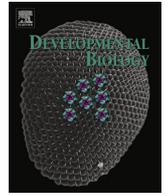




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FoxH1 mediates a Grg4 and Smad2 dependent transcriptional switch in Nodal signaling during *Xenopus* mesoderm development



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ABSTRACT

In the vertebrate blastula and gastrula the Nodal pathway is essential for formation of the primary germ layers and the organizer. Nodal autoregulatory feedback potentiates signaling activity, but mechanisms limiting embryonic Nodal ligand transcription are poorly understood. Here we describe a transcriptional switch mechanism mediated by FoxH1, the principle effector of Nodal autoregulation. FoxH1 contains a conserved engrailed homology (EH1) motif that mediates direct binding of groucho-related gene 4 (Grg4), a Groucho family corepressor. Nodal-dependent gene expression is suppressed by FoxH1, but enhanced by a FoxH1 EH1 mutant, indicating that the EH1 motif is necessary for repression. Grg4 blocks Nodal-induced mesodermal gene expression and Nodal autoregulation, suggesting that Grg4 limits Nodal pathway activity. Conversely, blocking Grg4 function in the ectoderm results in ectopic expression of Nodal target genes. FoxH1 and Grg4 occupy the Xnr1 enhancer, and Grg4 occupancy is dependent on the FoxH1 EH1 motif. Grg4 occupancy at the Xnr1 enhancer significantly decreases with Nodal activation or Smad2 overexpression, while FoxH1 occupancy is unaffected. These results suggest that Nodal-activated Smad2 physically displaces Grg4 from FoxH1, an essential feature of the transcriptional switch mechanism. In support of this model, when FoxH1 is unable to bind Smad2, Grg4 occupancy is maintained at the Xnr1 enhancer, even in the presence of Nodal signaling. Our findings reveal that FoxH1 mediates both activation and repression of Nodal gene expression. We propose that this transcriptional switch is essential to delimit Nodal pathway activity in vertebrate germ layer formation.

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1. Introduction

Nodal, a member of the TGF-beta superfamily of signaling molecules, initiates a critical signaling pathway in mesodermal and endodermal germ layer specification, organizer formation and

left-right patterning in all vertebrates [reviewed in Shen (2007)]. Loss of Nodal signaling in the mouse, zebrafish, or frog results in embryos that lack mesodermal and endodermal gene expression and subsequently fail to gastrulate (Agius et al., 2000; Conlon et al., 1991, 1994; Dougan et al., 2003; Gritsman et al., 1999; Larabell et al., 1996; Nagaso et al., 1999; New et al., 1997). Overexpression of Nodal ligands in the frog causes expansion of mesoderm at the expense of ectoderm, demonstrating that Nodal signaling must be excluded from the ectodermal region for proper embryonic patterning (Jones et al., 1995). Taken together, these results highlight the importance of the Nodal signaling pathway in establishing the embryonic germ layers and patterning the vertebrate axis.

Nodal functions in a concentration-dependent manner controlled by multiple positive and negative feedback mechanisms (Agius et al., 2000; Jones et al., 1995). In *Xenopus laevis*, Nodal signaling induces expression of the *Xenopus* Nodal-related ligand 1 (Xnr1) via an autoregulatory enhancer within the first intron (Osada et al., 2000). This autoregulatory loop amplifies small changes in signaling activity, requiring that the propagation of

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Nodal-ligand expression be limited to the mesendoderm in order to preserve proper patterning and germ layer formation. While several extracellular and intracellular inhibitors of Nodal signaling have been identified, it remains unknown how Nodal gene transcription is limited in germ layer formation.

Secreted Nodal ligands bind and activate heterodimeric receptor complexes, resulting in intracellular phosphorylation of the effector Smads, Smad2 and Smad3. Smad2/3, along with their co-Smad, Smad4, are recruited by the transcription factor FoxH1 to activate target genes [reviewed in Schier (2009)]. FoxH1 and Smads2/3/4 are maternally expressed ubiquitously throughout the blastula embryo (Chen et al., 1996; Chiu et al., 2014; Reid et al., 2012; Watanabe and Whitman, 1999). Morpholino knockdown of FoxH1 or inhibition of Smad2 activity in the zebrafish or frog greatly reduces mesendodermal gene expression and dramatically affects embryonic patterning (Hoodless et al., 1999; Kofron et al., 2004; Pei et al., 2007). Maternal knockdown of FoxH1 predictably decreases the expression of a number of mesodermal and organizer genes, but also increases the expression of two Nodal ligands, Xnr5 and Xnr6, revealing a repressive function for FoxH1 on select targets (Kofron et al., 2004). In the same study, FoxH1 activated a 3xARE (Activin response element) reporter at low concentrations, but repressed at higher concentrations, suggesting that FoxH1 can function as both a repressor and an activator depending upon dosage and context (Kofron et al., 2004). Consistent with a conserved role for FoxH1 in direct transcriptional repression, FoxH1 cooperates with Gsc to inhibit expression of Mix11 in the mouse gastrula (Hoodless et al., 2001). Recent comparisons of RNA-Seq and chromatin immunoprecipitation (ChIP-seq) studies in *Xenopus tropicalis* indicates that although FoxH1 and Smad2/3 positively regulate a number of Nodal target genes in the gastrula, FoxH1 also negatively regulates several genes at the same stage (Chiu et al., 2014). The molecular mechanisms that mediate the dual transcriptional output of FoxH1 have not previously been defined, and are the focus of this study.

