THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 34, pp. 24429–24440, August 23, 2013

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

Received for publication, February 19, 2013, and in revised form, June 27, 2013 Published, JBC Papers in Press, July 8, 2013, DOI 10.1074/jbc.M113.463083

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

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.

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 (1). 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 (1, 2).

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 (3). 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 (4–6). Although these factors are partially redundant (7, 8), GATA4 has been identified as a critical factor in cardiovascular development. Loss of GATA4 results in lethal cardiovascular defects in the mouse embryo (9–12). Depletion of *gata4* in the zebrafish by morpholino knockdown results in an unlooped heart tube (13), indicating that GATA4 has a conserved role in heart development 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 (14)). Among these interactions, the best characterized is between GATA4 and FOG2, a member of the Friend of GATA (FOG)[3] family of transcriptional regulators (15, 16). FOG proteins are unable to bind DNA and must instead bind GATA factors to regulate transcription (17). 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 *in vivo* phenocopies the loss of FOG2 (17, 18). 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 (15, 19–21). FOG proteins play critical roles in heart development in multiple vertebrate species. In the

[S] This article contains supplemental Tables S1–S5 and Figs. S1–S3.
[1] Supported by National Institutes of Health Grant T32 HL007971.
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[3] The abbreviations used are: FOG, Friend of GATA; bHLH, basic helix-loop-helix; hpf, hours post-fertilization.

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 familes (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), 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), $Gata4^{fl}$ floxed allele (38), CMV-Cre allele (39), $Nkx2.5^{Cre}$ allele (40), and $Atoh8^{\Delta ex1\text{-}2}$ allele (36). The $Atoh8^{GFP}$, $Atoh8^{\Delta ex1}$, and $Atoh8^{LacZ\Delta ex2}$ 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 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 $A_{600} = 0.6$ 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 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) $Atoh8^{\Delta ex1/\Delta ex1}$ 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.

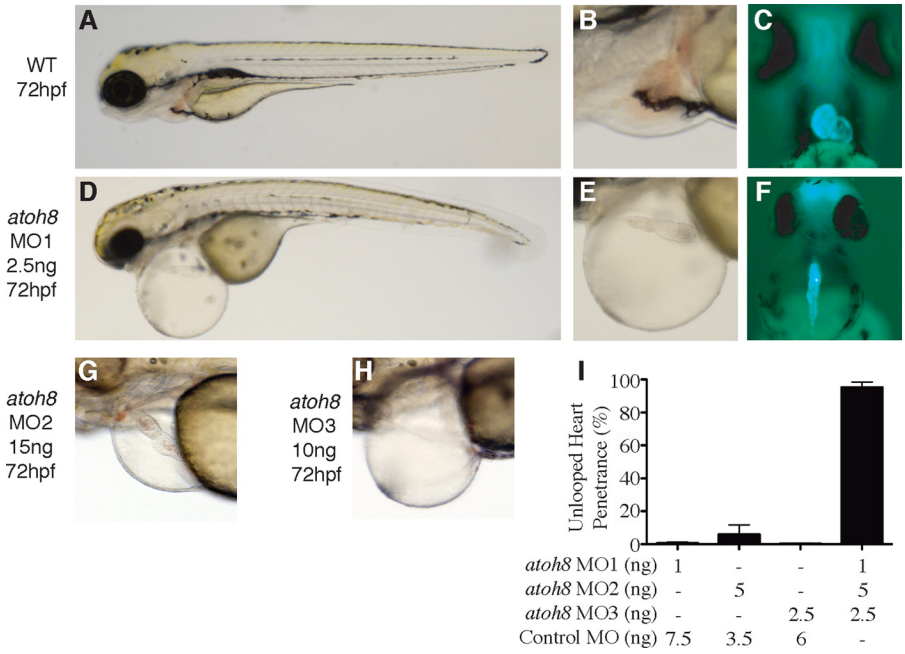

FIGURE 1. **Morpholino knockdown of *atoh8* results in a failure of zebrafish heart looping and pericardial edema.** *A–F*, one-cell zebrafish embryos were injected with *atoh8*-MO1 morpholino (*MO*) (*D–F*) and examined at 72 hpf in comparison to uninjected controls (*A–C*). Transgenic fish expressing myocardial GFP were used in *C* and *F*. *G* and *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; *error bars* represent S.E.

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 χ-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. 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 (Fig. 1, *G* 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. 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 *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

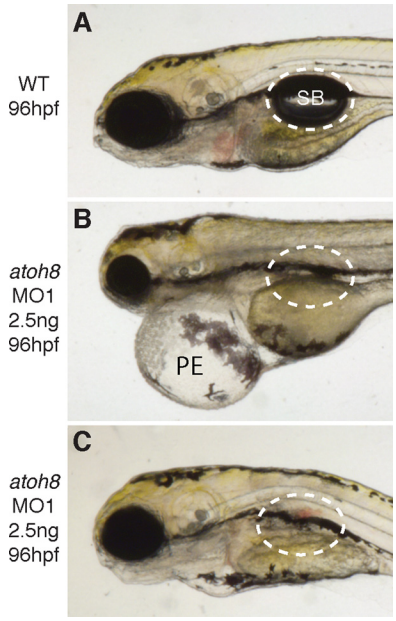

FIGURE 2. **Morpholino knockdown of *atoh8* prevents swim bladder inflation.** *A–C,* one-cell zebrafish embryos were injected with *atoh8*-MO1. Swim bladder inflation was scored at 96 hpf in injected embryos (*B* and *C*) *versus* wild-type (*WT*) embryos (*A*) at 96 hpf. Failure of swim bladder inflation was observed in morphant embryos with (*B*) and without (*C*) the heart phenotype. *SB*, swim bladder; *PE,* pericardial edema. Swim bladder location is outlined by *dashed line*.

