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Disrupting Foxh1-Groucho Interaction Reveals Robustness of Nodal-Based Embryonic Patterning

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Abstract

The winged-helix transcription factor Foxh1 is an essential regulator of Nodal signaling during the key developmental processes of gastrulation, anterior-posterior (A-P) patterning, and the derivation of left-right (L-R) asymmetry. Current models have Foxh1 bound to phospho-Smad2/3 (pSmad2/3) as a central transcriptional activator for genes targeted by Nodal signaling including *Nodal* itself, the feedback inhibitor *Lefty2*, and the positive transcriptional effector *Pitx2*. However, the conserved Engrailed homology-1 (EH1) motif present in Foxh1 suggests that modulated interaction with Groucho (Grg) co-repressors would allow Foxh1 to function as a transcriptional switch, toggling between transcriptional on and off states via pSmad2-Grg protein-switching, to ensure the properly timed initiation and suppression, and/or amplitude, of expression of *Nodal* and its target genes. We minimally mutated the Foxh1 EH1 motif, creating a novel *Foxh1*^{mEH1} allele to test directly the contribution of Foxh1-Grg-mediated repression on the transient, dynamic pattern of Nodal signaling in mice. All aspects of *Nodal* and its target gene expression in *Foxh1*^{mEH1/mEH1} embryos were equivalent to wild type. A-P patterning and organ situs in homozygous embryos and adult mice were also unaffected. The finding that Foxh1-Grg-mediated repression is not essential for *Nodal* expression during mouse embryogenesis suggests that other regulators compensate for the loss of repressive regulatory input that is mediated by Grg interactions. We suggest that the pervasive inductive properties of Nodal signaling exist within the context of a strongly buffered regulatory system that contributes to resilience and accuracy of its dynamic expression pattern.

Keywords

Nodal; Foxh1; Groucho; Transcriptional regulation; Embryonic patterning

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1. Introduction

Nodal is a key regulator of body axis formation and patterning in the developing embryo of a broad spectrum of animal species. Among vertebrates, Nodal signaling specifically plays conserved and essential roles in germ layer specification, gastrulation, anterior-posterior (A-P) axis formation, and left-right (L-R) patterning (Lu et al., 2001; Schier and Shen, 1999). As a morphogen belonging to the transforming growth factor β (TGF β) superfamily, Nodal signals through the type I (Alk4/5/7) and type II (ActRIIA/B) serine/threonine receptors, with the aid of the EGF-CFC family of co-receptors (Crypto/Cryptic in mouse) (Schier, 2009; Wu and Hill, 2009). The downstream phosphorylation of the signal transducers Smad2 and Smad3 leads to interactions with Smad4 and nuclear translocation, and binding of the forkhead box transcription factor Foxh1, to initiate transcription of downstream target genes. Cell fate determination is greatly influenced by the level of Nodal signaling, and therefore dynamic but precise regulation of Nodal signaling duration and amplitude is essential for proper embryogenesis (Schier, 2009).

Foxh1 is essential to the initiation and maintenance of the Nodal autoregulatory circuit by which Nodal signaling initiates the expression of *Nodal* itself, its feedback inhibitor *Lefty2*, and the homeodomain transcription factor *Pitx2*, an important effector of Nodal signaling (Norris et al., 2002; Saijoh et al., 2000; Shiratori et al., 2001). Foxh1 binds conserved sequences in a *cis*-regulatory region known as an asymmetric enhancer element (ASE; named for its ability to drive expression on the left but not right side of the embryo) that is present in all three Nodal autoregulatory circuit genes (*Nodal*, *Lefty2*, *Pitx2*) (Meno et al., 2001; Saijoh et al., 1999; Saijoh et al., 2000; Shiratori et al., 2001). The ASE, along with another enhancer, drives *Nodal* expression during early patterning events and then initiates the expression of *Nodal*, *Lefty2*, and *Pitx2* solely within the left lateral plate mesoderm (L LPM) during L-R patterning (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 1999; Shiratori et al., 2001). Deletion of the Foxh1 binding-sites, or deletion of the ASE as a whole in mouse, leads to decreased *Nodal* expression in the epiblast and complete loss of *Nodal*, *Lefty2*, and *Pitx2* expression in the L LPM (Adachi et al., 1999; Norris and Robertson, 1999; Norris et al., 2002; Saijoh et al., 2000; Shiratori et al., 2001). Foxh1 binding sites play a conserved role in the ASE as deletion of these sites also attenuated Nodal signaling in other species (Osada et al., 2000). Furthermore, the majority of embryos with a global deletion of *Foxh1* fail to orient the A-P axis appropriately, elongate the primitive streak, or form a node; together these defects cause embryonic lethality (Hoodless et al., 2001; Yamamoto et al., 2001). Embryos in which *Foxh1* was conditionally inactivated within the L LPM failed to express *Nodal*, *Lefty2*, and *Pitx2* in that tissue and exhibited right isomerism (Yamamoto et al., 2003).

The pSmad2/3-Smad4 complex binds to the Smad interaction domain (SID) on the C-terminus of Foxh1 (Chen et al., 1997; Weisberg et al., 1998). In addition to the SID, Foxh1 contains another more N-terminally located co-factor interaction motif—the 8 amino-acid Engrailed homology-1 (EH1) motif—that is recognized and bound by the Groucho/Groucho-related-gene/Transducin-like enhancer of split (Gro/Grg/TLE) family of co-repressors (Yaklichkin et al., 2007a). The Gro/Grg/TLE protein family comprises four full-length members: TLE1-4 in human and originally termed Grg1-4 in mouse; the latter

nomenclature we will use hereafter. Each member contains a conserved C-terminal WD-repeat domain that mediates interactions with transcription factors by recognizing two classes of motifs: the full EH1 or a smaller tetrapeptide (WRPW). It is currently unclear as to the direct mode by which Grg proteins repress transcription, but likely multiple mechanisms are used, and are dependent on biological context. One reported mechanism is the recruitment of histone deacetylases (HDACs) to Groucho-bound loci, promoting a closed chromatin conformation (Jennings and Ish-Horowicz, 2008).

The presence of both the EH1 motif and SID suggests that Foxh1 acts as a transcriptional switch, toggling between activator or repressor states by competitive partner-switching between pSmad2/Smad4 and Grg. This is an attractive mechanism for rapid and precise transcriptional control of Nodal signaling, for example, during the dynamically changing expression pattern of *Nodal* in both the epiblast and L LPM. At embryonic day (E) 5.5, Foxh1-dependent *Nodal* expression is initially throughout the epiblast and visceral endoderm, and then resolves to the prospective posterior side where it is expressed within the elongating primitive streak, becoming terminated by E7.5 (expression in the node at this time is Foxh1-independent) (Norris and Robertson, 1999; Adachi et al. 1999). Expression reinitiates exclusively in the L LPM at the 2–3 somite-stage. Within hours, *Nodal* expression, as well as the expression of its target and feedback inhibitor *Lefty2*, traverses the L LPM in a wave-like pattern (for data from frog embryos: Ohi and Wright, 2007), initiating in the posterior L LPM adjacent to the node and then rapidly expanding toward the anterior boundary of the LPM before becoming extinguished by 6–8 somite stage (Collignon et al., 1996; Lowe et al., 1996; Meno et al. 1996; Norris et al., 2002). During stages of L-R patterning in mouse, *Foxh1*, as well as *Grg3* and *Grg4*, are expressed bilaterally in the LPM (Koop et al., 1996; Leon and Lobe, 1997; Weisberg et al., 1998). Colocalization of the proposed switch components in the R LPM, which does not express *Nodal* but is competent to respond to Nodal signaling (Ohi and Wright, 2007; Yamamoto et al., 2003), as well as in the L LPM that expresses *Nodal* under rapidly changing spatiotemporal control, further supports the idea that Foxh1 is a transcriptional switch that participates in repressing *Nodal* transcription. Foxh1 bifunctionality that is conveyed by interchangeable binding of pSmad2 and Grg could facilitate rapid and precise tailoring of target gene transcription to varying thresholds of external instructive signals, as compared to mechanisms that rely on distinct complexes for transcriptional activation and repression (Cinnamon and Paroush, 2008).