Here we identify a previously undescribed mechanism for FoxH1-dependent repression in the Nodal signaling pathway. We and others have found that FoxH1 contains a conserved EH1 motif that mediates interaction with Grg4, a member of the Groucho family of corepressor proteins (Halstead and Wright, 2015; Yaklichkin et al., 2007b). Grg4, which is maternally expressed and ubiquitous in the early embryo, represses transcription through recruitment of histone deacetylases (HDACs) (Choudhury et al., 1997; Turki-Judeh and Courey, 2012). Misexpression of Grg4 blocks Nodal mediated gene expression and autoregulation, while inhibition of Grg4 activity leads to ectopic expression of Nodal target genes and aberrant mesoderm formation. We further provide evidence that FoxH1 mediates a transcriptional-switch mechanism; Smad2 displaces Grg4 and relieves repression at a FoxH1-bound enhancer upon initiation of Nodal signaling. This additional function of FoxH1 is likely essential to limit the spatial expression of mesendodermal genes during germ layer formation in the blastula and gastrula embryo.

2. Materials and methods

2.1. Embryo manipulation and microinjection

Xenopus embryos were collected, fertilized, injected and cultured as previously described (Yao and Kessler, 2001). Templates for *in vitro* transcription were pCS2-Xnr1 (Chen et al., 1996), pCS2-myc-FoxH1 (Chen et al., 1996), pCS2-myc-FoxH1A6 (this study), pGlo-myc-Grg4, pGlo-myc-Grg5 (Roose et al., 1998), pCS2-GST-FoxH1 (this study) and pCS2-GST-FoxH1A6 (this study), pCS2-FoxH1 (this study), pCS2-FoxH1A6 (this study), and pCS2-

FoxH1 Δ SID (this study). For HDAC-treated ectoderm, explants were prepared at the blastula stage and cultured in $0.5 \times$ MMR supplemented with either 2 mM valproic acid (VPA) or 2 mM sodium butyrate. Explants were cultured until the early gastrula stage and collected for RT-PCR analysis.

2.2. Plasmid constructs

The plasmid for pCS2-myc-FoxH1-A6 was generated using site directed mutagenesis of pCS2-myc-FoxH1. For pCS2-GST-FoxH1, pCS2-GST-FoxH1A6, pCS2-FoxH1 and pCS2-FoxH1A6, full-length open reading frames for wild type or mutant FoxH1 were amplified from pCS2-myc-FoxH1 or pCS2-myc-FoxH1A6 and inserted C-terminal to the GST tag in pCS2-GST (Yaklichkin et al., 2007a) or into the pCS2+vector. For pCS2-FoxH1 Δ SID, the Smad Interaction Domain (SID) of FoxH1 (Germain et al., 2000) was deleted from pCS2-FoxH1 using outward directed PCR and subsequent re-ligation of the resulting PCR product. All plasmids were verified by sequencing and *in vitro* translation assays, and immunohistochemistry and western blots when tagged.

2.3. Protein interaction assays

One-cell stage embryos were injected with mRNA encoding GST, GST-FoxH1, or GST-FoxH1A6 fusion proteins alone, or in combination with myc-Grg4 mRNA (Roose et al., 1998). The GST pull-down assay was performed as previously described (Yaklichkin et al., 2007a).

2.4. *In situ* hybridization, histology and immunocytochemistry

For whole-mount *in situ* hybridization, embryos were fixed and hybridized with antisense digoxigenin-labeled RNA probes as previously described (Bae et al., 2011; Pineda-Salgado et al., 2005). Templates for *in situ* probes were pBSSK-Xnr5 and pBSSK-Xnr6 (Takahashi et al., 2000), pCS2-Chd (Sasai et al., 1994), and pGEM-Xbra (Wilson and Melton, 1994). Embryos were scored for reduced or wild-type gene expression. Immunocytochemistry was performed as previously described (Sive et al., 2000) using the 9E10 anti-myc monoclonal antibody.

2.5. Reverse transcription-polymerase chain reaction

For RT-PCR, total RNA was isolated from ectodermal explants using the RNAqueous kit (Ambion), and cDNA synthesis and PCR were performed as described (Wilson and Melton, 1994). Radiolabeled PCR products were resolved on 5% native polyacrylamide gels. PCR primers and cycle parameters were as described for EF1 α , Xbra, Xwnt8, Gsc (Wilson and Melton, 1994), Xnr1, Xnr2 (Sampath et al., 1997), Xnr4 (Joseph and Melton, 1997) and Derriere (Sun et al., 1999). Quantitative PCR was performed as previously described (Blythe et al., 2009) using established primer sets for amplifying transcript from Xnr1, Xnr5, Xnr6 and Xbra (Kofron et al., 1999; Sun et al., 1999). Primers for Chd were from the *Xenopus* Molecular Marker Resource (Xenbase). Statistical analysis of quantitative PCR data, as well as all other quantitative data in this study, was performed using the Student's *t*-test1.

2.6. Luciferase reporter assay

One-cell stage *Xenopus* embryos were injected in the animal pole with *in vitro* transcribed mRNA encoding myc-FoxH1, myc-Grg4, or myc-FoxH1A6. At the two-cell stage, one blastomere was injected with 100 pg of pGL3-3XARE-Luciferase (Chen et al., 1996; Vize, 1996) containing firefly luciferase under the control of a multimerized Mix.2 Activin Response Element in combination

with 10 pg of pGL3-CMV-*Renilla* as an internal control. Luciferase activity was assayed as previously described (Bae et al., 2011).

