Functions of atoh8 and Mekk3 in Cardiovascular Development

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Abstract
The transcription factor ATOH8 and the kinase MEKK3 are evolutionary conserved proteins with known expression within the cardiovascular system. However, the roles of these proteins in the developing heart are undefined. We used a combination of mutant mouse models, morpholino gene suppression in the zebrafish, and cell culture to determine the role of these two proteins in cardiovascular system. Our experiments with ATOH8 reveal a direct interaction between ATOH8 and the cardiovascular transcription factors FOG2 and GATA4. This interaction is required in vivo in the developing zebrafish heart, where Atoh8 functions with Gata and Fog factors to promote proper cardiac looping. However, our genetic studies in the mouse show that ATOH8 is not required for cardiovascular development and has a much weaker genetic interaction with GATA4 in mammals. These results identify a novel interaction and role for Atoh8 in the zebrafish heart and also definitively exclude a requirement for ATOH8 in mammalian development. Our experiments with MEKK3 reveal a requirement for this protein in regulating endocardial growth factors that promote myocardial growth. We show that MEKK3 interacts with CCM2L, an endocardial protein known to regulate growth factor production in the endocardium in conjunction with the transmembrane protein HEG. Deletion of MEKK3 in the endocardium leads to embryonic death secondary to decreased myocardium, likely due to decreased myocardial proliferation. MEKK3 genetically interacts with HEG in vivo, providing additional evidence for coordinated function between MEKK3 and the HEG-CCM complex. These findings highlight a new role for MEKK3 in cardiovascular development and begin to identify the molecular mechanism underlying endocardial growth factor support for myocardial growth.

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FUNCTIONS OF ATOH8 AND MEKK3 IN CARDIOVASCULAR DEVELOPMENT

David R. Rawnsley

A DISSERTATION in Cell and Molecular Biology
Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

FUNCTIONS OF ATOH8 AND MEKK3 IN CARDIOVASCULAR DEVELOPMENT

David R. Rawnsley
Mark L. Kahn

The transcription factor ATOH8 and the kinase MEKK3 are evolutionary conserved proteins with known expression within the cardiovascular system. However, the roles of these proteins in the developing heart are undefined. We used a combination of mutant mouse models, morpholino gene suppression in the zebrafish, and cell culture to determine the role of these two proteins in cardiovascular system. Our experiments with ATOH8 reveal a direct interaction between ATOH8 and the cardiovascular transcription factors FOG2 and GATA4. This interaction is required in vivo in the developing zebrafish heart, where Atoh8 functions with Gata and Fog factors to promote proper cardiac looping. However, our genetic studies in the mouse show that ATOH8 is not required for cardiovascular development and has a much weaker genetic interaction with GATA4 in mammals. These results identify a novel interaction and role for Atoh8 in the zebrafish heart and also definitively exclude a requirement for ATOH8 in mammalian development. Our experiments with MEKK3 reveal a requirement for this protein in regulating endocardial growth factors that promote myocardial growth. We show that MEKK3 interacts with CCM2L, an endocardial protein known to regulate growth factor production in the endocardium in conjunction with the transmembrane protein HEG.
Deletion of MEKK3 in the endocardium leads to embryonic death secondary to decreased myocardium, likely due to decreased myocardial proliferation. MEKK3 genetically interacts with HEG \textit{in vivo}, providing additional evidence for coordinated function between MEKK3 and the HEG-CCM complex. These findings highlight a new role for MEKK3 in cardiovascular development and begin to identify the molecular mechanism underlying endocardial growth factor support for myocardial growth.
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Chapter 1: Introduction

Summary

This chapter will present an introduction to the two projects discussed in this dissertation: 1) my studies on interactions between ATOH8 and GATA4-FOG2 in development, and 2) my studies on the regulation of the endocardial growth factors by the MEKK3 and the HEG-CCM complex. As these two projects are unrelated, this chapter will be separated into two parts. In the first half, I will discuss the prior studies performed on the transcriptional factor ATOH8. I will then discuss the known functions and interactions of GATA4 and FOG2, two interacting partners of ATOH8, in the heart and the lungs. In the second half of this introduction I will discuss pro-growth signaling from the endocardium to the myocardium and also reciprocal signaling from the myocardium to the endocardium. I will briefly discuss cerebral cavernous malformations (CCM) and the CCM complex that underlies this disorder. I will then discuss the additional CCM complex proteins that our lab has identified and the role that these proteins play in endocardial growth factor signaling.

Introduction: Atoh8

In vitro and in vivo studies of ATOH8 in vertebrates

The gene Atonal homolog 8 (Atoh8) encodes the protein ATOH8, a basic helix-loop-helix (bHLH) transcription factor. bHLH factors are defined by the presence of a basic-helix-loop-helix domain in which the basic region binds DNA and the helix-loop-helix region mediates dimerization to a second bHLH protein (1). Within the hierarchy
of bHLH factors, *Atoh8* is the sole mammalian member of the Net family (2). The ATOH8 bHLH domain shares 43-57% identity on the amino acid level with the bHLH domains of proteins in the Atonal, NeuroD, and Neurogenin families, and the bHLH domain is highly conserved across vertebrate ATOH8 homologues (3). Unlike many genes within the atonal superfamily that are encoded by a single exon, *Atoh8* has a unique three-exon gene structure that is conserved from zebrafish to mammals (4). The conserved gene structure and conserved sequence of the bHLH domain suggest the possibility of an evolutionary conserved role for *Atoh8* in vertebrates.

Several studies have attempted to ascribe functions to ATOH8 through both in *vitro* and in *vivo* approaches. The first in *vitro* study overexpressed ATOH8 by retroviral infection in murine retinal explants, which resulted in a decrease in glial cells and an increase in rod cells, suggesting that ATOH8 may regulate neuronal-glial fate determination (3). In *vitro* studies of ATOH8 function in the pancreatic cell lines indicate that ATOH8 can physically interact with other bHLH factors, such as NEUROG3 and NEUROD1, and can function as a transcriptional repressor of pancreatic genes, suggesting a potential role in pancreatic development (5). Studies of cultured human endothelial cells detected an upregulation of the human orthologue *ATOH8* in response to fluid flow, suggesting that ATOH8 may function in the endothelial response to shear stress (6). All of these studies have been limited by being in *vitro* rather than in *vivo* approaches, and it remains unclear whether ATOH8 has any required in *vivo* activity in neural, pancreatic, or endothelial development or function.

Two studies have attempted to determine the in *vivo* requirements for ATOH8. In the zebrafish, morpholino gene suppression studies of Atoh8 were performed to
determine the requirement in the developing fish (7). Morphant embryos developed severe body plan malformations and died between 24 and 72 hours post fertilization (hpf). Morphant embryos were observed to have impaired somite development, with decreased expression of myogenic genes such as *myoD* and *myogenein*. Morphant embryos also had a decreased number of neuronal cells in the retina, a finding possibly consistent of the role in neural development that had previously been ascribed to murine *Atoh8* in retinal explant studies (3).

A second group attempted to determine the role of *Atoh8* in mammals by deriving an *Atoh8* mutant mouse (5). This allele has a 10 kb deleted region containing exon 1, exon 2, and the intervening intron 1; exons 1 and 2 contain nearly all of the *Atoh8* coding sequence. Mice homozygous for this allele were found to be dead by E8.5 and reportedly appeared to have arrested shortly after gastrulation. This early lethality has precluded the use of these mice in studying the developmental or postnatal requirement for ATOH8, and the role for this gene in mammals has remained ambiguous.

**GATA4 and FOG2 in heart and lung development**

The GATA transcription factor family regulates the development of multiple organ systems in vertebrates. GATA factors are characterized by binding to the DNA binding motif WGATAR and by structural conservation of two zinc fingers (8). Among the six vertebrate GATA factors, GATA4, GATA5, and GATA6 regulate multiple steps in the development of the vertebrate heart, as well as several reproductive and endodermal tissues (9-11). Although these factors are partially redundant (12,13), GATA4 has been identified as a critical factor in cardiovascular development. *GATA4*−/−
embryos develop cardia bifida and die by E9.5 (14,15), and both endothelial and myocardial-specific Gata4 deletions exhibit lethal embryonic cardiac defects (16,17). Gata4 heterozygosity is sufficient to cause cardiac defects on certain genetic background, as shown by Gata4\(^{+/-}\) mice that develop septal defects and ventricular hypoplasia when bred into a C57BL/6 background (18,19). Familial congenital heart defects have also been linked to GATA4 heterozygosity, demonstrating that conservation of GATA4’s critical role in the heart extends to humans (19-21). The cardiac requirement for GATA4 also extends to the developing zebrafish heart. Although gata5 appears to the most critical GATA factor in the zebrafish cardiogenesis (22), morpholino gene suppression of Gata4 results in an unlooped heart tube (23), indicating that GATA4 has a conserved requirement in heart development that extends from fish to mammals.

GATA4 does not operate in isolation in cardiac development and has been shown to interact with other cardiac transcription factors (e.g. Tbx5 (20)). Among these interactions, the best characterized is that between GATA4 and FOG2, a member of the Friend-of-GATA (FOG) family of transcriptional regulators (24,25). FOG proteins are unable to bind DNA and must instead bind GATA factors in order to regulate transcription (26). GATA factors bind FOG proteins via a highly conserved sequence on the N-terminal GATA zinc finger, and a Gata4 point mutation that disrupts this interaction \textit{in vivo} phenocopies the loss of FOG2 (26,27). Thus, the major developmental role of FOG2 is dependent on binding to GATA4. Interactions with FOG factors have been shown to exert both positive and negative effects on GATA transcriptional activity that depend on the cellular context (24,28-30). FOG proteins play critical roles in heart development in multiple vertebrate species. In the mouse, loss of FOG2 results in cardiac
defects and embryonic death (31-33). In the zebrafish, loss of Fog1, the Fog factor expressed in the heart, results in a failure of heart looping (34). Like GATA4, mutations in FOG2 have been linked to human congenital heart disease (35), making further study of GATA-FOG function and identification of additional GATA-FOG interacting partners an important goal for understanding human disease.

In addition to their roles in heart development, FOG2 and GATA4 have additional roles in the developing lung. A forward genetic screen in mice identified a point mutation in Fog2 that results in disrupted diaphragmatic development and pulmonary hypoplasia that was particularly severe in the accessory and right medial lobes (36). This pulmonary role for FOG2 has also been observed in human patients, where FOG2 point mutations have been linked to neonatal death from pulmonary aplasia and congenital diaphragmatic hernia (36). Given FOG2’s dependence on a GATA factor for transcriptional function, subsequent studies in the lung attempted to identify the GATA partners of FOG2. Examination of lungs from mice homozygous for either Gata4 or Gata6 point mutant alleles that could not bind FOG2 revealed that the GATA4-FOG2 interaction was critical for proper lung development (37), whereas the GATA6-FOG2 interaction was not required. In line with this observation, Gata4 and Fog2 expression in the pulmonary system is limited to the mesenchyme of the developing lung (36), whereas Gata6 expression is much more extensive (18,38). These results suggest a specific role for the GATA4-FOG2 complex in pulmonary mesenchyme development, although there is no evidence regarding which targets GATA4 and FOG2 regulate in the mesenchyme or what other interacting partners they may have in this tissue.
Conclusions and Outstanding Questions

ATOH8 is a transcription factor with a bHLH domain that is highly conserved across vertebrate species. This sequence conservation suggests a conserved requirement for ATOH8 in these species. Although several reports have attempted to ascribe functions to ATOH8, these studies have been limited by the flaws of the current in vivo loss-of-function models. The zebrafish morpholino experiments provide evidence for a requirement for Atoh8 in muscle and retinal development. However, the severe structural defects in these embryos may mask more subtle phenotypes, and using lower doses of the morpholinos may identify additional requirements for Atoh8. In the mouse, the early embryonic death of the reported mutant mouse at gastrulation prevents any definitive determination of the required roles for Atoh8 in mammals. To further study Atoh8, additional mutant mouse models are required, particularly conditional alleles that will allow us to bypass early lethality and enable us to derive tissue-specific deletions to determine where and when Atoh8 is acting.

In the case of GATA4 and FOG2, the role of these proteins has been extensively studied in the heart. However, it remains unclear what controls FOG2 binding to GATA4—when does GATA4 bind FOG2, and when it is independent of FOG2? One possibility is that other transcription factors may modulate FOG2-GATA4 binding and promote or inhibit formation of FOG2-GATA4 complexes at different sites throughout the genome. It would therefore be interesting to identify transcription factors that are capable of physically interacting with GATA4-FOG2 and also capable of functionally interacting with these proteins in vivo.
Introduction: CCM, MEKK3, and Endocardial Growth Factors

Endocardial-myocardial signaling

The inner wall of the myocardium of the heart is lined by endocardium, a specialized form of endothelium. In addition to performing the traditional roles of endothelium, such as separating circulating blood cells from cardiomyocytes and inhibiting inappropriate activation of coagulation, the endocardium also produces multiple growth factors that promote cardiomyocyte survival, proliferation, and maturation. Concurrently with these endocardial signals, the myocardium produces signals promoting endocardial development, creating a signaling cross talk that is necessary for the development of both cell types within the heart.

Previous studies have identified the protein neuregulin and FGF factors as critical endocardial growth factors. Neuregulin is a member of the epidermal growth factor (EGF) family; endocardial neuregulin activates the myocardial receptors ErbB2 and ErbB4 to promote cardiomyocyte survival and proliferation, and ventricular trabeculation (39,40). Null mutations of neuregulin or its receptors lead to reduced myocardial proliferation, decreased ventricular trabeculation, and embryonic death by E10.5 (41-43). Similar findings have been observed with endocardial fibroblast growth factors (FGF) and their associated FGF receptors (FGFR) in the myocardium. FGF9, FGF16, and FGF20 are expressed from the endocardium during cardiac development and activate the receptors Fgfr1 and Fgfr2 in the myocardium (44). FGF9−/− and FGF16−/− embryos both exhibit reduced myocardial proliferation (44,45); similar myocardial phenotypes are seen in mice lacking Fgfr1 and Fgfr2 in cardiomyocytes (44). It is unknown, however, what signals drive endocardial neuregulin and FGF production.
Reciprocal myocardial signals to the endocardium include vascular endothelial growth factor (VEGF) and angiopoietin-1 (ANG1). Myocardial VEGF, while not required for endocardium survival, promotes induction of endocardial cells into endothelial cells to form coronary arteries (46). Mice lacking myocardial ANG1 have a thin myocardium and fail to develop ventricular trabeculations (47). As ANG1 activates receptors on the endocardium, this result suggests that ANG1 promotes production of endocardial growth factors that then facilitate myocardial growth and development, although it is unknown what pathways ANG1 would activate in the endocardial cell to drive growth factor production.

While multiple endocardial-to-myocardial growth factor pathways have been identified, little is known about what regulates these pathways within the endocardium. Prior work in our lab has identified the HEG-CCM pathway as a novel regulator of endocardial growth factor production.

CCM Signaling and Endocardial Growth Factors

Cerebral cavernous malformations (CCM) are vascular lesions that develop within the vasculature of the central nervous system (CNS) and predispose patients to hemorrhagic stroke and focal neurological deficits (48). CCM lesions can either occur sporadically or occur as an inherited familial disorder. Patients with familial CCM lesions are heterozygous for CCM1, CCM2, or CCM3. CCM lesions form when a loss of heterozygosity occurs within the endothelium of the CNS (49-51); CCM lesions caused by mutations in any of the three genes are clinically indistinguishable, indicating that CCM1, CCM2, and CCM3 likely operate in a common pathway (48). In support of this
conclusion, biochemical studies have demonstrated that CCM1, CCM2, and CCM3 form a complex where CCM1 binds CCM2, which then binds CCM3 (52). Mice deficient for CCM1, CCM2, or CCM3 develop early lethal embryonic vascular defects (53-55), indicating that these proteins have a role in cardiovascular development in addition to their known role in CCM disease pathogenesis.

