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Characterization of an *lhx1a* transgenic reporter in zebrafish

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ABSTRACT The LIM-domain containing transcription factor, Lhx1, is involved in the regulation of early gastrulation cell movements, kidney organogenesis and other processes in vertebrate model organisms. To follow the expression of this gene in live embryos, we created transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) under the control of *lhx1a* regulatory regions. *Tg(lhx1a:EGFP)^{pt303}* recapitulates the expression of endogenous *lhx1a* beginning at early gastrula stages through 72 hours of development with only few exceptions. In addition, over-expression of the Nodal ligand, *ndr1*, results in the concomitant expansion of the transgene and endogenous *lhx1a* expression. Treatment of *Tg(lhx1a:EGFP)^{pt303}* embryos with the small molecule SB-431542, an inhibitor of Nodal signaling, results in the loss of both transgene and endogenous *lhx1a* expression. These experiments suggest that *Tg(lhx1a:EGFP)^{pt303}* is regulated in a manner similar to endogenous *lhx1a*. Therefore, this reporter can be utilized not only for monitoring *lhx1a* expression, but also for numerous applications, including chemical genetics screening.

KEY WORDS: *lhx1a, transgene, zebrafish, kidney, gastrulation*

Introduction

LIM domain-containing proteins have been implicated in numerous biological processes including cell-fate determination, tissue-specific gene expression, neuronal pathfinding, and actin organization. This motif was originally identified within LIM-homeodomain proteins (LHX), a family of proteins of which Lhx1 (formerly known as Lim1) is a member (Dawid *et al.* 1998). *Lhx1* plays a number of roles during the course of vertebrate development, including an important early role in gastrulation. It is expressed in the organizer region (and its functional equivalents) in *Xenopus*, mice, and zebrafish (Shawlot and Behringer 1995; Taira *et al.* 1992; Toyama and Dawid 1997), and *Lhx1* deficiency in both mice and *Xenopus* results in the loss of all cephalic structures (Hukriede *et al.* 2003; Kodjabachian *et al.* 2001; Shawlot and Behringer 1995).

Lhx1 also regulates the development of a number of organs, including the kidney. The vertebrate kidney develops from the intermediate mesoderm (IM), and *Lhx1* serves as a marker of progenitor cells in this tissue (Carroll and Vize 1999; Karavanov *et al.* 1996; Toyama and Dawid 1997). In the mouse it continues to be expressed in both the mesonephric and metanephric kidneys where it is required for nephron formation. Loss of *Lhx1* in mice results in the disruption of IM differentiation and the absence of functional kidneys (Shawlot and Behringer 1995).

There are multiple pathways that regulate *Lhx1* expression during development, including Nodal, which acts in the early embryo. Members of the Nodal signaling family are TGF-β ligands that serve as inducers of both mesoderm and endoderm and also pattern the dorsal-ventral axis of the vertebrate embryo (Schier and Talbot 2005). Nodals signal through EGF-CFC co-receptors and type I and II Activin receptors. In zebrafish, over-expression of synthetic mouse nodal RNA leads to ectopic expression of *lhx1a* in embryos beginning at 30% epiboly (Toyama *et al.* 1995). In addition, a mutation in the EGF-CFC, *one-eyed pinhead* (*oep*),

Abbreviations used in this paper: EGFP, enhanced green fluorescent protein; hpf, hours post fertilization; IM, intermediate mesoderm; Lhx1, LIM-homeobox 1; ndr1, nodal-related 1.

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results in the complete loss of *lhx1a* expression during gastrulation (Watanabe *et al.* 2002).

Because of the numerous roles that *Lhx1* plays throughout development, we created an EGFP transgene in zebrafish to follow *lhx1a* expression *in vivo,* and to further our understanding of its regulation during gastrula stages and pronephric development. *Tg(lhx1a:EGFP)*^{pt303} expression recapitulates *lhx1a* expression at all stages analyzed, and modulation of the Nodal signaling pathway, either by over-expression of *ndr1* or treatment with a small molecule inhibitor, confirms that this transgene is regulated in the same manner as the endogenous *lhx1a* gene. Therefore, *Tg(lhx1a:EGFP)*^{pt303} should serve as a useful resource for investigating gastrulation and mechanisms of cell-fate specification within the kidney.