Recent work in *Xenopus* fits the proposal of FoxH1 as such a transcriptional switch with respect to regulating *Nodal* expression, and with pSmad2 and Grg4 acting as co-activator and co-repressor, respectively. Overexpression of Grg4 in *Xenopus* embryos strongly reduced Nodal-dependent transcriptional activation, and chromatin-immunoprecipitation (ChIP) showed Grg4 occupancy at the *Nodal* ASE that depended upon the EH1 motif of FoxH1. ASE occupancy by Grg4 was greatly decreased with *Nodal* or *Smad2* overexpression (D. S. Kessler, personal communication). These results suggest that pSmad2 displaces Grg4 in response to Nodal signaling, and that complementary Grg4 displacement of pSmad2 causes transcriptional repression.

Transcriptional switching has been documented to control target gene transcription in other developmental signaling pathways such as Notch and Wnt (Bray and Furriols, 2001; Daniels

and Weis, 2005). For Wnt signaling, β -catenin and Grg co-repressors have overlapping binding domains on Tcf/Lef and compete with each other for occupancy at the locus (Daniels and Weis, 2005). The EH1 motif and SID do not overlap in Foxh1 and it is not known if pSmad2 and Grg engage in direct steric competition in the complete transcriptional complex. Alternatively, unknown components of the transcriptional complex might promote removal of pSmad2/Smad4 and/or Grg, leading to a mutual interference model that is highly dependent upon the levels of activator or repressor.

Knowledge about mechanisms regulating transcriptional repression of *Nodal* expression is greatly lacking. To determine if Foxh1 is conserved as a transcriptional switch and to identify the developmental consequences of removing the Foxh1-Grg repression arm of this switch, we developed a novel mouse model carrying a new mutant allele, *Foxh1*^{mEH1}, in which the interaction between Foxh1 and Grg is perturbed. Manipulations made to the *Foxh1*^{mEH1} locus were carefully controlled for, by deriving a control line carrying identical insertions while maintaining a wild-type EH1 motif. This study is the first in mouse to address the role of Foxh1 as a transcriptional-switch repressor of *Nodal* expression. Our findings support the idea that the mammalian embryo has evolved a robust and potentially diverse set of mechanisms to control-Nodal signaling levels and ensure successful germ-layer development and embryonic patterning.

2. Results

2.1. Derivation of *Foxh1*^{mEH1/mEH1} mice

In an effort to disrupt binding between Foxh1 and Grg co-repressors, we mutated the Foxh1 EH1 motif, FSIKSLLG. Before mutating the motif in mouse, three different EH1 mutations (eight amino-acid deletion, Ala substitution **AAAAAALG**, or point mutation **ESIKSLLG**) were tested for their effect on the production and stability of Foxh1. Western blot analyses on lysates collected from *Xenopus* embryos that were injected with RNA encoding these Foxh1 variants confirmed that the EH1 mutations did not destabilize or over-stabilize Foxh1 (data not shown). The single amino-acid substitution of F-to-E was chosen because it introduced the fewest Foxh1 sequence alterations and would, therefore, be least likely to affect the overall folding or activator functions of Foxh1 (Fig. 1A), an important consideration when moving forward into the derivation of mutant mouse strains. This minimal mutation has been shown to fully disrupt the interaction between EH1-motif-containing proteins and Grg co-repressors in numerous species (Jennings et al., 2006; Jiménez et al., 1999; Yaklichkin et al., 2007b). Also, the phenylalanine (F) residue, which binds in a hydrophobic recess of the WD-repeat domain of Grg proteins (Jennings et al., 2006), is 100% conserved in all EH1 motifs of vertebrate Fox proteins (Yaklichkin et al., 2007a).

A single FLAG tag was added to the N-terminus of the mutant EH1 *Foxh1* allele (*Foxh1*^{mEH1}; notation of FLAG tag was avoided in this abbreviated nomenclature for simplicity) to aid in detecting Foxh1^{mEH1} protein over wild-type Foxh1, and to facilitate future chromatin-binding and target gene studies. The N-terminus was tagged in order to avoid affecting the conformation of, and binding of other factors to, the more C-terminally located SID. The N-terminal tag did not affect the production or stability of Foxh1 via

western-blot analysis of lysates collected from *Xenopus* embryos injected with RNA encoding FLAG-tagged Foxh1 (data not shown). A control mouse line, *Foxh1^F*, which is identical to *Foxh1^{mEH1}* except for its wild-type EH1 motif, was derived in parallel, for the most rigorous testing that any abnormal phenotype observed in *Foxh1^{mEH1}* mice did in fact arise because of the EH1 mutation and not the FLAG tag or residual exogenous sequences (Lox, FRT, or restriction enzyme sites) inserted into the *Foxh1* locus that remain after gene engineering (Fig. 1A; supplementary material Fig. S1).

Prior to deriving the *Foxh1^{mEH1}* and *Foxh1^F* mouse strains by inserting them into the *Foxh1^{LCA}* in mouse ES cells, the mesoderm-inducing and Grg-binding potential of each protein was characterized to confirm that the exogenous sequences did not disrupt Foxh1 activator functions, and that the EH1 mutation abrogated the binding to Grg co-repressors. RNA encoding Foxh1^F, Foxh1^{mEH1}, or true wild-type Foxh1 was injected into the animal region of 1-cell *Xenopus* embryos. Injected embryos were cultured until the start of gastrulation at which time animal caps were cut, cultured overnight, and assessed next day for their elongation, as a measure of the induction, via the Nodal signaling pathway, of mesendoderm and other tissues that undergo convergent extension. Compared to caps from uninjected embryos, which rounded up and became atypical epidermis, caps from all three injection conditions had elongated similarly (data not shown), indicating activation of the mesendoderm-inducing pathways currently held to be controlled by threshold-dependent Nodal signaling (for example: Jones et al., 1995). In addition, quantitative real-time PCR (qRT-PCR) analysis of these “induced animal caps” showed increased relative expression of the mesendodermal markers and Nodal target genes *brachyury* and *goosecoid*, as well as the mesodermal derivative *muscle actin* marker, compared to control caps from uninjected embryos. Conversely, animal caps from embryos producing Foxh1^{mEH1}, Foxh1^F, or wild-type Foxh1 had decreased relative expression of the ectodermal marker *epidermal keratin* compared to uninjected controls (data not shown). Both the animal cap and qRT-PCR assays indicated that addition of the FLAG tag and EH1 mutation did not compromise the ability of Foxh1 to cause mesendoderm induction.