Previous work in our lab identified two additional components of the CCM complex: Heart-of-glass (HEG) and cerebral cavernous malformation 2-like (CCM2L). HEG is a transmembrane protein that can interact with the CCM complex by binding CCM1 (52). Loss of the zebrafish homologue Heg has been reported to result in defective myocardial growth (56), phenocopying the loss of CCM1 and CCM2 in the zebrafish (57) and linking Heg to the CCM complex. In the mouse, loss of Heg results in death in approximately half of animals in late embryogenesis or early postnatal life from either hemorrhages within the pulmonary vasculature or from myocardial ruptures secondary to local patches of thin myocardium (52). Our lab has also identified CCM2L, a paralogue of CCM2 that retains CCM2’s ability to bind CCM1 but cannot interact with CCM3 (58). Ccm2l−/− mice are viable without overt defects; however, Heg−/− Ccm2l−/− double mutant mice are lethal by E10.5 from heart failure secondary to reduced myocardial proliferation (58). Expression of CCM2L and HEG at this time point is limited to the endocardium, indicating that the myocardial defects were due to a depletion of endocardial growth factors. Levels of FGF16 mRNA, a critical endocardial-to-myocardial growth factor (45,59), were found to be reduced in Heg−/− Ccm2l−/− hearts, indicating that endocardial HEG and CCM2L are required to generate FGF16 and promote myocardial growth.
Conclusions and Outstanding Questions

The past studies on HEG have revealed a role for this endocardial protein in regulating myocardial growth (52,56). This role has become much clearer with the recent report of the Heg\(^{+/−}\) Ccm2l\(^{+/−}\) double mutant mouse that dies by E10.5 from heart failure secondary to profound myocardial thinning (58). These mice have decreased FGF16 levels in their hearts, identifying the likely target of HEG and CCM2L and also identifying the mechanism through which myocardial growth is stimulated.

The central outstanding question of this project is identifying the mechanism through which HEG and CCM2L regulate growth factor production. HEG is localized to the membrane due to its transmembrane domain, and CCM2L is mostly localized near the membrane due to its interaction with CCM1 and CCM1’s interaction with HEG. As neither of these proteins have transcriptional function, there is no mechanism through which they could directly alter FGF16 transcription. We conclude that there must an associated pathway, likely binding CCM2L, that is modulating FGF16 transcription.
Chapter 2: ATOH8 regulates GATA4 and FOG2 during Vertebrate Development

The data in this Chapter have been published in the Journal of Biological Chemistry (60).

Summary

Atonal homolog 8 (Atoh8) is a bHLH transcription factor that is highly conserved in vertebrates. Here we show that zebrafish atoh8 is required for heart tube looping and swimbladder inflation, and that atoh8 specifically interacts with gata4 and zfpm1 (Fog1) in these developing tissues. We used co-immunoprecipitation studies to show that ATOH8 can form a physical complex with GATA4 and FOG2, suggesting a molecular mechanism explaining the genetic interaction in the zebrafish. In contrast to the role of atoh8 in the zebrafish, multiple Atoh8 mutant mouse mice models exhibit normal cardiac development, indicating that the cardiac function of Atoh8 is not required in the mouse. We showed there is increased early postnatal death in Gata4+/− Atoh8GFP/GFP mice, suggesting that genetic interaction between Gata4 and Atoh8 is conserved into mice. We were unable to detect structural or functional defect in the hearts or lungs of these mice, however, preventing us from determining the cause of death or site of GATA4-ATOH8 interaction in these mice.

Introduction

The gene Atonal homolog 8 (Atoh8) encodes the protein ATOH8, a basic helix-loop-helix (bHLH) transcription factor within the atonal superfamily. The sequence of the ATOH8 bHLH domain is highly conserved across vertebrate ATOH8 homologues
(3), and Atoh8 has a unique three-exon gene structure that is conserved from zebrafish to mammals and that is unique among bHLH factors in the atonal superfamily (4). The conserved gene structure and conserved sequence of the bHLH domain suggest the possibility of an evolutionary conserved role for Atoh8 in vertebrates.

As we previously discussed in Chapter 1, limited in vitro and in vivo studies have several potential functions for Atoh8. Morpholino knockdown of atoh8 in the zebrafish was reported to lead to severe developmental malformations and early embryonic death, in addition to specific defects in development of the somites and retina (7). The early death and severe defects seen in the atoh8 morphant embryos may mask more subtle defects in other aspects of zebrafish development; lower doses of morpholinos may bypass these early phenotypes and allow us to analyze the role of atoh8 in later embryonic stages.

In contrast to the zebrafish, the in vivo requirement for Atoh8 in mammals has remained elusive. One group has reported creation of an Atoh8 mutant allele with a deletion of exon 1, intron 1, and exon 2 (hereafter referred to as the Atoh8Δex1-2 allele) (5). Atoh8Δex1-2/Δex1-2 mice were reported to be lethal by E8.5 and to be arrested at gastrulation. This early lethality precluded further use of these mice in studying the developmental or postnatal requirement for ATOH8 in specific tissues.

Prior to the publication of the lethal Atoh8Δex1-2 mouse and prior to the start of my thesis project, our lab attempted to create an Atoh8 mutant mouse. The coding sequence of eGFP followed by a stop codon was inserted into the first exon, replacing the N-terminus of the Atoh8 sequence and creating the Atoh8GFP allele. Mice homozygous for this allele were viable on a C57BL/6-129/Sv mixed background, but exhibited runt
that developed by weaning and persisted into adulthood. When these mice were bred onto a pure C57BL/6 background, approximately 50% of the $Atoh8^{GFP/GFP}$ mice died between birth and weaning (data not shown). These results strongly contradicted the phenotype of the $Atoh8^{\Delta ex1-2/\Delta ex1-2}$ mouse, which was completely lethal prior to E8.5 (5).

We interpreted this discrepancy as likely being due to the degree of deletion in these two alleles, with the $Atoh8^{\Delta ex1-2}$ allele deleting multiple exons and the $Atoh8^{GFP}$ allele deleting only a portion of $Atoh8$ exon 1. Two different hypotheses appeared possible from this result. First, the $Atoh8^{\Delta ex1-2}$ allele could be a complete $Atoh8$ null allele and the $Atoh8^{GFP}$ allele could be a hypomorphic allele. In this interpretation, total loss of $Atoh8$ in $Atoh8^{\Delta ex1-2/\Delta ex1-2}$ mice leads to early embryonic lethality, whereas partial $Atoh8$ function in $Atoh8^{GFP/GFP}$ mice leads to runting and strain-dependent partial postnatal lethality.

Alternatively, the $Atoh8^{GFP/GFP}$ could be a true $Atoh8$ null allele, and $Atoh8^{\Delta ex1-2/\Delta ex1-2}$ mice could have an additional defect unrelated to loss of ATOH8 protein that resulted in its early embryonic lethality.

We hypothesized that ATOH8 had conserved and required functions in vertebrate development. In order to test this hypothesis, we took two separate approaches to examine $in$ $vivo$ ATOH8 function. First, we used morpholinos to knockdown $atoh8$ in zebrafish embryos to identify additional $in$ $vivo$ requirements for $atoh8$. Secondly, in order to identify the functions of $Atoh8$ in mammals, we generated and characterized additional $Atoh8$ mutant mice models to definitely resolve the discordant phenotypes between $Atoh8^{\Delta ex1-2/\Delta ex1-2}$ and $Atoh8^{GFP/GFP}$ mice and to determine the requirement for $Atoh8$ in mammalian development.
Results

*atoh8* is required for zebrafish heart looping and swim bladder development

There is a high degree of conservation between the mouse gene *Atoh8* and the zebrafish homolog *atoh8* (3), suggesting that there may be a conserved function for this gene across vertebrate species. Knockdown of *atoh8* by morpholino in the zebrafish has previously been reported to result in severe developmental malformations in skeletal muscle somites and in the retina and death by 72 hpf (7). We hypothesized that lower morpholino doses would reveal additional developmental roles for *atoh8*. We used the same translation-blocking morpholino, *atoh8*-MO1, as Yao et al and lowered the injected dose to 2.5 ng per embryo. At this dose embryos did not develop the retinal and skeletal muscle defects seen at higher doses and greater than 90% of embryos survived beyond 72 hpf. Approximately 75% of embryos injected with *atoh8*-MO1 at a 2.5 ng dose developed an unlooped heart tube and pericardial edema by 72 hpf (Figure 2.1.A-F). We attempted to rescue this phenotype with *atoh8* cRNA injection, but we were unable to rescue due to toxicity of the cRNA by 72 hpf (data not shown). To confirm that this heart phenotype was due to loss of *atoh8*, we injected two additional *atoh8* morpholinos—one targeting the splice donor site at the exon 1/intron 1 junction (*atoh8*-MO2) and an additional translation-blocking morpholino targeting the 5’UTR (*atoh8*-MO3). Each morpholino produced a similar heart tube looping defect (Figure 2.1.G-H). In contrast, an *atoh8*-MO1 morpholino with five point mutations failed to induce a heart looping defect (data not shown). To further test the specificity of the observed cardiac defects in *atoh8* knockdown zebrafish we lowered the doses of all three *atoh8* morpholinos and used them individually and in combination. At low doses single morpholinos induced
heart looping defects in less than 10% of embryos; when used in combination greater than 90% of embryos developed an unlooped heart (Figure 2.1.I). This synergy suggests that the heart phenotype is due to knockdown of the same target gene by all three morpholinos, indicating that this phenotype is due to specific loss of \textit{atoh8}. These results indicate that \textit{atoh8} is required for normal cardiac looping in zebrafish.

Morpholino knockdown of \textit{atoh8} also revealed that 95% of injected embryos failed to develop an inflated swimbladder by 96 hpf (Figure 2.2.A-C). This swimbladder phenotype was observed in embryos with the heart phenotype (Figure 2.2.B) as well as embryos without the phenotype (Figure 2.2.C), indicating that the swimbladder defect is highly penetrant and independent of the cardiac defect.

\textbf{Atoh8 interacts specifically with Gata4 and Zfpm1 in the developing zebrafish heart}

In order to identify candidate genes and pathways that interact with \textit{atoh8}, we looked for genes that exhibit similar heart and swimbladder defects in response to morpholino knockdown. Knockdown of \textit{gata4} results in an unlooped heart, pericardial edema, and an uninflated swimbladder (23). To determine whether a genetic interaction exists between \textit{atoh8} and \textit{gata4}, we injected zebrafish embryos with low dose \textit{gata4}-specific and \textit{atoh8}-specific morpholinos individually and in combination. Embryos were then scored to determine the penetrance of swimbladder and heart phenotypes to detect genetic interaction between \textit{atoh8} and \textit{gata4} (61,62). Co-injection of \textit{atoh8} and \textit{gata4} morpholinos resulted in a synergistic increase in the penetrance of both the swimbladder (Figure 2.3.A) and heart (Figure 2.3.B) phenotypes. These results suggest that \textit{atoh8} interacts with \textit{gata4} in the developing zebrafish swimbladder and heart.
GATA factors are critical members in the transcriptional network controlling vertebrate heart development (10). Within this transcriptional network, atoh8 may interact specifically with gata4, or it may genetically interact with multiple cardiac transcription factors. In order to determine the specificity of the atoh8-gata4 interaction, we examined whether atoh8 exhibited genetic interaction with other transcription factors. Heart looping defects in the zebrafish have been previously observed with morpholino knockdown of mef2ca (61), tbx-5a (63), and zfpm1 (encoding Fog1) (34). Small additive increases in the penetrance of the unlooped heart phenotype were observed when mef2ca (Figure 2.3.C) or tbx-5a (Figure 2.3.D) morpholinos were injected in combination with atoh8-MO1. In contrast, a larger synergistic increase was observed with co-injection of zfpm1 and atoh8 morpholinos (Figure 2.3.E). These results suggest that atoh8 specifically interacts with gata4 and zfpm1 in the developing zebrafish heart.

*gata4* and zfpm1 encode Gata4 and Fog1 proteins respectively. Previous studies have revealed a physical interaction between mouse GATA4 and FOG2 (28), and germline expression of a GATA4 point mutant that does not bind FOG2 phenocopies the loss of FOG2 and leads to cardiovascular death in mice (27). Thus the interaction between GATA and FOG factors is critical for heart development. To determine if a similar genetic interaction exists between *gata4* and *zfpm1* in the zebrafish, we co-injected low doses of the *gata4* and *zfpm1* morpholinos. *gata4-zfpm1* morpholino combinations conferred an increased penetrance of the heart looping defect to a degree similar to that observed with atoh8-gata4 and atoh8-zfpm1 morpholino combinations (Figure 2.3.F), suggesting that Gata4 and Fog1 also interact in the developing zebrafish heart.
Our results suggested that *atoh8* might act in concert with both *gata4* and *zfpm1*. To further test this hypothesis, morpholino doses were lowered further and combinatorial knockdown studies performed. At doses in which each individual morpholino induced heart looping in <5% of embryos (Figure 2.3.G), injection of all three morpholinos resulted in heart looping defects in approximately 90% of embryos (Figure 2.3.G). This powerful synergy between the three transcription factors suggests a strong interaction between *atoh8*, *gata4*, and *zfpm1* in the developing zebrafish heart.

**ATOH8 forms a biochemical complex with GATA4 and FOG2**

The strong genetic interaction observed between *atoh8*, *gata4* and *zfpm1* in the developing zebrafish suggested either that Atoh8 functions upstream or downstream of the Gata-Fog complex in a common genetic pathway (i.e. an epistatic relationship) or that these 3 transcription factors function together in a single complex (i.e. a biochemical relationship). qPCR studies of fish embryos injected with *atoh8* morpholinos failed to reveal changes in the expression levels of either *gata4* or *zfpm1* and morpholino knockdown of *gata4* or *zfpm1* did not alter *atoh8* levels (data not shown), suggesting that *atoh8* does not interact with *gata4* and *fog1* epistatically. To assess a direct, physical interaction between these transcription factors epitope-tagged mouse ATOH8, GATA4 and FOG2 proteins were co-expressed in HEK293T cells and a series of co-immunoprecipitation experiments performed. We were unable to immunoprecipitate FLAG-ATOH8 and V5-GATA4 together (Figure 2.4.A). However, immunoprecipitation of FLAG-ATOH8 was associated with co-immunoprecipitation of V5-FOG2 (Figure 2.4.A), and when all three proteins were co-expressed V5-GATA4 could be pulled down
with both FLAG-ATOH8 and V5-FOG2 (Figure 2.4.B). Finally, co-expression of
FLAG-ATOH8, V5-FOG2 and V5-GATA4-V217G, a GATA4 point mutant that has been shown to be unable to associate with FOG2 (27), confirmed that association of
GATA4 with ATOH8 is bridged by FOG2 (Figure 2.4.B). We attempted to confirm the
ATOH8-FOG2 interaction and assess a direct mechanism of interaction using GST-
ATOH8 and GST-FOG2 fusion protein binding assays, but we were unable to generate
the GST-FOG2 protein (perhaps due to the large size of FOG2) (data not shown). These
studies provided a biochemical explanation for the genetic interaction observed between
atoh8, gata4, and zfpm1 in the fish, and suggested that a similar relationship should exist
in the mouse.

atoh8 is weakly expressed in the zebrafish heart tube

Our co-immunoprecipitation experiments suggested that physical interaction
underlies the genetic interaction observed between atoh8, gata4 and zfpm1. Based on
this finding, we hypothesized that atoh8 is expressed in the zebrafish heart tube with
gata4 and zfpm1. In situ hybridization revealed atoh8 expression throughout the embryo
at 13 hpf (Figure 2.5.A-B), 30 hpf (Figure 2.5.C-D) and 48 hpf (Figure 2.5.F), consistent
with a previous report of atoh8 expression (7). Weak atoh8 expression was observed in
the heart tube at 30 hpf (Fig. 5C-D), overlapping with the cardiac expression of gata4
(Figure 2.5.E) and with the reported expression of zfpm1 (34). This expression data is
consistent with our hypothesis that a physical complex between the proteins Atoh8,
Gata4, and Fog1 in the zebrafish, and suggests this could be the molecular mechanism
underlying the genetic interaction we observe in the developing zebrafish.
**Atoh8** is not required for survival in the mouse

In order to determine the requirement for ATOH8 in the mouse, previous members of our lab had created and begun to characterize an *Atoh8* mutant mouse. The eGFP coding sequence followed by a stop codon was inserted into exon 1 to generate the *Atoh8*<sup>GFP</sup> allele (Figure 2.6.A); approximately 50% of the coding region of exon 1 was deleted in this allele. On a mixed genetic background, *Atoh8*<sup>GFP/GFP</sup> mice were reported to be viable but runted; when backcrossed onto a C57BL/6 genetic background, approximately half of *Atoh8*<sup>GFP/GFP</sup> mice were reported to die prior to weaning (data not shown). This phenotype differed significantly from a published report of an *Atoh8* mutant mouse from another lab (5). This group used recombination with a bacterial artificial chromosome (BAC) targeting vector to delete a 10 kilobase region of *Atoh8*, replacing exon 1, intron 1, and exon 2 with sequence for an eGFP-Cre fusion protein (the *Atoh8*<sup>Δex1-2</sup> allele). Mice homozygous for this *Atoh8*<sup>Δex1-2</sup> allele were reported to be lethal shortly after gastrulation, precluding use of these mice to determine the function of *Atoh8* in cardiac development.