Results

Generation of lhx1a *transgenic line*

A BAC clone containing the *lhx1a* genomic region was identified and an 8.8 kb fragment containing 5' untranslated sequence, exon 1, and part of exon 2 was isolated and subcloned (Fig. 1A). This fragment contains the first intron, which is important for transcriptional regulation of both the *Xlim1* and *lhx1a* genes in *Xenopus* and zebrafish, respectively (Rebbert and Dawid 1997; Watanabe *et al.* 2002). EGFP was inserted downstream of the start codon and the *lhx1a:EGFP* DNA fragment was cloned into the pI-SceI meganuclease vector for generating transgenic lines (Thermes *et al.* 2002). The injected embryos were raised to adulthood and screened for germline carriers by EGFP expression. Three founder transgenics were identified and while levels of expression varied between lines, the expression patterns were similar. Homozygous lines were created from these founders (allele designations: *pt301*, *pt302, pt303*). *Tg(lhx1a:EGFP)* fish are fecund and develop normally suggesting that the insertion of this transgene does not cause any deleterious effects. For all subsequent experiments, we used *Tg(lhx1a:EGFP)*^{pt303}, the line with the strongest expression.

Early expression of Tg(lhx1a:EGFP)^{pt303}

EGFP protein was first analyzed at shield stage where it is localized to the marginal cells (Fig. 1 B,C, bracket) and the shield (Fig. 1 B,C, arrow), the fish equivalent of Spemann's Organizer in *Xenopus* and the mouse node. By 5-somites, EGFP protein is seen predominantly in the notochord (Fig. 1D, arrowhead) and bilaterally in the intermediate mesoderm (IM) (Fig. 1D, arrow), the