To verify that the F-to-E mutation disrupted Foxh1-Grg binding, co-immunoprecipitation (co-IP) assays were performed with homogenized *Xenopus* gastrula embryos that had been injected at the 1-cell stage with RNA encoding Foxh1^F or Foxh1^{mEH1} alone, or in combination with Myc-tagged *Xenopus* Grg4 (^{Myc}Grg4). Foxh1^F and ^{Myc}Grg4 were reproducibly co-immunoprecipitated with an antibody against Foxh1, but the ability of ^{Myc}Grg4 to co-IP with Foxh1^{mEH1} was absent or prominently reduced, even under these overexpression conditions, indicating that the EH1 mutation greatly disrupted Foxh1-Grg4 physical interactions (n = 3 independent injection experiments and western blots) (supplementary material Fig. S2). As argued in the introduction above, the high conservation of the WD-repeat domain among Grg family members means that this mutation likely also disrupts any interactions with other Grg proteins. Based on published *in situ* hybridization expression patterns, *Grg3* and *Grg4* are the most likely candidates to function in the transcriptional switch because they, like *Foxh1*, are expressed symmetrically within the LPM during stages of asymmetric Nodal signaling (Koop et al., 1996; Leon and Lobe, 1997; Weisberg et al., 1998).

A loxed cassette acceptor (LCA) allele of the *Foxh1* locus (supplementary material Fig. S1) was established in ES cells, to allow insertion of *Foxh1^{mEH1}* and *Foxh1^F* alleles into the endogenous locus through the highly efficient method of recombinase-mediated cassette exchange (RMCE). Analysis of *Foxh1* genomic organization across multiple species helped to avoid potentially conserved *cis*-regulatory regions and locate the least disruptive points for inserting variant *Lox* sites into the *Foxh1* locus. *Foxh1* and its neighboring gene *Kifc2*, a kinesin superfamily member expressed exclusively in neural tissue, are reported to share an overlapping 3' untranslated region (UTR) (Liu et al., 1999). The lack of apparent phenotype in *Kifc2^{-/-}* suggests that *Kifc2* is dispensable for normal development and behavior (Yang et al., 2001); therefore, a *Lox2272* site was placed within its 3' UTR. Correct targeting of the *Foxh1* locus was verified by Southern-blot analysis of *Bam*HI-digested genomic DNA with two probes, a 3' probe external to the 3' homology arm and a 5' probe requiring derivation from within the 5' homology arm because a repetitive short interspersed nuclear element (SINE) lies just outside the homology arm (Fig. 1B; supplementary material Fig. S1). Chimeras were generated with the *Foxh1^{LCA}* mESC line, and Southern blotting and PCR analysis confirmed *Foxh1^{LCA}* germline transmission from them (Fig. 1C). Embryos homozygous for the *Foxh1^{LCA}* allele resembled *Foxh1^{-/-}* embryos (data not shown), further verifying that wild-type *Foxh1* was correctly targeted and replaced.

The *Foxh1^{mEH1}* and *Foxh1^F* RMCE replacement constructs were electroporated into the *Foxh1^{LCA}* mESC line, and correctly recombined clonal cell lines were injected into C57BL/6 blastocysts to generate *Foxh1^{mEH1}* and *Foxh1^F* chimeras. Mice homozygous for the *Foxh1^{mEH1}* or *Foxh1^F* allele were born at Mendelian ratio, survived to adulthood, and bred normally. The *Foxh1* expression pattern was unchanged in *Foxh1^{F/F}* and *Foxh1^{mEH1/mEH1}* embryos compared to wild-type embryos, indicating that the EH1 mutation and FLAG tag did not disrupt *Foxh1* transcription (supplementary material Fig. S3). *Foxh1^{F/F}* embryos were molecularly and morphologically identical to wild type, therefore *Foxh1^{F/F}* and wild-type animals were used interchangeably as control embryos.

2.2. Gastrulation and A-P patterning are unaffected in *Foxh1^{mEH1/mEH1}* embryos

Since *Foxh1*-dependent Nodal signaling is essential for proper gastrulation and A-P patterning, E6.75-7.25 *Foxh1^{mEH1/mEH1}* embryos were analyzed for defects in cell-lineage allocation and axis orientation. Previously characterized *Nodal* gain-of-function mutants, such as *Lefty2^{-/-}* embryos, present with an expanded primitive streak and excess mesoderm (Meno et al., 1999). If *Foxh1*-*Grg*-mediated repression is an important negative regulator of Nodal signaling at this stage, loss of the *Grg* interaction would be expected to cause increased and/or prolonged Nodal signaling within the epiblast, resulting in defects similar to those seen in *Lefty2^{-/-}* embryos. However, *Foxh1^{mEH1/mEH1}* embryos appeared morphologically normal (Fig. 2; see supplementary material Table S1 for *n* values). Any size difference between wild-type and *Foxh1^{mEH1/mEH1}* embryos is attributed to the size and stage variation routinely seen within and between litters at these early developmental stages (Downs and Davies, 1993). The morphological observations were confirmed molecularly by *in situ* hybridization analyses of *Nodal*, its downstream targets, and early patterning genes. No spatial or temporal defects in the expression of *Nodal* (Fig. 2A,B) or its downstream target *Lefty2* (Fig. 2C,D) were observed. *Otx2* expression, which begins in the visceral

endoderm and shifts anteriorly to mark the anterior ectoderm as gastrulation progresses (Zakin et al., 2000), was not altered in mutant embryos, suggesting that regionalized lineage allocation was normal in these embryos (Fig. 2G,H). Additionally, expression of the mesoderm and primitive-streak markers, and targets of Foxh1-dependent transcription, *Brachyury/T* and *Gooseoid* were unaffected in *Foxh1^{mEH1/mEH1}* embryos, confirming that the primitive streak was correctly patterned (Fig. 2E,F,I,J). Finally, the definitive endoderm marker *Foxa2*, whose expression is dependent on Foxh1 (Hoodless et al., 2001) and is disrupted in both loss- and gain-of-function Nodal mutants (Perea-Gomez et al., 2002; Vincent et al., 2003), was clearly expressed in the axial midline of early somite mutant embryos (Fig. 2K,L). Combined, these results show that early cell specification and patterning were not altered in *Foxh1^{mEH1/mEH1}* embryos, and that Foxh1-Grg-mediated repression is not a primary regulator of Nodal signaling at this stage.

2.3. L-R patterning is unaffected in *Foxh1^{mEH1/mEH1}* embryos

We next investigated if Foxh1-Grg-mediated repression regulates Nodal signaling during stages of L-R patterning. As discussed in the Introduction, *Nodal* expression, as well as that of *Lefty2* and *Pitx2*, is strikingly left-sided, quickly moving through the L LPM in a dynamic posterior-to-anterior wave of expression (Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996; Ohi and Wright, 2007). Bilateral expression of Foxh1, Smad2/4, and Groucho corepressors in the LPM (Koop et al., 1996; Leon and Lobe, 1997; Waldrip et al., 1998; Weisberg et al., 1998) suggests that Foxh1 transcriptional switching may regulate the spatiotemporal expression of Nodal circuit genes by initiating their expression in the L LPM and suppressing it in the R LPM. If the Foxh1-Grg repression arm negatively regulated Nodal signaling, it was predicted that disrupting this interaction would result in premature, prolonged, and/or R-sided expression of *Nodal* and its downstream targets. In all *Foxh1^{mEH1/mEH1}* embryos assayed, *Nodal* was solely L-sided, and not detected in the L LPM prior to 2–3 somites or beyond 8 somites (Fig. 3A,B; supplementary material Fig. S4A–D). As expected, *Nodal* expression in the node, which is controlled by a Foxh1-independent enhancer, was not altered (data not shown). In accordance with *Nodal* expression, no spatial or temporal defects were detected in *Lefty2* and *Pitx2* expression (Fig. 3C–F; supplementary material Fig. S4E–L). From these results we conclude that loss of Foxh1-Grg-mediated repression during L-R patterning can be overcome without compromising development.