observed in embryos with the heart phenotype (Fig. 2*B*) as well as embryos without the phenotype (Fig. 2*C*), 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* (50, 51). Co-injection of *atoh8* and *gata4* morpholinos resulted in a synergistic increase in the penetrance of both the swim bladder (Fig. 3*A*) and heart (Fig. 3*B*) 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 looping 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. 3*C*) or *tbx-5a* (Fig. 3*D*) morpholinos were injected in combination with *atoh8*-MO1. A larger synergistic increase was observed with co-injection of *zfpm1* and *atoh8* morpholinos (Fig. 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 (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. 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 further lowered and combinatorial knockdown studies were performed. At doses in which each individual morpholino induced heart looping in <5% of embryos (Fig. 3*G*), injection of all three morpholinos resulted in heart looping defects in ~90% of embryos (Fig. 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). 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. 4*A*). However, immunoprecipitation of FLAG-ATOH8 was associated with co-immunoprecipitation of V5-FOG2 (Fig. 4*A*), and when all three proteins were co-expressed V5-GATA4 could be pulled down with both FLAG-ATOH8 and V5-FOG2 (Fig. 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 (18), confirmed that association of GATA4 with ATOH8 is bridged by FOG2 (Fig. 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 provide a biochemical explanation for the genetic interaction observed between *atoh8*, *gata4*, and *zfpm1* in the fish.

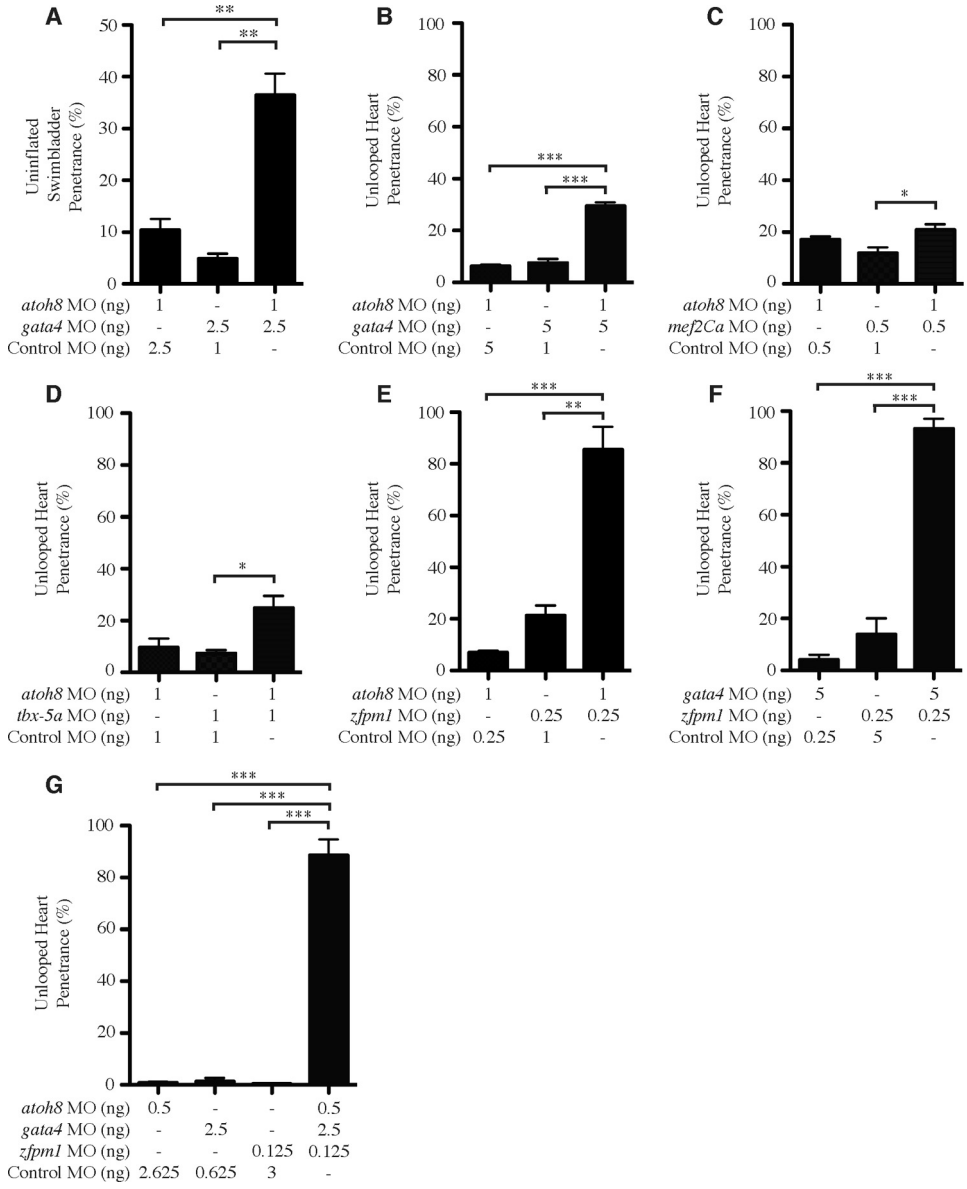

FIGURE 3. ***atoh8* specifically interacts with *gata4* and *zfpm1* in the zebrafish heart.** *A,* low doses of *atoh8*-MO1 and *gata4* MO were injected alone or in combination into one-cell embryos and scored at 96 hpf for failure of swim bladder inflation. *B–F*, low doses of morpholinos (*MO*) were 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; *error bars* represent S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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. 5*F*), 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. 5*E*).