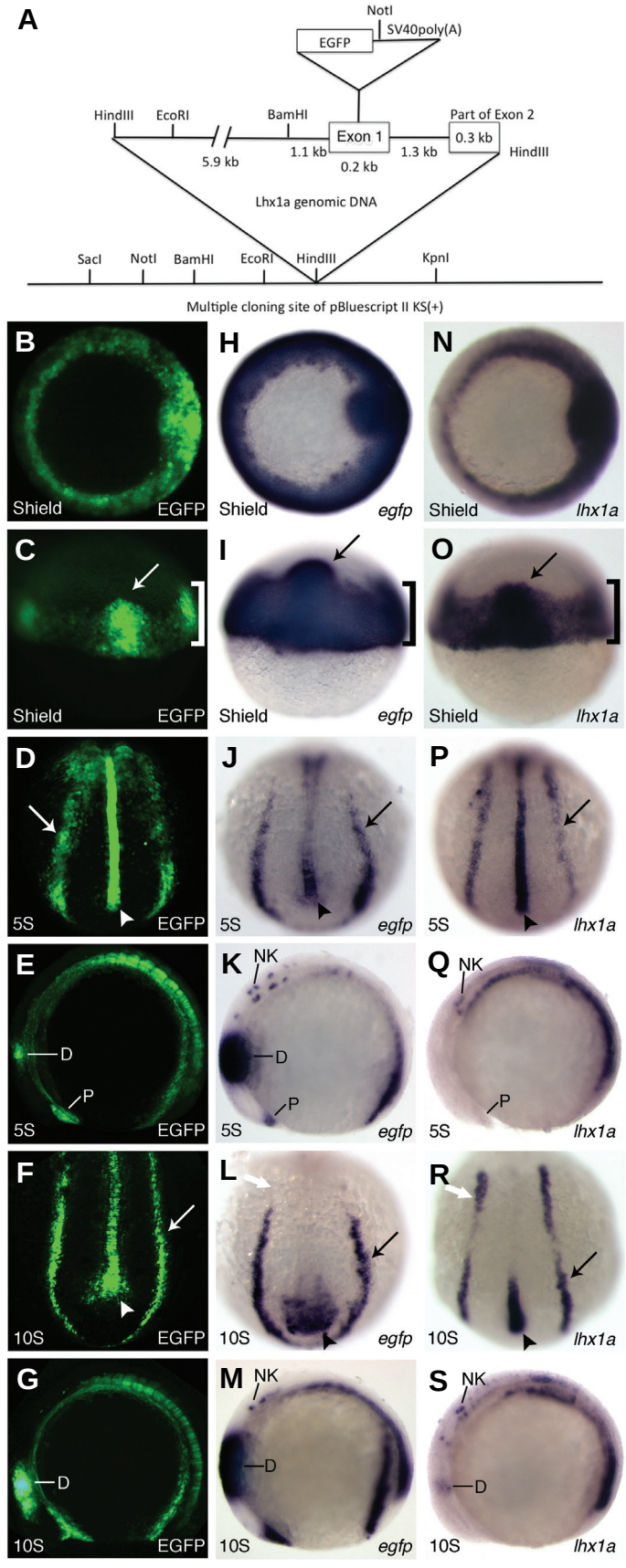

Fig. 1. Construct design and early *Tg(lhx1a:EGFP)*^{pt303} expression. **(A)** *Schematic of* lhx1a:EGFP *construct.* **(B-S)** Tg(lhx1a:EGFP)^{pt303} *embryos.* **(B-G)** Tg(lhx1a:EGFP)^{pt303} *expression.* **(H-M)** In situ *hybridization for* egfp. **(N-S)** In situ *hybridization for* lhx1a. **(B,H,N)** *Shield stage embryos, animal view.* **(C,I,O)** *Shield stage embryos, dorsal view. Arrow marks the shield and bracket denotes the marginal cells.* **(D,J,P)** *5-somite stage embryos, dorsal-posterior view. Arrow points to intermediate mesoderm and arrowhead marks the notochord.* **(E,K,Q)** *5-somite stage embryos, lateral view. Anterior is to the left.* **(F,L,R)** *10-somite stage embryos, dorsal-posterior view. Arrow points to the intermediate mesoderm and arrowhead marks the notochord. White arrow denotes anterior expression domain of* lhx1a *in the intermediate mesoderm.* **(G,M,S)** *10-somite stage embryos, lateral view. Anterior is to the left. Abbreviations: D, diencephalon; NK, neural keel; P, polster.*

tissue that will ultimately give rise to the kidney. There is also expression in the polster and the diencephalon (Fig. 1E). By 10-somites, EGFP protein remains in the most posterior region of the notochord as well as the IM (Fig. 1F, arrowhead and arrow, respectively). There is still strong expression in the diencephalon (Fig. 1G).

At the RNA level, *egfp* expression recapitulates what is seen with the protein, although some structures can be seen in finer detail through *in situ* hybridization. Similar to the protein, *egfp* mRNA is expressed in the marginal cells and the shield (Fig. 1 H,I, bracket and arrow, respectively). At 5-somites, expression is seen throughout the notochord and the IM (Fig. 1J, arrowhead and arrow, respectively). It is present in the polster and neural keel as well as in the diencephalon where expression is aberrantly strong (Fig. 1K). By 10-somites, down-regulation of *egfp* expression is beginning in the IM and the notochord (Fig. 1L, arrow and arrowhead, respectively). Expression is still present in the neural keel and remains strong in the diencephalon (Fig. 1M) at this stage.

The efficacy of this transgene lies in its ability to recapitulate endogenous *lhx1a* expression. For the majority of tissues examined, this appears to be the case. At shield stage, there is strong expression of endogenous *lhx1a* in the shield and the margin (Fig. 1 N,O, arrow and bracket, respectively). By 5-somites, expression is predominantly seen in the notochord and IM, although weak expression can also be seen in the polster and neural keel (Fig. 1P, arrowhead and arrow, respectively and 1Q). At 10-somites, *lhx1a* expression is down-regulated in the notochord and throughout most of the IM (Fig. 1R, arrowhead and arrow, respectively). While diencephalon expression is first reported at this stage, *lhx1a* expression remains in the neural keel (Fig. 1S).

The anterior IM expression of *lhx1a* (Fig. 1R, white arrow) ultimately contributes to proximal fates in the pronephric kidney, and presumably corresponds with the expression seen in the proximal tubule at 24 hours post fertilization (hpf) (Toyama and Dawid 1997). This expression domain is not found in the transgene (Fig. 1L, white arrow), although EGFP protein seems to perdure (Fig. 1F), suggesting variations between endogenous *lhx1a* expression and *Tg(lhx1a:EGFP)*^{pt303}. Overall, the expression of *Tg(lhx1a:EGFP)*^{pt303} agrees with the endogenous expression pattern of *lhx1a*. The main differences observed are earlier and stronger transgene expression in the diencephalon (Toyama and Dawid 1997; Toyama *et al.* 1995) and an absence of anterior IM expression at the 10-somite stage.