2.4. *Foxh1^{mEH1/mEH1}* embryos display normal L-R situs

Nodal signaling (*Nodal*, *Lefty2*, *Pitx2*) in the L LPM directs proper placement and anatomy of the visceral organs (Nakamura and Hamada, 2012). Alterations in *Nodal* expression, and thus signaling, such as right-sided or bilateral expression, can lead to multiple heart abnormalities, pulmonary isomerism, intestinal malrotation, and misplacement of visceral organs with respect to the midline. Heart abnormalities commonly observed in Nodal signaling mutants include dextrocardia, reversed heart looping, transposition of the great arteries, and ventricular septation (Bisgrove and Yost, 2001). Analysis of heart morphology and placement in mutant E9.5–E10.5 embryos revealed, in all embryos analyzed, stereotypically normal rightward looping and correct cardiac positioning left of midline (Fig. 4A,B). Functional assessment of heart morphology for septal and vessel-transposition

defects was done by injecting blue and yellow liquid latex, respectively, into the left and right ventricles of E15.5 *Foxh1*^{mEH1/mEH1} embryos to highlight the vasculature and aid in detecting cardiovascular defects. As seen in wild-type embryos, the pulmonary trunk passed ventrally to the aorta in all *Foxh1*^{mEH1/mEH1} embryos analyzed, indicating no transposition of the great arteries. Also, no ventricular septations were identified, as mixing of the yellow and blue latexes was not observed in the ventricles of these embryos (Fig. 4C,D). Overall morphology of visceral organs (lungs, liver, gut, stomach) appeared normal in *Foxh1*^{mEH1/mEH1} embryos (Fig. 4E–H). These results corroborate our early findings that showed no molecular defects in L-R patterning. Together, these data show that major disruption of the Foxh1-Grg interaction alone does not alter Nodal signaling in the mouse, and that, possibly, the role Foxh1-Grg-mediated repression plays in regulating *Nodal* transcription is masked by a highly buffered regulatory system, which compensates for its loss in an effort to maintain the spatiotemporal dynamics of Nodal signaling.

3. Discussion

This is the first study to use a precise amino-acid alteration to address the role of Foxh1-Grg-mediated repression in regulating Nodal signaling during early mouse development. We altered the endogenous *Foxh1* locus so that our experiments were performed under endogenous levels of gene and protein expression, rather than in an overexpression context, providing the most rigorous way to assay the direct influence of Grg-based repression acting through Foxh1. The minor modifications made to create the *Foxh1*^{mEH1} allele were carefully controlled for by the parallel construction of the “wild-type” *Foxh1*^F allele (note that both proteins also contained an N-terminal FLAG tag). We conclude that the blocked interaction between Foxh1 and Grg can be accommodated by adaptive gene regulatory mechanisms, such that there are no major effects on Nodal signaling at the level of target gene readout, or on overall body axis formation, anterior-posterior or left-right patterning. Our results suggest that there is substantial robustness in the mechanisms that have evolved to stabilize Nodal regulatory systems and lead to successful germ-layer development and embryonic patterning.

3.1. Buffered regulatory system potentially compensates for loss of Foxh1-Grg-mediated repression

Cell fate determination is qualitatively influenced by the level and duration of Nodal signaling. Experiments in *Xenopus* embryos showed that higher Nodal/Activin signaling induced the mesendoderm marker *gsc*, while lower levels induced the pan-mesodermal marker *xbra* (Agius et al., 2000; Gurdon et al., 1994; Jones et al., 1995). Dose-dependent responses also control mouse development (Robertson, 2014). In *Nodal* null embryos, little mesodermal differentiation occurs and there is subsequent failure to gastrulate, causing developmental arrest (Conlon et al., 1994; Zhou et al., 1993). Mesoderm induction is restored in mutants with reduced *Nodal* expression, but definitive endoderm is still absent (Norris et al., 2002; Vincent et al., 2003). Left-right patterning also depends upon the level of the Nodal signaling component *Pitx2*, a transcriptional effector that is essential for asymmetric organogenesis (Nakamura and Hamada, 2012). Experiments with *Pitx2* revealed a progressive gene-dosage requirement in organ formation and L-R patterning: lung and gut

required the highest level of *Pitx2* expression, followed by the heart, with the stomach requiring the least (Gage et al., 1999; Liu et al., 2001). Because proper embryonic patterning depends upon the precise titration of Nodal signaling, it is likely that buffered regulatory systems were developed to provide stability but also rheostat-like level control. The employment of such a system may be one explanation for the lack of phenotype in *Foxh^{mEH1/mEH1}* embryos.

Tissue-specific expression of *Nodal* is, in part, positively regulated by five different enhancers that drive expression in the epiblast, node, and LPM (Adachi et al., 1999; Papanayotou et al., 2014; Saijoh et al., 2005; Vincent et al., 2003). The dynamic expression in the epiblast and LPM is also strongly affected by a self-enhancement and lateral-inhibition (SELI) system through which Nodal enhances its own expression with a positive feed-forward loop that activates through the ASE. Nodal then rapidly initiates expression of its feedback antagonist *Lefty2* (Nakamura et al., 2006). *Lefty2* molecules, which have an inherent ability to travel faster and farther than Nodal, and have greater stability, help terminate *Nodal* expression in the L LPM, as well as prevent the *Nodal* autoregulatory loop from fully initiating in the R LPM (Marjoram and Wright, 2011; Müller et al., 2012; Nakamura et al., 2006). *Lefty2* inhibits Nodal signaling by either binding directly to the Nodal dimer, or competitively blocking access to the obligate EGF-CFC co-receptors (Chen and Shen, 2004). Such inhibition by *Lefty2* prevents phosphorylation and prevents Smad2 shuttling to the nucleus, shutting down Foxh1-dependent transcription of target genes.

We speculate that the *Nodal* locus could be more “open” in *Foxh1^{mEH1/mEH1}* embryos because of impaired interaction with Grg co-repressors. However, attenuation of Smad2 phosphorylation by *Lefty2* inhibition at the cell surface should still occur in mutant embryos, which would dampen the potential over-activation of *Nodal* expression. Thus, *Lefty2* would be an especially important component of the buffered regulatory system that compensates for the reduction/loss of Foxh1-Grg-mediated repression in *Foxh1^{mEH1}* mutant mice, preventing a mutant phenotype. Such buffering could be less apparent in the overexpression studies in *Xenopus* that implicate Foxh1 as a repressor of *Nodal* transcription (D. S. Kessler, personal communication), as compared to this study with *Foxh1^{mEH1}* being expressed from the endogenous locus.