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* ($Atoh8^{\Delta ex1-2}$) 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 $Atoh8^{GFP}$ allele (supplemental Fig. S1). In contrast to the $Atoh8^{\Delta ex1-2/\Delta ex1-2}$ mouse, $Atoh8^{GFP/GFP}$ mice were viable at the expected Mendelian ratio at postnatal day 14 (Table 1). However, GFP protein could not be detected in $Atoh8^{GFP/GFP}$ mice using either immunohistochemistry or Western blotting (data not shown).

Approximately half of exon 1 is left intact in the $Atoh8^{GFP}$ allele, suggesting residual ATOH8 function might explain the survival of $Atoh8^{GFP/GFP}$ mice compared with the lethality

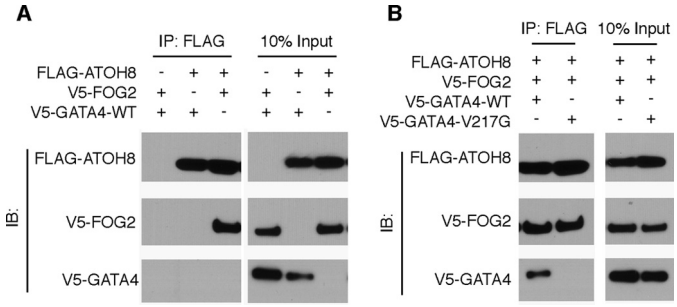

FIGURE 4. **ATOH8 physically interacts with FOG2 and indirectly with GATA4 via FOG2.** *A* and *B*, FLAG-ATOH8, V5-GATA4-WT, V5-GATA4-V217G, and V5-FOG2 were transiently expressed in HEK293T cells in the indicated combinations, immunoprecipitated (*IP*) with anti-FLAG antibody, and detected by Western blotting (*IB*) 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.

reported for $Atoh8^{\Delta ex1\text{-}2/\Delta ex1\text{-}2}$ mice. To rule out survival due to presence of part of *Atoh8* exon 1, we generated mice lacking all of *Atoh8* exon 1. LoxP sites were inserted to flank exon 1 and generate the $Atoh8^{fl}$ allele (supplemental Fig. S2); Cre-mediated recombination between these two sites deletes the entire first exon. $Atoh8^{fl/fl}$ mice were viable with no apparent defects. Transgenic mice expressing CMV-Cre (39) were used to recombine the $Atoh8^{fl}$ allele in the mouse germline cells and create the $Atoh8^{\Delta ex1}$ allele (supplemental Fig. S2). $Atoh8^{\Delta ex1/\Delta ex1}$ mice were viable at the expected Mendelian ratio at postnatal day 14 (Table 1). The viability of both $Atoh8^{GFP/GFP}$ and $Atoh8^{\Delta ex1/\Delta ex1}$ mice indicate that the first exon of *Atoh8* is not required for survival.

Analysis of *Atoh8* mRNA transcripts in $Atoh8^{\Delta ex1/\Delta ex1}$ mice using 5′ rapid amplification of cDNA ends (RACE) and examination of the EST database revealed the presence of *Atoh8* transcripts that lack exon 1 and include an occult exon in intron 1 spliced to exons 2 and 3 (data not shown). However, no open reading frame was identified that included the ATOH8 amino acids encoded by exons 2 and 3. To definitively address the role of *Atoh8* exon 2 and better characterize *Atoh8* expression in the mouse, we replaced exon 2 with an IRES-LacZ gene trap cassette to generate the $Atoh8^{LacZ\Delta ex2}$ allele (supplemental Fig. S3). $Atoh8^{LacZ\Delta ex2/LacZ\Delta ex2}$ mice were viable and present at the expected Mendelian ratio at postnatal day 14 (Table 1). These results indicate that neither exon 1 nor exon 2 of *Atoh8* is required for survival in the mouse. Because exons 1 and 2 encode virtually the entire coding sequence of *Atoh8*, these results indicate that the ATOH8 protein is not required in the mouse for survival, and that the embryonic lethality of $Atoh8^{\Delta ex1\text{-}2/\Delta ex1\text{-}2}$ 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 bacterial artificial chromosome targeting construct (discussed below).

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 may 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 (9, 10, 22). To test for genetic interaction between *Atoh8* and *Gata4* or *Zfpm2* we generated $Zfpm2^{+/-}Atoh8^{GFP/GFP}$ and $Gata4^{+/-}Atoh8^{GFP/GFP}$ animals. $Zfpm2^{+/-}Atoh8^{GFP/GFP}$ animals were viable and present at the expected Mendelian ratios at both postnatal day 1 (P1) and postnatal day 14 (P14) (supplemental Table S3). In contrast, a small decrease in the number of $Gata4^{+/-}Atoh8^{GFP/GFP}$ animals was observed at P1, and this deficit became more pronounced by P14 (Table 2). $Gata4^{+/-}Atoh8^{GFP/GFP}$ 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 $Gata4^{+/-}Atoh8^{GFP/GFP}$ 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 (supplemental Table S4). Thus loss of these animals 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 Heart 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 (27, 53). Although the cause of death was not identified in these studies, a higher incidence of cardiovascular defects (27, 53) was identified in $Gata4^{+/-}$ animals compared with wild-type littermates. 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 (Fig. 6*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 either histology or echocardiography (Fig. 6, *B* and *C*, and data not shown). 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* $Gata4^{fl/+}Atoh8^{GFP/GFP}$ 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 (supplemental Table S5), confirming that the lethality seen in $Gata4^{+/-}Atoh8^{GFP/GFP}$ mice is not due to more subtle cardiac defects.