Tg(lhx1a:EGFP)^{pt303} expression at 24 hpf through early larval stages

Tg(lhx1a:EGFP)^{pt303} embryos at 24 hpf have a dynamic expression pattern. EGFP protein is localized to the forebrain,

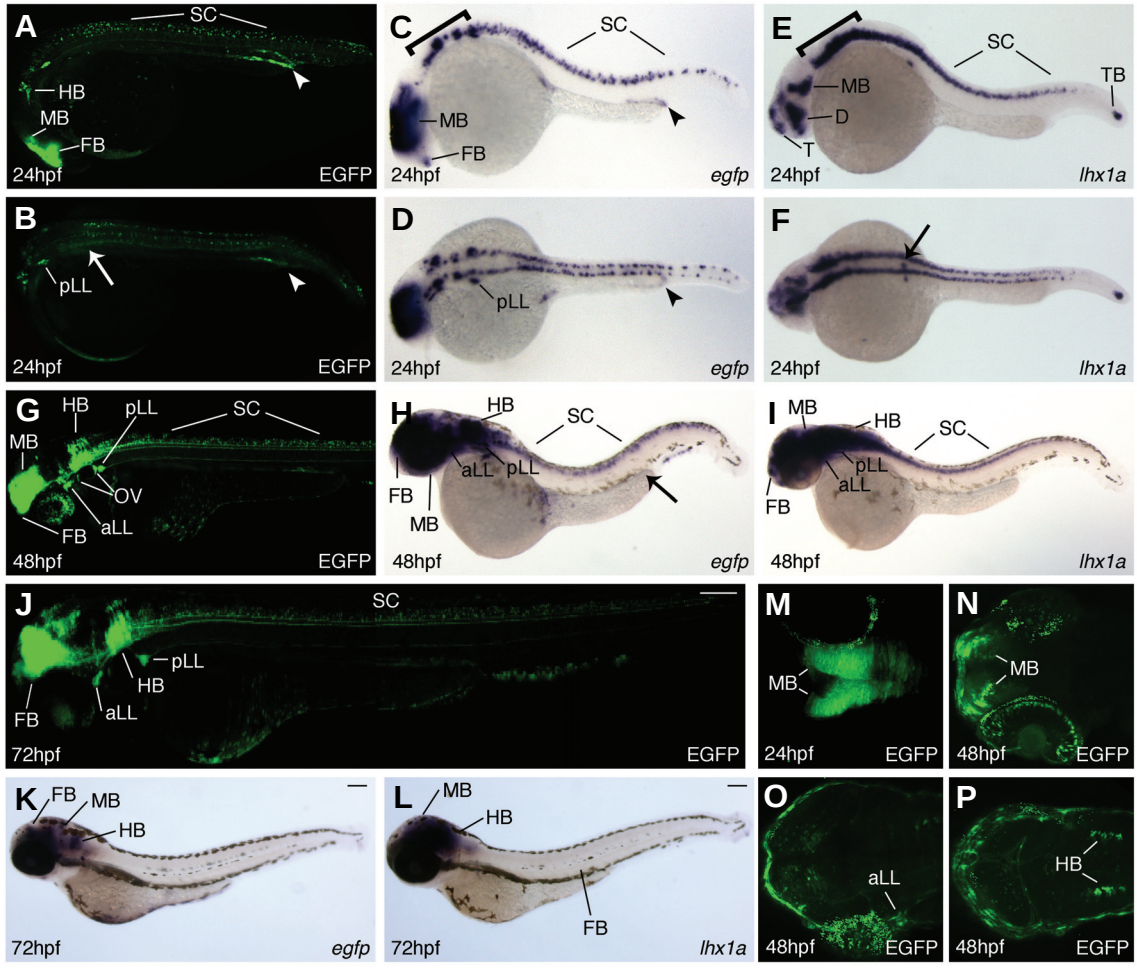

Fig. 2. Expression of *Tg(lhx1a:EGFP)*^{pt303} during later development. *All animals are oriented such that anterior is to the left.* **(A-P)** Tg(lhx1a:EGFP)^{pt303} *embryos and larvae.* **(A , B , G , J , M - P)** Tg(lhx1a:EGFP)^{pt303} *expression.* **(C,D,H,K)** In situ *hybridization for* egfp. **(E,F,I,L)** In situ *hybridization for* lhx1a. **(A-F)** *24 hpf embryos. Arrow marks position of pronephric proximal tubule and arrowhead delineates distal tubule and duct.* **(A,C,E)** *Lateral view, bracket denotes hindbrain.* **(B,D,F)** *Dorsal view.* **(G-I)** *48 hpf larvae, arrow in H marks distal tubule and duct in the kidney.* **(J-L)** *72 hpf larvae.* **(J)** *Montage of two images to display entire larvae.* **(M-P)** *Longitudinal optical sections of transgenic animals.* **(M)** *24hpf embryo.* **(N-P)** *48hpf larva. Abbreviations: aLL, anterior lateral line ganglia; FB, forebrain; HB, hindbrain; MB, midbrain; OV, otic vesicle; pLL, posterior lateral line ganglia; SC, spinal column; TB, tailbud.*