2.7. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (Blythe et al., 2009). One-cell embryos were injected with 250 pg of myc-FoxH1 mRNA or 8 ng myc-XGrg4 mRNA. An average of 65 embryos were collected at stage 10.25 and processed for ChIP. Immunoprecipitation was performed using polyclonal anti-myc antibody (Millipore, 06-549). Quantitative PCR was performed as described (Blythe et al., 2009) using primers designed to amplify the *Xnr1* intronic region 1 (Osada et al., 2000) (sequence: F: 5'-CACTGCTGTCTGAATGTTGGCCT-3' and R: 5'-AATTAGCCTGTCAACTGGGAACG-3') or a genomic region of the *Xnr1* 3' untranslated region (*Xnr1* 3'UTR) (sequence: F: 5'-AAGTTGTACCAACCCGAACGAGTG-3' and R: 5'-CAATCATCTCTTGGTGGTGCCCTCA-3'). Primers for *Ef1α* and *Xmcl2* were previously described (Blythe et al., 2009).

3. Results

3.1. FoxH1 and Grg4 physically interact via the EH1 motif

The presence of a highly conserved EH1 motif known to interact with Groucho family proteins suggests that FoxH1 may directly interact with the *Xenopus* Groucho corepressor, Grg4 (Fig. 1A) (Yaklichkin et al., 2007b). To confirm this interaction, mRNA encoding GST-tagged FoxH1 was injected into whole embryos along with mRNA encoding myc-tagged Grg4. Protein complexes were recovered from gastrula extracts with glutathione beads and the presence of GST-FoxH1 and myc-Grg4 were evaluated by Western blot (Fig. 1B). Detection of myc-Grg4 in the GST-FoxH1 precipitates demonstrates a physical interaction between Grg4 and FoxH1 (Fig. 1B). To determine if the EH1 motif of FoxH1 mediates this interaction, six of the seven residues within this motif were mutated to alanine to generate FoxH1A6 (Fig. 1A). The interaction between GST-FoxH1A6 and myc-Grg4 was greatly reduced, despite similar expression levels of GST-FoxH1 and GST-FoxH1A6 (Fig. 1B). Mutation of the EH1 motif did not alter the nuclear localization of FoxH1A6 (Suppl. Fig. 1A). These results demonstrate that the conserved EH1 motif mediates a direct physical interaction between FoxH1 and Grg4.

3.2. FoxH1 functions as an inhibitor of Nodal gene expression

The FoxH1-Grg4 interaction suggests that FoxH1 inhibits Nodal gene expression. Therefore, it would be expected that FoxH1 overexpression within the vegetal pole, an embryonic region high in Nodal activity [reviewed in Kimelman (2006)], would repress Nodal-dependent gene expression. A further prediction is that FoxH1A6, a mutant form of FoxH1 unable to interact with Groucho corepressors, would relieve repression resulting in upregulation of FoxH1 target genes. To test these predictions, mRNAs encoding myc-FoxH1 or myc-FoxH1A6 were injected into the vegetal pole of one-cell stage embryos. Expression of FoxH1 and FoxH1A6 was evaluated by Western blot to verify equivalent expression levels (Suppl. Fig. 1B). Nodal target gene expression was examined at the blastula and gastrula stages by quantitative RT-PCR and *in situ* hybridization (Fig. 2A–C). Expression of wild type FoxH1 significantly reduced levels of the early-expressed Nodal genes, *Xnr5* and *Xnr6*, at the late blastula stage as assayed by both qPCR (Fig. 2A) and *in situ* hybridization (Fig. 2B). When embryos expressing wild-type FoxH1 were evaluated at the gastrula stage, we found a significant decrease in expression of *Xnr1* by quantitative

