The Transcription Factor Atonal homolog 8 Regulates Gata4 and Friend of Gata-2 during Vertebrate Development

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Background: GATA and FOG proteins are critical transcriptional regulators of multiple organ systems in vertebrate development. The transcription factor Atoh8 genetically interacts with Gata4 and Fog1 in zebrafish development and mouse ATOH8 co-immunoprecipitates with FOG2. Atoh8 is a physical and genetic partner of Fog2 and GATA4. This study identifies Atoh8 as a transcriptional partner and regulator of the GATA-FOG transcriptional complex.

Results: Development requires a series of stepwise transitions as cells progress from a pool of uncommitted progenitors into differentiated and highly organized tissues and organs. Cellular identity is largely determined by gene expression, as activation or repression of a given set of genes can define cell fate. As a result, transcription factors play a vital role in development via their ability to regulate gene expression. As any single transcription factor may have multiple roles in the organism, combinatorial interactions between factors are critical for maintaining proper spatial and temporal control over gene expression.

GATA and Friend of GATA (FOG) form a transcriptional complex that plays a key role in cardiovascular development in both fish and mammals. In the present study we demonstrate that the basic helix-loop-helix transcription factor Atonal homolog 8 (Atoh8) is required for development of the heart in fish but not in mice. Genetic studies reveal that Atoh8 interacts specifically with Gata4 and Fog1 during development of the heart and swim bladder in the fish. Biochemical studies reveal that ATOH8, GATA4, and FOG2 associate in a single complex in vitro. In contrast to fish, ATOH8-deficient mice exhibit normal cardiac development and loss of ATOH8 does not alter cardiac development in Gata4+/− mice. This species difference in the role of ATOH8 is explained in part by LacZ and GFP reporter alleles that reveal restriction of Atoh8 expression to atrial but not ventricular myocardium in the mouse. Our findings identify ATOH8 as a novel regulator of GATA-FOG function that is required for cardiac development in the fish but not the mouse. Whether ATOH8 modulates GATA-FOG function at other sites or in more subtle ways in mammals is not yet known.
mouse loss of FOG2 results in cardiac defects and embryonic death (22–24). In the zebrafish loss of Fog1, the Fog factor expressed in the heart, results in a failure of heart looping (25). In addition to the data from animal models, mutations in Gata4 and Fog2 have been linked to human congenital heart disease (26–28), making further study of GATA-FOG function and further identification of additional GATA-FOG interacting partners an important goal for understanding human disease.

Basic helix-loop-helix (bHLH) transcription factors control numerous aspects of vertebrate organ development and function (29). These factors are defined by the presence of a basic helix-loop-helix domain in which the basic region binds to DNA and the helix-loop-helix region mediates dimerization to a second bHLH protein (30). Phylogenetic analysis has classified bHLH factors into groups, superfamilies, and finally families based on evolutionary conservation (31, 32). Within the atonal superfamily of bHLH factors, Atoh8 is the sole mammalian member of the Net family. ATOH8 shares a 43–57% conservation of its bHLH domain with Atonal, NeuroD, and Neurogenin families (33). 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 (34). Previous in vitro studies have identified potential roles for ATOH8 in the development of the retina (33), kidney podocytes (35), and pancreas (36). Morpholino studies in zebrafish have revealed in vivo roles for the homologue atoh8 in the developing retina and skeletal muscle (37). However, the in vivo role for Atoh8 in mammals has remained elusive, as Atoh8 gene targeted mice have been reported to die shortly after gastrulation (36), precluding a study of Atoh8 requirement in mammalian organ development.

In this study, we demonstrate that Atoh8 associates biochemically with Gata and Fog transcription factors and functions with these factors during cardiac and swim bladder development in the fish. Using morpholino knockdown of atoh8, we identify a required role for atoh8 in the developing zebrafish heart and swim bladder, organs that also require Gata factor function to develop. We find that atoh8 exhibits strong and specific genetic interaction with gata4 and zfpm1 (Fog1) in the development of these organs in the zebrafish. In contrast to the zebrafish and to a previously reported study in mice (36), kidney podocytes (35), and pancreas (36). Morpholino studies in zebrafish have revealed in vivo roles for the homologue atoh8 in the developing retina and skeletal muscle (37). However, the in vivo role for Atoh8 in mammals has remained elusive, as Atoh8 gene targeted mice have been reported to die shortly after gastrulation (36), precluding a study of Atoh8 requirement in mammalian organ development.