midbrain, and hindbrain (Fig. 2A). It is also expressed in the neurons that run the length of the spinal column (Fig. 2A). A dorsal view of a transgenic embryo at 24hpf also shows expression in the posterior lateral line ganglia (Fig. 2B). In the kidney, EGFP protein is present in the most distal region of the pronephric tubule and duct (Fig. 2 A,B, arrowhead). The expression domains of *egfp* mRNA coincide with those seen at the protein level. Expression is seen in the forebrain, midbrain, and hindbrain as well as in the neurons within the spinal cord (Fig. 2C). Strong expression is found within the posterior lateral line ganglia while kidney expression is limited to the distal tubule and duct (Fig. 2 C,D, arrowhead).

Endogenous *lhx1a* is found in the forebrain, specifically in the telencephalon and diencephalon, as well as in the midbrain and hindbrain (Fig. 2E, bracket marks hindbrain). It is also seen in the spinal cord neurons (Fig. 2E). Outside of the CNS, expression of *lhx1a* is restricted to the most proximal region of the pronephric tubule and to the tailbud (Fig. 2 E,F, arrow). Neither of these expression domains are conserved in *Tg(lhx1a:EGFP)*^{pt303} embryos at 24 hpf. Loss of proximal tubule expression (Fig. 2 C,D compared to 2 E,F) is consistent with the loss of anterior IM expression seen as early as 10 somites (Fig. 1L compared to 1R, white arrows). In addition, missing transgene expression in the tailbud (Fig. 2 C,D compared to 2 E,F) may be due to defuse, reduced *egfp* expression in the most posterior region of the notochord at 10 somites (Fig. 1L compared to 1R, arrowheads). These data suggest that additional regulatory elements may be necessary to completely mimic the endogenous expression pattern of *lhx1a*.

By 48 hpf, strong expression of *Tg(lhx1a:EGFP)*^{pt303} at both the protein and RNA level is seen in the CNS (Fig. 2 G,H). Expression is maintained in the posterior lateral line ganglia and is now apparent in the anterior lateral line ganglia (Fig. 2 G,H). Expression is also seen in the otic vesicle, which is a conserved expression domain in *Xenopus laevis* (Karavanov *et al.* 1996), but is difficult to detect at the mRNA level (Fig. 2 G,H). However, transgene expression is no longer readily apparent in the kidney (Fig. 2H, arrow). This expression pattern recapitulates what is seen for endogenous *lhx1a* at this stage (Fig. 2I). By 72 hpf, EGFP protein and *egfp* mRNA are still present in the brain (Fig. 2 J,K) but only the protein remains in spinal column neurons (Fig. 2J). Since endogenous *lhx1a* is no longer expressed in the neurons of the spinal column (Fig. 2L), this may represent perdurance of GFP protein in this tissue.

To analyze the expression of *Tg(lhx1a:EGFP)*^{pt303} in finer detail, we performed two-photon confocal microscopy on 24 hpf embryos and 48 hpf larvae, focusing on the CNS. At 24 hpf, transgene expression can be seen in both hemispheres of the midbrain (Fig. 2M) and this strong midbrain expression remains at 48 hpf (Fig. 2N). Sectioning deeper into the larva, expression in the anterior lateral line ganglia (Fig. 2O) and the hindbrain (Fig. 2P) becomes apparent. This transgene expression coincides with *lhx1a* expression previously shown in histological sections (Toyama and Dawid 1997). Overall, these data suggest that the expression of *Tg(lhx1a:EGFP)*^{pt303} recapitulates that of the endogenous *lhx1a* gene with only a few exceptions, and support the usefulness of this transgene for monitoring *lhx1a* during development in live embryos.