That a buffered system exerts meticulous control over Nodal signaling is suggested by the multiple Nodal signaling loss- and gain-of-function mutations that display incomplete penetrance or variable expressivity. For example, the severity of defects in *Foxh1^{-/-}* embryos varies greatly, from completely failed development of the embryo proper, to a less severe phenotype without midline structures (Hoodless et al., 2001; Yamamoto et al., 2001). Similarly, embryos lacking the co-receptor *Cryptic* have numerous defects in asymmetric organogenesis, but the specific organs affected differ among mutant embryos (Yan et al., 1999). Incomplete penetrance is seen in embryos that lack *Lefty2* in the LPM, with two-thirds of the animals dying a few days after birth from cardiac defects, which were not detected in mutants that survived to adulthood (Meno et al., 2001). Homozygous mutations in the Nodal antagonists *Lefty1* and *Cer1* alone do not cause defects in gastrulation (Meno et al., 1998; Simpson et al., 1999), but the double homozygous *Lefty1^{-/-};Cer1^{-/-}* mutants are 100% embryonic lethal (Perea-Gomez et al., 2002). These results demonstrate that loss of a

single negative regulator of Nodal signaling can be compensated for, and that removal of additional members of the buffered regulatory system is required to manifest an abnormal phenotype.

Therefore, it is possible that this new *Foxh1*^{mEH1/mEH1} condition represents a sensitized state in which relatively subtle future manipulation of other components of the buffered system could reveal patterning defects. Example experiments could test how the loss of Foxh1-Grg-mediated repression is compensated. For example, loss of *Lefty2* in the *Foxh1*^{mEH1} background may yield patterning deficits that have so far remained hidden under disruption of only the Foxh1-Grg interaction. While our *Foxh1*^{mEH1} amino-acid alteration removes a residue that is essential for tight Grg interaction (Jennings et al., 2006), we must remain open to the possibility that the primary explanation for no abnormal phenotype is that some low-level Foxh1^{mEH1}-Grg association is sufficient to effect substantial repression of *Nodal* transcription. The strength of the data indicating the importance of the phenylalanine residue for Grg interaction strongly suggests that any residual Grg binding might occur not through the highly disrupted EH1 motif, but indirectly through another member of the as-yet-uncharacterized transcriptional complex. An even simpler interpretation is that Grg-mediated repression of Nodal signaling is not conserved in the mouse. The sensitization experiments described above are pertinent and rely on our findings here as the foundation. In addition, experiments investigating the type of epigenetic repression marks present at the *Nodal* locus in *Foxh1*^{mEH1} mutant embryos compared to control embryos could help determine the extent of repression that remains in *Foxh1*^{mEH1} embryos.

3.2. Epigenetic regulation of Nodal signaling

The epigenetic landscape provides critical input onto a gene's transcriptional state, but almost nothing is known about such landscape alterations at the *Nodal* locus during development, or the degree to which epigenetic modifications contribute to Nodal signaling regulation. It is well documented that pSmad2/Smad4 recruits the co-activators p300 and CREB binding protein (CBP), which use intrinsic histone-acetyltransferase activity to promote an open-chromatin conformation (Attisano and Wrana, 2000). The ability of Foxh1 to interact with factors that recruit HDACs, which are capable of reversing the pSmad2/Smad4-founded markings, further supports Foxh1 transcriptional switching as a plausible epigenetic mode of modulating Nodal signaling.

Recent studies report roles for histone acetylation and methylation in the positive and negative regulation of Nodal signaling in mouse ESCs and in embryos. In mouse ESCs, Nodal-Smad2/3 signaling recruits Jmjd3, a H3^{k27me3}-specific demethylase, to Nodal target-genes to counteract the repressive effect of Polycomb repressive complex 2 (PRC2) (Dahle et al., 2010). On the other hand, studies in Medaka fish report physical interaction of Foxh1 with the PRC2 histone-modifier Ezh1, with *Ezh1* knockdown producing bilateral *Nodal* expression and L-R patterning defects (Arai et al., 2010). Because PRC2 can be recruited by Grg co-repressors (Patel et al., 2012), Foxh1, through interaction with Grg, could initiate longer-term transcriptional repression. Also, others have already implied that

HDACs could be part of the repression mechanism for *Nodal* expression in the R LPM (Carneiro et al., 2011).

Understanding whether or not the *Nodal* locus remains poised or repressed during time points when *Nodal* is not expressed may provide insight into the flexible regulation of Nodal signaling during embryogenesis, and the specific levels of signaling that transcriptionally activate the various sets of downstream targets. We also speculate that the Grg-mediated long-term repression of *Nodal* expression might be essential in preventing neoplastic reactivation of the Nodal signaling pathway. The *Foxh1^F* line will be useful for studying the epigenetic regulation occurring at the *Nodal* locus, and other ASE-containing loci, as well as for identifying novel targets of Foxh1.

4. Materials and Methods

4.1. Derivation of *Foxh1^{mEH1}* and *Foxh1^F* mice

The *Foxh1^{LCA}*, *Foxh1^F*, and *Foxh1^{mEH1}* mouse lines were derived with help from Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. A 129S6 *Foxh1^{LCA}* ES cell line was generated through homologous recombination of a targeting vector that was created by BAC recombineering with the BAC bMQ206P16 (BAC PAC Resources at C.H.O.R.I., Oakland, Ca.). Colonies surviving selection were screened by Southern blot analysis (5/137 colonies correctly targeted *Foxh1*) and karyotyped. *Foxh1^{LCA}* chimeras were generated to validate germline transmissibility of *Foxh1^{LCA}* ES cell clones. Exchange vectors for the *Foxh1^F* and *Foxh1^{mEH1}* alleles were generated by PCR-based site-directed mutagenesis of the *Foxh1* locus. Recombinase-mediated cassette exchange (RMCE) between the *Foxh1^{LCA}* ES cell clone 4B6 and exchange vectors was used to generate *Foxh1^F* and *Foxh1^{mEH1}* ES cell lines. Colonies surviving selection were screened with PCR and karyotyped. One clone for each line was injected into C57BL/6 embryos for generation of chimeras. Following the generation of founder mice for the *Foxh1^F* and *Foxh1^{mEH1}* alleles, hygromycin resistance selection cassettes were excised by mating to mice with FLPe (Rodriguez et al., 2000). FLP-mediated deletion was confirmed with the primers 5' AGC TGC CCA TTG TAG TAG C 3' and 5' CAA AGT GAG TTC CAG GAC A 3'. A schematic of the *Foxh1^{LCA}*, *Foxh1^F*, and *Foxh1^{mEH1}* alleles is provided in supplementary material Fig. S1.

4.2. Mouse husbandry

Animal handling was approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. *Foxh1^{mEH1}* and *Foxh1^F* animals were genotyped by PCR (primers 5' ACT TGG GAA ACC ACT TGG TC 3' and 5' TTG ACT CTT GAA CCT CCA GG 3'). Experimental embryos resulted from crosses between mice homozygous for the *Foxh1^{mEH1}*, *Foxh1^F*, or wild-type *Foxh1* allele.

