed the effects of \textit{mespaa} overexpression at the margin. The margin (where cells of the embryo meet the yolk) is the \textit{in vivo} site where mesendoderm specification is initiated. Our lab has previously shown that donor cells over-expressing \textit{gata5} placed in the margin are strongly biased towards cardiac contribution (Lou et al., 2011). Strikingly, \textit{mespaa} over-expressing cells, when placed at the margin, contributed to the myocardium at the same frequency as WT cells (25%). This suggests that in the context of the margin \textit{mespaa} expression does not have the ability to drive cells to a cardiac fate at a high frequency. This may be the consequence of inhibitory signals that act at the margin to restrict the cardiac lineage that are not present in the animal cap.
Figure 4.3 – Mesp over-expressing cells are capable of differentiating into endocardium, vascular endothelium and facial muscle.

(A-F) Host embryos at 48hpf generated from animal cap transplants of rhodamine dextran labelled kdrl:EGFP donor cells of WT (A-C) or mespaa over-expressing cells (D-F). (G) Percent of host embryos with GFP positive cells in the endocardium and vasculature or vasculature alone when different mesp family members were over-expressed in the transplant assay. (H-M) Host embryos at 48hpf generated from animal cap transplants of rhodamine dextran labelled acta:EGFP donor cells of WT (H-J) or mespaa over-expressing cells (K-M). (N) Percent of host embryos with GFP positive cells in the head when different mesp family members were over-expressed.
4.3.3  

*mespaa* is not required for zebrafish cardiac specification

Given the potent ability of *mespaa* to make cells placed in the animal cap competent to form cardiac tissues, I next evaluated if *mespaa* is essential for cardiac development. CRISPR-Cas9 mediated targeting was used to generate indel mutations in *mespaa*. The *mespaa*\(^{hsc11}\) allele, in which a premature STOP codon is located upstream of the bHLH domain, was chosen for further analysis as it would be expected to be a full loss-of-function allele (Figure 4.4A). This was confirmed in animal cap transplants via over-expression of the mutant *mespaa* in *myl7:EGFP* donor embryos, in which no myocardial contribution was observed (0/42, 0%). Unexpectedly, *mespaa*\(^{hsc11}\) homozygous mutant embryos displayed no overt embryological defects (Figure 4.4B-C). Both *nkx2.5* (cardiac progenitors) and *myl7* (differentiating cardiomyocytes) expression appeared normal in these embryos at 16 and 24hpf, respectively. Further, 48-120hpf *mespaa*\(^{hsc11}\) mutant embryos displayed no pericardial edema, a typical indicator of cardiovascular defects (Figure 4.4B-C,F,J). While in my over-expression assays *mespaa* demonstrated the most potent pro-cardiac activity, it of course is possible that the other *mesp* family members may play redundant functions in heart development. Furthermore, it is possible that even if they are not normally required in heart development, they may become upregulated to assume *mespaa* function when it is lost. To address these two questions, I decided to make use of morpholino antisense oligonucleotides to knockdown function of Mespab, Mespba or Mespbb in a WT background. All knockdown experiments were performed and imaged by a graduate student in the lab, Chris Onderisin. Individual morpholinos to the other *mesp* family members were found to have no effect on heart formation in a WT background as demonstrated by normal *myl7* expression at 48 hpf (Figure 4.4F-I). Furthermore, no effect on heart specification was observed when these morpholinos were injected into *mespaa*\(^{hsc11}\) null mutants, suggesting that they are not compensating for *mespaa* to regulate heart development in its absence (Figure4.4J-M). These results suggest that while overexpression of (in particular) *mespaa* can drive cardiac fate, *mesp* function may not be essential for zebrafish heart formation.
Figure 4.4 – Mespaa is not required for heart formation in zebrafish.

(A) Schematic of the mespaa<sup>hsc11</sup> null allele that was generated. (B-C) Whole embryo morphology of WT and mespaa<sup>hsc11</sup> null mutants at 48hpf. (D-E) nki2.5 expression in WT and
mespaa^{hsc11} null embryos at 16hpf. (F-M) myl7 expression in WT and mespaa^{hsc11} null mutant embryos alone or in combination with morpholinos against mespab, mespba or mespbb.