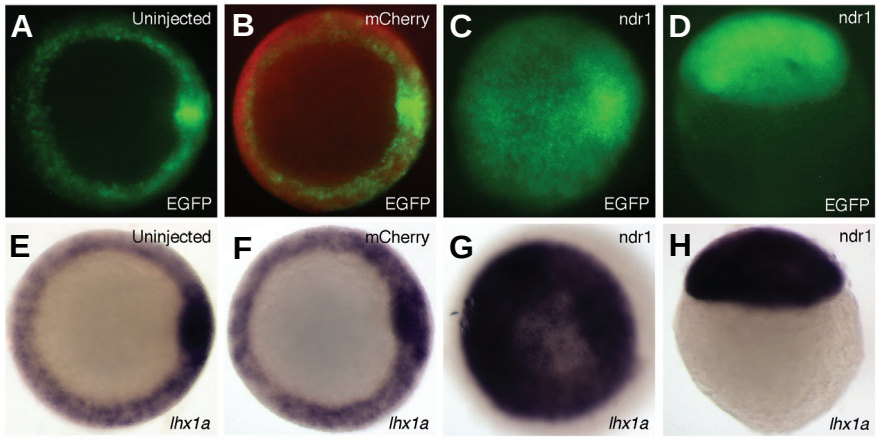

Fig. 3. Responsiveness of *Tg(lhx1a:EGFP)*^{pt303} to perturbations in Nodal signaling. (A-H) *Shield stage* Tg(lhx1a:EGFP)^{pt303} *embryos.* **(A-C,E-G)** *Animal view.* **(D,H)** *Lateral view.* **(A,E)** *Uninjected control embryos.* **(B,F)** *Embryos injected with 125pg* mCherry *RNA.* **(C,D,G,H)** *Embryos injected with 100pg* mCherry *RNA and 25pg* ndr1 *RNA.* **(A-D)** Tg(lhx1a:EGFP)^{pt303} *expression.* **(B)** *Overlay of* mCherry *expression (red) displays an embryo that was successfully injected.* **(E-H)** In situ *hybridization for* lhx1a.

Responsiveness of Tg(lhx1a:EGFP)^{pt303} *to modulation of Nodal signaling*

To test whether *Tg(lhx1a:EGFP)*^{pt303} is responsive to signaling pathways known to regulate endogenous *lhx1a* expression, we over-expressed *ndr1*, one of the zebrafish ligands of the Nodal pathway. It was previously shown that injection of low doses of *ndr1* resulted in ectopic expression of *lhx1a* in shield-stage embryos (Rebagliati *et al.* 1998). As compared to controls, injection of *Tg(lhx1a:EGFP)*^{pt303} embryos with 25pg *ndr1* mRNA led to an expansion of both transgene and endogenous *lhx1a* expression (Fig. 3). These data suggest that *Tg(lhx1a:EGFP)*^{pt303}, similar to the endogenous *lhx1a* gene, is responsive to over-expression of Nodal and is therefore regulated by this pathway.

In a complimentary experiment, we utilized a small molecule to inhibit the Nodal pathway. SB-431542 blocks the activity of the type I activin receptor-like kinases (ALK) ALK4, ALK5, and ALK7 in mammalian cell culture by preventing phosphorylation of downstream effector molecules Smad2 and Smad3 (Inman *et al.* 2002). This compound inhibits Nodal signaling in zebrafish embryos (Hagos and Dougan 2007), and at high doses phenocopies *sqt;cyc* double mutants, which eliminate early Nodal signals in the fish (Feldman *et al.* 1998; Hagos and Dougan 2007). As compared to controls, treatment of *Tg(lhx1a:EGFP)*^{pt303} embryos with 800μM SB-431542 led to a complete loss of transgene and endogenous *lhx1a* expression (Fig. 4 A-F). *Eve1*, a marker of the ventral side of the embryo, served as a negative control to show that treatment with SB-431542 does not disrupt all endogenous signaling events in the early embryo. As compared to controls, *eve1* expression is still present in SB-431542 treated embryos (Fig. 4 G-O). Taken together, these data suggest that the effect

seen with SB-431542 is specific, and that *Tg(lhx1a:EGFP)*^{pt303}, like endogenous *lhx1a*, is regulated by Nodal signaling and is capable of responding to perturbations in this pathway.

Discussion

Here we present evidence that the transgenic line, *Tg(lhx1a:EGFP)*^{pt303}, recapitulates the endogenous expression of *lhx1a* and is regulated in the same manner during early development in the zebrafish embryo. Strong expression of this transgene begins during gastrula stages. In addition, *Tg(lhx1a:EGFP)*^{pt303} is detected in the pronephric kidney during early somitogenesis, which to date is the earliest published expression of a zebrafish transgenic reporter in the nephric field. Comparison with previous data suggests that this nephric expression of *Tg(lhx1a:EGFP)*^{pt303} may serve as a marker of kidney progenitor cells (Serluca and Fishman 2001). There are some inconsistencies between the transgene and the endogenous expression pattern of *lhx1a*. Specifically, proximal tubule expression in the kidney and tailbud expression in the axial mesoderm are absent while diencephalon expression is aberrantly strong in the transgene. It is likely that regulatory regions are absent from this transgene, and this results in the discrepancies that we see between transgene and endogenous expression.