Due to the discrepant phenotypes between these *Atoh8*<sup>GFP</sup> and *Atoh8*<sup>Δex1-2</sup> mutant models, we hypothesized that the *Atoh8*<sup>GFP</sup> allele may be a hypomorphic allele with residual function. In order to create a definitive deletion of exon 1 of *Atoh8*, we designed a mutant allele deleting all of exon 1 of *Atoh8* (Figure 2.7.A). The 5’ loxP site was placed 617 base pairs upstream of the transcriptional start site; this position places the loxP outside a conserved region of sequence upstream of the *Atoh8* transcribed region that we suspected contain conserved regulatory elements and the *Atoh8* promoter. The 3’ loxP was placed in intron 1. Recombination between these sites deletes exon 1 and the
putative Atoh8 promoter. Atoh8Δββ mice were viable with no apparent defects.

Transgenic mice expressing CMV-Cre (64) were used to recombine the Atoh8β allele in the mouse germline cells and create the Atoh8Δex1 allele (Figure 2.7.A). Atoh8Δex1/ex1 mice were viable at the expected Mendelian ratio at postnatal day 14 on a mixed genetic background (Table 2.1) and did not display any overt phenotypes into adulthood. Atoh8Δex1/ex1 mice also failed to exhibit the runting previously reported Atoh8GFP/GFP mice, suggesting that the previous characterization of the Atoh8GFP/GFP mouse was incorrect. At this point, we attempted to reproduce the previous data showing postnatal death in Atoh8GFP/GFP mice on a C57BL/6 background. We were unable to reproduce this data, as Atoh8GFP/GFP mice were found at Mendelian ratios (Table 2.2) and exhibited no overt phenotypes. In summary, two different mutant alleles of Atoh8 exon 1 (Atoh8Δex1/ex1 and Atoh8GFP/GFP) do not affect viability and indicate that Atoh8 exon 1 is not required for survival in the mouse.

Although our Atoh8Δex1 allele removed exon 1 and the putative promoter of Atoh8, we were able to detect continued expression of Atoh8 exon 2 and 3 mRNA transcript in Atoh8Δex1/ex1 mice by RT-PCR, suggesting that alternative transcripts exist for Atoh8. In order to characterize these transcripts, we performed 5’ Rapid Amplification of cDNA Ends (5’RACE) on mRNA isolated from Atoh8Δex1/ex1 mice (data not shown). Using primers in Atoh8 exon 2, we identified an alternative exon in intron 1 that spliced into exon 2 and 3. There were no open reading frames in this transcript, however, indicating that this transcript could not encode any truncated ATOH8 protein.

Although we were unable to identify any open reading frames in our Atoh8Δex1/ex1 mice, it is possible that our 5’RACE experiments failed to identify all alternative
transcripts. We hypothesized that, in \textit{Atoh8}\textsuperscript{Δex1/Δex1} mice, such transcripts could splice into the intact exon 2 and possibly create an open reading frame and a partially functional ATOH8-like protein. This hypothesis would potentially explain the difference in phenotype between our \textit{Atoh8}\textsuperscript{Δex1/Δex1} mouse and the previously published \textit{Atoh8}\textsuperscript{Δex1-2/Δex1-2} mouse that lacks both exon 1 and exon 2. To test this hypothesis and definitively address the role of \textit{Atoh8} exon 2, we replaced exon 2 with an IRES-LacZ gene trap cassette to generate the \textit{Atoh8}\textsuperscript{LacZΔex2} allele (Figure 2.8.A). \textit{Atoh8}\textsuperscript{LacZΔex2/LacZΔex2} mice were viable and present at the expected Mendelian ratio at postnatal day 14 (Table 2.3). These results indicate that exon 2 of \textit{Atoh8} is not required for survival in the mouse. Since exons 1 and 2 encode virtually the entire coding sequence of \textit{Atoh8}, these results indicate that the ATOH8 protein is not required in the mouse for survival, and that the embryonic lethality of \textit{Atoh8}\textsuperscript{Δex1-2/Δex1-2} mice does not reflect a requirement for ATOH8 protein during mouse development but instead may reflect loss or alteration of another region due to the use of a BAC targeting construct.

\textit{Atoh8 expression is restricted to the atria, lung mesenchyme, and vascular smooth muscle in the mouse}

In order to identify possible functions for \textit{Atoh8} in the mouse, we determined the expression pattern of \textit{Atoh8}. Due to the cardiac phenotype we identified in the \textit{atoth8} morphant fish, we first focused on \textit{Atoh8} expression in the heart. Previous studies have reported cardiac expression of \textit{Atoh8} (3,5,65), but these studies have not defined the precise spatial or temporal expression pattern of \textit{Atoh8} within the heart. In order to determine the expression pattern of \textit{Atoh8}, we generated antibodies against an N-terminal
fragment of the ATOH8 protein. These antibodies were able to detect ATOH8 when overexpressed in cell culture but could not detect ATOH8 in mouse tissues using immunohistochemical staining (data not shown), and we were unable to use them to determine Atoh8 expression in vivo.

In lieu of an effective antibody, we used the Atoh8\textsuperscript{LacZ\textDelta ex2} IRES-LacZ gene trap allele as a reporter for Atoh8 expression. Whole mount X-gal staining of Atoh8\textsuperscript{LacZ\textDelta ex2/+} embryos at embryonic day 9.5 (E9.5) revealed LacZ expression in the developing brain, eye, somites, limb bud and branchial arches, while the heart was free of LacZ expression (Figure 2.9.A). This pattern was largely maintained at E12.5, with persistent LacZ expression in somites, brain, eye, and limb bud (Figure 2.9.B), but no expression in the developing heart or liver was detected (Figure 2.9.C). To determine whether Atoh8 is expressed in later stages of heart development, we performed whole mount X-gal staining on isolated Atoh8\textsuperscript{LacZ\textDelta ex2/+} hearts (Figure 2.9.D). At E16.5, strong LacZ expression was observed in both the aorta and pulmonary artery. There was weak staining of both the left and right atria; the ventricles were negative except for the developing coronary vessels. At postnatal day 1 (P1) strong expression in the aorta and pulmonary artery persisted, with increased expression in the coronaries and atria, and no staining of the ventricles. The vascular pattern in the great vessels and coronaries was maintained at P14. However, the atrial pattern was altered, with continued right atrial expression but an absence of expression in the left atrium. Whole mount X-gal staining of Atoh8\textsuperscript{LacZ\textDelta ex2/+} organs also revealed strong expression throughout the lung at both E16.5 and P1 (Figure 2.9.E).

In order to determine which cells express Atoh8 in the heart, we next used
immunohistochemistry to detect GFP expression from the $Atoh8^{\Delta ex1-2}$ nuclear GFP reporter allele (5). As was seen with whole mount LacZ staining of $Atoh8^{LacZ\Delta ex2/+}$ mice, nuclear GFP expression was detected throughout the atrial myocardium at E16.5 (Figure 2.9.H-I). In contrast to whole mount LacZ staining, GFP expression in the atria could also be detected at the earlier E12.5 time point (Figure 2.9.F-G), likely due to the higher sensitivity of detection for this reporter. At both time points, the ventricles displayed weaker expression than the atria (Fig. Figure 2.9.G, J). In the ventricles, nuclear GFP expression was limited to the layers of cardiomyocytes in closest proximity to the endocardium (Figure 2.9.J). These results indicate that cardiac expression of $Atoh8$ is primarily limited to the atria.

We next used the $Atoh8^{\Delta ex1-2/+}$ GFP reporter allele to further define the $Atoh8$ expression pattern in the lung and vasculature. The GFP reporter showed strong expression throughout the mesenchyme of the lung at E12.5 (Figure 2.9.K-L). In contrast, the epithelium was completely devoid of GFP expression at E12.5 (Figure 2.9.L). This pattern of expression was preserved at E16.5, with strong mesenchymal expression and no expression in the epithelium of either the proximal or distal airways (Figure 2.9.M-N).

$Atoh8^{\Delta ex1-2/+}$ GFP expression was observed in the vascular smooth muscle of the aorta and pulmonary artery (Figure 2.9.O-R), and in the smaller arteries of the lung (Figure 2.9.T). The endothelium of these vessels was noticeably free of GFP expression (Figure 2.9.Q), indicating that vascular $Atoh8$ expression is limited to the smooth muscle. As in ventricular myocardium, GFP expression appeared to be strongest in the smooth muscle cells directly underlying the endothelium (Figure 2.9.P-Q), suggesting that
muscle cell *Atoh8* expression may be regulated in some way by the endothelium. In contrast to the arterial expression pattern, the pulmonary veins were largely free of GFP expression (Figure 2.9.S). Thus studies of the *Atoh8*\textsuperscript{LacZΔex2} LacZ and *Atoh8*\textsuperscript{Δex1-2+/} GFP reporter alleles are consistent and demonstrate that *Atoh8* is specifically expressed in the atria of the heart, lung mesenchyme, and arterial vascular smooth muscle. These findings suggest that restricted gene expression in the mouse may explain the lack of an important role for *Atoh8* in mouse heart development.

*Atoh8* interacts weakly with *Gata4* in the mouse

Although our results indicated that there is not an absolute requirement for ATOH8 in the mouse, our studies in zebrafish embryos and our biochemical studies suggested that ATOH8 might be necessary for optimal GATA4 and/or FOG2 function in mice. Loss of *Gata4* or *Zfpm2* (encoding FOG2) in the mouse leads to severe cardiovascular defects and death at E9.5-10.5 and E13.5 respectively (14,15,31). To test for genetic interaction between *Atoh8* and *Gata4* or *Zfpm2* we generated *Zfpm2*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} and *Gata4*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} animals. *Zfpm2*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} animals were viable and present at expected Mendelian ratios at both postnatal day 1 (P1) and postnatal day 14 (P14) (Table 2.4). In contrast, a small decrease in the number of *Gata4*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} animals was observed at P1, and this deficit became more pronounced by P14 (Table 2.5). *Gata4*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} mice that survived past P14 were indistinguishable from littermates and displayed no overt phenotypes, indicating that death occurs in both the immediate neonatal (by P1) and early postnatal (P1-P14) periods.

The partial loss of *Gata4*\textsuperscript{+/-} *Atoh8*\textsuperscript{GFP/GFP} mice observed at P1 is consistent with
either embryonic or neonatal death. To more precisely determine the time of death, we
examined embryos in late embryogenesis to determine viability of the $Gata4^{+/−}$
$Atoh8^{GFP/GFP}$ animals in utero. In contrast to P1, there was no loss of $Gata4^{+/−}$
$Atoh8^{GFP/GFP}$ embryos at embryonic day 17.5 (E17.5) relative to control littermates
(Table 2.6). These results indicate that loss of $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice occurs between
E17.5 and P1. These results reveal that although ATOH8 is not essential for mouse
development, genetic interaction between $Gata4$ and $Atoh8$ is conserved from zebrafish
to mammals.

$Gata4^{+/−}Atoh8^{GFP/GFP}$ mice exhibit structurally normal hearts and lungs

We next sought to understand the small increase in mortality in $Gata4^{+/−}$
$Atoh8^{GFP/GFP}$ mice. $Gata4^{+/−}$ heterozygotes have been reported to exhibit partial postnatal
lethality when backcrossed onto a C57/BL6 background (18,19). Although the cause
of death was not identified in these studies, a higher incidence of cardiovascular defects
(18,19) was identified in $Gata4^{+/−}$ animals compared to wild-type littermates. In order to
determine whether $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice die due to cardiac defects, we examined
animals in late embryogenesis for these defects. $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice did not
exhibit a decrease in cardiac fractional shortening at E17.5 relative to littermates (Figure
2.10.A), indicating that there is no myocardial or contractile defect in these animals.
Structural abnormalities were also not detected in $Gata4^{+/−}Atoh8^{GFP/GFP}$ hearts by
histology (Figure 2.10.B-C). These results suggest that defective heart development is
not the cause of death in $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice. To further rule out a myocardial
cause of death in $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice, we generated $Nkx2.5-Cre Gata4^{+/−}$...
Atoh8\(^{GFP/GFP}\) mice. In these mice Gata4 heterozygosity is limited to the Nkx2.5-lineage cells, including the myocardium. There was no loss of Nkx2.5-Cre Gata4\(^{fl/+}\) Atoh8\(^{GFP/GFP}\) mice at P1 (Table 2.7), confirming that the lethality seen in Gata4\(^{+/}\)Atoh8\(^{GFP/GFP}\) mice is not due to more subtle cardiac defects.

Our zebrafish studies identified the swimbladder as a site of gata4 and atoh8 interaction. The mammalian lung is the closest evolutionary equivalent to the swimbladder; both organs share an origin from a common region of the endoderm (66) and express similar surfactant proteins for inflation (67). In addition, Gata4\(^{+/}\) heterozygotes on a C57/BL6 background have also been reported to exhibit an increase in the pulmonary defects (18). These results suggested that the increased lethality observed in Gata4\(^{+/}\)Atoh8\(^{GFP/GFP}\) animals may be due to impaired lung development. We were unable to detect gross structural abnormalities in Gata4\(^{+/}\)Atoh8\(^{GFP/GFP}\) lungs (Figure 2.11.A-B) or a statistically significant change in lung mass (Figure 2.11.C) at E18.5.

There was also no change in the expression of the type II cell marker surfactant protein-C (Sftpc) or the type I marker Aquaporin-5 (Aqp5) by quantitative RT-PCR (Figure 2.11.D) at E18.5, suggesting that both type I and type II cells are present at normal numbers. Although we could not identify morphologic defects in lung development, we next examined the expression of molecular factors involved in mesenchymal-to-epithelial signaling in Gata4\(^{+/}\)Atoh8\(^{GFP/GFP}\) animals because pulmonary expression of Atoh8 (Figure 2.9.K-N) and Gata4 (18,37) is limited to the lung mesenchyme. Mesenchymal expression of Wnt2, Fgf10, and Tbx4 has previously been shown to be required for proper lung development (68-70). Expression of these factors was downregulated in both Gata4\(^{+/}\) and Atoh8\(^{GFP/GFP}\) lungs at E12.5 (Figure 2.11.E), although we did not observe an
additional decrease in the expression of these factors in \( Gata4^{+/+}, Atoh8^{GFP/GFP} \) compound mutants. We also observed a decrease in the mRNA levels of the mesenchymal transcription factor \( Twist1 \) in \( Atoh8^{GFP/GFP} \) lungs, and \( Twist1 \) mRNA levels were further decreased in \( Gata4^{+-}, Atoh8^{GFP/GFP} \) (Figure 2.11.E). These results suggest that \( Gata4 \) and \( Atoh8 \) regulate gene expression in the developing lung mesenchyme. However, the lack of any structural defects in the lung indicates that that the essential role for Atoh8 in zebrafish swimbladder development is not conserved in the mammalian lung.

**Discussion**

Our studies revealed essential roles for Atoh8 in zebrafish cardiac and swim bladder development that are performed in concert with Gata and Fog transcription factors. Biochemical studies indicate that ATOH8, GATA, and FOG2 can form a physical complex, suggesting that this molecular mechanism underlies the genetic interactions observed in the developing zebrafish heart. However, our Atoh8 loss-of-function studies in the mouse indicate that Atoh8 is not required for survival in mammals and that Atoh8 does not have a required role in mammalian cardiac development. Expression analysis of Atoh8 in mice indicate that cardiovascular expression of Atoh8 is primarily limited to atrial cardiomyocytes and vascular smooth muscle, possibly explaining the lack of a requirement for Atoh8 in the developing heart. Our finding of partial postnatal lethality in \( Gata4^{+/+}, Atoh8^{GFP/GFP} \) demonstrates that a weak genetic interaction between \( Gata4 \) and \( Atoh8 \) exists in mammals, but we were not able to link this lethality to defects in either the heart or lungs.
Previous studies of ATOH8 function in vivo have identified essential roles in both zebrafish and mouse early embryogenesis (5,7). While our studies identify an additional essential role in zebrafish development for cardiac looping, we find that neither the first nor second exon of Atoh8 is required for mouse development or postnatal survival. Since virtually the entire coding sequence of Atoh8 is contained within these two exons, these findings demonstrate definitively that the ATOH8 protein is not required for mouse development or survival. This result conflicts with a previous report of early embryonic lethality in the Atoh8Δex1-2 mouse lacking both exon 1 and 2 and intervening intron 1 (5). There exist several alternative explanations for the lethality seen in the Atoh8Δex1-2 mouse.