4.3. *Xenopus* embryo manipulations and microinjections

Plasmids for generating RNA in vitro were generated by inserting cDNAs encoding Foxh1^F or Foxh1^{mEH1} into pCS2+ (from Dr. Dave Turner, University of Michigan). An injection construct encoding Myc-tagged *Xenopus* Grg4 (^{Myc}Grg4) was a gift from Dr. Daniel

Kessler, University of Pennsylvania. Capped RNA was produced using the mMessage mMachinE kit (Ambion). RNA encoding Foxh1^F or Foxh1^{mEH1} (2.0 ng) was injected alone or in combination with MycGrg4 (2.0 ng) into the animal caps of 1–2 cell *Xenopus* embryos. Embryos were cultured in 1x Steinberg's solution until stage 10, when they were frozen and stored at –20°C for use in co-immunoprecipitations (co-IPs). Embryos were staged according to (Nieuwkoop and Faber, 1967).

4.4. Co-immunoprecipitation and immunoblotting

Ten *Xenopus* embryos were used in each co-IP. Embryos were homogenized (buffer: 20 mM Tris.HCl pH 8.0, 2.0 mM EDTA, 5.0 mM EGTA, 0.5% NP-40, 1x protease inhibitor cocktail [Roche]), incubated on ice 10 minutes, and centrifuged at 2,000 × g for 5 minutes at 4°C. Centrifuged lysates were removed from the pellet and pellicle and incubated with 4 µg rabbit anti-Foxh1 (ab49133, Abcam) overnight at 4°C mixing end-over-end on a hematological mixer. Lysate-antibody mixtures were then incubated with magnetic Dynabeads Protein G (Life Technologies) for 4–5 hours at 4°C on a hematological mixer. After incubation, beads were boiled in 4x sample reducing buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue). Samples were separated on NuPage 10% Bis-Tris gels (Life Technologies), transferred onto PVDF (0.45 µM, Millipore), and blocked with 7–10% non-fat dry milk dissolved in TBS-Tween (0.1%) for 30–45 minutes at room temperature. Membranes were incubated with monoclonal mouse anti-FLAG M2 (1:3,000; 200472, Agilent Technologies) or rabbit anti-Myc (1:3,000; 06-549, Millipore) in blocking solution overnight at 4°C. Specifically bound primary antibodies were detected with HRP-conjugated goat anti-mouse IgG₁ (1:7,500; sc2969, Santa Cruz Biotechnology) or Clean-Blot IP detection reagent HRP (1:4,000; 21230, Thermo Scientific) for 45 minutes at room temperature. Amersham ECL Prime western blotting detection reagent (GE Healthcare) was used for chemiluminescence and membranes were exposed to film (GenHunter) for various time periods.

4.5. In situ hybridizations

Embryos were staged based on morphology according to (Downs and Davies, 1993). Whole-mount *in situ* hybridization was as described (Belo et al., 1997), except antibody was used at 1:5,000, with DIG labeled *Nodal*, *Lefty2*, *Pitx2*, *Foxh1*, *Otx2*, *Brachyury/T*, *Gsc*, and *Foxa2* RNA probes. Developed embryos were imaged with a Leica M165 FC microscope.

4.6. Latex injections

Ventricles of E15.5 embryos were injected with colored, liquid latex (Connecticut Valley Biological Supply) as described (Oh and Li, 1997). Hearts were allowed to beat several times to help circulate latex before fixing whole embryos in 4% paraformaldehyde at 4°C. Embryos were imaged with a Leica M165 FC microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Foxh1-mediated repression of *Nodal* in gastrulation, left-right asymmetry addressed in mouse.
- Grg-binding motif (EH1) in Foxh1 altered to block Groucho (Grg) interaction.
- EH1 mutation greatly disrupts Foxh1 binding to Grg co-repressors.
- Disrupting Foxh1-Grg interaction does not alter *Nodal* expression or embryogenesis.
- Buffered regulatory system likely compensates for reduced Foxh1-Grg binding.

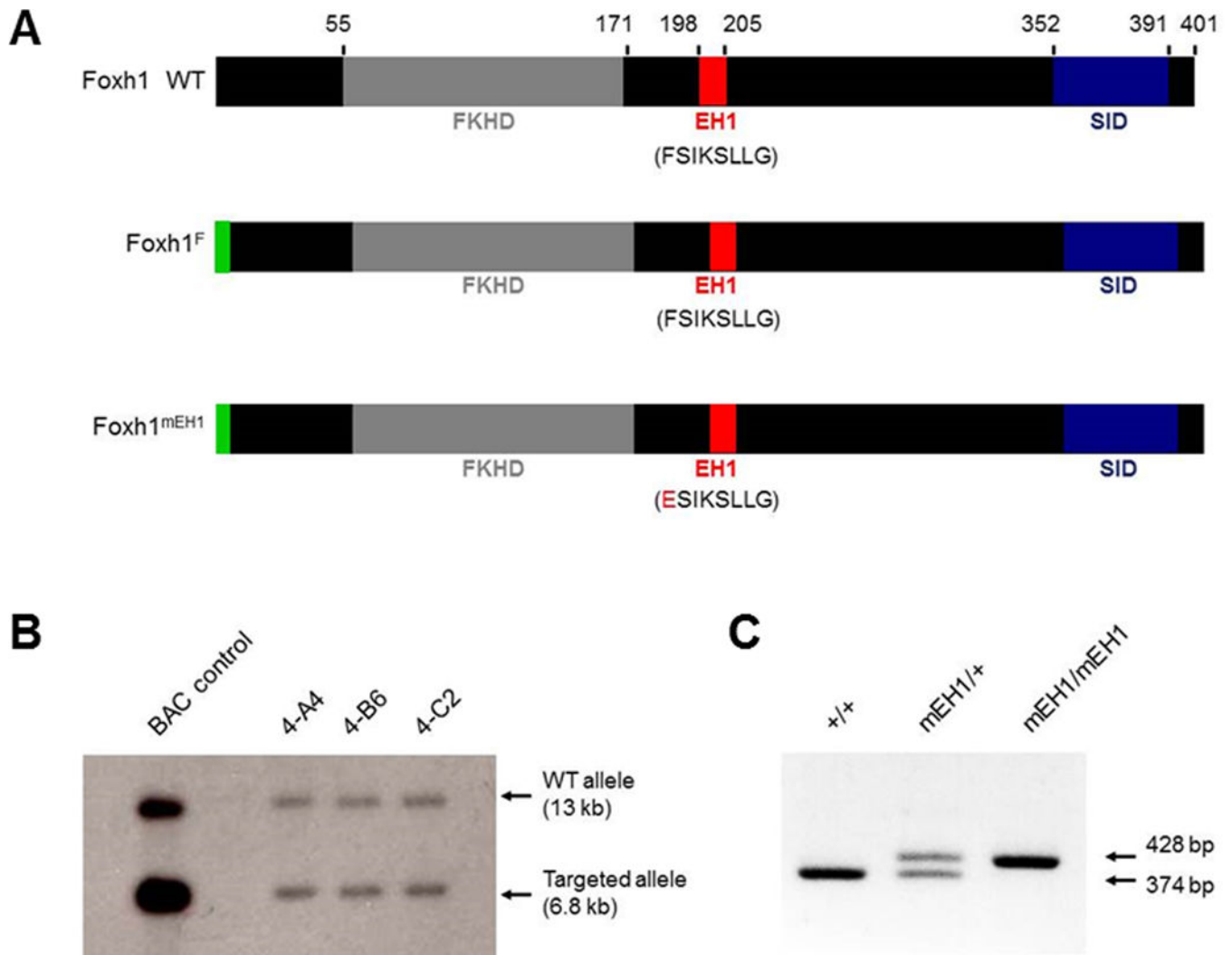


Fig. 1. Gene targeting and manipulation of Foxh1 EH1 domain. (A) The locations of FLAG tag (green), forkhead DNA-binding domain (gray), EH1 motif (red), and Smad-interacting domain (blue) in wild-type and variant Foxh1 proteins. Numbers denote amino acid position. Altered EH1 amino acids in Foxh1^{mEH1} are in red. (B) Southern blot analysis showing correct targeting of *Foxh1* locus in three independent mESC clonal cell lines heterozygous for *Foxh1*^{LCA} allele. (C) PCR analysis of offspring derived from *Foxh1*^{mEH1} chimeras detects two bands, 428 bp and 374 bp, for the *Foxh1*^{mEH1} and wild-type *Foxh1* alleles, respectively.