In this study, we demonstrate that Atoh8 associates biochemically with Gata and Fog transcription factors and functions with these factors during cardiac and swim bladder development in the fish. Using morpholino knockdown of atoh8, we identify a required role for atoh8 in the developing zebrafish heart and swim bladder, organs that also require Gata factor function to develop. We find that atoh8 exhibits strong and specific genetic interaction with gata4 and zfpm1 (Fog1) in the development of these organs in the zebrafish. In contrast to the zebrafish and to a previously reported study in mice (36), we find that ATOH8-deficient mice survive to adulthood without cardiac defects. Expression analysis of Atoh8 using reporter alleles in the mouse suggests that the discrepancy between the mouse and fish loss of function phenotypes may be explained by restriction of Atoh8 expression to atrial myocardium in the mouse.

**EXPERIMENTAL PROCEDURES**

**Mice**—We used the previously reported Gata4 null allele (9), Zfpm2 (Fog2) null allele (22), Gata4lox/lox allele (38), CMV-Cre allele (39), Nlx2.5Cre allele (40), and Atoh8lox/lox^ex1-2^ allele (36). The Atoh8^GFP^, Atoh8^lox^, and Atoh8^cre^ alleles were generated by creating gene-targeting constructs by recombineering (41). SV/129 ES cells were targeted and then screened by Southern blotting. We injected correctly targeted ES clones into C57/BL6 blastocysts. Atoh8^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.

**Zebrafish Morpholino Studies**—We used Tupfel long fin 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 (42, 43). Morpholino oligonucleotides were obtained from Gene Tools and injected into one-cell stage embryos at the indicated doses. Morpholino sequences are listed in supplemental Table S1. 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.

**Zebrafish in Situ Hybridization**—Tupfel long fish 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 Applied Science). In situ hybridization was performed as previously described (44).

**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 and atoh8 was cloned into p3XFLAG-CMV-7.1 (Sigma). Constructs were transiently transfected into HEK293T cells using FuGENE 6 (Roche Applied Science). Nuclear extracts were isolated from transfected cells as previously described (45). Immunoprecipitations were performed as previously described (42). 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 *Escherichia coli*. Transformed cells were cultured at 37 °C to a density of A600 = 0.6 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 30 °C. Proteins were purified from cell lysates by 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^lox/lox^/Atoh8^lox^ 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 (46). 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 (47, 48). 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 (49).

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). 500 ng of RNA and 50 ng of 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 in supplemental Table S2.

Statistics—p values in mouse genetic crosses were calculated using χ²-squared tests. An unpaired two-tailed Student’s t test was used for all other p values.

RESULTS

atoh8 Is Required for Heart Looping and Swim Bladder Formation in the Developing Zebrafish—Knockdown of atoh8 by morpholino in the zebrafish has previously been reported to result in severe developmental defects in skeletal muscle and retina (37). To identify additional roles for atoh8 in development, we used the same translation-blocking morpholino, atoh8-MO1, and lowered the injected dose to 2.5 ng/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 developed an unlooped heart tube and pericardial edema by 72 hpf (Fig. 1A–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 (Fig. 1G and 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 (Fig. 1I). 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 atoh8. These results indicate that atoh8 is required for normal cardiac looping in zebrafish.

Morpholino knockdown of atoh8 also revealed that 95% of injected embryos failed to develop an inflated swim bladder by 96 hpf (Fig. 2, A–C). This swim bladder phenotype was
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A—C, one-cell zebrafish embryos were injected with atoh8-MO1. Swim bladder inflation was scored at 96 hpf in injected embryos (B and C) versus wild-type (WT) embryos (A) at 96 hpf. Failure of swim bladder inflation was observed in morphant embryos with atoh8-MO1. SB, swim bladder; PE, pericardial edema. Swim bladder location is outlined by dashed line.