While performing my loss-of-function analyses, I noticed an elevated prevalence of laterality defects in cardiac looping. Chris Onderisin, a graduate student in the lab, performed detailed analysis of this and the results are as following. While normal (rightward) cardiac looping proceeded in the vast majority (99.4%) of WT embryos, mespaa^{hsc11} mutant embryos displayed an elevated (18%) frequency of reversed (leftward) looping (Figure 4.5B-C). Morpholino mediated knockdown of Mespab, Mespba or Mespbb in WT embryos also displayed laterality defects and exacerbated the phenotype of mespaa^{hsc11} mutants (Figure 4.5C). This suggests that while not playing an essential role in cardiac progenitor specification, the mesp family plays a key role in cardiac looping. Examination of the expression of lefty1/2, a marker of left-right asymmetry, revealed significant defects in mesp deficient embryos (Figure 4.5H). Interestingly, loss of mespaa appeared to increase the percentage of embryos displaying bilateral lefty1/2 expression while loss of the other mesp family members appeared to increase the percentage of embryos with absent lefty1/2 expression.
Figure 4.5 – Cardiac looping in Mesp deficient embryos is perturbed

(A-B) Normal and reversed heart looping demonstrated with myl7 expression at 48hpf. A demarcates the atrium and V indicates the ventricle. (C) Percent of embryos displaying normal or reversed heart looping when deficient for different mesp family members alone or in combination when compared to WT. (D-G) The four categories of lefty1/2 expression observed
in WT and Mesp deficient embryos. (H) Percent of embryos displaying the different *lefty1/2* expression patterns in WT and Mesp deficient embryos.

### 4.3.4 *mespaa* is required early in gastrulation for pro-cardiac activity

The *mespaa* over-expression/animal cap assay provides a useful tool to examine key questions in the earliest steps of cardiac specification. Using this system, I decided to investigate the temporal requirement for *mespaa* in cardiogenesis. To address this question, a *mespaa-ecr* (Ecdysone Receptor) fusion was generated, allowing regulated nuclear localization of *mespaa* in response to the addition of tebufenozide (Esengil, Chang, Mich, & Chen, 2007). Animal cap transplants were repeated using *myl7:EGFP* donor embryos injected with *mespaa-ecr* RNA, with tebufenozide added to post-transplantation hosts at various time points (Figure 4.6A). As I found that injection of *mespaa-ecr* RNA resulted in leaky (present without tebufenozide addition) pro-cardiac activity, graduate student Chris Onderisin generated a mutant *mespaa-Δnls-ecr* fusion construct in which two putative nuclear localization sequences in the *mespaa* sequence were disrupted (see Methods). I then performed animal cap transplants using *mespaa-Δnls-ecr* expressing cells and found that they did not result in any contribution to the heart in the absence of tebufenozide (Figure 4.6B). Interestingly, while addition of tebufenozide at 4hpf was sufficient to promote myocardial contribution (20% of transplants - the NLS mutant had attenuated activity), addition at 6hpf resulted in very little pro-cardiac activity and addition in 8hpf had no effect (Figure 4.6B). This suggests that *mespaa* is required early in development, at the onset of gastrulation (before 6hpf) for pro-cardiac activity.

I next wanted to examine how the behavior of cells driven to a cardiac fate is altered by *mespaa*. A post-doctoral fellow in the lab, Dr. Ana Aleksandrova, performed the following experiments to address this question. Co-transplantation of *mespaa* and *mespab* over-expressing cells, distinguishable by different lineage labels, was used to directly compare transplanted cells driven to a cardiac fate (by *mespaa*) to those where this does not occur (*mespab*). Transplants were imaged over a time course from the start of gastrulation at 5.5 hpf up until mid-somite stages at 16.5 hpf. *mespab* over-expressing cells did not exhibit any unique behaviours throughout this time course and remained dispersed at the site of transplantation, with no indication of migration
or clustering (Figure 4.6C-F, red cells). Interestingly, *mespaa* over-expressing cells exhibited similar behaviours to *mespab* expressing cells during gastrulation and remained relatively immotile. However, upon the completion of gastrulation *mespaa* over-expressing cells began to cluster together and display signs of unique behaviour (Figure 4.6C-F, green cells). By 16.5 hpf very tight clusters of *mespaa* over-expressing cells were observed (denoted by arrows), in contrast to *mespab* over-expressing cells which remained dispersed. Together this suggests that *mespaa* over-expressing cells display unique cellular behaviours, but only after the completion of gastrulation.
Figure 4.6 – Mespaa is required early in gastrulation for pro-cardiac activity but over-expressing cells do not exhibit unique cellular behaviours until after its completion.