First, it is possible that removal of intron 1 in Atoh8Δex1-2 mice may have deleted a critical non-coding element within this intron. Second, it is possible that this discrepancy could reflect differences in strain background and the effect of modifier genes. This is unlikely as both Atoh8Δex1-2 and Atoh8GFP mice were studied after being back-crossed more than 7 generations onto a pure C57Bl/6 background. Finally, it is possible that this difference reflects disruption of a genetic element outside the Atoh8 locus in the Atoh8Δex1-2 mouse. The Atoh8Δex1-2 mouse was created by gene targeting of ES cells using a bacterial artificial chromosome (BAC) targeting vector, an approach that uses much longer recombination arms than conventional gene targeting. With this approach recombination can take place over a much larger area that, unlike gene targeting with conventional vectors, cannot be fully assessed by PCR or Southern blot analysis of genomic DNA following recombination. Thus it seems most likely that mutations outside the coding region of Atoh8 are responsible for the embryonic lethality of Atoh8Δex1-2 mice.
Our studies reveal a striking requirement for Atoh8 during early cardiac development in the zebrafish, where it functions in close association with Gata4 and Fog1 to regulate cardiac looping (Figure 2.1 and 2.3). Zebrafish atoh8 displays a high degree of sequence conservation with its murine orthologue Atoh8, particularly within the bHLH domain (4), suggesting the possibility of a conserved role for Atoh8 in cardiac development. This possibility is strengthened by the recent identification of an ultra-conserved cardiac enhancer in the second intron of Atoh8 present in both fish and mice (71), and by our finding that murine ATOH8 interacts with FOG2 and GATA4 biochemically. In contrast to Atoh8-deficient fish, however, mice lacking ATOH8 are viable and do not display defects in heart development or function, even when put on a Gata4+/- background to further stress the putative transcriptional mechanism. One explanation for this species difference appears to lie in the highly specific and restricted pattern of Atoh8 expression in the mouse heart. Using two different reporter alleles, we detect Atoh8 predominantly within the atria during development and persistent expression that becomes primarily restricted to the right atrium in mature animals. These results suggest a shift in ATOH8 function from a broad role in regulating early cardiac morphogenesis in the zebrafish to a more specific role in atrial development and/or function in mammals, and perhaps one that is more important in the mature than developing heart.

In addition to identifying an essential role for atoh8 in the development of the zebrafish heart and swimbladder, our studies reveal strong and specific genetic interaction between atoh8 and gata4 in the development of these tissues. This genetic interaction is also weakly observed in mammals, as Gata4+/−Atoh8GFP/GFP mice exhibit a
partially lethal phenotype. This phenotype is not due to a cardiac defect, as these animals have functionally and structurally normal hearts. In addition, we were unable to reproduce the lethality seen in $Gata4^{+/−}Atoh8^{GFP/GFP}$ mice with myocardial-specific deletion, further ruling out the heart as the cause of death. We could not determine the basis for the compound lethality observed in mice, but the neonatal timing of this lethality, our studies identifying the lung mesenchyme as a site of strong $Atoh8$ and $Gata4$ expression, and the small changes in mesenchymal-to-epithelial signaling observed in the developing lung of ATOH8-deficient embryos suggest that subtle defects in lung function around the time of birth may be causal.

Our biochemical studies demonstrate that mouse ATOH8, FOG2 and GATA4 are capable of forming a single protein complex in vitro, suggesting that ATOH8 may regulate GATA and FOG function in mammals as well as fish. However, extensive genetic studies to define such an interaction have very little requisite interaction during development despite the important roles previously demonstrated for GATA4 and FOG2. As suggested above, part of the explanation for this species difference appears to lie in the restricted expression pattern of $Atoh8$ in the heart, the tissue in which GATA4 and FOG2 play required roles during development. Another explanation for this difference may lie in the expression and function of GATA and FOG in the mouse versus the zebrafish. Previous studies using either hypomorphic $Gata4$ alleles or $Gata4^{+/−}$ animals have revealed that partial loss of GATA4 is sufficient to confer a lethal phenotype (18,19,72). In contrast, lethality in $Fog2$ heterozygotes has not been reported, suggesting that larger reductions in FOG2 levels may be necessary to confer phenotypes in the mouse. Since our biochemical studies implicate FOG as the bridge between ATOH8 and
GATA, more insight into the role of ATOH8 may require a better understanding of the in vivo roles of FOG and its mechanism of action.

Materials and Methods

Mice

We used the previously reported Gata4 null allele (14), Zfpm2 (Fog2) null allele (31), Gata4fl floxed allele (73), CMV-Cre allele (64), Nkx2.5Cre allele (74), and Atoh8\textsuperscript{Δex1-2} allele (5). The Atoh8\textsuperscript{GFP}, Atoh8\textsuperscript{Δex1}, and Atoh8\textsuperscript{LacZ,Δex2} alleles were generated by creating gene-targeting constructs by recombineering (75). SV/129 ES cells were targeted and then screened by Southern blotting. We injected correctly targeted ES clones into C57/BL6 blastocysts. Atoh8\textsuperscript{GFP/GFP} mice were backcrossed onto a C57/BL6 background. All other mouse experiments were done in mixed genetic backgrounds. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols. The following genotyping primers were used:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atoh8GFP-WT-F</td>
<td>5'- GACACAGCCAGGGAATTCC -3’</td>
</tr>
<tr>
<td>Atoh8GFP-GFP-R</td>
<td>5'- GCAGAAGAAGGCATCAAGG -3’</td>
</tr>
<tr>
<td>Atoh8GFP-R</td>
<td>5'- GCAGAAGAAGGCATCAAGG -3’</td>
</tr>
<tr>
<td>Atoh8flox-F</td>
<td>5'- AAGCATGGGTGTTCTCCAGATTC -3’</td>
</tr>
<tr>
<td>Atoh8flox-R</td>
<td>5'- GGCTGGTCAATTGCATCAACTG -3’</td>
</tr>
<tr>
<td>Atoh8Δex1-F</td>
<td>5'- AAGCATGGGTGTTCTCCAGATTC -3’</td>
</tr>
<tr>
<td>Atoh8Δex1-R</td>
<td>5'- AGAGCGAAAGGTCGTGCTC -3’</td>
</tr>
<tr>
<td>Atoh8Δex2-F</td>
<td>5'- TGGGAATAGCTGAGCAGGAT -3’</td>
</tr>
<tr>
<td>Atoh8Δex2-WT-R</td>
<td>5'- CACACAACCTGGAGAAGCTGA -3’</td>
</tr>
<tr>
<td>Atoh8Δex2-LacZ-R</td>
<td>5'- ACCTGGTTGCATGGAGGAG -3’</td>
</tr>
<tr>
<td>GATA4-F</td>
<td>5'- CGAGACTAGTGAGACGTC -3’</td>
</tr>
<tr>
<td>GATA4-R</td>
<td>5'- ACTGGGTCTGCCATGTATCC -3’</td>
</tr>
<tr>
<td>Fog2-WT-F</td>
<td>5'- GCCAGATCTAATTCAAACAGCGTC -3’</td>
</tr>
<tr>
<td>Fog2-Neo-F</td>
<td>5'- GCCTGCTTCTTACTGAAGGCTTTT -3’</td>
</tr>
<tr>
<td>Fog2-R</td>
<td>5'- CGTACCAGAAAGATTCTGTCC -3’</td>
</tr>
</tbody>
</table>
**Zebrafish morpholino studies**

We used Tupfel long fin (TLF) strain zebrafish for all studies except for the transgenic cardiac GFP studies. For the cardiac GFP studies, a previously described transgenic cardiac reporter zebrafish line was used (76,77). Morpholino oligonucleotides were obtained from Gene Tools and injected into one-cell-stage embryos at the indicated doses. For all images, embryos were mounted in 2% methylcellulose, and bright field and GFP images were acquired using an Olympus MVX10 microscope with an Olympus DP72 camera. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols. Morpholino sequences are listed below:

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gata4</td>
<td>5’- TCCACAGGTGAGCGATTATTGCTCC -3’</td>
</tr>
<tr>
<td>zfpm1</td>
<td>5’- TCATGTCCCCCTACCTCACTGGCA -3’</td>
</tr>
<tr>
<td>tbx5a</td>
<td>5’- CCTCCTCTTCCAAAGTACAGTCC -3’</td>
</tr>
<tr>
<td>mef2ca</td>
<td>5’- GAAAAGTGCTCTCACTGTCGCCCAT -3’</td>
</tr>
<tr>
<td>atoh8-MO1</td>
<td>5’- GTTTAGATGTGGGTCTTCTTATTCG -3’</td>
</tr>
<tr>
<td>atoh8-MO2</td>
<td>5’- CCGTGGGAATTACCTGCTTTTCTGA -3’</td>
</tr>
<tr>
<td>atoh8-MO3</td>
<td>5’- GATGTCAGGCTGACTAAAGTCC -3’</td>
</tr>
<tr>
<td>atoh8-MO1missense</td>
<td>5’- GTTTACATCTGCGTTGATTTCG -3’</td>
</tr>
<tr>
<td>Control</td>
<td>5’- CCTCTTACCTCAGTTACAATTTATA -3’</td>
</tr>
</tbody>
</table>

32
Zebrasfish in situ hybridization

Tupfel long fin (TLF) strain zebrafish were used for all experiments. For the gata4 and atoh8 probes, the coding region of each transcript was amplified from 48 hpf zebrafish cDNA and cloned into pcDNA3. Probes were synthesized using a DIG RNA labeling kit (Roche). In situ hybridization was performed as previous described (78).

Co-immunoprecipitation studies

cDNAs encoding GATA4, and FOG2 were cloned into pcDNA3.1 (Invitrogen); V5 epitope tags were added during cloning. The GATA4-V217G point mutation was introduced by site-directed mutagenesis. cDNA encoding ATOH8 was cloned into p3XFLAG-CMV-7.1 (Sigma). Constructs were transiently transfected into HEK293T cells using Fugene 6 (Roche). Nuclear extracts were isolated from transfected cells as previously described (79). Immunoprecipitations were performed as previously described (76). Flag-tagged Atoh8 was detected with HRP-conjugated anti-Flag-M2 antibody (1:1000, Sigma). V5-tagged proteins were detected with monoclonal mouse anti-V5 antibody (1:5000, Invitrogen) and HRP-conjugated goat anti-mouse IgG antibody (1:5,000 Jackson ImmunoResearch Laboratories Inc.)

GST fusion protein studies

cDNAs encoding ATOH8 and FOG2 were cloned into pGEX-4T-1 (GE Healthcare Life Sciences) and transformed into BL21 E. coli. Transformed cells were cultured at 37C to a density of A_{600}=0.6 and induced with 0.1mM IPTG for 4 hours at
30C. Proteins were purified from cell lysates by using a Bulk GST Purification Module kit (GE Healthcare Life Sciences).

**5’ rapid amplification of cDNA ends (RACE)**

Total RNA was isolated from postnatal day 14 (P14) Atoh8\(^{Δex1/Δex1}\) heart and liver tissue using Trizol (Invitrogen). 5’ cDNA fragments were generated and amplified using the SMARTer RACE cDNA Amplification Kit (Clontech). Following amplification, fragments were separated by gel electrophoresis and purified (Qiagen). Isolated DNA fragments were cloned into pCR2.1-TOPO by TOPO-TA cloning (Invitrogen). Single clones were isolated and sequenced to identify the cDNA fragments.

**Whole mount X-gal staining**

Whole embryos or organs were dissected at the indicated ages. Tissues were fixed and stained as previously described (80). Images were acquired using an Olympus MVX10 microscope with an Olympus DP72 camera.

**Histology and immunostaining**

Mouse embryos at the indicated developmental stages were dissected, fixed in paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. We performed immunostaining and hematoxylin-eosin staining. Histological techniques were performed as previously described (81,82). For immunostaining, a goat polyclonal antibody against GFP (1:250, Abcam) was used. Bright field and fluorescent images were acquired using a Nikon Eclipse 80i microscope.
Fetal echocardiography

Trans-uterine embryonic ultrasound was performed using a high-resolution Vevo 770 micro-ultrasound system (VisualSonics Inc.) as previously reported (83).

Gene expression studies

E12.5 lung buds and E18.5 lungs were dissected from embryonic mice. For E12.5 lung buds, six lung buds were pooled together; for the E18.5 lungs, the right lung was used. RNA was isolated from the tissue using Trizol (Invitrogen). 500ng of RNA and 50ng random hexamer primers were then used to synthesize cDNA using the SuperScript First-Strand Synthesis System (Invitogen). Quantitative RT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). RT-PCR primers are listed below:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactant Protein C-Forward</td>
<td>5’- ACCCTGTGTGGAGAGCTACCA -3’</td>
</tr>
<tr>
<td>Surfactant Protein C-Reverse</td>
<td>5’- TTTGCGGAGGGTCTTTTCTT -3’</td>
</tr>
<tr>
<td>Aquaporin 5-Forward</td>
<td>5’- ATGAACCCAGCCCGATCTTT -3’</td>
</tr>
<tr>
<td>Aquaporin 5-Reverse</td>
<td>5’- ACATCGTGTCCTACCCAGAAG -3’</td>
</tr>
<tr>
<td>Wnt2-Forward</td>
<td>5’- TCTTGAACAAAGAATGCAAGTGCA -3’</td>
</tr>
<tr>
<td>Wnt2-Reverse</td>
<td>5’- GAGATAGTCGCTGTTTTCCTGAA -3’</td>
</tr>
<tr>
<td>FGF10-Forward</td>
<td>5’- TGGATGAAGTTATCTGCACAT -3’</td>
</tr>
<tr>
<td>FGF10-Reverse</td>
<td>5’- GAGAGTTGACCTTCATACCAAATTTCC -3’</td>
</tr>
<tr>
<td>Tbx4-Forward</td>
<td>5’- TGCCCCCTCAACACCTTTCCCA -3’</td>
</tr>
<tr>
<td>Tbx4-Reverse</td>
<td>5’- TGGGGGTACCTCAGTTGCAA -3’</td>
</tr>
<tr>
<td>Twist1-Forward</td>
<td>5’- CCGACAAGCTGAGCAAGAT -3’</td>
</tr>
<tr>
<td>Twist1-Reverse</td>
<td>5’- GACCTGAGTACAGGAAGTGC -3’</td>
</tr>
<tr>
<td>GAPDH-Forward</td>
<td>5’- ATCAACCATCTTCACCAAGAGCAG -3’</td>
</tr>
<tr>
<td>GAPDH-Reverse</td>
<td>5’- GCCCTTCCACAATGCCAAG -3’</td>
</tr>
</tbody>
</table>
Statistics

P values in mouse genetic crosses were calculated using chi-squared tests. An unpaired two-tailed Student’s t-test was used for all other p-values.
Figure 2.1. Morpholino knockdown of *atoh8* results in a failure of zebrafish heart looping and pericardial edema.

(A-F) One-cell zebrafish embryos were injected with *atoh8*-MO1 morpholino (D-F) and examined at 72 hours post fertilization (hpf) in comparison to uninjected controls (A-C). Transgenic fish expressing myocardial GFP were used in C and F. (G-H) One-cell zebrafish embryos were injected with *atoh8*-MO2 (G) and *atoh8*-MO3 (H) and examined at 72 hpf. (I) Low doses of the three *atoh8* morpholinos were injected independently and in combination into one-cell embryos and then scored at 72 hpf for an unlooped heart tube. Graph in I shows the mean of three injections; errors bars represent SEM.
Figure 2.2. Morpholino knockdown of atoh8 prevents swimbladder inflation. (A-C) One cell zebrafish embryos were injected with atoh8-MO1. Swimbladder inflation was scored at 96 hpf in injected embryos (B-C) versus wild-type (WT) embryos (A) at 96 hpf. Failure of swimbladder inflation was observed in morphant embryos with (B) and without (C) the heart phenotype. SB=swimbladder, PE=pericardial edema. Swimbladder location is outlined by dashed line.
Figure 2.3. *atoh8* specifically interacts with *gata4* and *zfpm1* in the zebrafish heart.
Figure 2.3. *atoh8* specifically interacts with *gata4* and *zfpm1* in the zebrafish heart.

(A) Low doses of *atoh8*-MO1 and *gata4* MO were injected alone or in combination into one-cell embryos and scored at 96hpf for failure of swimbladder inflation. (B-F) Low doses of morpholinos were injected alone or in combination into one-cell embryos. Embryos were scored at 72 hpf for the unlooped heart tube phenotype. The following combinations were used: *atoh8*-MO1 + *gata4* (B), *atoh8*-MO1 + *mef2ca* (C), *atoh8*-MO1 + *tbx5-a* (D), *atoh8*-MO1 + *zfpm1* (E), and *gata4* + *zfpm1* (F). (G) Doses for *atoh8*-MO1, *zfpm1*, and *gata4* morpholinos were lowered as indicated and injected individually or in combination into one-cell embryos. Embryos were scored at 72 hpf for the unlooped heart tube phenotype. For all experiments, control morpholino was used to equalize amount of the total morpholino used in each injection. Graphs show the mean of 3 injections with >50 embryos per injection; errors bars represent SEM. * P<0.05, ** P<0.01, *** P<0.001
Figure 2.4. ATOH8 physically interacts with FOG2 and indirectly with GATA4 via FOG2.