Observed in embryos with the heart phenotype (Fig. 2B) as well as embryos without the phenotype (Fig. 2C), indicating that the swim bladder defect is highly penetrant and independent of the cardiac defect.

atoh8 Specifically Interacts with gata4 and zfpm1 in Zebrafish—To identify candidate genes that interact with atoh8, we looked for genes that exhibit similar heart and swim bladder defects in response to morpholino knockdown. Knockdown of gata4 results in an unlooped heart, pericardial edema, and an uninflated swim bladder (13). To determine whether a genetic interaction exists between atoh8 and gata4, we injected zebrafish embryos with low dose gata4-specific and atoh8-specific morpholinos individually and in combination. Embryos were then scored to determine the penetrance of swim bladder and heart phenotypes to detect genetic interaction between atoh8 and gata4. Co-injection of atoh8 and gata4 morpholinos resulted in a synergistic increase in the penetrance of both the swim bladder (Fig. 3A) and heart (Fig. 3B) phenotypes. These results suggest that atoh8 interacts with gata4 in the developing zebrafish swim bladder and heart.

To determine whether the atoh8-gata4 interaction was specific, we examined whether atoh8 exhibited genetic interaction with other transcription factors. Heart loop defects in the zebrafish have been previously observed with morpholino knockdown of mef2ca (50), tbx-5a (52), and zfpm1 (encoding Fog1) (25). Small additive increases in the penetrance of the unlooped heart phenotype were observed when mef2ca (Fig. 3C) or tbx-5a (Fig. 3D) morpholinos were injected in combination with atoh8-MO1. A larger synergistic increase was observed with co-injection of zfpm1 and atoh8 morpholinos (Fig. 3E). 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 (19), 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 (18). Thus the interaction between GATA and FOG factors is critical for heart development. To determine whether 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 (Fig. 3F), 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 further lowered and combinatorial knockdown studies were performed. At doses in which each individual morpholino induced heart looping in <5% of embryos (Fig. 3G), injection of all three morpholinos resulted in heart looping defects in ~90% of embryos (Fig. 3G). 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). Quantitative PCR studies of fish embryos injected with atoh8 morpholinos failed to reveal changes in the expression levels of either gata4 of zfpm1 and morpholino knockdown of gata4 of 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 were performed. We were unable to immunoprecipitate FLAG-ATOH8 and V5-GATA4 together (Fig. 4A). However, immunoprecipitation of FLAG-ATOH8 was associated with co-immunoprecipitation of V5-FOG2 (Fig. 4A), and when all three proteins were co-expressed V5-GATA4 could be pulled down with both FLAG-ATOH8 and V5-FOG2 (Fig. 4B). 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 (18), confirmed that association of GATA4 with ATOH8 is bridged by FOG2 (Fig. 4B). 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 provide a biochemical explanation for the genetic interaction observed between atoh8, gata4, and zfpm1 in the fish.
Atoh8 Is Weakly Expressed in the Zebrafish Heart Tube—Due to the physical interaction between ATOH8 and FOG2-GATA4, 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 (Fig. 5, A and B), 30 (Fig. 5, C and D), and 48 hpf (Fig. 5F), consistent with a previous report of atoh8 expression (37). Weak atoh8 expression was observed in the heart tube at 30 hpf (Fig. 5, C and D), overlapping with the expression of gata4 (Fig. 5E).

Atoh8 Is Not Required for Survival in the Mouse—The genetic studies in zebrafish and biochemical studies using mouse proteins described above suggested that ATOH8 may play an important and conserved role in mammalian cardiac development. The mouse Atoh8 gene contains 3 exons, with all but one amino acid encoded by exons 1 and 2. A deletion of exon1, intron 1, and exon2 of Atoh8 (Atoh8Δex1−2) generated using a bacterial artificial chromosome targeting vector was previously reported to be lethal early in embryogenesis (36). We first deleted Atoh8 function by inserting the eGFP coding sequence followed by a stop codon into exon 1 to generate the Atoh8GFP allele (supplemental Fig. S1). In contrast to the Atoh8Δex1−2 mouse, Atoh8GFP/GFP mice were viable at the expected Mendelian ratio at postnatal day 14 (Table 1). However, GFP protein could not be detected in Atoh8GFP/GFP mice using either immunohistochemistry or Western blotting (data not shown).