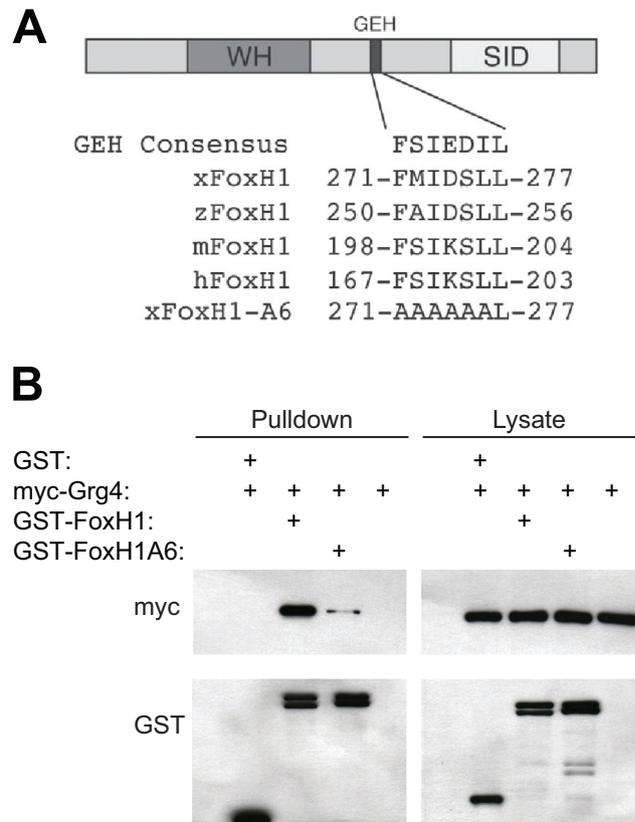


Fig. 1. FoxH1 contains a conserved EH1 motif that mediates a physical interaction with Grg4. (A) A diagram of the FoxH1 protein shows the location of the EH1 motif between the winged helix (WH) DNA binding domain and the Smad Interaction Domain (SID). This motif is highly conserved among multiple species (*Xenopus*, zebrafish, mouse and human). In the FoxH1A6 mutant, 6 of the 7 residues that comprise the EH1 motif have been mutated to alanine. (B) FoxH1 physically interacts with Grg4. mRNA encoding GST, GST-FoxH1 or GST-FoxH1A6 (1 ng) was injected along with myc-Grg4 mRNA (5 ng) into single cell embryos, which were collected at the gastrula stage for GST pulldown assays (Left). Top panel represents an anti-myc western blot, showing the physical interaction of myc-Grg4 with GST-FoxH1 (lane 3), which is reduced with mutation of the EH1 motif in FoxH1A6 (lane 4). Lower panel is an anti-GST western indicating recovery of GST proteins by pulldown. (Right) Control western blots showing approximately equal expression of myc-tagged and GST-tagged proteins in the starting lysates.

RT-PCR (Fig. 2A), suggesting that FoxH1 can negatively regulate Nodal expression. Similarly, the expression of mesodermal genes downstream of Nodal, including *Brachyury* (*Xbra*), *Chordin* (*Chd*) and *Gooseoid* (*Gsc*), was reduced (Fig. 2A and C), indicating that FoxH1 inhibits both Nodal autoregulation and Nodal-dependent mesodermal gene expression. In contrast, expression of an equal dose of FoxH1A6 (Suppl. Fig. 1B) resulted in a reproducible increase in the expression of *Xnr5* and *Xnr6*—albeit not to the level of statistical significance—and had no effect on expression of *Xnr1*. Overexpression of FoxH1A6 had no effect on *Chd* and *Gsc* expression, and resulted in a slight increase in the expression of *Xbra* (Fig. 2A and C). As predicted by these experiments, ventral expression of FoxH1 does not affect dorsoanterior development, whereas ventral expression of FoxH1A6 weakly dorsalized embryos (DAI=6, data not shown). Taken together, the results suggest that FoxH1 recruits Grg4 via the EH1 motif to repress Nodal target genes.

3.3. Grg4 inhibits Nodal pathway activity

To determine whether Grg4 is sufficient to repress Nodal pathway activity, Nodal induction of mesodermal target genes was assessed in the presence of overexpressed Grg4 (Fig. 3A).