Our zebrafish studies identified the swim bladder as a site of *gata4* and *atoh8* interaction. The mammalian lung is the closest evolutionary equivalent to the swim bladder; both organs share an origin from a common region of the endoderm (54) and express similar surfactant proteins for inflation (55). In addition, $Gata4^{+/-}$ heterozygotes on a C57/BL6 background have also been reported to exhibit an increase in the pulmonary defects (53). These results suggested that the increased lethality observed in $Gata4^{+/-}Atoh8^{GFP/GFP}$ animals may be due to

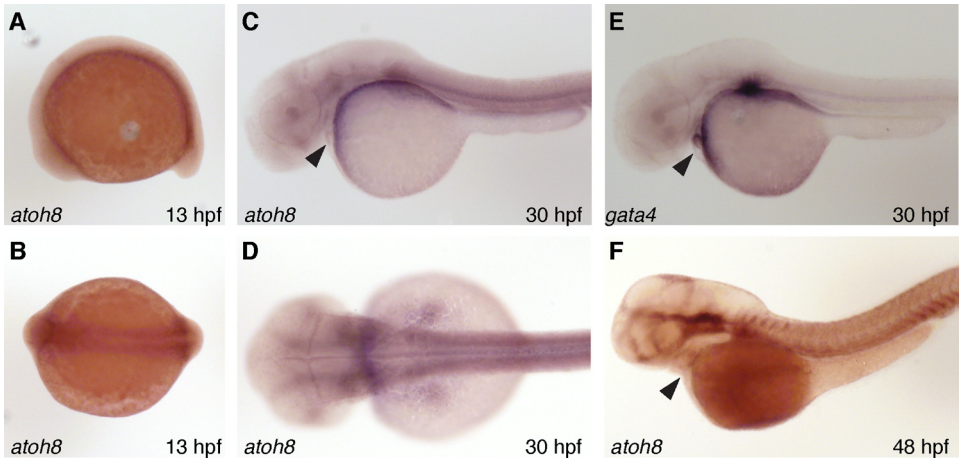

FIGURE 5. ***atoh8* expression in the zebrafish.** *A* and *B*, *atoh8* expression by *in situ* hybridization at 13 hpf. Lateral (*A*) and dorsal (*B*) views are shown. *C–E*, *in situ* hybridization for *atoh8* (*C* and *D*) and *gata4* (*E*) at 30 hpf. Lateral (*C*) and dorsal (*D*) views are shown 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.

TABLE 1
Survival of *Atoh8* homozygous mutant mice at postnatal day 14

Cross: $Atoh8^{\Delta GFP/+}$ X $Atoh8^{GFP/+}$			
Genotype	Number	Observed %	Mendelian %
$Atoh8^{+/+}$	28	34%	25%
$Atoh8^{GFP/+}$	34	40%	50%
$Atoh8^{GFP/GFP}$	22	26%	25%
Total	84		P=0.14

Cross: $Atoh8^{\Delta ex1/+}$ X $Atoh8^{\Delta ex1/+}$			
Genotype	Number	Observed %	Mendelian %
$Atoh8^{+/+}$	43	30%	25%
$Atoh8^{\Delta ex1/+}$	70	48%	50%
$Atoh8^{\Delta ex1/\Delta ex1}$	32	22%	25%
Total	145		P=0.39

Cross: $Atoh8^{LacZ\Delta ex2/+}$ X $Atoh8^{LacZ\Delta ex2/+}$			
Genotype	Number	Observed %	Mendelian %
$Atoh8^{+/+}$	17	26%	25%
$Atoh8^{LacZ\Delta ex2/+}$	29	45%	50%
$Atoh8^{LacZ\Delta ex2/LacZ\Delta ex2}$	19	29%	25%
Total	65		P=0.64

TABLE 2
Survival of $Gata4^{+/-}$ $Atoh8^{GFP/GFP}$ mice at postnatal day 1 (P1) and postnatal day 14 (P14)

Cross: $Atoh8^{\Delta GFP/+}$ X $Gata4^{+/-}$ $Atoh8^{GFP/+}$		Age: P1	
Genotype	Number	Observed %	Mendelian %
$Atoh8^{+/+}$	40	18%	12.5%
$Atoh8^{GFP/+}$	66	30%	25%
$Atoh8^{GFP/GFP}$	25	11%	12.5%
$Gata4^{+/-}$ $Atoh8^{+/+}$	24	11%	12.5%
$Gata4^{+/-}$ $Atoh8^{GFP/+}$	49	22%	25%
$Gata4^{+/-}$ $Atoh8^{GFP/GFP}$	15	7%	12.5%
Total	219		P=0.0106

Cross: $Atoh8^{\Delta GFP/+}$ X $Gata4^{+/-}$ $Atoh8^{GFP/+}$		Age: P14	
Genotype	Number	Observed %	Mendelian %
$Atoh8^{+/+}$	43	22%	12.5%
$Atoh8^{GFP/+}$	69	35%	25%
$Atoh8^{GFP/GFP}$	23	12%	12.5%
$Gata4^{+/-}$ $Atoh8^{+/+}$	21	11%	12.5%
$Gata4^{+/-}$ $Atoh8^{GFP/+}$	35	18%	25%
$Gata4^{+/-}$ $Atoh8^{GFP/GFP}$	8	4%	12.5%
Total	199		P<0.0001

impaired lung development. We were unable to detect gross structural abnormalities in $Gata4^{+/-}Atoh8^{GFP/GFP}$ lungs (Fig. 7, *A* and *B*) or a statistically significant change in lung mass (Fig. 7*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 (Fig. 7*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 *Gata4* expression in the lung is limited to the mesenchyme (53, 56). Mesenchymal expression of *Wnt2*, *Fgf10*, and *Tbx4* has previously been shown to be required for proper lung development (57–59). Expression of these factors was down-regulated in both $Gata4^{+/-}$ and $Atoh8^{GFP/GFP}$ lungs at E12.5 (Fig. 7*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 $Gata4^{+/-}Atoh8^{GFP/GFP}$ (Fig. 7*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 the essential role for Atoh8 in zebrafish swim bladder development is not conserved in the mammalian lung.