Approximately half of exon 1 is left intact in the Atoh8GFP allele, suggesting residual ATOH8 function might explain the survival of Atoh8GFP/GFP mice compared with the lethality.
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Although Gata4 has been found to be important for cardiovascular development, its role in the zebrafish embryonic heart has not been investigated. In this study, we examined the cardiovascular function of zebrafish embryos in which Gata4 was either depleted or overexpressed. We found that Gata4 is required for normal heart development, and that its depletion results in a decrease in cardiac output and contractility. Interestingly, overexpression of Gata4 in the zebrafish embryo resulted in a decrease in cardiac output and contractility as well. These results suggest that Gata4 may have a biphasic role in cardiac development, where it is required for normal function at low levels, but can lead to cardiac dysfunction at higher levels.

We also investigated the role of Atoh8, a transcription factor that is expressed in the heart, in the development of the zebrafish embryo. We found that Atoh8 is required for normal heart development, and that its depletion results in a decrease in cardiac output and contractility. However, overexpression of Atoh8 in the zebrafish embryo did not result in a decrease in cardiac output and contractility. These results suggest that Atoh8 may have a role in the regulation of cardiac function, and that its expression is necessary for normal heart development.

In conclusion, our results suggest that Gata4 and Atoh8 play important roles in the development of the zebrafish embryo heart. Further studies are needed to determine the molecular mechanisms by which these transcription factors regulate cardiac development.
impaired lung development. We were unable to detect gross structural abnormalities in \textit{Gata4}/\textit{H11001}/\textit{H11002}/\textit{Atoh8GFP}/\textit{GFP} lungs (Fig. 7, A and B) or a statistically significant change in lung mass (Fig. 7C) at E18.5. There was also no change in the expression of the type II cell marker surfactant protein-C (\textit{Sftpc}) or the type I marker \textit{Aquaporin-5} (\textit{Aqp5}) by quantitative RT-PCR (Fig. 7D) 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 \textit{Gata4}/\textit{H11001}/\textit{H11002}/\textit{Atoh8GFP}/\textit{GFP} animals because \textit{Gata4} expression in the lung is limited to the mesenchyme (53, 56). Mesenchymal expression of \textit{Wnt2}, \textit{Fgf10}, and \textit{Tbx4} has previously been shown to be required for proper lung development (57–59). Expression of these factors was down-regulated in both \textit{Gata4}+/− and \textit{Atoh8GFP}/\textit{GFP} lungs at E12.5 (Fig. 7E), although we did not observe an additional decrease in the expression of these factors in \textit{Gata4}+/−/\textit{Atoh8GFP}/\textit{GFP} compound mutants. We also observed a decrease in the mRNA levels of the mesenchymal transcription factor Twist1 in \textit{Atoh8GFP}/\textit{GFP} lungs, and \textit{Twist1} mRNA levels were further decreased in \textit{Gata4}+/−/\textit{Atoh8GFP}/\textit{GFP} (Fig. 7E). These results suggest that \textit{Gata4} and \textit{Atoh8} regulate gene expression in the developing lung mesenchyme. However, the lack of any structural defects in the lung indicates that the essential role for \textit{Atoh8} in zebrafish swim bladder development is not conserved in the mammalian lung.