(A) Schematic outlining the strategy for exploring the temporal requirement for mespaa in pro-cardiac activity. (B) Percent of host embryos with GFP+ cells at 48hpf after animal cap transplantation of mespaa-Δnls-ecr over-expressing cells with tebufenozide addition at different
time points. Data are represented as means ± SEM. (C-F) Time-lapse images of animal cap transplants of mespaa and mespab over-expressing cells together at different stages in development.

4.3.5 Mespaa sits upstream of gata5/6 during cardiac progenitor development

Given the position of Gata5/6 near the top of the transcriptional network governing cardiac specification in zebrafish, I next examined how Mespaa fits into this hierarchy. Donor myl7:EGFP embryos were co-injected with mespaa RNA and morpholinos targeting gata5/6, and the ability of these cells to contribute to the heart in host embryos was evaluated. Interestingly, these cells were not able to contribute to the myocardium (0%), as indicated by the lack of GFP +’ve cells in the heart of host embryos (Figure 4.7A-C). I next evaluated if these cells could contribute to the endocardium by using kdr1:EGFP donor embryos. Interestingly, mespaa over-expressing cells also injected with gata5/6 morpholinos were not able to contribute to the endocardium at a high frequency as compared to mespaa over-expressing controls, and in transplants where contribution was evident only a small number of GFP +’ve endocardial cells were apparent (Figure 4.7D-J). This is in stark contrast to mespaa over-expressing cells alone where in many cases the entire endocardium was made up of donor cells (Figure 4.7E). In contrast, mespaa over-expressing cells injected with gata5/6 morpholinos remained capable to contribute to the vasculature at a high frequency (Figure 4.7D-J). These results suggest that gata5/6 lie downstream of mespaa with respect to cardiac (cardiomyocyte and endocardium) development but not for formation of the non-cardiac endothelium.
Figure 4.7- Mespaa sits upstream of gata5/6 with respect to cardiac, but not endothelial, development.
(A-C) Animal cap transplants of myl7-EGFP donor embryos over-expressing mespaa and co-injected with gata5/6 MOs. (D-J) Animal cap transplants of kdrl-EGFP mespaa over-expressing cells co-injected with gata5/6 MOs. Area of the heart is outlined with a dashed line. Arrows indicate kdrl-EGFP positive cells in the heart. Quantification of contribution is provided in (J).

4.3.6 mespaa over-expressing cells activate gata5 expression after the completion of gastrulation.

Given the key role of gata5/6 in the pro-cardiac activity of Mespaa, I next examined when gata5 expression is initiated in response to mespaa overexpression. While a role for Gata5/6 in cardiac specification in zebrafish is clear, the temporal nature of this activity remains unknown. To address this question I made use of a gata5:EGFP BAC transgenic line that was generously provided by the lab of Dr. Kenneth Poss (K. Kikuchi et al., 2011). I first wanted to confirm that the transgenic line faithfully re-capitulated gata5 expression during and after gastrulation. Chris Onderisin performed in situ hybridization looking at both gata5 and gfp expression in embryos from this transgenic line at 6,10 and 14hpf. At all three stages while gfp expression was stronger than gata5, expression occurred in a near identical pattern validating the use of this line for further analysis (Figure 4.8).
Figure 4.8 – *gata5* and *gfp* are expressed in the same pattern in *gata5:EGFP* transgenic embryos.

(A-F) *gata5* and *gfp* expression in *gata5:EGFP* embryos at 6, 10 and 14 hours post fertilization. Embryos are viewed from the animal cap, lateral and dorsal sides.
To determine when *gata5* was first expressed in *mespaa* over-expressing cells, transplanted cells were first examined at bud stage (10hpf), at the completion of gastrulation. Interestingly, GFP expression was not observed in any hosts at this stage either when control or *mespaa* over-expressing *gata5:EGFP* donor cells were used (Figure 4.9A-B). Examination of transplant embryos at gastrula stages (6-10hpf) similarly did not reveal earlier GFP expression. In contrast, examination of embryos at the 6-8 somite stage (12-13hpf) revealed robust GFP expression in donor cells over-expressing *mespaa*, but not in WT (un-injected) donor cells (Figure 4.9C-D).