Atoh8 Expression Is Restricted to the Atria, Lung Mesenchyme, and Vascular Smooth Muscle in the Mouse—To identify the cellular site of interaction of *Gata4* and *Atoh8* in the mouse, we determined the expression pattern of *Atoh8*. Due to the cardiac phenotype we identified in the *atoh8* morphant fish and the defined role for GATA4 in the cardiovascular system (9, 11, 12), we first focused on *Atoh8* expression in the heart. Previous studies have reported cardiac expression of *Atoh8* (33, 35, 36), but these studies have not defined the precise spatial or temporal expression pattern of *Atoh8* within the heart. To determine the expression pattern of *Atoh8*, we generated antibodies against an N-terminal fragment of the ATOH8 protein. These antibodies were able to detect ATOH8 when overexpressed cell

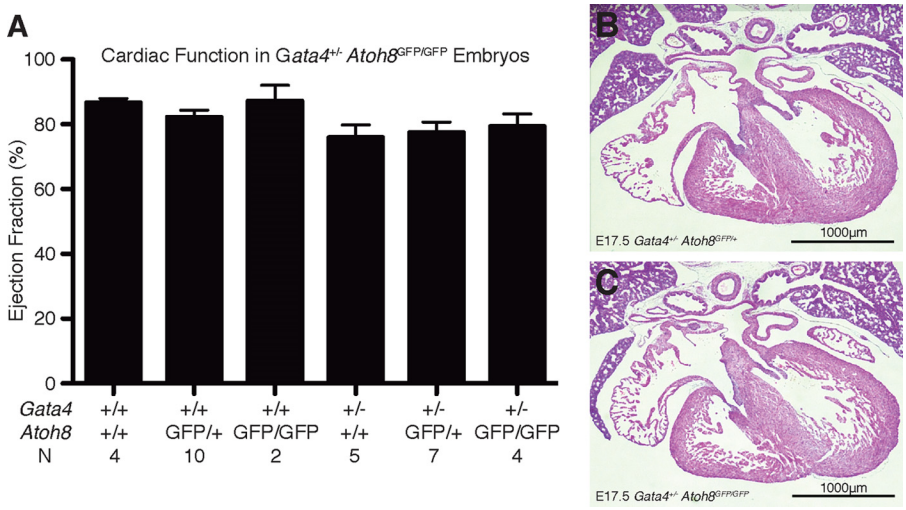

FIGURE 6. ***Gata4*$^{+/-}$*Atoh8*$^{GFP/GFP}$ embryonic hearts are functionally and structurally normal.** *A*, ejection fraction was determined for *Gata4*$^{+/-}$*Atoh8*$^{GFP/GFP}$ and littermates at E17.5. *n* for each genotype is shown in panel. Graph shows mean of all samples of that genotype; *error bars* represent S.E. *B* and *C*, hematoxylin-eosin staining was performed on E17.5 heart sections from control (*B*) and *Gata4*$^{+/-}$*Atoh8*$^{GFP/GFP}$ (*C*) embryos. Scale is shown in each panel.

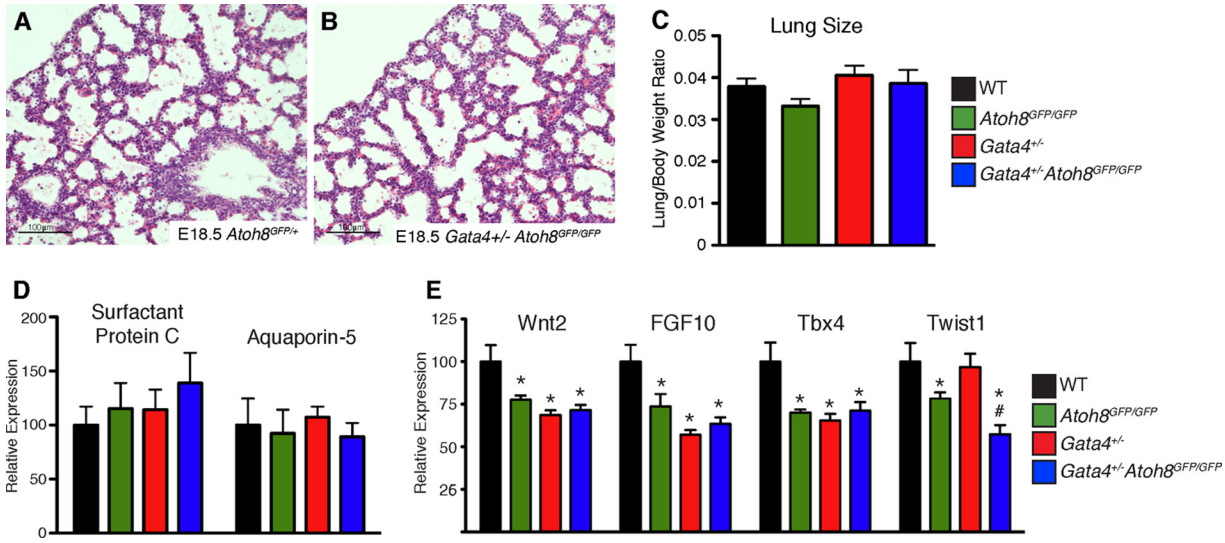

FIGURE 7. ***Gata4*$^{+/-}$*Atoh8*$^{GFP/GFP}$ lungs appear structurally normal but display defects in mesenchymal-epithelial signaling.** *A* and *B*, hematoxylin-eosin staining was performed on E18.5 lung sections from control (*A*) and *Gata4*$^{+/-}$*Atoh8*$^{GFP/GFP}$ (*B*) embryos. Scale is shown in each panel. *C*, lung/body weight ratios were measured at E18.5 to compare lung growth between the 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; *error bars* represent S.E. *, $p < 0.05$ compared with WT; #, $p < 0.05$ compared with *Atoh8*$^{GFP/GFP}$.