(A-B) FLAG-ATOH8, V5-GATA4-WT, V5-GATA4-V217G, and V5-FOG2 were transiently expressed in HEK293T cells in the indicated combinations, immunoprecipitated with anti-FLAG antibody, and detected by Western blotting with anti-Flag or anti-V5 antibodies as indicated. V5-GATA4-WT is wild-type GATA4 with a V5 epitope tag. V5-GATA4-V217G is a GATA4 mutant with a V5 epitope tag and a valine-to-glycine point mutation at residue 217 that abolishes FOG2-GATA4 interaction.
Figure 2.5.  

*atoh8* expression in the zebrafish.

(A-B) *atoh8* expression by in situ hybridization at 13 hours post fertilization (hpf). Lateral (A) and dorsal (B) views are shown. (C-E) In situ hybridization for *atoh8* (C-D) and *gata4* (E) at 30 hpf. Lateral (C) and dorsal (D) views are shown for *atoh8*. Arrowheads in C and E indicate heart tubes. (F) Expression of *atoh8* at 48 hpf by in situ hybridization. Arrowhead in F indicates heart.
Figure 2.6. Targeting strategy for the $Atoh8^{GFP}$ allele.

The eGFP coding sequence followed by a stop codon was inserted into exon 1. The PGK-Neo cassette was removed \textit{in vivo} by crossing these mice to a FLP-expressing mouse line to promote excision between the FRT sites (blue circles). (B) Southern blot with Ndel digest to detect correct targeting in embryonic stem cells is shown.
Figure 2.7. Targeting strategy for the \textit{Atoh8}^{flo}\textsuperscript{f} and \textit{Atoh8}^{Δex1}\textsuperscript{f} alleles.

(A) LoxP sites were inserted to flank exon 1 of the \textit{Atoh8} gene. The 5’ loxP was inserted upstream of a conserved genomic region extending 517 bp 5’ of the transcriptional start site. \textit{Atoh8}^{Δex1}\textsuperscript{f} allele was generated by Cre-mediated recombination of the \textit{Atoh8}^{flo}\textsuperscript{f} allele in the mouse germline using CMV-Cre mouse line. Green triangles are LoxP sites; blue circles are FRT sites. (B) Southern blot with SpeI digest to detect correct targeting in embryonic stem cells. (C) Reverse-transcriptase PCR using primers in \textit{Atoh8} exons 1 and 3 to confirm loss of exon 1 transcripts in \textit{Atoh8}^{Δex1/Δex1}\textsuperscript{f} in the hearts of postnatal day 21 mice.
Table 2.1. Survival of $Atoh8^{\Delta ex1/\Delta ex1}$ mice at postnatal day 14.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed (%)</th>
<th>Mendelian (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Atoh8^{+/+}$</td>
<td>43</td>
<td>30%</td>
<td>25%</td>
</tr>
<tr>
<td>$Atoh8^{\Delta ex1/+}$</td>
<td>70</td>
<td>48%</td>
<td>50%</td>
</tr>
<tr>
<td>$Atoh8^{\Delta ex1/\Delta ex1}$</td>
<td>32</td>
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<tr>
<td>Total</td>
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Table 2.2. Survival of $Atoh8^{GFP/GFP}$ mice at postnatal day 14.

<table>
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<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Atoh8^{++}$</td>
<td>28</td>
<td>34%</td>
<td>25%</td>
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<tr>
<td>$Atoh8^{GFP/+}$</td>
<td>34</td>
<td>40%</td>
<td>50%</td>
</tr>
<tr>
<td>$Atoh8^{GFP/GFP}$</td>
<td>22</td>
<td>26%</td>
<td>25%</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td></td>
<td>P=0.14</td>
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</table>
Figure 2.8. Targeting strategy for the \(Atoh8^{\text{LacZ} \Delta \text{ex}2}\) allele. (A) An IRES-LacZ gene trap cassette was inserted in place of \(Atoh8\) exon 2 to generate the \(Atoh8^{\text{LacZ} \Delta \text{ex}2}\) allele. The first three nucleotides of exon 2 were left in place to preserve the endogenous splice acceptor site. Green triangles are LoxP sites; blue circles are FRT sites. (B) Southern blot with Ncol digest was used to detect correct targeting in embryonic stem cells. (C) Reverse-transcriptase PCR using primers in \(Atoh8\) exons 1 and 3 to confirm loss of transcripts in \(Atoh8^{\text{LacZ} \Delta \text{ex}2/LacZ} \Delta \text{ex}2\) in the hearts of postnatal day 21 mice.
Table 2.3. Survival of $Atoh8^{LacZ\Delta ex2/LacZ\Delta ex2/+}$ mutant mice at postnatal day 14.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Atoh8^{+/+}$</td>
<td>17</td>
<td>26%</td>
<td>25%</td>
</tr>
<tr>
<td>$Atoh8^{LacZ\Delta ex2/+}$</td>
<td>29</td>
<td>45%</td>
<td>50%</td>
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<tr>
<td>$Atoh8^{LacZ\Delta ex2/LacZ\Delta ex2}$</td>
<td>19</td>
<td>29%</td>
<td>25%</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>P=0.64</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.9. *Atoh8* is expressed in the atria, lung mesenchyme, and vascular smooth muscle.
Figure 2.9. *Atoh8* is expressed in the atria, lung mesenchyme, and vascular smooth muscle. (A-C) Whole mount X-gal staining was performed on *Atoh8*\(^{\text{LacZ}_{\text{ex}2/+}}\) embryos at E9.5 (A) and E12.5 (B-C). C shows a magnified image of the boxed region in B with forelimb removed. (D-E) Whole mount X-gal staining was performed at the indicated stages on isolated *Atoh8*\(^{\text{LacZ}_{\text{ex}2/+}}\) hearts (D) and *Atoh8*\(^{\text{LacZ}_{\text{ex}2/+}}\) lungs (E). (F-T) Anti-GFP immunostaining of heart sections from *Atoh8*\(^{\text{ex1-2/+}}\) mice at E12.5 (F-G, K-L, O) and E16.5 (H-J, M-N, P-T). Horseradish peroxidase staining was used in panels F-P and R-T; FITC staining was used to panel Q. Arrowheads in Q indicate endothelial cells lacking GFP expression. Scale is shown in each panel. H=heart, Liv=liver, Ao=aorta, PA=pulmonary artery, RA=right atrium, RV=right ventricle, LA=left atrium, LV=left ventricle, IVS=interventricular septum, ProxAir=proximal airway, DA=distal airway, PV=pulmonary vein, Art=artery.
Table 2.4. Survival of Zfpm2\textsuperscript{+/−} Atoh8\textsuperscript{GFP/GFP} mice at postnatal day 1 (P1) and postnatal day 14 (P14).

Cross: Atoh8\textsuperscript{AGFP/+} X Zfpm2\textsuperscript{+/−} Atoh8\textsuperscript{GFP/+}

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
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<td>Atoh8\textsuperscript{+/+}</td>
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</tr>
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<td>Atoh8\textsuperscript{GFP/+}</td>
<td>16</td>
<td>40%</td>
<td>25%</td>
</tr>
<tr>
<td>Atoh8\textsuperscript{GFP/GFP}</td>
<td>5</td>
<td>12%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Zfpm2\textsuperscript{+/−} Atoh8\textsuperscript{+/+}</td>
<td>2</td>
<td>5%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Zfpm2\textsuperscript{+/−} Atoh8\textsuperscript{GFP/+}</td>
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<td>25%</td>
</tr>
<tr>
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<tr>
<td>Total</td>
<td>41</td>
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Age: P1

Cross: Atoh8\textsuperscript{AGFP/+} X Zfpm2\textsuperscript{+/−} Atoh8\textsuperscript{GFP/+}

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<th>Number</th>
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<th>Mendelian %</th>
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<td>25%</td>
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<tr>
<td>Atoh8\textsuperscript{GFP/GFP}</td>
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<td>9%</td>
<td>12.5%</td>
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<td>25%</td>
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Table 2.5. Survival of *Gata4*<sup>+/−</sup> *Atoh8*<sup>GFP/GFP</sup> mice at postnatal day 1 (P1) and postnatal day 14 (P14).

<table>
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<tr>
<th>Cross: <em>Atoh8</em>&lt;sup&gt;GFP/+&lt;/sup&gt; X <em>Gata4</em>&lt;sup&gt;+/−&lt;/sup&gt; <em>Atoh8</em>&lt;sup&gt;GFP/+&lt;/sup&gt;</th>
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<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
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<td></td>
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<td>11%</td>
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<td></td>
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<td>11%</td>
<td>12.5%</td>
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<tr>
<td></td>
<td><strong>Gata4</strong>&lt;sup&gt;−/−&lt;/sup&gt; <em>Atoh8</em>*&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>49</td>
<td>22%</td>
<td>25%</td>
</tr>
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<td><strong>Gata4</strong>&lt;sup&gt;−/−&lt;/sup&gt; <em>Atoh8</em>*&lt;sup&gt;GFP/GFP&lt;/sup&gt;</td>
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<td>Total</td>
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<td><strong>P=0.0106</strong></td>
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<table>
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<th>Cross: <em>Atoh8</em>&lt;sup&gt;GFP/+&lt;/sup&gt; X <em>Gata4</em>&lt;sup&gt;+/−&lt;/sup&gt; <em>Atoh8</em>&lt;sup&gt;GFP/+&lt;/sup&gt;</th>
<th>Genotype</th>
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<tr>
<td></td>
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<td>23</td>
<td>12%</td>
<td>12.5%</td>
</tr>
<tr>
<td></td>
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<td></td>
<td><strong>Gata4</strong>&lt;sup&gt;−/−&lt;/sup&gt; <em>Atoh8</em>*&lt;sup&gt;+/+&lt;/sup&gt;</td>
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<td>25%</td>
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<td><strong>Gata4</strong>&lt;sup&gt;−/−&lt;/sup&gt; <em>Atoh8</em>*&lt;sup&gt;GFP/GFP&lt;/sup&gt;</td>
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<td><strong>P&lt;0.0001</strong></td>
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Table 2.6. Viability of $Gata4^{+/+} Atoh8^{GFP/GFP}$ embryos at embryonic day 17.5.

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<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
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</thead>
<tbody>
<tr>
<td>$Atoh8^{+/+}$</td>
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<td>10%</td>
<td>12.5%</td>
</tr>
<tr>
<td>$Atoh8^{GFP/+}$</td>
<td>11</td>
<td>27%</td>
<td>25%</td>
</tr>
<tr>
<td>$Atoh8^{GFP/GFP}$</td>
<td>2</td>
<td>5%</td>
<td>12.5%</td>
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<tr>
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<td>19%</td>
<td>12.5%</td>
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<tr>
<td>$Gata4^{+/-} Atoh8^{GFP/+}$</td>
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<td>25%</td>
</tr>
<tr>
<td>$Gata4^{+/-} Atoh8^{GFP/GFP}$</td>
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<td>12%</td>
<td>12.5%</td>
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<tr>
<td>Total</td>
<td>41</td>
<td>P=0.56</td>
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</table>
Figure 2.10. *Gata4*<sup>+/−</sup>*Atoh8*<sup>GFP/GFP</sup> embryonic hearts are functionally and structurally normal. (A) Ejection fraction was determined for *Gata4*<sup>+/−</sup>*Atoh8*<sup>GFP/GFP</sup> and littermates at E17.5. N for each genotype is shown in panel. Graph shows mean of all samples of that genotype; errors bars represent SEM. (B-C) Hematoxylin-eosin staining was performed on E17.5 heart sections from control (B) and *Gata4*<sup>+/−</sup>*Atoh8*<sup>GFP/GFP</sup> (C) embryos. Scale is shown in each panel.
Table 2.7. Survival of Nkx2.5-Cre Gata4\(^{fl/+}\) Atoh8\(^{GFP/GFP}\) embryos at postnatal day 1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata4(^{fl/+}) Atoh8(^{GFP/+})</td>
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<tr>
<td>Gata4(^{fl/+}) Atoh8(^{GFP/GFP})</td>
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<td>32%</td>
<td>25%</td>
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<tr>
<td>Nkx2.5-Cre Gata4(^{fl/+}) Atoh8(^{GFP/+})</td>
<td>2</td>
<td>7%</td>
<td>25%</td>
</tr>
<tr>
<td>Nkx2.5-Cre Gata4(^{fl/+}) Atoh8(^{GFP/GFP})</td>
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<td>36%</td>
<td>25%</td>
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<tr>
<td><strong>Total</strong></td>
<td>28</td>
<td></td>
<td>P=0.14</td>
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Figure 2.11. *Gata4*<sup>+/−</sup>*Atoh8<sup>GFP/GFP</sup> lungs appear structurally normal but display defects in mesenchymal-epithelial signaling.

(A-B) Hematoxylin-eosin staining was performed on E18.5 lung sections from control (A) and *Gata4*<sup>+/−</sup>*Atoh8<sup>GFP/GFP</sup> (B) embryos. Scale is shown in each panel. (C) Lung/body weight ratios were measured at E18.5 to compare lung growth between indicated genotypes. N=3-6 embryos per genotype. (D) Surfactant Protein C and Aquaporin 5 expression in E18.5 lungs. N=3 lungs/genotype. (E) Wnt2, Fgf10, Tbx4, and Twist1 expression in E12.5 lung buds. N=6 lung buds/genotype. All graphs show the mean; errors bars represent SEM. * P<0.05 compared to WT, # P<0.05 compared to *Atoh8<sup>GFP/GFP</sup>.*
Chapter 3: Endocardial MEKK3 regulates myocardial growth

Summary

Previous work has identified the proteins Heart-of-glass (HEG) and cerebral cavernous malformation 2-like (CCM2L) as endocardial factors that are required to generate endocardial growth factors and promote myocardial growth (58). How HEG and CCM2L regulate growth factor production has remained unclear. Here we identify the MAPK cascade component MEKK3 as the likely link between HEG-CCM2L and growth factor production. We show that MEKK3 interacts with CCM2L in co-immunoprecipitation experiments and that MEKK3 is required for CCM2L regulation of FGF transcription in cell culture. We then establish that loss of endocardial MEKK3 leads to a lethal loss of myocardial growth, copying the phenotype seen Heg\(^{-/-}\) Ccm2l\(^{-/-}\) mice and suggesting that MEKK3 is involved in the same pathway as HEG and CCM2L. We then demonstrate genetic interaction between Heg and Map3k3 (encoding MEKK3), further connecting MEKK3 to the HEG-CCM pathway \textit{in vivo}.

Introduction

Cerebral cavernous malformations (CCM) are vascular lesions that develop within the central nervous system that can lead to hemorrhagic stroke (48). CCM lesions are linked to mutations in CCM1, CCM2, or CCM3 (49-51); biochemical studies have demonstrated that these proteins form a complex where CCM1 binds CCM2, which then binds CCM3 (52). Genetic studies of these genes in mouse models have reveal a
requirement for these genes in early vascular development in addition to their role in CCM pathogenesis.

In our lab’s previous work on CCM, we identified two additional components of the CCM complex: Heart-of-glass (HEG) and cerebral cavernous malformation 2-like (CCM2L). Neither of HEG nor CCM2L is involved in traditional CCM lesion formation. Heg is a transmembrane protein that can interact with the CCM complex by binding CCM1 (52). Loss of the zebrafish homologue Heg has been reported to result in defective myocardial growth (56); in the mouse, loss of Heg results in death in half of the animals by weaning from either pulmonary hemorrhage or from myocardial ruptures secondary to local patches of thin myocardium (52). CCM2L is a paralogue of CCM2 can interact with CCM1 but cannot bind with CCM3 (58). Ccm2l−/− mice are viable; however, Heg−/− Ccm2l−/− double mutant mice are lethal by E10.5 from heart failure secondary to reduced myocardial proliferation (58). Heg−/− Ccm2l−/− embryos had normal appearing endocardium but were found to have reduced levels of FGF16, an endocardial-to-myocardial growth factor that has previously been linked to myocardial proliferation (45,59).

Although our lab identified a requirement for HEG and CCM2L in regulating endocardial FGF16 transcription, the mechanism for this regulation remains unclear. HEG is embedded with the cell membrane and CCM2L is presumably in a complex with CCM1 and HEG at the membrane, leading us to conclude that HEG and CCM2L must facilitate some type of signaling pathway that regulates growth factor transcription. We identified a likely candidate in MEKK3, a MAPK kinase kinase kinase. MEKK3 has previously been shown to physically interact with CCM2 (84), suggesting it could also be
a binding partner of CCM2L. Mice lacking MEKK3 are dead by E10.5 with widespread vascular defects (85). These mice also exhibited myocardial thinning, although these defects could be secondary to the vascular defects, precluding a clear understanding of the role of MEKK3 in the heart.