Given the heterogeneity of donor cells in transplant embryos, in a complementary approach FACS was used to isolate rhodamine-labeled donor cells at different stages of development, which were examined and quantified for GFP expression. Confirming previous observations, no GFP signal was observed in cells at 10hpf in either *mespaa* over-expressing cells or WT controls (Figure 4.10A-B). A low number (1.4%) of *mespaa* over-expressing donor cells demonstrated GFP expression at 11.5hpf, with this increasing to 6.4% at 13hpf (Figure 4.10C-F). Control cells at all stages did not display any GFP expression. These results suggest that *mespaa* over-expressing cells do not induce *gata5* expression until the completion of gastrulation. Therefore *gata5*, while expressed during early gastrulation stages, may not be required for cardiac specification until close to the end or after the completion of gastrulation. These experiments were carried out with the help of Dr. Ana Aleksandrova who did all confocal imaging and a graduate student in the lab Xuefei Yuan who performed the flow activated cell sorting (FACS).
Figure 4.9 – *mespaa* over-expressing cells do not induce expression of a *gata5-EGFP* reporter until after gastrulation

(A-D) Host embryos with transplanted WT or *mespaa* overexpressing donor cells (*gata5:EGFP*, labelled with rhodamine dextran) at the bud stage (A-B) and the 6-8 somite stage (C-D).
Figure 4.10 – *mespaa* over-expressing cells do not induce expression of a *gata5:EGFP* reporter until after gastrulation when examined by FACS

(A-F) Plot of FACS sorted rhodamine dextran labelled *gata5:EGFP* donor cells from host embryos at different stages. Cells were either WT (A,C,E) or *mespaa* overexpressing cells (B,D,F).
4.4 Discussion

4.4.1 Mespaa is a conserved regulator of cardiac development in zebrafish

In this chapter I provide evidence implicating Mespaa as a conserved but non-essential regulator of cardiac development in zebrafish. Surprisingly, despite duplication of this gene family in zebrafish, Mespaa is the sole Mesp with strong cardiogenic potential. Consistent with recent findings in mice and cultured stem cells (Chan et al., 2013), I find that Mespaa does not act as a cardiac-specific regulator but instead is able to drive the development of multiple mesodermal lineages, including vascular endothelium and facial muscle. Interestingly, despite a conserved function in the promotion of ectopic cardiac progenitor development, I find that mespaa is not essential for zebrafish heart development. Depletion of Mespaa along with other Mesp family members does not seem to have any consequence on cardiovascular formation. Altogether these results provide much needed resolution to the question of whether mesp genes play a role in zebrafish heart development and clarifies the regulatory hierarchy of cardiovascular specification in this system and others.

The non-essential nature of mespaa in zebrafish cardiac development raises a number of interesting questions. In mice, Mesp1 is absolutely essential for the migration of the cardiac mesoderm, and Mesp1/2 double mutants completely lack a heart (Kitajima et al., 2000; Y Saga et al., 1999). In the absence of Mesp function in mice, cardiac progenitors, along with other mesodermal tissues fail to properly ingress during gastrulation. The fact that heart development occurs normally in zebrafish mesp depleted embryos suggests that other genes instead fulfill these key functions. While Nodal signaling has been demonstrated to be essential for this process, the downstream targets which drive this process in fish are completely unknown (Carmany-Rampey & Schier, 2001). While in my previous chapter I demonstrate that the Aplnr is important for the proper induction of these downstream targets, the identification of which targets are critical in this process requires future study. It should be noted that in the absence of a quadruple mespaa/ab/ba/bb mutant, it cannot be definitively determined that mesp is not required for cardiac development. Despite this, I believe that this may in fact be the case. Based on my findings, mespaa, and to a lesser extent mespbb, are the only two mesp family members able to drive cardiac formation in the zebrafish embryo. The fact that depletion of the two
together has no consequence on embryonic development is quite compelling. Furthermore, depletion of the other two \textit{mesp} family members in a \textit{mespaa} mutant background did not yield any major defects in cardiac specification. It is unlikely that these observations reflect a simple failure of the morpholinos to knock down gene function given that the knockdown of individual \textit{mesps} in \textit{mespaa} mutants increased the penetrance of cardiac laterality defects.