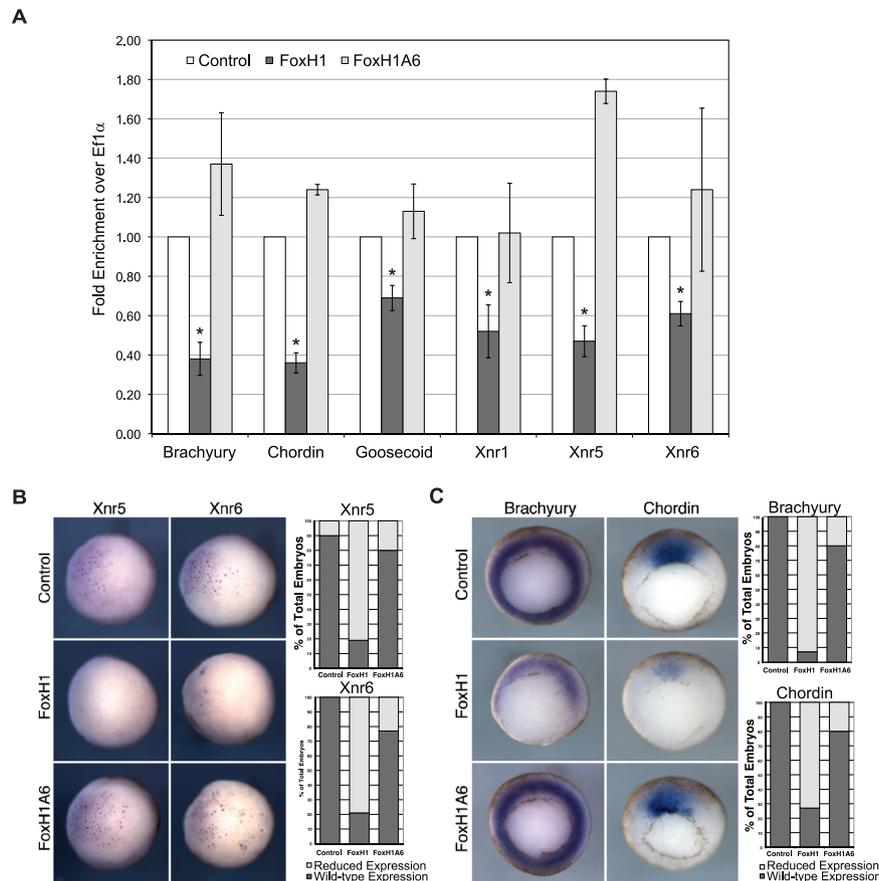


Fig. 2. FoxH1 represses Nodal target genes at blastula and gastrula stages. (A) FoxH1, but not FoxH1A6, represses expression of endogenous mesodermal and Nodal genes. Embryos were injected in the vegetal pole with mRNA encoding myc-FoxH1 or myc-FoxH1A6 (250 pg). cDNA was prepared from pooled whole embryos collected at blastula stage (for Xnr5 and Xnr6) and gastrula stage (for Xbra, Chd and Gsc). qPCR was performed for the mesodermal marker *Brachyury*, the organizer genes *Chordin* and *Goosecoid*, and the Nodal ligands Xnr1, Xnr5 and Xnr6. The data presented are the combined results of three independent biological replicates, and have been normalized to expression of a housekeeping gene, *Efl1α*. Error bars represent standard error. * indicates $p < 0.05$ by Student's *t*-test. (B) FoxH1, but not FoxH1A6, inhibits expression of Xnr5 and Xnr6 in the blastula vegetal pole. Embryos injected in the vegetal pole with mRNA encoding myc-FoxH1 or myc-FoxH1A6 (250 pg) were collected at the late blastula stage and subjected to *in situ* hybridization for either Xnr5 or Xnr6. (C) FoxH1, but not FoxH1A6, blocks expression of Xbra and Chd in the gastrula. Embryos were injected in the vegetal pole with mRNA encoding myc-FoxH1 or myc-FoxH1A6 (250 pg) and collected at the mid-gastrula stage. *In situ* hybridization was performed to analyze expression of Xbra and Chd. 100 embryos were analyzed for each condition, and the percentage of embryos displaying normal (dark gray bar) or reduced (light gray bar) expression was quantified in the bar graphs to the right. Expression of FoxH1 reduced the expression of Xnr5, Xnr6, Xbra and Chd.

mRNA encoding the Nodal ligand Xnr1 alone or combined with mRNA encoding myc-Grg4 was injected in the animal pole of one-cell stage embryos. Ectodermal explants were prepared at the early blastula stage, cultured until the gastrula stage, and assessed for mesodermal gene expression by RT-PCR (Fig. 3A). Xnr1 induced expression of Nodal ligands, including Xnr1, Xnr2, Xnr4 and *Derriere* (*Der*) (Fig. 3A). Xnr1 also induced expression of Xbra, Xwnt8, and Gsc (Fig. 3A). Co-expression of myc-Grg4 dramatically reduced expression of all Nodal-induced genes and disrupted Nodal autoregulatory activation of Xnr1 transcription (Fig. 3A). Grg4 repressed not only Nodal gene expression, but also downstream mesoendodermal genes. Mesoderm induction by eFGF, a Smad-independent signaling pathway, was unaffected by coexpression of myc-Grg4, confirming that Grg4 acts specifically within the Nodal pathway (Suppl. Fig. 2).

Nodal target gene expression must be excluded from the ectodermal germ layer to maintain proper germ layer patterning and organization (Jones et al., 1995). To determine whether Grg4 represses Nodal pathway activity in the ectoderm and prevents inappropriate activation of mesodermal genes, Groucho-related gene 5 (Grg5) was used to antagonize Grg4 function (Beagle and Johnson, 2010; Brantjes et al., 2001). Grg5 lacks the canonical HDAC-recruitment “WD” domain of Grg4, and thus, relieves Grg4-dependent repression, resulting in upregulation of Grg4 target

genes (Brantjes et al., 2001). Unlike myc-Grg4, myc-Grg5 robustly induced expression of Xbra, Xwnt8, Gsc, and the Nodal ligands Xnr1 and *Der* in ectodermal explants (Fig. 3A and B). The Grg5-expressing explants underwent convergent extension and elongated, consistent with mesodermal cell fate, while control or Grg4-expressing explants remained spherical and formed atypical epidermis (Fig. 3C) (Symes and Smith, 1987). The induction of mesoderm by Grg5 is consistent with a role for Grg4 in ectodermal inhibition of Nodal autoregulation and downstream mesodermal gene activation.

The ability of both FoxH1 and Grg4 to repress Nodal target genes suggests that these proteins cooperate to directly repress FoxH1-dependent transcription. Luciferase assays using the 3xARE reporter—comprising three tandem Activin/Nodal response elements from the Mix.2 enhancer driving expression of luciferase—were performed in ectodermal explants lacking endogenous Nodal activity (Chen et al., 1996; Vize, 1996). FoxH1 mRNA injection resulted in a nearly 3-fold increase in reporter activity compared to controls (Fig. 3D). Previous studies also found that FoxH1 expression alone can activate expression of the 3xARE reporter (Kofron et al., 2004), which we suggest is due to weak recruitment of endogenous Smad2/3 by FoxH1 even in the absence of Nodal signaling. Coexpression of Grg4 with FoxH1 abolished 3xARE reporter activation (Fig. 3D). Similar to FoxH1, FoxH1A6 increased