We hypothesized that HEG-CCM2L control endocardial growth factor production by regulating MEKK3. To test this hypothesis, we investigated whether MEKK3 could physically interact with CCM2L and regulate CCM2L activation of FGF16 transcription. We then investigated whether endocardial MEKK3 was required for myocardial growth in the developing heart.

**Results**

**MEKK3 can physically interact with either CCM2 or CCM2L**

In order to identify signaling pathways that could connect the HEG-CCM complex with transcription of FGF growth factors, we looked at known interacting partners with CCM proteins. MEKK3 (encoded by the gene *Map3k3*) is a MAPK kinase kinase kinase that has previously been reported to be required for vascular development (85) and that has been reported to physically interact with CCM2 (84). Because of the high homology between CCM2 and CCM2L, we hypothesized that MEKK3 could physically interact with CCM2L. In order to test this hypothesis, we performed co-immunoprecipitation experiments to detect physical interaction between MEKK3 and either CCM2 or CCM2L. We overexpressed HA-tagged MEKK3 (HA-MEKK3) with either FLAG-tagged CCM2 (FLAG-CCM2) or FLAG-tagged CCM2L (FLAG-CCM2L) in HEK293T cells and then performed immunoprecipitations with anti-FLAG antibodies.
HA-MEKK3 co-immunoprecipitated with either FLAG-CCM2 or FLAG-CCM2L (Figure 3.1), indicating that MEKK3 can physically interact with CCM2L in addition to CCM2. These results are compatible with a mechanism where MEKK3 physically interacts with CCM2L and facilitates signaling from the HEG-CCM1-CCM2L complex.

**CCM2L increases FGF16 transcription via a MEKK3-dependent mechanism**

Having demonstrated that MEKK3 could physically interact with CCM2L, we next asked whether MEKK3 was required for CCM2L regulation of FGF transcription. Our lab previously developed a cell culture model where adenoviral-mediated overexpression of CCM2L in human microvascular endovascular cells (HMVEC) leads to an upregulation of FGF16 transcription (58). In order to determine whether MEKK3 is required for this regulation of FGF16 expression, we infected HMVECs with CCM2L adenovirus or control LacZ adenovirus in the presence of MEKK3 siRNA or scramble control siRNA. Adenoviral overexpression of CCM2L results in an approximately three-fold increase in FGF16 expression (Figure 3.2). siRNA-mediated knockdown of MEKK3 in HMVECs blocks this increase in FGF16 (Figure 3.2). This result indicates that CCM2L regulates FGF16 via a MEKK3-dependent mechanism and supports our hypothesis that MEKK3 functions in a signaling pathway between HEG-CCM2L and FGF transcription.
**Nfatc1-Cre is limited to the developing endocardium and is not expressed in the endothelium outside the heart**

Having shown that MEKK3 is required for CCM2L regulation of FGF16 transcription in cell culture, we next asked whether MEKK3 is required *in vivo* in the endocardium of the developing heart. MEKK3 is encoded by the gene *Map3k3*, and *Map3k3* mutants are reported to be lethal by embryonic day 10.5-11.0 with widespread defects in cardiac and vascular development (85). Although conditional *Map3k3* alleles have been developed, the lack of a mouse Cre allele that specifically deletes in the endocardium while sparing the rest of the endothelium has prevented a study of the endocardial requirement for MEKK3. However, a group recently has published a report of an endocardial-specific Cre allele, generated by insertion of an IRES-Cre cassette into the 3’UTR of the *Nfatc1* gene (hereafter referred to as *Nfatc1Cre*) (46). Within the endothelial lineage, this allele is reported to delete within only the endocardium and in the coronary vasculature endothelium that is later derived from endocardium, making this allele an ideal tool for determining the endocardial requirement for MEKK3.

In order to validate the use of the *Nfatc1Cre* allele as an endocardial-specific Cre deletor, we performed lineage analysis using the *Nfatc1Cre* allele and a previously described *R26R-EYFP* reporter allele (86). Immunohistochemistry for EYFP and the endothelial marker PECAM was used to determine the contribution of *Nfatc1* to the endocardium and endothelium in multiple vascular beds. At embryonic day 10.5 (E10.5), *Nfatc1Cre* labels cells throughout the endocardium of the ventricle, atrium, and atrioventricular cushions (Figure 3.3.A). EYFP reporter expression extends into the proximal portion of the heart outflow tract (Figure 3.3.B), but it is excluded from the
remainder of the aortic sac and branchial arch arteries (Figure 3.3.B-C), indicating that 
\( Nfatc1^{Cre} \) is limited to the endocardium and spares other endothelium at E10.5.

In order to determine the endothelial activity for \( Nfatc1^{Cre} \) throughout 
embryogenesis, we performed additional lineage tracing experiments on \( Nfatc1^{Cre} R26R-EYFP \) neonates on postnatal day 1 (P1). EYFP reporter expression overlapped with 
PECAM expression at P1 throughout the endocardium (Figure 3.3.D-G). EYFP reporter 
expression was also seen in PECAM+ capillary and coronary artery endothelium in the 
heart (Figure 3.3.D-G), a finding consistent with a previous report that endocardial cells 
contribute to a significant portion of the cardiac vasculature (46). \( Nfatc1^{Cre} \) lineage cells 
also contributed to other endocardium-derived structures, including the cardiac valves 
(Figure 3.3.H) and the endothelium of the proximal aorta (Figure 3.3.I). We next 
examined non-cardiac endothelium in P1 embryos to determine whether the \( Nfatc1^{Cre} \) 
allele was active in these cells. In contrast to the widespread expression of the EYFP 
reporter in endocardial lineages, there is no EYFP expression in the endothelium of the 
liver (Figure 3.3.J-K) or kidney (Figure 3.3.L-M) at P1, indicating that the \( Nfatc1^{Cre} \) 
allele is not active in endothelial cells other than the endocardium and the cardiac 
vasculature. These results demonstrate that the expression of \( Nfatc1^{Cre} \) allele is limited to 
the endocardium and that this Cre allele can be used to create an endocardial-specific 
deletion of MEKK3.

**Endocardial MEKK3 is required for mouse survival and myocardial development**

In order to determine whether MEKK3 is required in the endocardium, we 
combined the \( Nfatc1^{Cre} \) conditional allele with a previously reported \( Map3k3 \) (encoding
MEKK3) floxed allele in which loxP sites flank exons 12-13, which encode several subdomains of the MEKK3 kinase domain (87). Deletion of these exons has been shown to result in a kinase-dead form of MEKK3 (87), and thus cells homozygous for this mutation will be null for functional MEKK3. We were unable to find any Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> neonates when litters were genotyped at birth (Table 3.1), indicating that loss of endocardial MEKK3 results in death during embryogenesis. Timed matings were then performed to determine when Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos died. No viable Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> animals were found at E14.5 (Table 3.2), indicating that endocardial loss of MEKK3 is lethal before this time point. At E10.5, Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos were found at approximately 80% of the expected rate (Table 3.2), indicating that most Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos are viable at this embryonic stage. By E11.5, the number of Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos had decreased to less than 40% of the Mendelian prediction (Table 3.2). Although additional crosses are need to determine the viability of these embryos at E12.5 and E13.5, these results suggest that endocardial deletion of MEKK3 leads to embryonic loss beginning at E10.5-E11.5, with loss of all embryos by E14.5.

In order to determine the cause of death in Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos, we used hematoxylin & eosin (H&E) staining to examine the structure of viable Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos at E10.5 and E11.5. Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos had profound myocardial thinning relative to littermate controls at E10.5 (Figure 3.4.A-D) and E11.5 (Figure 3.4.E-H), indicating that endocardial loss of MEKK3 has a deleterious effect on myocardial development. In contrast, the endocardium was indistinguishable between Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup> embryos and littermate controls (Figure 3.4.A-D, see arrowheads), suggesting that endocardial development proceeds normally in these embryos. These
results indicate that endocardial loss of MEKK3 results in lethal defects in myocardial
development. This result is nearly identical to the phenotype our lab previously observed
in Heg^{−/−}Ccm2l^{−/−} mouse (58). This is consistent with our hypothesis that MEKK3
functions downstream of HEG and CCM2L to promote endocardial production of growth
factors to facilitate myocardial growth, although further analysis of Nfatc1^{Cre} Map3k3^{fl/fl}
embryos is needed to determine whether these animals have the same defects in
proliferation and endocardial FGF production as were seen in Heg^{−/−}Ccm2l^{−/−} embryos.

**Endocardial CCM1 is required for survival in the embryonic mouse**

Although the endothelial functions of CCM1, CCM2, and CCM3 in development
have been studied in mice with endothelial-specific (Tie2^{Cre}) deletions of these genes, the
function of these proteins in the endocardium has remained unclear due to very early
embryonic death that occurs secondary to vascular defects (53-55). It would be
particularly interesting to examine embryos lacking endocardial CCM1 for defects
similar to Heg^{−/−}Ccm2l^{−/−} and Nfatc1^{Cre} Map3k3^{fl/fl} embryos, due to the fact that we
suspect CCM1 forms a complex with HEG and CCM2L. Our Nfatc1^{Cre} lineage trace and
our positive results with Nfatc1^{Cre} Map3k3^{fl/fl} embryos suggest that the Nfatc1^{Cre} allele
could be used to determine the endocardial requirement for CCM proteins. In order to
determine the function of CCM1 in the endocardium, we combined the Nfatc1^{Cre} allele
with the Krit1^{fl} allele (Krit1 encodes CCM1). Nfatc1^{Cre} Krit1^{fl/fl} mice are not found at
birth (Table 3.3), indicating that endocardial CCM1 is required for survival. We are now
undertaking timed matings to characterize the embryonic defects present in these mice.
and to determine whether endocardial CCM1 has a similar role in myocardial growth as HEG, CCM2L, and MEKK3.

**Heg and Map3k3 genetically interact in the endothelium and endocardium**

In our previous studies of HEG and CCM proteins, our lab has utilized compound genetic mutants to demonstrate genetic interaction between different members of this complex. For example, in our studies of the Heg mutant mouse, our lab showed that Heg\(^{-/-}\) CCM2\(^{+/-}\) mice phenocopy the embryonic vascular defects of CCM2\(^{-/-}\) mice (52), implicating Heg in this developmental process. Such interactions provide evidence for shared function *in vivo*. Because endocardial-specific deletion of MEKK3 displays a similar phenotype to the Heg\(^{-/-}\) Ccm2l\(^{-/-}\) mouse, we hypothesized that Map3k3 (encoding MEKK3) would exhibit genetic interaction with Ccm2l and Heg. To test this hypothesis, we generated Ccm2l\(^{+/-}\) Map3k3\(^{+/-}\) and Heg\(^{-/-}\) Map3k3\(^{+/-}\). Ccm2l\(^{-/-}\) Map3k3\(^{+/-}\) mice were viable and had no overt phenotypes (data not shown). Viable Heg\(^{-/-}\) Map3k3\(^{+/-}\) mice, however, were not found at birth (Table 3.4). Timed matings revealed that Heg\(^{-/-}\) Map3k3\(^{+/-}\) die between E12.5 and E14.5 (Table 3.4). At E12.5 Heg\(^{-/-}\) Map3k3\(^{+/-}\) embryos exhibited profound myocardial thinning relative to littermate controls (Figure 3.5), although this phenotype was not completely penetrant. Viable Heg\(^{-/-}\) Map3k3\(^{+/-}\) animals at E13.5 did not have a decrease in heart function at this time point (Figure 3.6). Given that our genetic crosses indicate that all animals are dead by E14.5, this result suggests that cardiac function is preserved in these animals until E13.5 and rapidly deteriorates by E14.5.
The embryonic lethality and myocardial thinning that we observe in Heg\(^{\text{-}}\)Map3k3\(^{\text{+/+}}\) animals demonstrate that a genetic interaction exists between Heg and Map3k3. We hypothesized that this interaction was taking place within the endothelium and possibly specifically within the endocardium. In order to determine what cell type underlies this interaction, we used Tie2\(^{\text{Cre}}\) and Nfatc1\(^{\text{Cre}}\) conditional alleles to respectively generate Tie2\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) and Nfatc1\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice. In these mice, Heg deletion is global while Map3k3 heterozygosity is limited to all endothelium (with Tie2\(^{\text{Cre}}\)) or to only endocardium (with Nfatc1\(^{\text{Cre}}\)). If the Heg\(^{\text{-}}\) Map3k3\(^{\text{+/+}}\) interaction we observe is dependent on endocardial function of Map3k3, we would predict Nfatc1\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice would copy the phenotype of Heg\(^{\text{-}}\) Map3k3\(^{\text{+/+}}\) mice; if it is dependent on pan-endothelial function of Map3k3, we would predict Tie2\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice would copy the phenotype of Heg\(^{\text{-}}\) Map3k3\(^{\text{+/+}}\) mice. In our matings Tie2\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice were lethal at P1 and almost completely lethal at E14.5 (Table 3.5), mimicking the lethality we observe in Heg\(^{\text{-}}\) Map3k3\(^{\text{+/+}}\) mice. In contrast, approximately half of the Nfatc1\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice were viable at E14.5 and at P1 (Table 3.6). These results suggest that the lethality we observed from genetic interaction between Heg and Map3k3 is partially due to endocardial defects (as shown by the 50% lethality in Nfatc1\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice) and partially due to other endothelial defects (as shown by the increased lethality seen in Tie2\(^{\text{Cre}}\) Heg\(^{\text{-}}\) Map3k3\(^{\text{fl/+}}\) mice). We are performing histology on these conditional mice to determine whether they have similar cardiac pathology to Heg\(^{\text{-}}\) Map3k3\(^{\text{+/+}}\) mice.
Discussion

Our studies of MEKK3 support our hypothesis that MEKK3 operates downstream of HEG and CCM2L to facilitate growth factor production in the endocardium to support myocardial growth. We demonstrate that MEKK3 can physically interact with CCM2L and that MEKK3 is required in endothelial cell culture models for CCM2L-stimulated FGF16 production. Our \textit{Nfatc1}\textsuperscript{Cre} \textit{Map3k3}\textsuperscript{fl/fl} mouse studies demonstrate that endocardial MEKK3 is required for myocardial growth, a finding consistent with a role for MEKK3 as an activator of endocardial growth factor production. Finally, we identify genetic interaction between \textit{Heg} and \textit{Map3k3}, providing \textit{in vivo} evidence connecting MEKK3 to the HEG-CCM complex.

While previous studies of \textit{Map3k3}\textsuperscript{−/−} embryos had reported myocardial thinning defects with loss of MEKK3 (85), it was unclear if this defect was due to a loss of endocardial or myocardial MEKK3, or if it was a secondary consequence of global vasculature defects. Our studies with the \textit{Nfatc1}\textsuperscript{Cre} \textit{Map3k3}\textsuperscript{fl/fl} mice clearly identify an endocardial requirement for MEKK3 in facilitating myocardial growth. Our lineage trace experiments with the \textit{Nfatc1}\textsuperscript{Cre} \textit{R26R-EYFP} mice demonstrate that \textit{Nfatc1}\textsuperscript{Cre} activity is limited to the endocardium and indicate that the phenotypes in \textit{Nfatc1}\textsuperscript{Cre} \textit{Map3k3}\textsuperscript{fl/fl} mice are due to endocardial loss of MEKK3. In addition, the normal appearance of the endocardium in \textit{Nfatc1}\textsuperscript{Cre} \textit{Map3k3}\textsuperscript{fl/fl} suggests that the phenotype is due to a specific loss of endocardial growth factors rather than a loss of endocardial cells. This phenotype—loss of myocardium with normal endocardium—is a close copy of the \textit{Heg}\textsuperscript{−/−} \textit{Ccm2l}\textsuperscript{−/−} phenotype, which supports our hypothesis that MEKK3 acts downstream of HEG and CCM2L to facilitate growth factor production. Further studies are now needed to
confirm that $Nfatc1^{Cre}$ $Map3k3^f{^/-}$ embryos have the same type of molecular defects as $Heg^{+/-}$ $Ccm2l^+/-$ embryos. For example, we need to determine whether reduced FGF16 expression underlies the phenotype in $Nfatc1^{Cre}$ $Map3k3^f{^/-}$ embryos or whether changes in other signals are responsible.