\textbf{4.4.2 Mespaa and the core cardiac transcription factor network}

The ability of \textit{mespaa} to promote cardiac fates in cells placed in the animal cap is quite unique. This phenomenon has been observed only once before through the over-expression of \textit{gata5} (Lou et al., 2011). Other core cardiac transcription factors such as \textit{nkx2.5}, \textit{hand2} and \textit{mef2cb} do not recapitulate this activity (A.R.D. and I.C.S. unpublished observations). What is also intriguing is the variety of cell types that \textit{mespaa} over-expressing cells can contribute to including vascular endothelium and facial muscle. This is in contrast to previous studies on \textit{gata5}, in which over-expressing cells demonstrated a more restricted potential, best illustrated by the lack of contribution to the vascular endothelium but ability to contribute to the endocardium, cardiomyocytes and smooth muscle of the heart (Lou et al., 2011). This along with other lines of evidence presented earlier confirm findings in other model systems suggesting that \textit{mesp} sits upstream of the core cardiac regulatory network. This taken together with the fact that Mesp function is not required for cardiac progenitor specification leads me to propose a model in which Nodal signaling in zebrafish bypasses the activation of \textit{mesps} and instead directly acts on Gata5/6 which in turn drive heart development (Figure 4.11). Nodal regulation of \textit{gata5} expression has previously been documented and as mentioned earlier loss of Nodal signaling results in the complete loss of the heart as well (Dougan et al., 2003; Reiter, Verkade, & Stainier, 2001).

It is also interesting to note the differences observed in the ability of the different zebrafish Mesp isoforms to promote various cell fates. Given the high conservation of sequence in the bHLH domain, how these differing activities is conferred to various Mesps is likely due to motifs in the divergent N- and C- terminal portions of these proteins. While swaps of either the whole N- or C- terminal region of the protein did not reveal one or the other to be uniquely cardiogenic, more detailed mutation analysis will be required to tease out the particular motifs that confer the unique ability of Mespaa to make cardiac tissues. This will be of interest as understanding these
differences may shed additional light on the mechanisms of early cardiac progenitor specification.

4.4.3 Zebrafish mesp family members regulate cardiac left-right asymmetry

One of the surprising results from my mesp family loss of function analysis was the finding that left-right asymmetry was disrupted in both mespaa mutants and mespab, mespba and mespbb morphants. Left-right asymmetry is established in zebrafish through the counterclockwise flow of fluid in Kupffer’s vesicle which induces the left sided expression of the Nodal ligand southpaw and the right sided expression of the Nodal antagonist charon (Hashimoto et al., 2004; Matsui & Bessho, 2012). Southpaw expression in turn drives the asymmetric expression of downstream targets including lefty1 and lefty2 (Long, Ahmad, & Rebagliati, 2003). Interestingly
I found defects in the expression of *lefty1/2* in *mesp* depleted embryos suggesting a global defect in left-right asymmetry not restricted to the heart. This suggests that the perturbation in cardiac laterality is caused by an earlier defect in left-right asymmetry and not during the time of looping itself. This defect is intriguing given that zebrafish *mesp* family members do not display expression in the dorsal forerunner cells, the precursors of Kupffer’s vesicle, or in or around the vesicle itself once it is formed (Cutty et al., 2012; Sawada et al., 2000). *mesp* expression is however observed around the margin during gastrulation in the cells that will give rise to the lateral plate mesoderm where asymmetric expression of *southpaw* and its downstream targets occurs. I hypothesize that *mesp* expression is required to poise these cells to properly respond to the signals conveyed by Kupffer’s vesicle or perhaps for the induction of downstream targets. Expression analysis of *southpaw* and the negative regulator *charon* in Mesp deficient embryos will shed light on its role in the molecular regulation of left-right asymmetry.

It should be noted that knockdown of both Mespba and Mespbb resulted in defects in cardiac laterality. This is surprising given their lack of high levels of expression until somitogenesis where they are expressed in the developing somites. It is possible that low levels of expression undetectable by in situ hybridization are essential for left-right asymmetry or perhaps morpholino knockdown of either isoform is having an off target effect on the other *mesp* family members. Individual loss of function mutants should be generated to distinguish between these two possibilities. Another interesting observation was that while loss of *mespaa* resulted in an increase in the number of embryos with bilateral expression of *lefty1/2*, depletion of the other *mesp* family members alone resulted in an increase in the percent of embryos with completely absent expression. Interestingly, when the other three *mesp* family members were depleted in a *mespaa* mutant background the enhancement in the number of embryos with bilateral expression was obtained. This appears to suggest a difference in function between *mespaa* and the other *mesps* in regulating left-right asymmetry and requires further investigation. The complete absence of *lefty1/2* expression suggests a defect in the activity of *southpaw* as mutants lack *lefty1/2* expression (Noël et al., 2013). In contrast the bilateral expression of *lefty1/2* would point towards either a defect in the embryonic midline or an earlier defect in left-right specification (Lenhart, Lin, Titus, Postlethwait, & Burdine, 2011; Superina, Borovina, & Ciruna, 2014). Analysis of *charon* and *southpaw* expression and the examination of fluid flow in Kupffer’s vesicle may shed light on the differences in these phenotypes. Given the importance of
establishing left-right asymmetry for proper human development and the numerous congenital malformations that result from its disruption, investigation into whether the human MESP genes may be implicated in heterotaxia will be of great interest (Peeters & Devriendt, 2006).