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*$^{LacZ\Delta ex2}$ IRES-*LacZ* gene trap allele as a reporter for *Atoh8* expression. Whole mount X-gal staining of *Atoh8*$^{LacZ\Delta ex2/+}$ 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. 8*A*). This pattern was largely maintained at E12.5, with persistent LacZ expression in somites, brain, eye, and limb bud (Fig. 8*B*), but no expression in the developing heart or liver was detected (Fig. 8*C*). To determine whether *Atoh8* is expressed in later stages of heart development, we isolated and performed whole mount X-gal staining on *Atoh8*$^{LacZ\Delta ex2/+}$ hearts (Fig. 8*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 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 *Atoh8*$^{LacZ\Delta ex2/+}$ organs also revealed strong expression throughout the lung at both E16.5 and P1 (Fig. 8*E*).

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 (36). As was seen with whole mount LacZ staining of *Atoh8*$^{LacZ\Delta ex2/+}$ mice, nuclear GFP expression was detected throughout the atrial

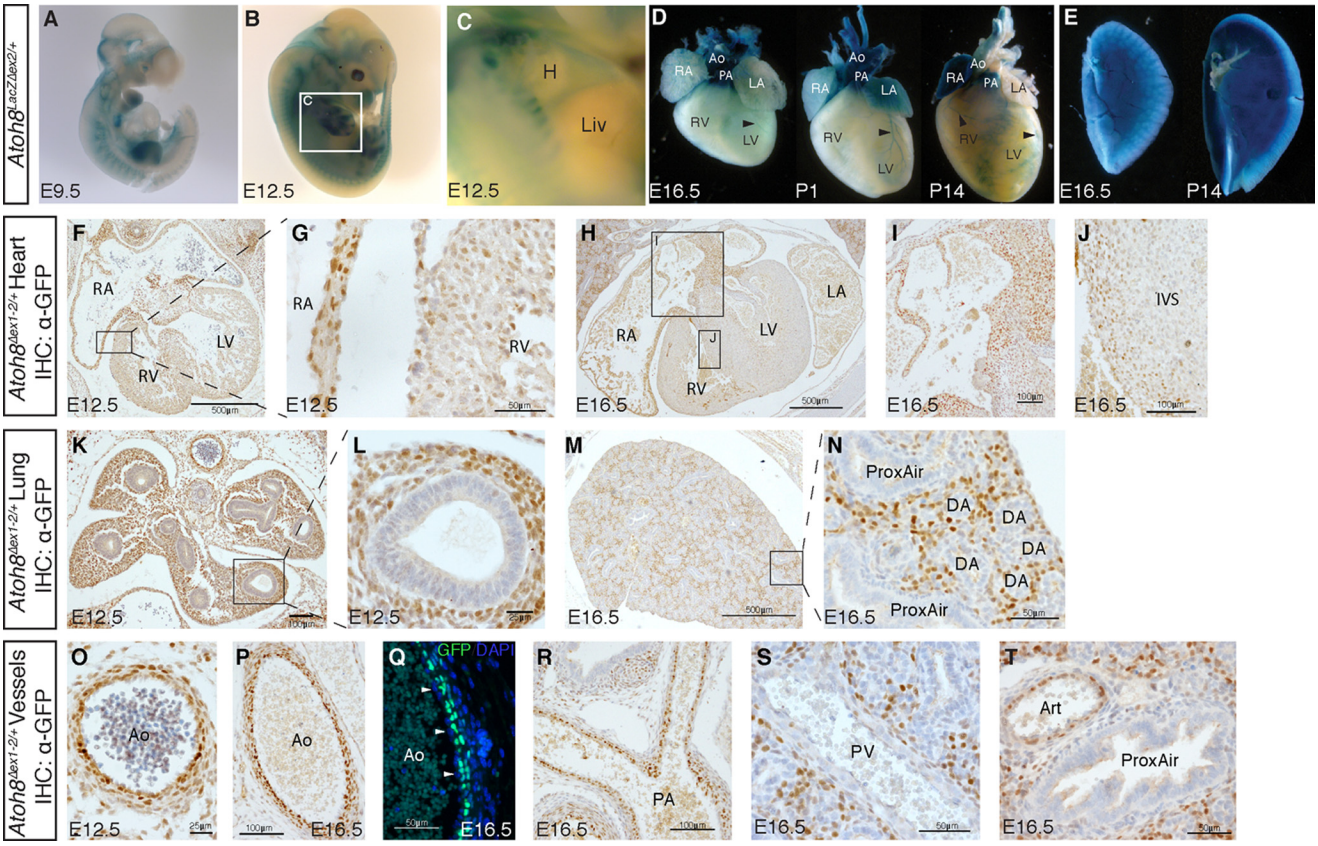

FIGURE 8. **Atoh8 is expressed in the atria, lung mesenchyme, and vascular smooth muscle.** *A–C*, whole mount X-gal staining was performed on $Atoh8^{LacZ\Delta ex2/+}$ embryos at E9.5 (*A*) and E12.5 (*B* and *C*). *C* shows a magnified image of the boxed region in *B* with forelimb removed. *D* and *E*, whole mount X-gal staining was performed at the indicated stages on isolated $Atoh8^{LacZ\Delta ex2/+}$ hearts (*D*) and $Atoh8^{LacZ\Delta ex2/+}$ lungs (*E*). *F–T*, anti-GFP immunostaining of heart sections from $Atoh8^{\Delta ex1\text{-}2/+}$ mice at E12.5 (*F*, *G*, *K*, *L*, and *O*) and E16.5 (*H–J*, *M*, *N*, and *P–T*). Horseradish peroxidase staining was used in *panels F–P* and *R–T*; FITC staining was used in *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.