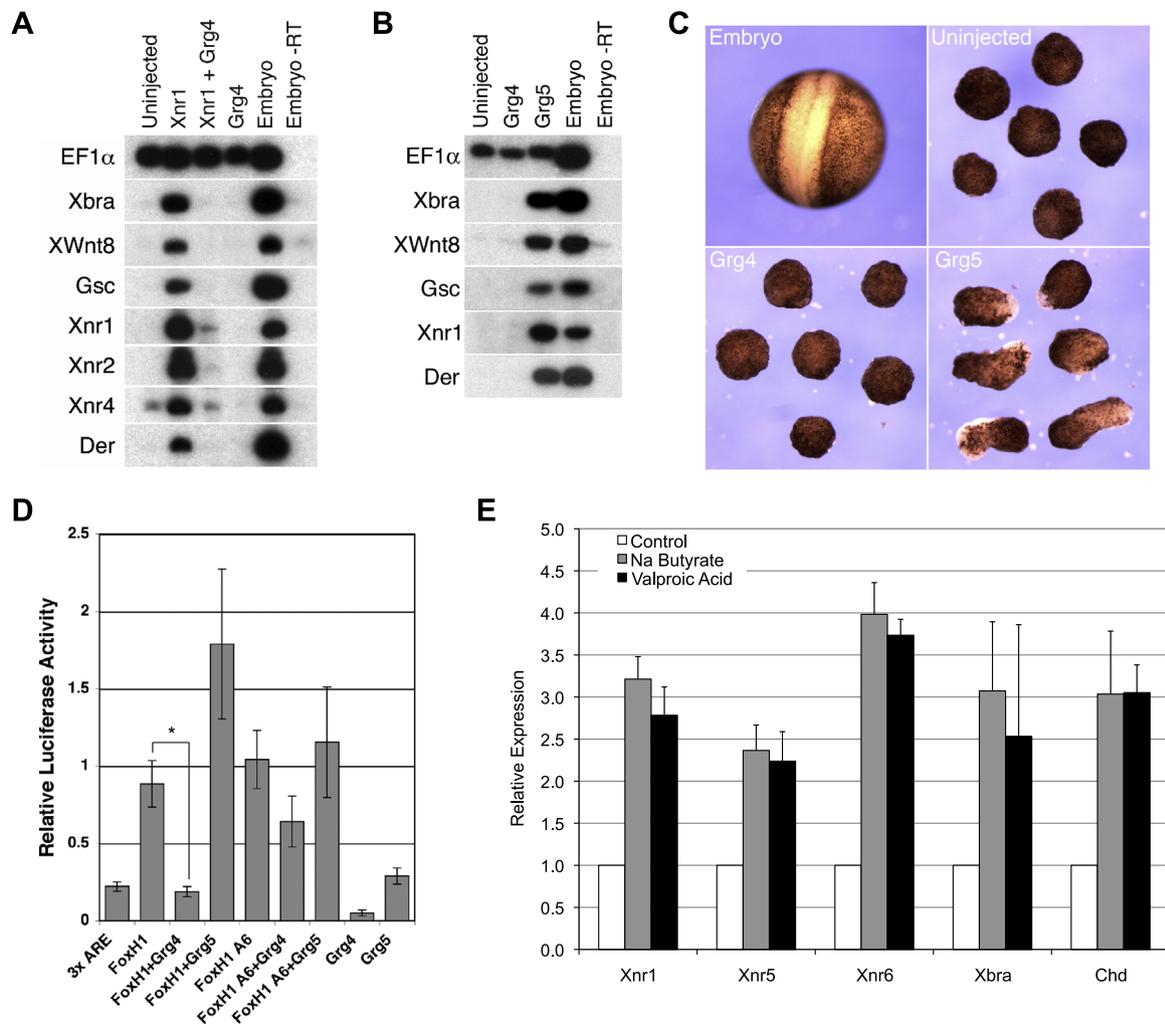


Fig. 3. Grg4 inhibits Nodal-dependent mesoderm induction. (A) mRNA encoding Xnr1 (30 pg) or mRNA encoding myc-Grg4 (5 ng), or a combination of the two, were injected into the animal pole of one-cell stage embryos. Animal caps were prepared at blastula stage and analyzed for the expression of mesodermal and Nodal gene expression by RT-PCR at gastrula stage. Xnr1 induced expression of Xbra, Xwnt8 and the organizer gene Gsc, as well as Xnr1, Xnr2, Xnr4 and Der. Coexpression of myc-Grg4 blocked the upregulation of all these genes. Ef1 α served as a control for RNA recovery and loading controls. Uninjected animal caps and PCR from a cDNA sample made without reverse transcriptase (Embryo-RT) showed no amplification. Whole embryo cDNA was used as a positive control. (B) Grg5 expression is sufficient to induce mesoderm in animal pole explants. mRNA encoding myc-Grg4 or myc-Grg5 (5 ng) was injected into the animal pole at the one-cell stage, and explants were isolated as described. Grg5 induced the mesodermal genes, as well as Xnr1 and Der. (C) Grg5 expression is sufficient to induce convergent extension. mRNA encoding myc-Grg4 or myc-Grg5 (5 ng) was injected into the animal pole of one-cell stage embryos. Explants were isolated at the blastula stage and allowed to develop until the neurula stage. Pictured is an unmanipulated embryo, which serves as a control for staging, and representative control, myc-Grg4, and myc-Grg5 injected explants. (D) Grg4 and Grg5 modulate the expression of a FoxH1-dependent reporter. The 3xARE luciferase reporter plasmid (100 pg) was injected along with a Renilla luciferase control plasmid (10 pg) at the one-cell stage, followed by single-blastomere injection at the two cell stage with mRNA encoding myc-FoxH1 or myc-FoxH1A6 (250 pg) alone, or in combination with myc-Grg4 or myc-Grg5 mRNA (5 ng). The 3xARE reporter alone served as a control for basal activity. Data shown represents four independent experiments and error bars represent standard error. * indicates $p < 0.05$ as compared to FoxH1 alone. (E) HDAC inhibition induces Nodal and mesodermal gene expression in ectoderm. Animal explants prepared from blastula embryos were cultured for 2 h in media containing 2 mM sodium butyrate (Na Butyrate – gray bars) or 2 mM valproic acid (black bars). cDNA was prepared from treated and untreated caps and qPCR was performed to assay expression of the Xnr1, Xnr5, Xnr6, Xbra, and Chd. Gene expression is normalized to Ef1 α and is shown as fold increase in expression over untreated caps. Error bars represent standard error in four independent experiments.