### 4.4.4 Mespaa over-expressing cells are a model for cardiac progenitor development in zebrafish

Given the unique ability of *mespaa* over-expressing cells to make cells competent to form cardiac tissues with a high frequency, I performed a number of experiments designed to exploit this assay to gain novel insights into cardiac progenitor formation. While *mespaa* over-expressing cells certainly do not exactly recapitulate endogenous cardiac progenitor development, at the very least several interesting observations were made that merit further study. I found that Mespaa pro-cardiac activity was dependent on early (before 8hpf) expression, a time window coincident with when previous elegant work has shown that cell fates are plastic in the zebrafish embryo (R. K. Ho & Kimmel, 1993). How and when cell fate is first established in the cardiac lineage remains an open question. The finding that Mespaa is required early in gastrulation for pro-cardiac activity is supported by recent clonal lineage analyses suggesting that cardiac progenitors have formed unique cell populations early in mouse gastrulation (Devine et al., 2014). Furthermore, findings that the timing of *mesp* expression early in gastrulation differentiates the first and second heart field adds additional support that cardiac specification may in fact happen very early (Lescroart et al., 2014).

It was interesting to note that *gata5* (or at least *gata5:EGFP*) induction did not occur until after the completion of gastrulation in *mespaa* over-expressing cells, which was surprising given the expression of *gata5* in mesendodermal progenitors before and during gastrulation. Given the relatively long period of developmental time that is present between the requirement for Mespaa (4-5hpf) and the induction of *gata5* (>10hpf) it may be that the role of *mespaa* may be more complex than the simple transcriptional activation of *gata5/6* expression. One hypothesis is that Mesp may be required to “poise” cardiac progenitors for the later induction of the core cardiac transcriptional network. Recent evidence suggests that a subset of *Mesp1* expressing cells subsequently activate the expression of the SWI/SNF chromatin re-modelling complex sub-unit *Smarcd3* in cells that will ultimately contribute to the heart (Devine et al., 2014). Our lab has also previously documented a role for *smarcd3b* in zebrafish cardiovascular development (Lou,
Deshwar, Crump, & Scott, 2011). An intriguing possibility is that perhaps Mesp serves to induce the expression of Smarcd3b to facilitate the generation of a chromatin state poised for the activation of the cardiac program by Gata5. Future studies will be required to address this question. Ultimately, determining the precise mechanism through which Mespaa regulates cardiac fate will require a detailed temporal analysis of its target genes.

4.4.5 Summary

In summary, I find that while not essential for heart development in zebrafish, mesp family members, particularly mespaa, is an extremely potent driver of cardiac fate. Furthermore, I uncover a novel role for the mesp family in left-right asymmetry and make several surprising insights into cardiac progenitor development. Specifically I find that while mespaa expression is required early in gastrulation for pro-cardiac activity gata5 expression is not required until after the completion of epiboly. Altogether my results clarify the role of mesp in the regulatory network responsible for cardiac progenitor formation in zebrafish and implicate alternative pathways in the early specification and migration of these cells. Further study will be required to understand the molecular mechanisms that must compensate for the absence of mesp function in zebrafish with respect to heart development.
5.1 Conclusions and Future Directions

Altogether, I have made significant strides in addressing several of the outstanding questions in early cardiac progenitor development. Through the study of the Aplnr, I have found that the delicate regulation of Nodal signaling is required for the proper initiation of the transcriptional program required to drive the migration of cardiac progenitor cells and proper heart development. In doing so, I have found that the Aplnr plays a more general role in early embryonic development as opposed to playing a specific role in cardiac progenitor migration. Furthermore, I have identified a completely novel function of the Aplnr and its newly discovered ligand Apela. My work on the Mesp family of transcription factors has also revealed a number of novel findings on the formation of cardiac progenitor cells. Consistent with findings in other model systems, Mesp does not specifically regulate the formation of cardiac progenitors but instead seems to direct the creation of multiple mesodermal lineages. Interestingly, Mesp does not appear to be essential for cardiac progenitor formation in zebrafish. Finally, I found that its ability to generate cardiac progenitor cells is contingent on expression during early gastrulation providing additional evidence that it plays a role in the early specification of these cells.