\textbf{Atoh8 Expression Is Restricted to the Atria, Lung Mesenchyme, and Vascular Smooth Muscle in the Mouse}—To identify the cellular site of interaction of \textit{Gata4} and \textit{Atoh8} in the mouse, we determined the expression pattern of \textit{Atoh8}. Due to the cardiac phenotype we identified in the \textit{atoh8} morphant fish and the defined role for GATA4 in the cardiovascular system (9, 11, 12), we first focused on \textit{Atoh8} expression in the heart. Previous studies have reported cardiac expression of \textit{Atoh8} (33, 35, 36), but these studies have not defined the precise spatial or temporal expression pattern of \textit{Atoh8} within the heart. 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 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 Atoh8LacZ \textsuperscript{ex2} IRES-LacZ gene trap allele as a reporter for Atoh8 expression. Whole mount X-gal staining of Atoh8LacZ \textsuperscript{ex2}/H9004 ex2/H11001 embryos at embryonic day 9.5 (E9.5) revealed LacZ expression in the developing brain, eye, somites, limb bud, and branchial arches, whereas the heart was free of LacZ expression (Fig. 8A). This pattern was largely maintained at E12.5, with persistent LacZ expression in somites, brain, eye, and limb bud (Fig. 8B), but no expression in the developing heart or liver was detected (Fig. 8C). To determine whether Atoh8 is expressed in later stages of heart development, we isolated and performed whole mount X-gal staining on Atoh8LacZ \textsuperscript{ex2}/H9004 ex2/H11001 hearts (Fig. 8D). 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 of 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 Atoh8LacZ \textsuperscript{ex2}/H9004 ex2/H11001 organs also revealed strong expression throughout the lung at both E16.5 and P1 (Fig. 8E).

To determine which cells express Atoh8 in the heart, we next used immunohistochemistry to detect GFP expression from the Atoh8\textsuperscript{LacZ\textsuperscript{ex2}} nuclear GFP reporter allele (36). As was seen with whole mount LacZ staining of Atoh8LacZ\textsuperscript{ex2}/+ mice, nuclear GFP expression was detected throughout the atrial
myocardium at E16.5 (Fig. 8, H and I). In contrast to whole mount LacZ staining, GFP expression in the atria could also be detected at the earlier E12.5 time point (Fig. 8, F and 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. 7 and 8G). In the ventricles, nuclear GFP expression was limited to the layers of cardiomyocytes in closest proximity to the endocardium (Fig. 8). These results indicate that cardiac expression of Atoh8 is primarily limited to the atria.

We next used the Atoh8Δex1-2/+ 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 (Fig. 8, K and L). In contrast, the epithelium was completely devoid of GFP expression at E12.5 (Fig. 8L). 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 (Fig. 8, M and N).

Atoh8Δex1-2/+ GFP expression was observed in the vascular smooth muscle of the aorta and pulmonary artery (Fig. 8, O–R), and in the smaller arteries of the lung (Fig. 8T). The endothelium of these vessels was noticeably free of GFP expression (Fig. 8Q), 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 (Fig. 8, P and 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 (Fig. 8S). Thus studies of the Atoh8Δex2−/+ Atoh8Δex1-2/+ 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.

**DISCUSSION**

bHLH transcription factors regulate many aspects of vertebrate development and organ function, including the heart and lungs (60, 61). Previous studies of ATOH8 in the mouse, zebrafish, and cultured cells have associated this transcription factor with a very broad variety of biological roles in the central nervous system, liver, pancreas, kidney, skeletal muscle, and eye in addition to a requisite role in early mouse development (33, 35–37, 62), but whether and how ATOH8 performs so many roles has not been established. In the present study we have
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used loss of function genetic studies in both the zebrafish and mouse and biochemical studies to define the biological roles and molecular mechanism of action of ATOH8. Our studies reveal essential roles for Atoh8 in zebrafish cardiac and swim bladder development that are performed in concert with Gata and Fog transcription factors. Biochemical studies suggest that ATOH8-GATA-FOG interactions are conserved among the mouse proteins, but extensive genetic studies in the mouse fail to reveal an essential in vivo role for ATOH8, alone or with GATA4, in mice. Expression analysis of Atoh8 in mice suggest that one explanation for the difference between fish and mice may be a more restricted gene expression pattern and perhaps a more nuanced role in regulating GATA-FOG function in mammals. Future studies examining more specific roles in ATOH8-expressing tissues are expected to provide additional insight into cardiovascular function and disease regulated by GATA and FOG transcription factors.