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*J* and 8*G*). In the ventricles, nuclear GFP expression was limited to the layers of cardiomyocytes in closest proximity to the endocardium (Fig. 8*J*). These results indicate that cardiac expression of *Atoh8* is primarily limited to the atria.

We next used the $Atoh8^{\Delta ex1\text{-}2/+}$ 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. 8*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 (Fig. 8, *M* and *N*).

$Atoh8^{\Delta ex1\text{-}2/+}$ 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. 8*T*). The endothelium of these vessels was noticeably free of GFP expression (Fig. 8*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 (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. 8*S*). Thus studies of the $Atoh8^{LacZ\Delta ex2}$ LacZ and $Atoh8^{\Delta ex1\text{-}2/+}$ 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

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 $Atoh8^{\Delta ex1\text{-}2}$ mouse lacking both exon 1 and 2 and intervening intron 1 (36). There exist several alternative explanations for the lethality seen in the $Atoh8^{\Delta ex1\text{-}2}$ mouse. First, it is possible that removal of intron 1 in $Atoh8^{\Delta ex1\text{-}2}$ 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 $Atoh8^{\Delta ex1\text{-}2}$ and $Atoh8^{GFP}$ 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 $Atoh8^{\Delta ex1\text{-}2}$ mouse. The $Atoh8^{\Delta ex1\text{-}2}$ 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 $Atoh8^{\Delta ex1\text{-}2}$ 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 $Gata4^{+/-}Atoh8^{GFP/GFP}$ 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 $Gata4^{+/-}Atoh8^{GFP/GFP}$ 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 $Gata4^{+/-}Atoh8^{GFP/GFP}$ 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 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.

Acknowledgments—We acknowledge Drs. Ed Morrisey, Klaus Kaestner, Mitch Weiss, Gerd Blobel, and the members of the Kahn lab for valuable suggestions and guidance during these experiments. We thank Dr. Eric Svensson for providing the Zfpm2 mutant mice.

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., Piaseckyj, C. M., Burghard, E., Fialkovich, J. J., 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 GATA-4, -5 and -6 in vertebrate heart development. *Semin. Cell Dev. Biol.* **16,** 83–94
6. Zaytouni, T., Efimenko, E. E., and Tevosian, 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., Richardson, 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., Juraszek, 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., Ma, Q., Springer, Z., Izumo, S., Tabin, C. J., and Pu, W. T. (2006) Development of heart valves requires Gata4 expression in endothelial-derived cells. *Development* **133,** 3607–3618
13. Holtzinger, A., and Evans, T. (2005) Gata4 regulates the formation of multiple organs. *Development* **132,** 4005–4014
14. Garg, V., Kathiriya, I. S., Barnes, R., Schluterman, M. K., King, I. N., Butler, C. A., Rothrock, C. R., Eapen, R. S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J. C., and Srivastava, D. (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424,** 443–447
15. Svensson, E. C., Tufts, R. L., Polk, C. E., and Leiden, J. M. (1999) Molecular cloning of FOG-2. A modulator of transcription factor GATA-4 in cardiomyocytes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 956–961
16. Tevosian, S. G., Deconinck, A. E., Cantor, A. B., Rieff, H. I., Fujiwara, Y., Corfas, G., and Orkin, S. H. (1999) FOG-2. A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 950–955
17. Cantor, A. B., and Orkin, S. H. (2005) Coregulation of GATA factors by the Friend of GATA (FOG) family of multitype zinc finger proteins. *Semin. Cell Dev. Biol.* **16,** 117–128
18. Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D., and Orkin, S. H. (2001) Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev.* **15,** 839–844
19. Lu, J. R., McKinsey, T. A., Xu, H., Wang, D. Z., Richardson, J. A., and Olson, E. N. (1999) FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. *Mol. Cell. Biol.* **19,** 4495–4502
20. Manuylov, N. L., and Tevosian, S. G. (2009) Cardiac expression of Tnnt1 requires the GATA4-FOG2 transcription complex. *Scientific World J.* **9,** 575–587
21. Svensson, E. C., Huggins, G. S., Dardik, F. B., Polk, C. E., and Leiden, J. M. (2000) A functionally conserved N-terminal domain of the friend of GATA-2 (FOG-2) protein represses GATA4-dependent transcription. *J. Biol. Chem.* **275,** 20762–20769
22. Svensson, E. C., Huggins, G. S., Lin, H., Clendenin, C., Jiang, F., Tufts, R., Dardik, F. B., and Leiden, J. M. (2000) A syndrome of tricuspid atresia in mice with a targeted mutation of the gene encoding Fog-2. *Nat. Genet.* **25,** 353–356
23. Tevosian, S. G., Deconinck, A. E., Tanaka, M., Schinke, M., Litovsky, S. H., Izumo, S., Fujiwara, Y., and Orkin, S. H. (2000) FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101,** 729–739
24. Zhou, B., Ma, Q., Kong, S. W., Hu, Y., Campbell, P. H., McGowan, F. X., Ackerman, K. G., Wu, B., Zhou, B., Tevosian, S. G., and Pu, W. T. (2009) Fog2 is critical for cardiac function and maintenance of coronary vasculature in the adult mouse heart. *J. Clin. Invest.* **119,** 1462–1476
25. Walton, R. Z., Bruce, A. E., Olivey, H. E., Najib, K., Johnson, V., Earley, J. U., Ho, R. K., and Svensson, E. C. (2006) Fog1 is required for cardiac looping in zebrafish. *Dev. Biol.* **289,** 482–493
26. De Luca, A., Sarkozy, A., Ferese, R., Consoli, F., Lepri, F., Dentici, 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
27. 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
28. 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
29. Massari, M. E., and Murre, C. (2000) Helix-loop-helix proteins. Regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* **20,** 429–440
30. Phillips, S. E. (1994) Built by association. Structure and function of helix-loop-helix DNA-binding proteins. *Structure* **2,** 1–4
31. 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
32. 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
33. 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
34. Chen, J., Dai, F., Balakrishnan-Renuka, A., Leese, F., Schempp, 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
35. 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
36. Lynn, F. C., Sanchez, L., Gomis, R., German, 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,** e2430
37. Yao, J., Zhou, J., Liu, Q., Lu, D., Wang, L., Qiao, X., and Jia, W. (2010)