expression of luciferase 4-fold (Fig. 3D). However, unlike wild-type FoxH1, coexpression of Grg4 had little effect on FoxH1A6-induced reporter expression (Fig. 3D). If FoxH1 recruits Grg4 to regulate the 3xARE reporter, the coexpression of Grg5 would be expected to relieve this repression. Coexpression of FoxH1 and Grg5 induced higher activity than that observed for FoxH1 alone (Fig. 3D). Coexpression of Grg5 with FoxH1A6 did not change reporter activity (Fig. 3D), consistent with a requirement for the EH1 motif in Groucho-mediated gene regulation. Similar results were obtained using the Xnr1 intron 1 enhancer-luciferase reporter (Osada et al., 2000) (Suppl. Fig. 3). These results demonstrate that FoxH1 and Grg4 interaction *via* the EH1 motif to represses gene expression.

Groucho proteins repress gene expression through HDAC recruitment (Brantjes et al., 2001). To verify that a Groucho-HDAC complex regulates mesodermal gene expression in the ectoderm,

ectodermal explants were isolated at the blastula stage and were cultured with HDAC inhibitors valproic acid (VPA) or sodium butyrate. Treatment of ectodermal explants with VPA or sodium butyrate significantly enhanced expression of the Nodal-related genes Xnr1, Xnr5, and Xnr6 and increased expression of the Nodal target genes Xbra and Chd, indicating a role for HDAC activity in the repression of mesodermal genes that are targets of Nodal signaling (Fig. 3E). This requirement for HDAC activity in the repression of Nodal target genes is consistent with a FoxH1-Grg4-dependent mechanism for target gene repression.

3.4. FoxH1 and Grg4 occupy the Xnr1 enhancer

FoxH1 is thought to activate transcription only when bound by activated Smad2/3 in response to Nodal signaling. The physical

and functional interaction between FoxH1 and Grg4 identified here suggests that a FoxH1-Grg4 repressive complex occupies Nodal target enhancers in the absence of Nodal signaling. Such repressor activity would be particularly important in the ectodermal germ layer to prevent ectopic mesoderm induction through Nodal autoregulation (Osada et al., 2000). The Nodal autoregulatory loop amplifies low-level Nodal signals, and if not properly regulated could cause an increase of Nodal activity and expansion of mesendoderm into the ectodermal domain. A FoxH1-Grg4 complex would be expected to occupy the intron 1 enhancer of *Xnr1* in the absence of Nodal activity, and be displaced by formation of a FoxH1-Smad2/3 activation complex in response to Nodal signals. To establish that FoxH1, Grg4 and Smad2/3 can occupy the same genomic location within the endogenous *Xnr1* enhancer, chromatin immunoprecipitation (ChIP) was performed with whole *Xenopus* embryos (Blythe et al., 2009). mRNAs encoding myc-FoxH1 and myc-Grg4 were expressed in one-cell embryos, which were fixed at the early gastrula stage. Immunoprecipitation was performed against the myc tag, followed by qPCR with primers flanking three FoxH1 binding sites within the *Xnr1* enhancer. ChIP results demonstrated that both FoxH1 and Grg4 occupy the *Xnr1* enhancer (Fig. 4A and B). ChIP of uninjected embryos using an antibody recognizing endogenous Smad2/3 confirmed that Smad2/3 occupy the same genomic region within the *Xnr1* enhancer (Suppl. Fig. 4A).

To further test the hypothesis that FoxH1 mediates the interaction of Grg4 with the *Xnr1* enhancer, ChIP was performed on embryos expressing myc-Grg4 in combination with FoxH1 or FoxH1A6. Myc-Grg4 occupies the *Xnr1* enhancer with a 2-fold enrichment over the control *Xnr1* 3'UTR (Fig. 4C). Grg4 occupancy is further increased (~5-fold) when mRNA encoding FoxH1 is coexpressed (Fig. 4C). Coexpression of myc-Grg4 with FoxH1A6, however, did not result in a significant increase of Grg4 occupancy at the *Xnr1* enhancer (Fig. 4C), indicating that the FoxH1 EH1 motif mediates Grg4 occupancy at the *Xnr1* enhancer.

To confirm that the Smad Interaction Domain (SID) of FoxH1 mediates the interaction of Smad2/3 with the *Xnr1* enhancer (Germain et al., 2000), FoxH1 or FoxH1ΔSID was expressed in whole embryos, followed by ChIP for endogenous Smad2/3 (Suppl. Fig. 4B). In unmanipulated embryos, Smad2/3 had a 6-fold

enrichment in occupancy at the *Xnr1* enhancer that was further enriched to approximately 11-fold with coexpression of FoxH1 (Suppl. Fig. 4B). Expression of FoxH1ΔSID, however, did not enhance Smad2/3 occupancy, which remained similar to that observed in unmanipulated embryos (Suppl. Fig. 4B). A similar result was obtained using myc-Smad2 coexpressed with FoxH1 or FoxH1ΔSID (data not shown). Myc-Smad2 occupancy increased about 2-fold in the presence of untagged FoxH1, but did not increase with coexpression of FoxH1ΔSID (data not shown). FoxH1 thus mediates the interactions of both the corepressor Grg4 and the coactivator Smad2/3 with the *Xnr1* enhancer, supporting a dual function for FoxH1 as either a repressor or activator depending on the interacting coregulator.