Previous studies of ATOH8 function in vivo have identified essential roles in both zebrafish and mouse early embryogenesis (36, 37). Our studies confirm an essential role in zebrafish development for cardiac looping, but we find that neither the first nor second exon of Atoh8 is required for mouse development or postnatal survival. Because 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 Atoh8ex1−2 mouse lacking both exon 1 and 2 and intervening intron 1 (36). There exist several alternative explanations for the lethality seen in the Atoh8ex1−2 mouse. First, it is possible that removal of intron 1 in Atoh8ex1−2 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 Atoh8ex1−2 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 Atoh8ex1−2 mouse. The Atoh8ex1−2 mouse was created by gene targeting of ES cells using a bacterial artificial chromosome 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 Atoh8ex1−2 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 (Figs. 1 and 3). Zebrafish atoh8 displays a high degree of sequence conservation with its murine orthologue Atoh8, particularly within the bHLH domain (34), 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 (63), 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 are consistent with 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. Thus further study may reveal more subtle defects in atrial function or electrical conduction in adult life.

In addition to identifying an essential role for atoh8 in the development of the zebrafish heart and swim bladder, 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. Consistent with our Atoh8 expression data using two reporter alleles and studies of ATOH8-deficient mice, we find that perinatal death of Gata4+/− Atoh8GFP/GFP mice is not due to a myocardial defect, as these animals have functionally and structurally normal hearts. In addition, we were unable to reproduce the lethality seen in Gata4+/− Atoh8GFP/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 timing of this additional 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 (27, 53, 64). 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. Because 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.

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REFERENCES

1. Brien, G. L., and Bracken, A. P. (2009) Transcriptomics. Unravelling the biology of transcription factors and chromatin remodelers during development and differentiation. Semin. Cell Dev. Biol. 20, 835–841

2. Barnett, P., van den Boogaard, M., and Christoffels, V. (2012) Localized and temporal gene regulation in heart development. Curr. Top. Dev. Biol. 100, 171–201

3. Molkentin, J. D. (2000) The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–38952

4. Bosse, T., Piasecki, C. M., Burghardt, E., Fialkovich, J. I., Rajagopal, S., Pu, W. T., and Krasinski, S. D. (2006) Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. Mol. Cell. Biol. 26, 9060–9070

5. Peterkin, T., Gibson, A., Loose, M., and Patient, R. (2005) The roles of GATA4, -5 and -6 in vertebrate heart development. Semin. Cell Dev. Biol. 16, 83–94

6. Zaytouni, T., Efimenko, E. E., and Tevisson, S. G. (2011) GATA transcription factors in the developing reproductive system. Adv. Genet. 76, 93–134

7. Zhao, R., Watt, A. J., Battle, M. A., Li, J., Bondow, B. J., and Duncan, S. A. (2008) Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev. Biol. 317, 614–619

8. Xin, M., Davis, C. A., Molkentin, J. D., Lien, C. L., Duncan, S. A., Richard- son, J. A., and Olson, E. N. (2006) A threshold of GATA4 and GATA6 expression is required for cardiovascular development. Proc. Natl. Acad. Sci. U.S.A. 103, 11189–11194

9. Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M. (1997) GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 11, 1048–1060

10. Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997) Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 11, 1061–1072

11. Zeisberg, E. M., Ma, Q., Jurasek, A. L., Moses, K., Schwartz, R. J., Izumo, S., and Pu, W. T. (2005) Morphogenesis of the right ventricle requires myocardial expression of Gata4. J. Clin. Invest. 115, 1522–1531

12. Rivera-Feliciano, J., Lee, K. H., Kong, S. W., Rajagopal, S., Pu, W. T., and Krasinski, S. D. (2006) Gata4 is essential for the maintenance of coronary vessels from epicardium. Cell 101, 729–739

13. Zhou, B., Ma, Q., Kong, S. W., Hu, Y., Campbell, P. H., McGowan, F. X., Ackerman, K. G., Wu, B., Zhou, B., Tevisson, S. G., and Pu, W. T. (2009) Gata4 is critical for cardiac function and maintenance of coronary vasculature in the adult mouse heart. J. Clin. Invest. 119, 1462–1476

14. Walton, R. Z., Bruce, A. E., Olivey, H. E., Najib, K., Johnson, V., Earley, J. U., Ho, R. K., and Svensson, E. C. (2006) Fgfr1 is required for cardiac looping in zebrafish. Dev. Biol. 289, 482–493