In our $Heg^{+/-}$ $Map3k3^{+/-}$ compound mutant embryos, we observed complete embryonic lethality by E14.5, a much more severe phenotype than $Heg^{+/-}$ embryos, which largely survive into the postnatal period (52). This lethality is a clear sign of genetic interaction between $Heg$ and $Map3k3$, serving as possible in vivo evidence linking MEKK3 to the HEG-CCM complex. It is difficult, however, to determine how whether $Heg$ and $Map3k3$ are interacting in the endocardium or in other sites. We observe myocardial thinning some $Heg^{+/-}$ $Map3k3^{+/-}$ embryos, suggesting that myocardial growth may be impaired in these embryos, as it is in $Nfatc1^{Cre}$ $Map3k3^f{^/-}$ or $Heg^{+/-}$ $Ccm2l^+/-$ embryos, although the phenotype is much less penetrant in $Heg^{+/-}$ $Map3k3^{+/-}$ embryos. However, cardiac function was preserved in viable $Heg^{+/-}$ $Map3k3^{+/-}$ embryos at E13.5, arguing against a cardiac cause of death in these animals. A similar discrepancy is observed in $Tie2^{Cre}$ $Heg^{+/-}$ $Map3k3^{+/-}$ versus $Nfatc1^{Cre}$ $Heg^{+/-}$ $Map3k3^f{^/-}$ mice. Embryonic death in endothelial ($Tie2^{Cre}$) mutants is nearly completely penetrant, copying the phenotype in $Heg^{+/-}$ $Map3k3^{+/-}$ embryos, whereas endocardial ($Nfatc1^{Cre}$) mutants have a 50% reduction in death. The most likely explanation for this outcome is two separate causes of death—an endocardial cause death and a separate vascular endothelial cause—in $Heg^{+/-}$ $Map3k3^{+/-}$ embryos. In this model, a portion of the $Heg^{+/-}$ $Map3k3^{+/-}$ population develops lethal myocardial thinning secondary to endocardial defects; this explains the thin $Heg^{+/-}$ $Map3k3^{+/-}$ hearts and the partial lethality present in $Nfatc1^{Cre}$ $Heg^{+/-}$ $Map3k3^f{^/-}$
embryos. The rest of the $\text{Heg}^{-/-}\text{Map3k3}^{+/+}$ population escapes this cardiac defect.

However, these survivors likely develops a second defect within the vasculature that results in death at E14.5—this would explain the embryonic death we see in all $\text{Heg}^{-/-}\text{Map3k3}^{+/+}$ embryos and nearly all $\text{Tie2}^{\text{Cre}}\text{Heg}^{-/-}\text{Map3k3}^{\text{fl/+}}$ embryos. Further study is needed to examine the histology of these conditional embryos to more completely characterize the defects present in these animals.

**Materials and Methods**

**Mice**

We used the previously reported $\text{Map3k3}$ floxed allele (14), $\text{Heg}$ mutant allele (52), $\text{Tie2}^{\text{Cre}}$ allele (88), $\text{Nfatc1}^{\text{Cre}}$ allele (46) and $\text{R26R-EYFP}$ allele (86). The $\text{Krit1}^{\text{fl}}$ is unpublished and was provided by our collaborator Dean Li from the University of Utah.

All mouse experiments were done in mixed genetic backgrounds. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols. The following genotyping primers were used:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map3k3-WT-F</td>
<td>5’- CATTTTTGCCAGGCAGTG -3’</td>
</tr>
<tr>
<td>Map3k3-FL-R</td>
<td>5’- CTCTATAGCAGGAGAGAC -3’</td>
</tr>
<tr>
<td>Map3k3-KO-R</td>
<td>5’- AGACTCACTGTCAGAGACC -3’</td>
</tr>
<tr>
<td>Heg-WT-F</td>
<td>5’- CCTCCCCGCGGTCCACAACCTAT -3’</td>
</tr>
<tr>
<td>Heg-WT-R</td>
<td>5’- AGACTCTCCTACCAGGGCTCA -3’</td>
</tr>
<tr>
<td>Heg-LacZ-R</td>
<td>5’- GGCTCAGTCGGTGGCGAGT -3’</td>
</tr>
<tr>
<td>Krit1-F</td>
<td>5’- TGCTCCATTCCCTCCTCCACTCAC -3’</td>
</tr>
<tr>
<td>Krit1-R</td>
<td>5’- AAACCAGCAGTCTCAACTAATCGG -3’</td>
</tr>
<tr>
<td>YFP-WT-F</td>
<td>5’- AAAGTGCTCTGAGTTATTAT -3’</td>
</tr>
<tr>
<td>YFP-WT-R</td>
<td>5’- GCGAAGAGTTTGGTCTCAACC -3’</td>
</tr>
<tr>
<td>YFP-YFP-R</td>
<td>5’- GGAGCGGGAAATGATATG -3’</td>
</tr>
<tr>
<td>Cre-F</td>
<td>5’- GAACCTGATGAGCATTCTCCAGGA -3’</td>
</tr>
<tr>
<td>Cre-R</td>
<td>5’- CAGAGTCATCCTTAGCGCCGTAAA -3’</td>
</tr>
</tbody>
</table>
Co-immunoprecipitation studies

We used the previously described HA-MEKK3 expression vector (89) and previously described FLAG-CCM2 and FLAG-CCM2L vectors (58) in our immunoprecipitation experiments. Constructs were transiently transfected into HEK293T cells using Fugene 6 (Roche). Cellular lysis and immunoprecipitations were performed as previously described (76). FLAG-tagged CCM2 and CCM2L were detected with HRP-conjugated anti-FLAG-M2 antibody (1:1000, Sigma). HA-tagged proteins were detected with monoclonal mouse anti-HA antibody (1:10000, Covance) and HRP-conjugated goat anti-mouse IgG antibody (1:5,000 Jackson ImmunoResearch Laboratories Inc.).

Adenoviral Expression and siRNA studies in HMVECs

siRNA against human MEKK3 (Invitrogen) and scramble control siRNA (Invitrogen) were transfected into human microvascular endothelial cells (HMVEC) using RNAiMAX (Invitrogen). 24 hours after transfections, cells were infected with adenovirus expressing HA-CCM2L or LacZ. Adenovirus dose was 2000gc/cell. RNA was harvested from these cells 48 hours after adenoviral transfection using Trizol (Invitrogen). 1000ng of RNA and 50ng random hexamer primers were then used to synthesize cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). RT-PCR primers are listed below:
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFGF16-Forward</td>
<td>5’- AGTGGACTCTGGCCTGTACCT -3’</td>
</tr>
<tr>
<td>hFGF16-Reverse</td>
<td>5’- TTTGTTCCAGGGCCACGTAATA -3’</td>
</tr>
</tbody>
</table>

**Histology and immunostaining**

Mouse embryos at the indicated developmental stages were dissected, fixed in paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. We performed immunostaining and hematoxylin-eosin staining. Histological techniques were performed as previously described (81,82). For immunostaining, a goat polyclonal antibody against GFP (1:500, Abcam) was used to detect YFP, and a rat monoclonal antibody against PECAM (1:500, BD Pharmingen). Bright field and fluorescent images were acquired using a Nikon Eclipse 80i microscope.

**Fetal echocardiography**

Trans-uterine embryonic ultrasound was performed using a high-resolution Vevo 770 micro-ultrasound system (VisualSonics Inc.) as previously reported (83).

**Statistics**

P values in mouse genetic crosses were calculated using chi-squared tests. An unpaired two-tailed Student’s t-test was used for all other p-values.
Figure 3.1. MEKK3 physically interacts with CCM2 and CCM2L.

<table>
<thead>
<tr>
<th></th>
<th>IP: Flag</th>
<th>Lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-MEKK3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flag-CCM2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flag-CCM2L</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(A) HA-MEKK3, Flag-CCM2, and Flag-CCM2L were transiently expressed in HEK293T in the indicated combinations, immunoprecipitated with anti-Flag antibodies and detected by Western blotting with the indicated antibodies.
Figure 3.2. Overexpression of CCM2L upregulates FGF16 in human microvascular endothelial cells via a MEKK3-dependent mechanism.

(A) Adenoviral overexpression of CCM2L increases FGF16 mRNA levels in human microvascular endothelial cells (HMVEC). siRNA-mediated knockdown of MEKK3 blocks this upregulation. HMVECs were transfected with indicated siRNA (MEKK3 or scramble control) and infected with indicated adenoviruses 24 hours later. Cells were harvested 24 hours after adenoviral infection. Graph shows mean of 4 replicates +/- sem. *** P < 0.001.
Figure 3.3. *Nfatc1*<sup>Cre</sup> deletes specifically in the endocardium and coronary endothelium.
Figure 3.3. *Nfatc1*<sup>Cre</sup> deletes specifically in the endocardium and coronary endothelium. (A-C) *Nfatc1*<sup>Cre</sup> lineage tracing with the *R26R-YFP* reporter labels the E10.5 endocardium while sparing the aorta and brachial arch arteries. (D-I) *Nfatc1*<sup>Cre</sup> lineage cells contribute to the endocardium, cardiac valves, and the coronary endothelium at P1. *R26R-YFP* reporter expression overlaps with PECAM expression in the endocardium and coronary endothelium (D-G). YFP expression is also seen in endocardium-derived structures such as the cardiac valves (H) and the proximal aortic endothelium (I). (J-M) *Nfatc1*<sup>Cre</sup> lineage cells do not contribute to PECAM+ endothelial cells in the liver (J-K) or kidney (L-M) vascular beds. K and M show higher magnification images of J and L, respectively. Abbreviations: Atr=atrium, AVC=atrioventricular cushion, Vent=common ventricle, Ao=aortic sac, BAA=branchial arch artery, LV=left ventricle, RV=right ventricle, CA=coronary artery.
Table 3.1. Survival of $Nfatc1^{Cre/+} Map3k3^{fl/-}$ mice at P1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Map3k3^{fl/+}$</td>
<td>14</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>$Map3k3^{fl/-}$</td>
<td>19</td>
<td>33%</td>
<td>25%</td>
</tr>
<tr>
<td>$Nfatc1^{Cre/+} Map3k3^{fl/+}$</td>
<td>21</td>
<td>42%</td>
<td>25%</td>
</tr>
<tr>
<td>$Nfatc1^{Cre/+} Map3k3^{fl/-}$</td>
<td>0</td>
<td>0%</td>
<td>25%</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td></td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>
Table 3.2. Embryonic survival of \( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/+}} \) mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Map3k3}^{\text{fl/+}} )</td>
<td>29</td>
<td>29%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Map3k3}^{\text{fl/-}} )</td>
<td>29</td>
<td>29%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/+}} )</td>
<td>23</td>
<td>23%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/-}} )</td>
<td>19</td>
<td>19%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
<td><strong>P=0.410</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
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<tbody>
<tr>
<td>( \text{Map3k3}^{\text{fl/+}} )</td>
<td>15</td>
<td>27%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Map3k3}^{\text{fl/-}} )</td>
<td>23</td>
<td>42%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/+}} )</td>
<td>12</td>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/-}} )</td>
<td>5</td>
<td>9%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>55</td>
<td><strong>P=0.007</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Map3k3}^{\text{fl/+}} )</td>
<td>16</td>
<td>31%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Map3k3}^{\text{fl/-}} )</td>
<td>11</td>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/+}} )</td>
<td>24</td>
<td>47%</td>
<td>25%</td>
</tr>
<tr>
<td>( \text{Nfatc1}^{\text{Cre}+/} \text{Map3k3}^{\text{fl/-}} )</td>
<td>0</td>
<td>0%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>51</td>
<td><strong>P&lt;0.001</strong></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4. Nfatc1\textsuperscript{Cre} Map3k3\textsuperscript{fl/-} embryos exhibit myocardial thinning.

E10.5

A

B

C

D

E11.5

E

F

G

H
Figure 3.4. *Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup>* embryos exhibit myocardial thinning. (A-D) *Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup>* embryos exhibit myocardial thinning relative to littermates at E10.5. The endocardium (arrowheads in A-D) is intact and indistinguishable between *Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup>* embryos and controls. B and D are higher magnification images of boxed region in A and C, respectively. (E-H) *Nfatc1<sup>Cre</sup> Map3k3<sup>fl/fl</sup>* embryos exhibit myocardial thinning relative to littermates at E11.5. F and H are higher magnification images of boxed region in E and G, respectively. Scale bars are 100µm in A, C, E, G, and 50µm in B, D, F, H.
Table 3.3. Survival of \( Nfatc1^{Cre/+} \) \( Krit1^{fl/+} \) mice at P1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Observed %</th>
<th>Mendelian %</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Krit1^{fl/+} )</td>
<td>13</td>
<td>48%</td>
<td>25%</td>
</tr>
<tr>
<td>( Krit1^{fl/fl} )</td>
<td>6</td>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>( Nfatc1^{Cre/+} ) ( Krit1^{fl/+} )</td>
<td>8</td>
<td>30%</td>
<td>25%</td>
</tr>
<tr>
<td>( Nfatc1^{Cre/+} ) ( Krit1^{fl/fl} )</td>
<td>0</td>
<td>0%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>27</td>
<td></td>
<td><strong>P=0.005</strong></td>
</tr>
</tbody>
</table>
Table 3.4. Survival of Heg\(^{-/-}\) Map3k3\(^{+/-}\) mice.

<table>
<thead>
<tr>
<th>Age</th>
<th>Heg(^{+/-})</th>
<th>Heg(^{-/-})</th>
<th>Heg(^{+/-}) Map3k3(^{+/-})</th>
<th>Heg(^{-/-}) Map3k3(^{+/-})</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12.5</td>
<td>14</td>
<td>11</td>
<td>10</td>
<td>7</td>
<td>42</td>
<td>0.50</td>
</tr>
<tr>
<td>E13.5</td>
<td>20</td>
<td>16</td>
<td>15</td>
<td>7</td>
<td>58</td>
<td>0.10</td>
</tr>
<tr>
<td>E14.5</td>
<td>19</td>
<td>17</td>
<td>13</td>
<td>0</td>
<td>49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P1</td>
<td>27</td>
<td>15</td>
<td>32</td>
<td>0</td>
<td>74</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 3.5. *Heg*<sup>-/-</sup> *Map3k3<sup>+/−</sup>* hearts exhibit myocardial thinning at E12.5.

*Heg*<sup>-/-</sup> *Map3k3<sup>+/−</sup>* hearts exhibit myocardial thinning at E12.5. (A-D)

*Heg*<sup>-/-</sup> *Map3k3<sup>+/−</sup>* hearts (C-D) exhibit myocardial thinning relative to littermate controls (A-B). B and D show higher magnification image of ventricular wall from A and C, respectively. Scale bars are 100µm in A, C, and 50µm in B, D.
Figure 3.6. Cardiac function is normal in E13.5 *Heg-/- Map3k3+/-* embryos.

Fractional shortening was measured on E13.5 embryos by echocardiography. Dots show fractional shortening for individual embryo; graph lines show mean +/- sem for each genotype. No significant changes were observed between the genotypes.
Table 3.5. Survival of $\text{Tie2}^{\text{Cre}} \, \text{Heg}^{-/-} \, \text{Map3k3}^{0/+}$ mice.

<table>
<thead>
<tr>
<th>Age</th>
<th>$\text{Heg}^{+/-} , \text{Map3k3}^{0/+}$</th>
<th>$\text{Heg}^{+/-} , \text{Heg}^{+/-} , \text{Map3k3}^{0/+}$</th>
<th>$\text{Tie2}^{\text{Cre}} , \text{Heg}^{+/-} , \text{Map3k3}^{0/+}$</th>
<th>$\text{Tie2}^{\text{Cre}} , \text{Heg}^{-/-} , \text{Map3k3}^{0/+}$</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
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<td>20</td>
<td>18</td>
<td>2</td>
<td>55</td>
<td>0.003</td>
</tr>
<tr>
<td>P1</td>
<td>17</td>
<td>14</td>
<td>21</td>
<td>0</td>
<td>52</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
### Table 3.6. Survival of \( \text{Nfatc1}^{\text{Cre}} \text{ Heg}^{-/-} \text{ Map3k3}^{+/+} \) mice.