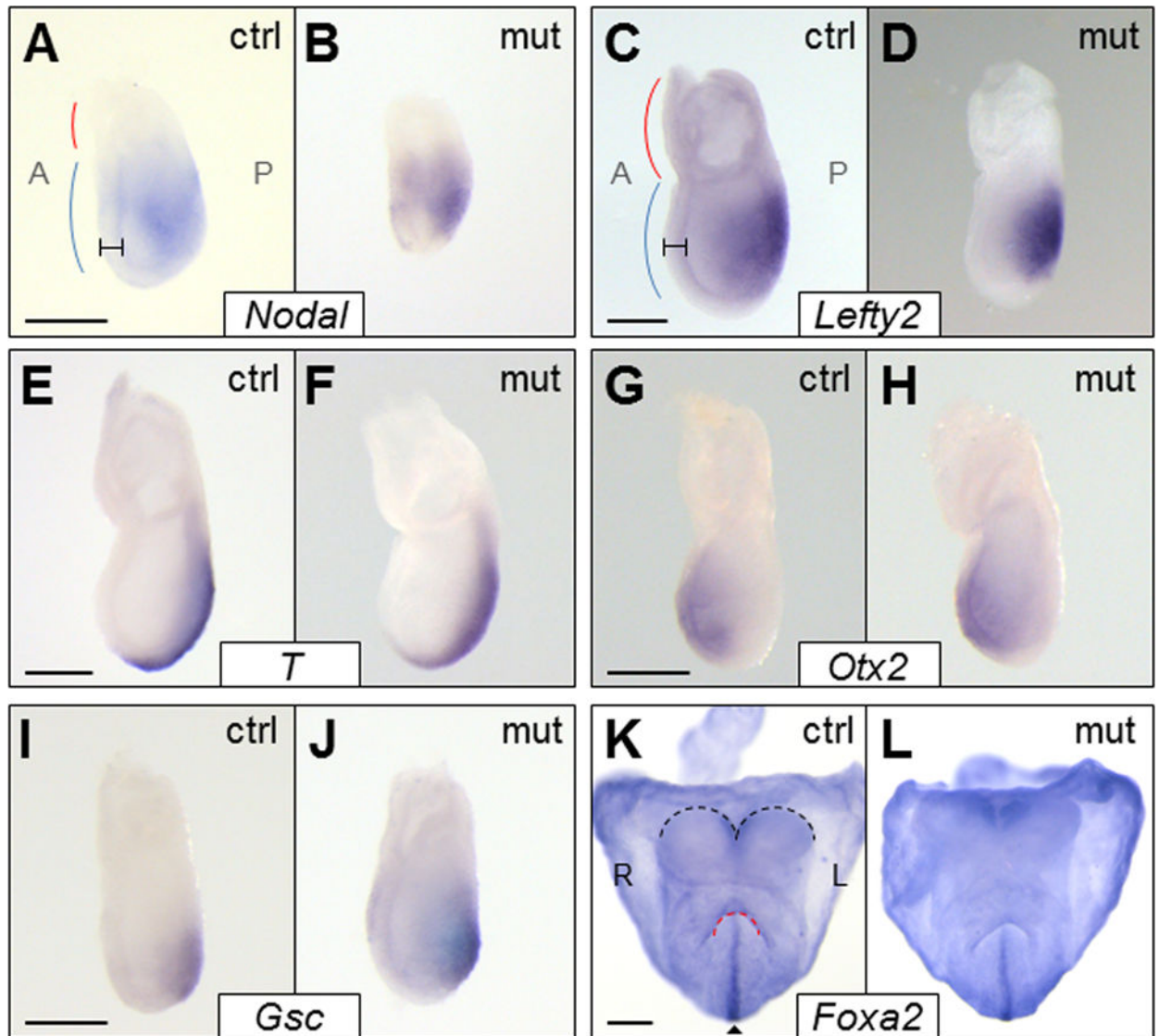


Fig. 2.

A-P patterning and gastrulation were unaffected in *Foxh1^{mEH1/mEH1}* embryos. Expression of multiple A-P patterning genes was examined by whole-mount *in situ* hybridization in control (ctrl represents wild-type or *Foxh1^{F/F}*) or *Foxh1^{mEH1/mEH1}* (mut) E6.75-E7.25 embryos. The expression patterns of *Nodal* (A,B), *Lefty2* (C,D), *Brachyury/T* (E,F), *Otx2* (G,H), and *Gsc* (I,J) were similar between control and *Foxh1^{mEH1/mEH1}* embryos. Red and blue lines mark representative location of extraembryonic tissue and embryo proper, respectively, in (A) E6.75 and (C) E7.25 embryos. Hash marks denote definitive endoderm. (K,L) *Foxa2* expression was unaffected in *Foxh1^{mEH1/mEH1}*, confirming midline (arrowhead) integrity in mutant embryos. Black and red lines outline headfolds and foregut pocket, respectively. Embryo orientations: (A–J) Lateral views. (K–L) Anterior views. Scale bars: 200 μ m. A, anterior; L, left; P, posterior; R, right.