Our data demonstrate that *Tg(lhx1a:EGFP)*^{pt303} responds to alterations in the Nodal pathway, suggesting that it is regulated in a manner similar to the endogenous gene. *Lhx1a* plays a number of important roles throughout development, and this transgene will serve as a useful tool not only for gaining insight into gene regulation but also for trying to understand some of the earliest steps of cell-fate specification. Finally, since zebrafish are well-suited for small molecule screening (Vogt *et al.* 2009), this transgene could be used to screen for compounds that affect early gastrulation or kidney progenitor cells.

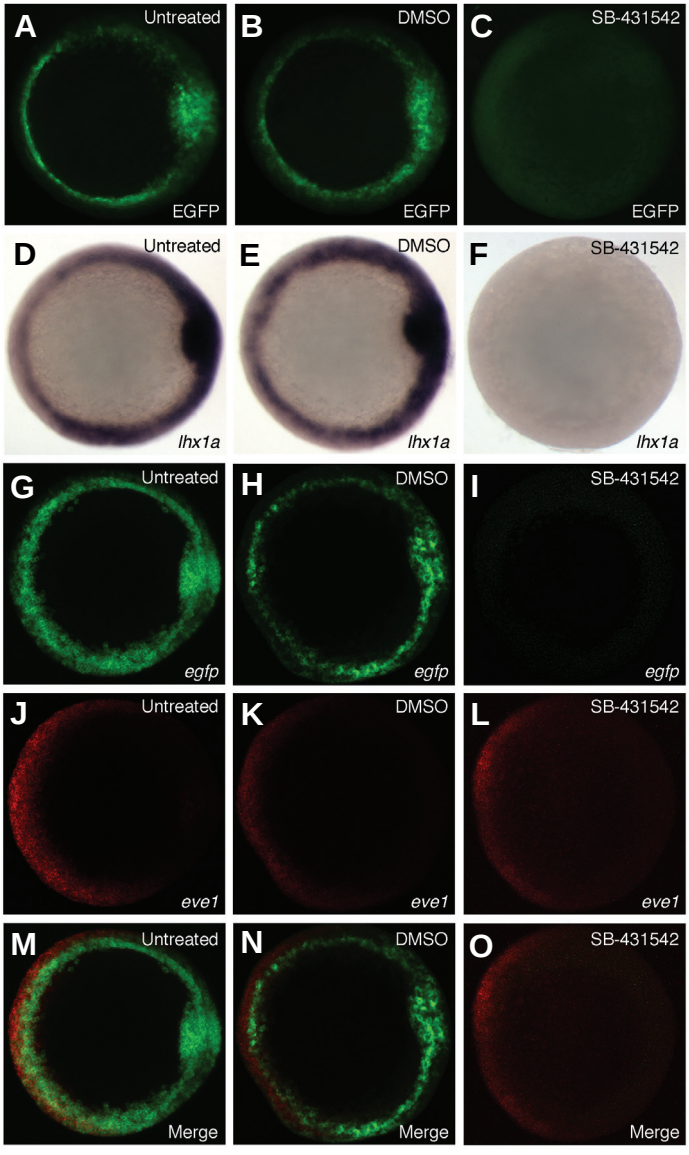

Fig. 4. Modulation of *Tg(lhx1a:EGFP)*^{pt303} expression by SB-431542. **(A-O)** *Shield stage* Tg(lhx1a:EGFP)^{pt303} *embryos, animal view.* **(A,D,G,J,M)** *Untreated control embryos.* **(B,E,H,K,N)** *Embryos treated with 0.8% DMSO.* **(C,F,I,L,O)** *Embryos treated with 800 μM SB-431542.* **(A-C)** Tg(lhx1a:EGFP)^{pt303} *expression.* **(D-F)** In situ *hybridization for* lhx1a. **(G-O)** *Fluorescent* in situ *hybridization.* **(G-I)** egfp. **(J-L)** eve1. **(M-O)** *Merged images.*

Materials and Methods

Zebrafish husbandry

Embryos expressing the *Tg(lhx1a:EGFP)* transgene were obtained from incrossing homozygous adults. Embryos were maintained at 28.5°C and were staged according to Kimmel *et al.* (Kimmel *et al.* 1995).