<table>
<thead>
<tr>
<th>Cross: Heg(^{+/+}) X Heg(^{+/+}) Map3k3(^{+/+})</th>
<th>Age</th>
<th>Heg(^{+/+}) Map3k3(^{+/+})</th>
<th>Heg(^{+/+}) Map3k3(^{+/+})</th>
<th>Nfatc1(^{\text{Cre}}) Heg(^{+/+}) Map3k3(^{+/+})</th>
<th>Nfatc1(^{\text{Cre}}) Heg(^{-/-}) Map3k3(^{+/+})</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>4</td>
<td>30</td>
<td>0.423</td>
<td></td>
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<tr>
<td>P1</td>
<td>12</td>
<td>14</td>
<td>20</td>
<td>6</td>
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Chapter 4: Conclusions and Future Directions

Summary

ATOH8 is a highly conserved bHLH transcription factor in vertebrates whose \textit{in vivo} role is largely undefined. In Chapter 2, I utilized a combination of zebrafish and mouse models to characterize the requirements and functions of ATOH8. Through morpholino studies in the zebrafish, I identified a requirement for \textit{atoh8} in the developing zebrafish in regulating heart looping and swimbladder inflation. I further demonstrated that \textit{atoh8} specifically interacts with \textit{gata4} and \textit{zfpm1} (encoding Fog1) in zebrafish heart development. These results, combined with co-immunoprecipitation experiments showing that murine ATOH8 physically interacts with murine FOG2, suggest that Atoh8 functions as a co-factor with the Gata4-Fog1 complex in zebrafish. However, multiple \textit{Atoh8} mutant mouse models failed to exhibit any overt phenotype, in contrast to the zebrafish. Partial postnatal lethality was observed in \textit{Gata4} and \textit{Atoh8} compound mutants, suggesting that a weak genetic interaction may exist in the mouse, but to a much-reduced extent relative to the zebrafish.

Prior work in the Kahn lab had identified the transmembrane protein HEG and the adaptor protein CCM2L as required endocardial factors for proper generation of FGF growth signals for the myocardium. How HEG and CCM2L regulate growth factor production, however, had remained unclear. In Chapter 3, I identify MEKK3 as a likely intermediary between HEG-CCM2L and growth factor production. I show that MEKK3 is capable of physically interacting with CCM2L and is required for CCM2L regulation of FGF production \textit{in vitro}. I further show that endocardial MEKK3 is required for
myocardial proliferation, linking MEKK3 to endocardial growth factor signaling. I also identify genetic interaction between Heg and Map3k3 (encoding MEKK3) in compound mutant mice, further linking MEKK3 to the HEG-CCM complex in vivo. It remains to be determined which growth signals are specifically disrupted in MEKK3 endocardial mutants, what signals activate MEKK3 in the endocardium, and what pathways are activated downstream of MEKK3 within the endocardium.

**Future Directions: Atoh8**

My studies on Atoh8 in murine development have definitively shown that Atoh8 is not required for survival in developing mammals. Furthermore, although we do see weak genetic interaction between the Atoh8 and Gata4 in the mouse, the phenotypic penetrance of this interaction is very weak and I was unable to identify a clear cause of death in these animals. Given that the Kahn lab focuses on mammalian cardiovascular development, it is unlikely that we will continue to study Atoh8. However, I have identified two potential future directions that may be of interest to other groups: 1) determining the mechanism through which zebrafish atoh8 regulates cardiac development, and 2) possible functions of Atoh8 in adult mammals.

**The role of atoh8 in the zebrafish heart**

My morpholino gene suppression studies clearly show that zebrafish heart looping is dependent on Atoh8. I did not, however, identify molecular targets or pathways that were disrupted in the morphant hearts. Heart looping defects have been linked to multiple causes, including disruption of cardiac transcription factors (23,34,61,63,90),
cardiac ion channels (91) and cardiac structural proteins (92). Given the robust synergy we observe between Atoh8 and Gata4-Fog1 (Figure 2.3), there is likely a shared mechanism between Atoh8 and these other genes in regulating heart looping.

Unfortunately, the studies to date in the zebrafish have not identified the targets of Gata4 (23) or Fog1 (34) in heart development, so there are no clear candidates. I therefore propose examining the expression pattern of multiple developmental cardiac transcription factors such as hand2, tbx5a, mef2c, gata5 and nkh2.5 by in situ hybridization in order to determine if the specification of cardiomyocyte progenitors is preserved in atoh8, gata4, and zfpm1 (Fog1) morphant embryos. I would propose further examination of the expression of cardiac structural proteins such as cardiac actin and myosin genes to determine whether proper development from progenitor to differentiated cardiomyocyte is preserved. Given the lack of cardiac defects in Atoh8 mutant mice, it is unlikely that these processes are conserved into mammals. However, these studies may reveal how these factors contribute to heart looping and also may provide insight into how Atoh8, Gata4, and Fog1 interact.

A possible role for Atoh8 in the adult iron metabolism

Although my studies showed that Atoh8 is not required for development and survival, the high degree of conservation in ATOH8 sequence suggests evolutionary pressure to preserve this protein and argues for some role for Atoh8 in mammals. Given the lack of a requirement for Atoh8 in the embryo, it is plausible to infer that the requirement for Atoh8 may be limited to the adult animal. Such requirements may be difficult to observe in adult mice in the laboratory setting, particularly if Atoh8 is required
in stress responses that a laboratory mouse may be unlikely to encounter. Based on my work here and previously reported studies on Atoh8, I propose studying the potential Atoh8 function in the regulation of iron metabolism in the adult liver.

Iron metabolism is regulated by multiple inputs, such as hepatic and circulating iron levels, erythropoietic activity, and inflammation (93). In the liver, excess iron levels are detected by an unknown sensor, which increases hepatic production of BMP6 (94). BMP6 increases production of hepcidin, a regulatory protein that inhibits iron absorption and iron export from the intestine and promotes sequestration of iron within reticuloendothelial macrophages (93). Atoh8 has been shown to be upregulated in the mouse liver following excess iron accumulation secondary to either dietary iron overload or genetic deletion of hepcidin (95), indicating that Atoh8 may be part of the hepatic response program to iron overload. Hepatic Atoh8 expression is also dependent on BMP6-Smad4 signaling (95), suggesting that Atoh8 may be a direct target of this signaling pathway. The mutant mice models we have generated for the Atoh8 project are ideally suited for testing the requirement for ATOH8 in iron metabolism. I propose examining the hepatic and circulating iron levels in adult Atoh8^{Δex1/Δex1} mice vs. control littermates, on both a normal chow diet and an iron-enriched diet. If differences in iron levels are observed in ATOH8-deficient animals, I propose examining the levels of hepcidin and BMP6 to attempt to determine the mechanism by which ATOH8 affects iron regulation. Intriguingly, a previous report identified GATA4 as a transcriptional regulator of hepcidin (96); it is possible that the genetic and physical interactions I have identified between ATOH8 and GATA4 in my work may be applicable in the context of iron regulation.
**Future Directions: MEKK3**

Our studies here have clearly shown that MEKK3 can interact with CCM2L and regulate its function, and have also demonstrated that MEKK3 has a required role in the endocardium that is very similar to the role of HEG and CCM2L in this tissue. Future studies are now needed to further characterize the defects in \( Nfatc1^{Cre} Map3k3^{fl/} \) mice, to identify the factors upstream and downstream of MEKK3 in the endocardium, and to investigate the role of other CCM proteins in the endocardium in development.

**Characterizing the defect in \( Nfatc1^{Cre} Map3k3^{fl/} \) mice**

My experiments with the \( Nfatc1^{Cre} Map3k3^{fl/} \) mice demonstrate that embryonic lethality at E11.5 and severe myocardial thinning result from endocardial loss of MEKK3. This phenotype is remarkably similar to the phenotype previously reported by our lab in \( Heg^{+/} Ccm2l^{+/} \) embryos (58). Future experiments are needed to fully characterize the \( Nfatc1^{Cre} Map3k3^{fl/} \) phenotype to determine why endocardial loss of this protein results in a myocardial defect. First, we need to determine why there is a decrease in myocardial tissue—is this a decrease in proliferation, or an increase in apoptosis? We will use bromodeoxyuridine (Brdu) pulse labeling and TUNEL staining to determine the respective rate of proliferation and apoptosis in the endocardium and myocardium of \( Nfatc1^{Cre} Map3k3^{fl/} \) hearts. Preliminary immunostaining suggests that proliferation is reduced within the myocardium of these animals with no changes in endocardial proliferation or in apoptosis in either tissue (data not shown), which would agree with the reported result in \( Heg^{+/} Ccm2l^{+/} \) embryos. Additional analysis is needed.
to quantify this proliferative change to confirm that there is a significant change in 
\( Nfatc1^{Cre} \ Map3k3^{fl/} \) hearts.

Following my studies on proliferation and apoptosis, I propose to examine how loss of endocardial MEKK3 leads to myocardial defects. Given the physical separation between the endocardium and myocardium at E10.5-11.5, it is reasonable to conclude that there is a deficiency in a secreted endocardial factor. In the study of \( Heg^{-/-} \ Ccm2^{-/-} \) embryos, the decreased myocardial growth was attributed to a decrease in endocardial FGF16 transcription (58), which had previously been shown to be required for myocardial growth (45,59). It is therefore reasonable to start with FGF16 and the related factors FGF9 and FGF20 as initial candidates. However, myocardial growth has been shown to be dependent on multiple growth factor signals in addition to FGFs, such as neuregulin (43) and Notch (97). I would therefore like to do transcriptional profiling of whole E10.5 \( Nfatc1^{Cre} \ Map3k3^{fl/} \) hearts to screen for changes in multiple signaling pathways. If I am able to identify a signal as a plausible cause for the defects in \( Nfatc1^{Cre} \ Map3k3^{fl/} \) hearts, I will attempt to rescue the defect using heart explant cultures. In these experiments, explanted hearts are cultured with replacement cytokines to rescue a proliferation defect; this technique has previously been used to rescue hearts deficient in FGFs (98) or neuregulin (97) to link proliferation specifically to deficits in these endocardial growth factors.

**Identifying the pathways downstream of MEKK3**

MEKK3 is a MAPK kinase kinase kinase and has no known transcriptional activity; it is likely that there are downstream targets of MEKK3 within the endocardial
cell that convey a signal that ultimately promotes increased transcription of growth factor genes. Although the *in vivo* endothelial targets of MEKK3 have not been identified, MEKK3 has been shown to be upstream of the MAPK kinases ERK5 and p38 in cultured endothelial cells (99). In immune cells, where it has been studied more extensively, MEKK3 has been shown to have additional roles as an upstream activator of the MAPK kinase JNK and also as an activator of NF-KB pathway (100). These pathways are our best candidates for possible downstream targets of MEKK3 in the endocardium. We propose three approaches to identifying which target pathway is critical in these cells.

First, we will examine the phosphorylation of MAPK and NF-KB pathway components in E10.5 *Nfatc1*Cre*Map3k3fl/fl* hearts to screen for decreased activation of these pathways with endocardial loss of MEKK3. While this approach allows me to look directly at the *in vivo* situation, it is potentially complicated by the small number of cells in an E10.5 heart, which would limit my source material, and by the presence of non-endocardial cells (primarily myocardium), which may have phosphorylated MAPK proteins and mask changes in the endocardium. To address the latter concern, I could attempt to sort endocardium from these hearts to isolate a pure population, although I would still be limited by the number of endocardial cells.

In addition to investigating phosphorylation *in vivo*, I will also use our adenoviral-CCM2L HMVEC expression system (Figure 3.2) to identify possible MEKK3 targets. In this system, overexpression of CCM2L increases FGF16 transcription. Suppression of MEKK3 with siRNA inhibits this increase, indicating that CCM2L is likely acting through MEKK3 and suggesting that this cell culture system may be a somewhat similar model of the *in vivo* pathway in the endocardium. I will use siRNA against potential
targets of MEKK3 in the endocardium in order to identify which proteins are required for this CCM2L-induced upregulation of FGF16 levels.

Finally, I can ablate candidate genes in the endocardium using the \textit{Nfatc1}^{Cre} allele and screen for phenotypes similar to the \textit{Nfatc1}^{Cre} \textit{Map3k3}^{flo/-} mouse. This approach is the best tool for confirming that the candidate is truly operating in the same path as MEKK3; however, the large number of possible candidates precludes pursuing this approach on a large scale and I will therefore limit this approach to our most likely candidates. At this point, I plan to pursue this approach with the MAPK kinase ERK5; I suspect ERK5 because it has been shown regulated by MEKK3 in the endothelium (99), and because endothelial-specific ERK5 mutants are highly similar to MEKK3 mutants (85,101). If my other approaches identify other MAPK and NF-KB pathways as being better candidates than ERK5, we will make endocardial deletions of those genes.

**Identifying the upstream activator of MEKK3**

In addition to identifying the downstream targets of MEKK3 in the endocardium, I would like to identify potential upstream signals that activate MEKK3, presumably through an activating phosphorylation of MEKK3. Although HEG and the CCM proteins genetically and/or physically interact with MEKK3, none of these proteins have intrinsic kinase activity and therefore would be unable to directly activate MEKK3. In order to identify potential activators of MEKK3, I will attempt to detect proteins that physically interact with MEKK3 and the HEG/CCM complex, which I hypothesize may interact with MEKK3 in the endocardium. I will create a lentiviral construct expressing FLAG-HA-HEG or FLAG-HA-MEKK3 in a doxycycline-inducible manner. I will use this
lentivirus to express tagged proteins in endothelial cells such as HMVECs, and then titrate the level of expression to endogenous levels via the doxycycline-inducible system. I will then immunoprecipitate the tagged proteins via their double epitope tags, and perform mass spectrometry to identify binding partners of HEG and MEKK3.

As an alternative to the traditional immunoprecipitate mass spectrometry approach, I am also using a recently reported biotin-labeling strategy to identify HEG and MEKK3 interactors (102). In this approach, a mutated promiscuous biotin ligase is fused to the protein of interest. The biotin ligase biotinylates other proteins in the vicinity of the fusion protein; interacting proteins should be enriched when biotinylated proteins are immunoprecipitated from the cell lysate. This approach is particularly attractive for identifying HEG interactors, as the lysis conditions necessary to liberate HEG from the cell membrane may disrupt protein-protein interactions, whereas a biotin-tag should be permanent even if interactions are disrupted by lysis. However, there may be more background noise with this approach, as non-interacting proteins in the same subcellular domain as HEG or MEKK3 may also be labeled.

**Determining the endocardial functions of CCM proteins**

My studies with the *Nfatc1*\(^{Cre}\) *Map3k3*\(^{fl/–}\) mouse revealed a clear requirement for MEKK3 in the endocardium that had been previously been hidden by the early embryonic death imposed by vascular defects in constitutive and endothelial (*Tie2*\(^{Cre}\)) deletions of this gene. I would like to pursue a similar approach with CCM1 and CCM2. Global and endothelial-specific mutants of CCM1 and CCM2 are lethal at E9.5 due to defects in development of the branchial arch arteries (54,55). This results in a failure to
connect the heart outflow tract with the descending aorta, which prevents effective circulation. My lineage trace experiments show that \( Nfatc1^{Cre} \) expression is not present in the branchial arch arteries (Figure 3.3), indicating that deletion of CCM1 or CCM2 with this Cre allele should bypass this early lethal vascular defect. I will generate \( Nfatc1^{Cre} Krit1^{β/β} \) and \( Nfatc1^{Cre} Ccm2^{β/β} \) to delete CCM1 and CCM2, respectively, in the endocardium. Preliminary results showing an absence of viable \( Nfatc1^{Cre} Krit1^{β/β} \) animals at birth (Table 3.3) indicate that CCM1 is required at some point in the endocardium in embryogenesis; timed matings are needed to determine when and why these animals die. I will pursue similar crosses with the \( Ccm2^{β} \) allele to determine the requirement for CCM2. It is possible that CCM2 may be redundant with its paralogue CCM2L, which is expressed in the embryonic endocardium (58). To account for this possibility, I will also examine \( Nfatc1^{Cre} Ccm2^{β/β} Ccm2L^{−/−} \) double mutant animals. If MEKK3 interacts with CCM1 or CCM2 in the endocardium, it is possible that the endocardial deletions of CCM1 or CCM2 will phenocopy endocardial deletion of MEKK3. Alternatively, I may see other defects within the endocardium, or lethal defects in the development of the coronary endothelium that arises from endocardium in later embryogenesis (Figure 3.3).

**Concluding remarks**

Our studies of ATOH8 clearly define the requirements for ATOH8 between different vertebrate species. While there is a requirement for \( atoh8 \) in the developing zebrafish heart, our \( Atoh8 \) null mice definitively demonstrate that this requirement is not preserved in mammalian development. In a similar manner, the robust genetic interaction
we observe between Atoh8 and Gata/Fog proteins in the zebrafish development is much reduced in the mouse. Further studies will be needed to determine if and where ATOH8 may function in the adult mammal, and to determine whether ATOH8 interacts with GATA or FOG factors in these processes outside of development.

Our studies of MEKK3 indicate that this kinase has a critical role in the endocardium to facilitate myocardial growth, likely by promoting growth factor production. This finding provides a mechanism to explain the phenotype that was observed in HEG-CCM2L double mutant mice, and identifies a role for MEKK3 in the endocardium that had previously been masked by the early lethal vascular defects of prior deletions of this gene. Future studies are needed to define the upstream activators and downstream targets of MEKK3 within the endocardium, and to further define the functions and interactions of the HEG-CCM-MEKK3 complex within the endocardium and within the remainder of the endothelium.
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