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 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 loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* **23,** 5080–5081
40. Moses, K. A., DeMayo, F., Braun, R. M., Reecy, 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 recombineering-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., Wang, H., Cheng, Y., Billin, A. N., Hardison, R. C., Mackay, J. P., and Blobel, G. A. (2011) Bromodomain protein Brd3 associates with acetylated GATA1 to promote its chromatin occupancy at erythroid target genes. *Proc. Natl. Acad. Sci. U.S.A.* **108,** E159–E168
46. Shu, W., Jiang, Y. Q., Lu, M. M., and Morrisey, E. E. (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* **129,** 4831–4842
47. Bertozzi, C. C., Schmaier, A. A., Mericko, P., Hess, P. R., Zou, Z., Chen, M., Chen, C. Y., Xu, B., Lu, M. M., Zhou, D., Sebzda, E., Santore, M. T., Merianos, D. J., Stadtfeld, M., Flake, A. W., Graf, T., Skoda, R., Maltzman, J. S., Koretzky, G. A., and Kahn, M. L. (2010) Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. *Blood* **116,** 661–670
48. Li, S., Wang, Y., Zhang, Y., Lu, M. M., DeMayo, F. J., Dekker, J. D., Tucker, P. W., and Morrisey, E. E. (2012) Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. *Development* **139,** 2500–2509
49. Lee, J. S., Yu, Q., Shin, J. T., Sebzda, E., Bertozzi, C., Chen, M., Mericko, P., Stadtfeld, M., Zhou, D., Cheng, L., Graf, T., MacRae, C. A., Lepore, J. J., Lo, C. W., and Kahn, M. L. (2006) Klf2 is an essential regulator of vascular hemodynamic forces *in vivo*. *Dev. Cell* **11,** 845–857
50. Ghosh, T. K., Song, F. F., Packham, E. A., Buxton, S., Robinson, T. E., Ronksley, J., Self, T., Bonser, A. J., and Brook, J. D. (2009) Physical interaction between TBX5 and MEF2C is required for early heart development. *Mol. Cell. Biol.* **29,** 2205–2218
51. Gore, A. V., Swift, M. R., Cha, Y. R., Lo, B., McKinney, M. C., Li, W., Castranova, D., Davis, A., Mukouyama, Y. S., and Weinstein, B. M. (2011) Rspo1/Wnt signaling promotes angiogenesis via Vegfc/Vegfr3. *Development* **138,** 4875–4886
52. Garrity, D. M., Childs, S., and Fishman, M. C. (2002) The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129,** 4635–4645
53. Jay, P. Y., Bielinska, M., Erlich, 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
54. 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
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
56. 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
57. 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. *Nat. Genet.* **21,** 138–141
58. Sakiyama, J., Yamagishi, A., and Kuroiwa, A. (2003) Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development. *Development* **130,** 1225–1234
59. Goss, A. M., Tian, Y., Tsukiyama, T., Cohen, E. D., Zhou, D., Lu, M. M., Yamaguchi, T. P., and Morrisey, E. E. (2009) Wnt2/2b and β-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev. Cell* **17,** 290–298
60. Conway, S. J., Firulli, B., and Firulli, A. B. (2010) A bHLH code for cardiac morphogenesis. *Pediatr. Cardiol.* **31,** 318–324
61. Costa, R. H., Kalinichenko, V. V., and Lim, L. (2001) Transcription factors in mouse lung development and function. *Am. J. Physiol. Lung Cell Mol. Physiol.* **280,** L823–L838
62. Kautz, L., Meynard, D., Monnier, A., Darnaud, V., Bouvet, R., Wang, R. H., Deng, C., Vaulont, S., Mosser, J., Coppin, H., and Roth, M. P. (2008) Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. *Blood* **112,** 1503–1509
63. Blow, M. J., McCulley, D. J., Li, Z., Zhang, T., Akiyama, J. A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., Afzal, V., Bristow, J., Ren, B., Black, B. L., Rubin, E. M., Visel, A., and Pennacchio, L. A. (2010) ChIP-Seq identification of weakly conserved heart enhancers. *Nat. Genet.* **42,** 806–810
64. Pu, W. T., Ishiwata, T., Juraszek, A. L., Ma, Q., and Izumo, S. (2004) GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev. Biol.* **275,** 235–244