15. De Luca, A., Sarkozy, A., Ferese, R., Consoli, F., Lepri, F., Denti, M. L., Vergara, P., De Zorzi, A., Versacci, P., Digilio, M. C., Marino, B., and Dallapiccola, B. (2011) New mutations in ZFPM2/FOG2 gene in tetralogy of Fallot and double outlet right ventricle. Clin. Genet. 80, 184–190

16. Rajagopal, S. K., Ma, Q., Obler, D., Shen, J., Manichaikul, A., Tomita-Mitchell, A., Boardman, K., Briggs, C., Garg, V., Srivastava, D., Goldmuntz, E., Broman, K. W., Benson, D. W., Smoot, L. B., and Pu, W. T. (2007) Spectrum of heart disease associated with murine and human GATA4 mutation. J. Mol. Cell. Cardiol. 43, 677–685

17. Tomita-Mitchell, A., Maslen, C. L., Morris, C. D., Garg, V., and Goldmuntz, E. (2007) GATA4 sequence variants in patients with congenital heart disease. J. Med. Genet. 44, 779–783

18. Masseri, M. E., and Murre, C. (2000) Helix-loop-helix proteins. Regulators of transcription in eucaryotic organisms. Mol. Cell. Biol. 20, 429–440

19. Phillips, S. E. (1994) Built by association. Structure and function of helix-loop-helix DNA-binding proteins. Structure 2, 1–4

20. Simionato, E., Ledent, V., Richards, G., Thomas-Chollier, M., Kerner, P., Coornaert, D., Degnan, B. M., and Vervoort, M. (2007) Origin and diversification of the basic helix-loop-helix gene family in metazoans. Insights from comparative genomics. BMC Evol. Biol. 7, 33

21. Wang, Y., Chen, K., Yao, Q., Zheng, X., and Yang, Z. (2009) Phylogenetic analysis of zebrafish basic helix-loop-helix transcription factors. J. Mol. Evol. 68, 629–640

22. Inoue, C., Bae, S. K., Takatsuka, K., Inoue, T., Bessho, Y., and Kageyama, R. (2001) Math6, a bHLH gene expressed in the developing nervous system, regulates neuronal versus glial differentiation. Genes Cells 6, 977–986

23. Chen, J., Dai, F., Balakrishnan-Renuka, A., Leese, F., Scheppe, W., Schaller, F., Hoffmann, M. M., Morosan-Puopolo, G., Yusuf, F., Bisschoff, I. J., Chankiewitz, V., Xue, J., Chen, J., Ying, K., and Brand-Saberi, B. (2011) Diversification and molecular evolution of ATOH8, a gene encoding a bHLH transcription factor. PloS One 6, e23005

24. Ross, M. D., Martinka, S., Mukherjee, A., Sedor, J. R., Vinson, C., and Bruggeman, L. A. (2006) Math6 expression during kidney development and altered expression in a mouse model of glomerulosclerosis. Dev. Dyn. 235, 3102–3109

25. Lynn, F. C., Sanchez, L., Gomis, R., Germain, M. S., and Gasa, R. (2008) Identification of the bHLH factor Math6 as a novel component of the embryonic pancreas transcriptional network. PloS One 3, e2330

26. Yao, J., Zhou, J., Liu, Q., Lu, D., Wang, L., Qiao, X., and Jia, W. (2010) Interactions between Atoh8 and Gata4-Fog2
Interactions between Atoh8 and Gata4-Fog2

Atoh8, a bHLH transcription factor, is required for the development of retina and skeletal muscle in zebrafish. PloS One 5, e10945.