Tg(lhx1a:EGFP) construct design

An 8.8kb genomic region of the *lhx1a* locus was obtained from BAC 184f16 by HindIII digest and sub-cloned into pBluescript II KS(+). The upstream 5.9kb BamHI fragment was removed from this vector to facilitate the introduction of an AscI site, at base pair 12 downstream of the initiation codon, into the remaining 2.9kb fragment. This allowed for the insertion of EGFP in-frame with the first three amino acids of Lhx1a. Sequencing confirmed that no spurious mutations were created by site-directed mutagenesis. EGFP was amplified from the pEGFP-1 vector using primers with MluI sites at their 5' ends, an enzyme compatible with AscI. Following insertion of EGFP into the construct, the 5.9kb BamHI fragment was returned to generate the original 8.8kb genomic fragment in pBluescript II KS(+). The entire *lhx1a:EGFP* fragment was then cloned into the pI-SceI vector by HindIII digest. 25pg of *lhx1a:EGFP*/pI-SceI plasmid DNA was injected into 1-cell stage embryos along with the I-SceI restriction enzyme (NEB) (Thermes *et al.* 2002). These injected embryos were raised to adulthood and screened for EGFP expression in known *lhx1a* expression domains. Three independent transgenic lines were isolated; *Tg(lhx1a:EGFP)*^{pt301}, *Tg(lhx1a:EGFP)*^{pt302}, and *Tg(lhx1a:EGFP)*^{pt303}. All three lines displayed expression in the same domains, with slight variability in overall expression levels.

In situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) and processed for whole mount *in situ* hybridization as described (Toyama and Dawid 1997) or for fluorescent *in situ* hybridization (FISH) (Schoenebeck *et al.* 2007), with the following modifications. Embryos were incubated in Cy3-tyramide or fluorescein-tyramide solution for one hour. RNA probes for *lhx1a*, *egfp*, and *eve1* were used for this study.

Zebrafish mRNA microinjection

mCherry/pCS2^{+} was linearized with SacII and *ndr1*/pCS2^{+} was linear-

ized with NotI. The mMessage mMachine SP6 kit (Ambion) was used to transcribe capped RNA for both genes as per manufacture's instructions. *Tg(lhx1a:EGFP)*^{pt303} embryos were injected with either 125pg *mCherry* mRNA or 100pg *mCherry* and 25pg *ndr1* mRNA at the 1-cell stage. All embryos that expressed *mCherry* at shield stage were either fixed in 4% PFA or were analyzed for transgene expression.

Chemical treatment of transgenic embryos

SB-431542 (4- [4-(1,3 benzodioxol-5-yl)-5-(2-pyridinyl-1*H*-imidazol-2-yl]benzamide) (Sigma) was stored as a 100mM stock in DMSO at -20°C. *Tg(lhx1a:EGFP)*^{pt303} embryos at equivalent stages were arrayed into 12-well plates (maximum of 25/well) and were treated with 1.5mL of E3, 0.8% DMSO or 1.5mL of E3, 0.8% DMSO, 800 µM SB-431542 between the 256 and 512-cell stage (Hagos and Dougan 2007). Drug or vehicle was removed at shield stage by washing five times with E3 media, and embryos were either fixed in 4% PFA or were analyzed for transgene expression.

Zebrafish Imaging

Tg(lhx1a:EGFP)^{pt303} embryos (shield through 24 hpf) were held in place on glass bottom culture dishes (MatTek Corporation) with 1% low melting point agarose. Larvae (48 and 72 hpf) were immobilized with tricaine and placed on similar dishes. *Tg(lhx1a:EGFP)*^{pt303} expression was imaged with either a Zeiss 510 Meta or a Leica TCS SP5 confocal microscope. Z-stacks were taken for each image and projections were made using Image J. For *Tg(lhx1a:EGFP)*^{pt303} embryos treated with SB-431542 or injected with *ndr1* and/or *mCherry* RNA, images were taken with a Leica M205 FA epifluorescent microscope. Images were further analyzed with Adobe Photoshop CS3.

For optical sectioning, *Tg(lhx1a:EGFP)*^{pt303} embryos and larvae (24hpf and 48hpf, respectively) were fixed in 4% PFA for 1 hour at room temperature. They were then positioned in a depression slide containing 3% methylcellulose and imaged with an Olympus Fluoview FV1000 MPE system. Images were analyzed in the same manner as above.

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