It should be noted that the aplnr and mesp are perhaps the only two genes that have been directly implicated in cardiac progenitor development during gastrulation. It is surprising then that neither of these genes plays a specific role in the formation of cardiac progenitors but instead regulate cell populations with a variety of fates. Together this suggests that while cardiac progenitor populations certainly seem to exist during gastrulation, it is unlikely that they possess uniquely expressed transcription factors or receptors. Instead it seems more likely that they possess a unique transcriptional and epigenetic signature that can only be appreciated at a more global level.

In general, this raises the question of how the embryonic margin in zebrafish gives rise to the diverse set of cell types of both the mesoderm and endoderm. During gastrulation all margin specific genes are expressed in one of the three stereotypical patterns: the speckled pattern denoting endodermal expression typical of sox17 and sox32, expressed all the way around the
margin (*aplnra*), or expressed around the margin but absent at the embryonic shield (*mespa*, *mespab* and *aplnrb*). It is surprising that this extremely homogenous set of expression patterns results in a diverse set of cell types including cardiac progenitors. A more detailed analysis of the margin is required to better understand how cardiac progenitors are specified and maintained. Single cell expression analysis could be performed to identify unique clonal populations that are present at the margin. Given the regional expression of Nodals, Bmps and Wnts in the early embryo, it is likely that various regions possess unique levels of expression of downstream targets unappreciated by in situ hybridization analysis. Furthermore, by studying the transcription factor occupancy of regulatory regions by the transcriptional effectors of these signaling pathways, it may be possible to decipher the unique molecular events contributing to each lineage. Recent advances in performing chromatin immunoprecipitation on small populations of cells and huge leaps in single cell sequencing technology together may make this possible (Grün & van Oudenaarden, 2015).

To complement the aforementioned studies, more detailed analysis needs to be done at the protein level for both the Aplnr and Mesp to better understand their mechanisms of action. Unique antibodies for each respective protein are essential to be able to appreciate their role in cardiac progenitor development at a higher resolution. While both genes display relatively uniform marginal expression, whether or not this holds true at the protein level remains unknown. In the case of the Aplnr, the ability to live image Aplnr protein dynamics would be invaluable. Whether the Aplnr spends more time at the extracellular membrane or internally is unknown and knowing if it localizes to particular subcellular compartments would be invaluable in understanding its mechanisms of action. In the case of both the Aplnr and Mesp, being able to isolate these proteins in vivo would allow for the identification of associated proteins which enable their function. Both the transcriptional binding partners of Mesp and members of the Aplnr signaling complex are relatively unknown. In the case of the Aplnr, fluorescence resonance energy transfer (FRET) technology could be used to look at the interaction of the receptor with g-protein signaling molecules such as B-arrestin to tease out the details of this process.

In addition to more detailed analysis at the protein level of Aplnr and Mesp, it will be interesting to look at the changes in gene expression that occur over time in the embryo in their absence.
RNA-sequencing of *aplnra/b* and *mespaa* mutant embryos could be conducted at multiple time points over the course of gastrulation and early somitogenesis to gain an appreciation for how gene expression fluctuates over this time. In the case of the Aplnr, the dynamics of Nodal target genes will be interesting to study in this manner. Whether all targets ultimately recover to their wild type expression levels or stay reduced over the entire course of gastrulation is unknown. Differences in the expression dynamics of these targets will be critical in understanding the subtleties of the *aplnr* mutant phenotype. In the case of Mesp, it remains unclear how it may work to orchestrate proper left right asymmetry of the heart. Detailed expression analysis may reveal the transcriptional changes and ultimately the exact molecular mechanisms that are guiding this process. Further expression analysis of Mesp perturbation may also reveal insights into the process of cardiac progenitor specification. RNA-sequencing analysis of *mespaa* over-expressing cells may yield a number of interesting downstream targets important in cardiac development. Looking at the intersection of these genes with those found from *gata5* over-expression may be useful in identifying a more restricted list of relevant genes that could then be followed up on for functional analysis.