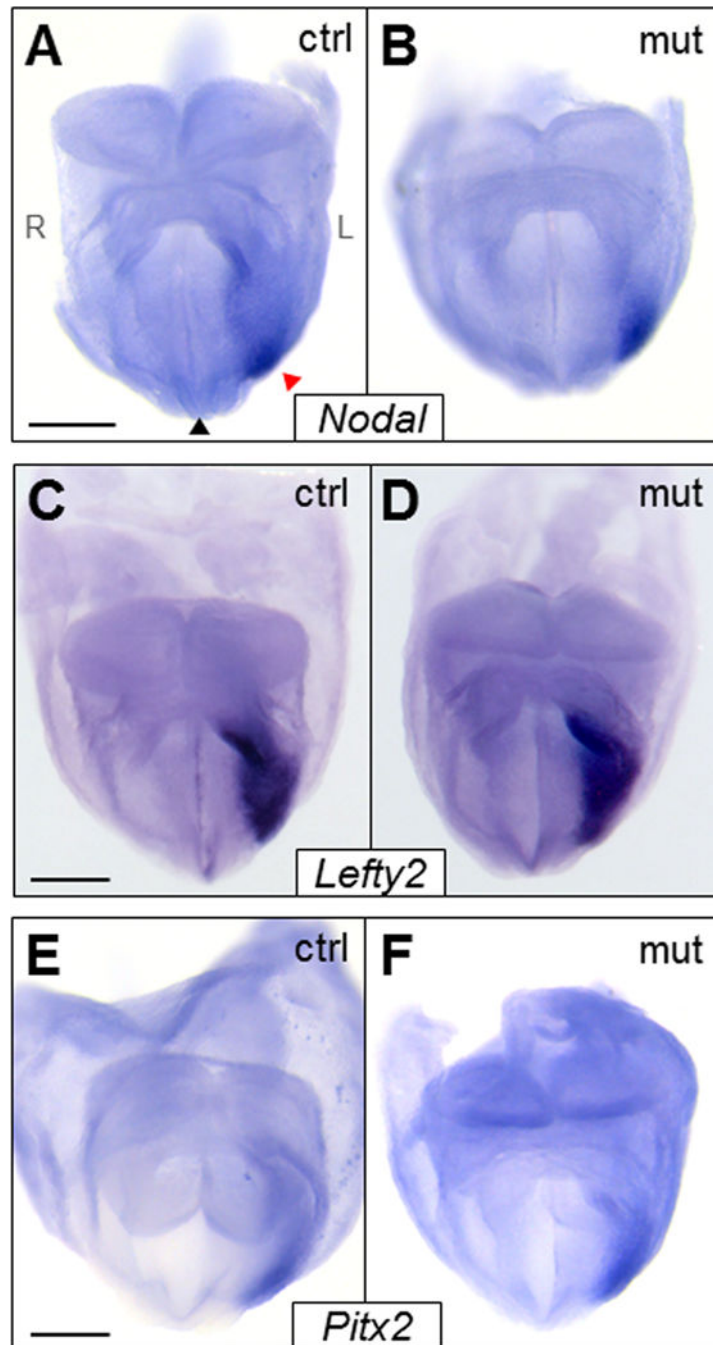
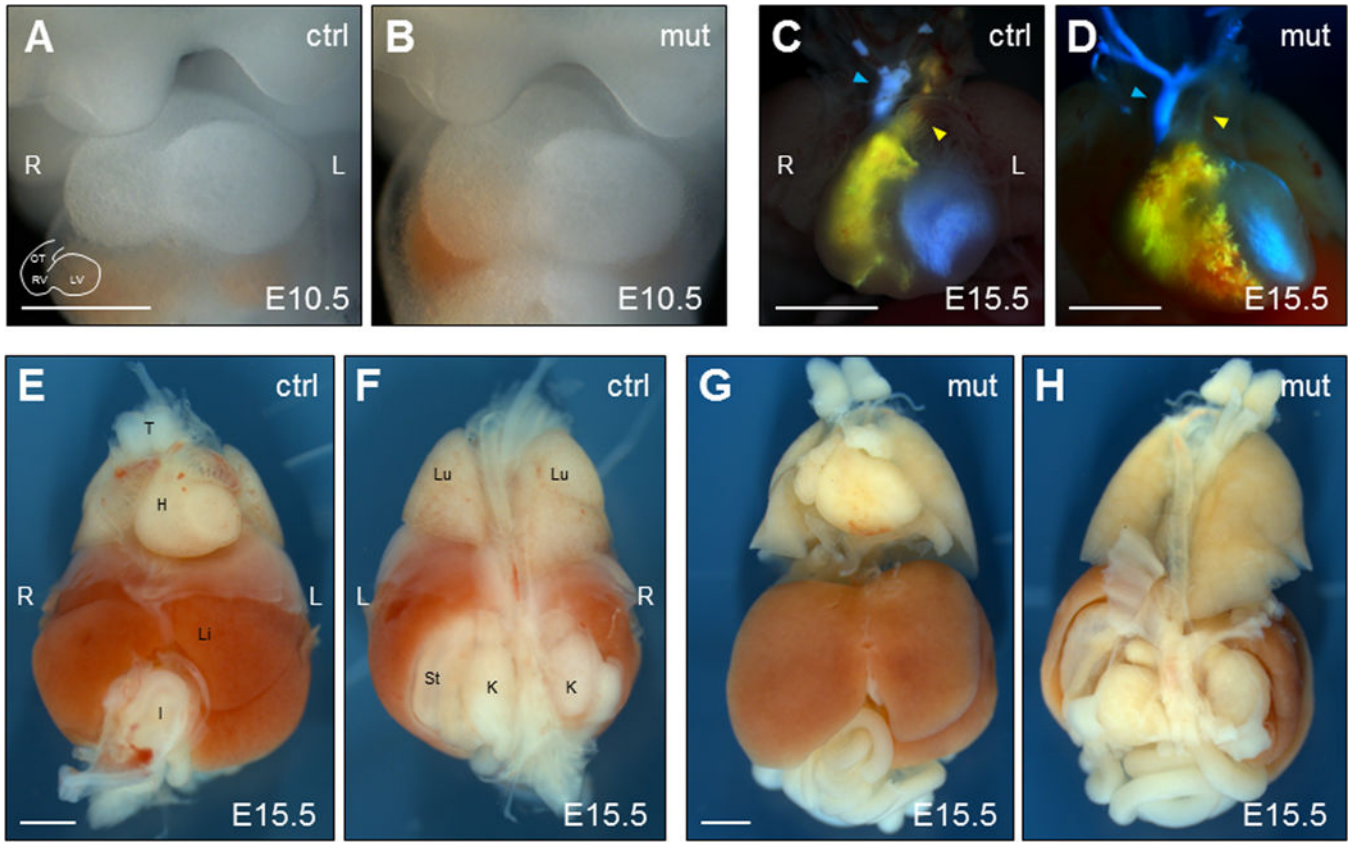


Fig. 3. Expression of L-R patterning genes was unaffected in *Foxh1*^{mEH1/mEH1} embryos. (A–F) Whole-mount *in situ* hybridization analysis of L-R patterning genes (A,B) *Nodal*, (C,D) *Lefty2*, and (E,F) *Pitx2* in the left LPM of control or *Foxh1*^{mEH1/mEH1} embryos at E8.25. No aberrant expression of L-R patterning genes was detected in mutant embryos. Black and red arrowheads indicate midline and LPM, respectively. Embryos orientations: (A–F) anterior views. Scale bars: 200 μ m. L, left; R, right.

**Fig. 4.**

Internal organ situs and morphology was normal in *Foxh1*^{mEH1/mEH1} embryos. (A,B) Normal dextral looping of the heart was observed in E10.5 *Foxh1*^{mEH1/mEH1} embryos. (C,D) Different colors of liquid latex were injected into the right (yellow) and left (blue) ventricles of E15.5 control and *Foxh1*^{mEH1/mEH1} embryos to highlight potential defects in ventricular septation, formation and positioning of the great arteries and aortic arch. No latex mixing, which would have indicated septation defects, was observed in *Foxh1*^{mEH1/mEH1} embryos. Also, the pulmonary trunk (yellow arrowhead) always appeared ventral to the aorta (blue arrowhead), and aortic branching was equivalent to control embryos. (E–H) Overall organ placement and morphology was unaffected in *Foxh1*^{mEH1/mEH1} embryos at E15.5. Embryo orientations: (A–E, G) Ventral views. (F, H) Dorsal views. Scale bars: (A,B) 500 μ m; (C–H) 1 mm. H, heart; I, intestine; K, kidney; L, left; Li, liver; Lu, lung; LV, left ventricle; OT, outflow tract; RV, right ventricle; St, stomach; T, thymus.