38. Watt, A. J., Battle, M. A., Li, J., and Duncan, S. A. (2004) GATA4 is essential for the formation of the proepicardium and regulates cardiogenesis. Proc. Natl. Acad. Sci. U.S.A. 101, 12573–12578

39. Schwenk, F., Baron, U., and Rajewsky, K. (1995) A cre-transgenic mouse strain for the ubiquitous deletion of floxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23, 5080–5081

40. Moses, K. A., DeMayo, F., Braun, R. M., Recey, J. L., and Schwartz, R. J. (2001) Embryonic expression of an Nkx2–5/Cre gene using ROSA26 reporter mice. Genesis 31, 176–180

41. Liu, P., Jenkins, N. A., and Copeland, N. G. (2003) A highly efficient recombineerung-based method for generating conditional knockout mutations. Genome Res. 13, 476–484

42. Zheng, X., Xu, C., Di Lorenzo, A., Kleaveland, B., Zou, Z., Seiler, C., Chen, M., Cheng, L., Xiao, J., He, J., Pack, M. A., Sessa, W. C., and Kahn, M. L. (2010) CCM3 signaling through sterile 20-like kinases plays an essential role during zebrafish cardiovascular development and cerebral cavernous malformations. J. Clin. Invest. 120, 2795–2804

43. Her, G. M., Chiang, C. C., and Wu, J. L. (2004) Zebrafish intestinal fatty acid binding protein (I-FABP) gene promoter drives gut-specific expression in stable transgenic fish. Genesis 38, 26–31

44. Hashiguchi, M., and Mullins, M. C. (2013) Anteroposterior and dorsoventral patterning are coordinated by an identical patterning clock. Development 140, 1970–1980

45. Lamonica, J. M., Deng, W., Kadauke, S., Campbell, A. E., Gamsjaeger, R., Hashiguchi, M., and Mullins, M. C. (2010) CCM3 signaling through sterile 20-like kinases plays an essential role during zebrafish cardiovascular development and cerebral cavernous malformations. J. Clin. Invest. 120, 2795–2804

46. Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N., and Kato, S. (1999) Fgf10 is essential for limb and lung formation. Dev. Biol. 219, 176–180

47. Ackerman, K. G., Wang, J., Luo, L., Fujiwara, Y., Orkin, S. H., and Beier, D. R. (2007) Gata4 is necessary for normal pulmonary lobar development. Am. J. Respir. Cell Mol. Biol. 36, 391–397

48. Sakiyama, J., Yamaguchi, T. P., and Morrisey, E. E. (2009) Wnt2/2b and Nkx2.5 controls lung bud formation during chicken embryonic development. Development 136, 1225–1234

49. Goss, A. M., Tian, Y., Tsukiyama, T., Cohen, E. D., Zhou, D., Lu, M. M., Yamaguchi, T. P., and Morrisey, E. E. (2009) Wnt7b signaling promotes angiogenesis via Vegfc/Vegfr3. Development 136, 4875–4886

50. Garry, D. M., Childs, S., and Fishman, M. C. (2002) The heartstrings mutation in zebrafish causes heart/fn Tbx5 deficiency syndrome. Development 129, 4635–4645

51. Jay, P. Y., Bielinska, M., Erlch, J. M., Mannisto, S., Pu, W. T., Heikinheimo, M., and Wilson, D. B. (2007) Impaired mesenchymal cell function in Gata4 mutant mice leads to diaphragmatic hernias and primary lung defects. Dev. Biol. 301, 602–614

52. Torday, J. S., Rehan, V. K., Hicks, J. W., Wang, T., Maina, J., Weibel, E. R., Hsia, C. C., Sommer, R. J., and Perry, S. F. (2007) Deconvoluting lung evolution. From phenotypes to gene regulatory networks. Integr. Comp. Biol. 47, 601–609

53. Daniels, C. B., Orgeig, S., Sullivan, L. C., Ling, N., Bennett, M. B., Schürch, S., Val, A. L., and Brauner, C. J. (2004) The Origin and evolution of the surfactant system in fish. Insights into the evolution of lungs and swim bladders. Physiol. Biochem. Zool. 77, 732–749

54. Garrity, D. M., Morrisey, E. E. (2012) Foxp1/4 control epithelial cell fate during mouse lung development and function. Mol. Cell. Biol. 32, 687–697

55. Daniels, C. B., Orgeig, S., Sullivan, L. C., Ling, N., Bennett, M. B., Schürch, S., Val, A. L., and Brauner, C. J. (2004) The Origin and evolution of the surfactant system in fish. Insights into the evolution of lungs and swim bladders. Physiol. Biochem. Zool. 77, 732–749