While both *aplnra/b* and *mespaa* are expressed ubiquitously in cells that will ultimately form the heart during gastrulation, the role of these two proteins in first vs second heart field development remains to be addressed. In mice *Aplnr* expression has been documented in the second heart field and it is possible that the Aplnr plays a role in second heart field formation (D’Aniello et al., 2013). The generation of conditional alleles using Crispr-Cas technology will be required to address this question in order to specifically target *aplnra* and *aplnrb* in either a temporal or spatially restricted pattern. Previous studies have identified that the timing of *Mesp1* expression delineates the first vs second year field progenitors in the mouse (Lescroart et al., 2014). Whether or not this is true in the zebrafish embryo could be investigated in future studies and this timing could then be exploited to profile these cells and better understand the divergence of these two lineages. In order to investigate this question a *mespaa* reporter would be required and could be generated using Crispr-Cas technology to knock-in a GFP coding sequence in the *mespaa* locus. One question that was not addressed in the mouse study is whether or not the timing of *Mesp1* expression actually drives the divergence of these two lineages or simply acts as a read out. By using my *mespaaΔnls-ecr* construct I could add tebufenozide at different time...
points and observe if the timing of induction biases the contribution of cells to structures derived from the first or second heart field. This same approach could be used to test if timing of induction also biases these cells to different mesodermal fates as observed in cell culture (Chan et al., 2013).

While both proteins have been known to play a role in the formation of the heart, an intriguing possibility is a role for either gene in cardiac regeneration. The zebrafish heart possesses the unique and remarkable ability to completely regenerate up to 20% of the ventricle when resected with no lasting scar or injury (Poss, Wilson, & Keating, 2002). Understanding this phenomenon has been an intense area of research over the past decade given the potential applications toward healing the human heart after ischemic injury. It has been demonstrated that the regeneration of the heart occurs through the dedifferentiation of existing cardiomyocytes and a reactivation of gata4 expression (Jopling et al., 2010; K. Kikuchi et al., 2010). Given the requirement for the re-differentiation of existing mature cardiac cells into the various cardiac cell types required to heal the injury, it is possible that the Aplnr and/or Mesp may play a role in this process. Both seem to be able to have a significant effect on the fate of mesodermal progenitors in the embryo and this could also be the case in the regenerating heart. Investigation of this question is potentially easy to explore given the ability of null mespaa and aplnrb mutant embryos to be raised to adulthood. Regeneration studies should be performed on these fish in comparison to WT to establish if these proteins may be important in this process.

The ability of null mespaa and aplnrb/apela mutant fish to be raised to adulthood also provides several intriguing possibilities for large scale small molecule screens using these embryos. Zebrafish embryos are particularly amenable to these types of screens given their large numbers per clutch, external fertilization and transparency. Small molecules can simply be diluted into the surrounding egg medium which makes screening possible without significant manual labor and can often be automated using robotic technology (Hao, Williams, Webb, & Hong, 2010). Given the variable and sometimes absent penetrance of the aplnrb and apela mutant phenotypes, null embryos could be exposed to small molecules to look for ones that increase the penetrance of the phenotype or yield interesting different phenotypes that are not observed in WT controls. This would provide a relatively un-biased tool to identify pathways that are acting in concert with these genes. It may not only reveal pathways that are important in the aplnr/apela regulation of
cardiac development, but may indicate other previously undocumented or more subtle roles for these genes in development.

In the case of *mespaa* null embryos, small molecule screens could be useful for identifying interacting pathways in left-right asymmetry and also to identify possible sources of redundancy that may be maintaining proper cardiac specification in their absence. While I have found that the *mesp* family no longer appears to be required for cardiac progenitor formation in zebrafish, small molecules that generate cardiac defects in *mespaa* null embryos but not WT controls may reveal genes/pathways that now compensate for *mesp* in the zebrafish embryo. This would provide interesting insight into the evolutionary divergence of the cardiac transcriptional hierarchy in fish. Screens could also be performed on *mespaa* null mutant embryos to look for molecules that exacerbate the left-right asymmetry phenotype or alternatively provide rescue. Since this phenotype is more subtle and will require large numbers in order to distinguish differences among samples, this will only be amenable to a smaller number of candidate molecules.

In conclusion, the role of the Aplnr and Mesp in the development of cardiac progenitor cells has been clarified and expanded. Future work is now required to understand their role in this process at a more exhaustive level and a number of interesting questions remain. Ultimately, a detailed appreciation of this phenomenon will be required if we are to harness it for the effective prevention and treatment of congenital and adult heart disease.
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