3.5. FoxH1 acts as a transcriptional switch in Nodal autoregulation

The ability of FoxH1 to function as both a transcriptional activator and repressor points to a role as a transcriptional switch in the Nodal pathway. To determine whether the association of Grg4 with FoxH1 at the *Xnr1* enhancer is disrupted by Nodal pathway activation, ChIP was performed for myc-FoxH1, myc-Grg4, or endogenous Smad2/3 in the absence or presence of exogenous Nodal signals. FoxH1 occupancy of the *Xnr1* enhancer increased slightly in response to pathway activation from 70-fold to 120-fold enrichment (Fig. 5A). As expected, Smad2/3 occupancy significantly increased in response to Nodal signals (7-fold to 18-fold enrichment) (Fig. 5C). Occupancy by p300, a histone-modifying enzyme known to bind Smad2/3, was similarly enhanced in response to Nodal activity (Suppl. Fig. 4C) (Ross et al., 2006; Tu and Luo, 2007). Conversely, Nodal pathway activation significantly reduced myc-Grg4 occupancy at the *Xnr1* enhancer (7-fold to 3-fold enrichment) (Fig. 5B). This signal-dependent recruitment of a coactivator and displacement of a corepressor are core features of the transcriptional switch mechanism.

The presence in FoxH1 of a SID and an EH1 motif (Fig. 1A) suggests that Smad2/3 interacts with Grg4 when binding to FoxH1 to physically displace the corepressor. Smad2 overexpression, in the absence of exogenous Nodal signals, has previously been shown to activate mesoendodermal gene expression (Hoodless et al., 1999). To determine if Smad2 overexpression is sufficient to

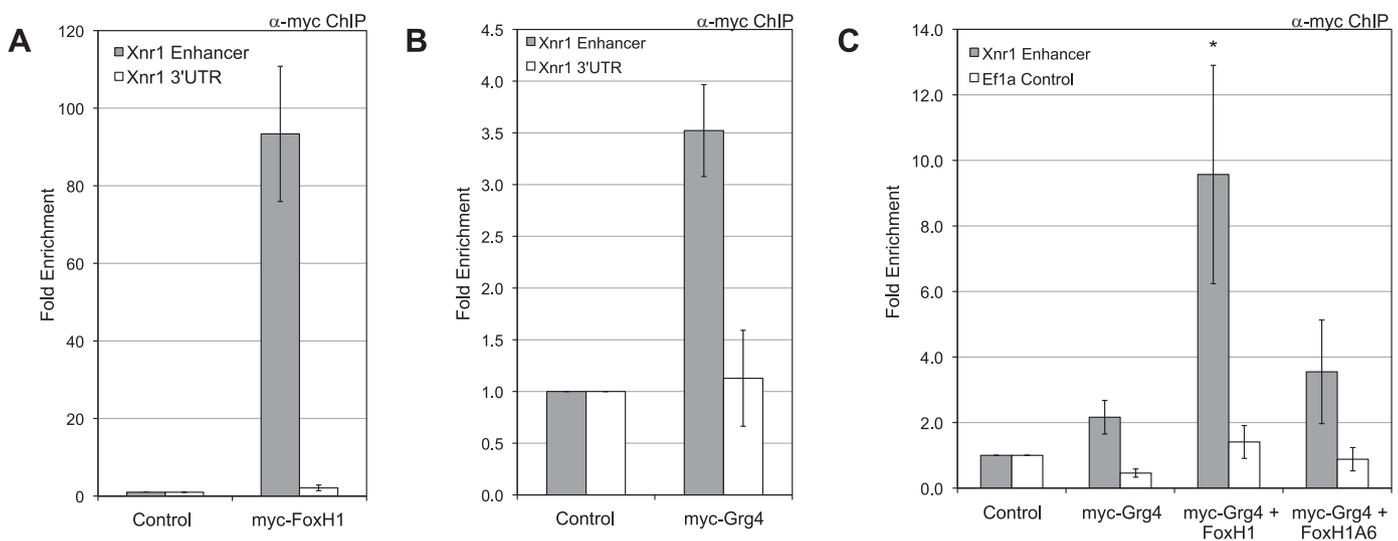


Fig. 4. Grg4 occupies the endogenous *Xnr1* Enhancer through interaction with the FoxH1 EH1 motif. Occupancy at the *Xnr1* enhancer was evaluated by chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) of embryos injected with (A) 250 pg myc-FoxH1 or (B) 8 ng myc-Grg4. Immunoprecipitation using anti-myc antibody was also performed on uninjected embryos (Control). Each result shown represents three independent experiments. The white bars represent qPCR for genomic *Xnr1* 3'UTR as a control. (C) Genomic DNA fragments recovered by ChIP from embryos injected with myc-Grg4 alone (8 ng) or in combination with untagged FoxH1 or FoxH1A6 (250 pg) were evaluated by qPCR for the *Xnr1* Intron 1 enhancer (gray bars). The white bars represent qPCR for genomic *Ef1a* as a control. The data shown represent three independent biological replicates. * indicates that $p < 0.05$ as compared to myc-Grg4 alone.

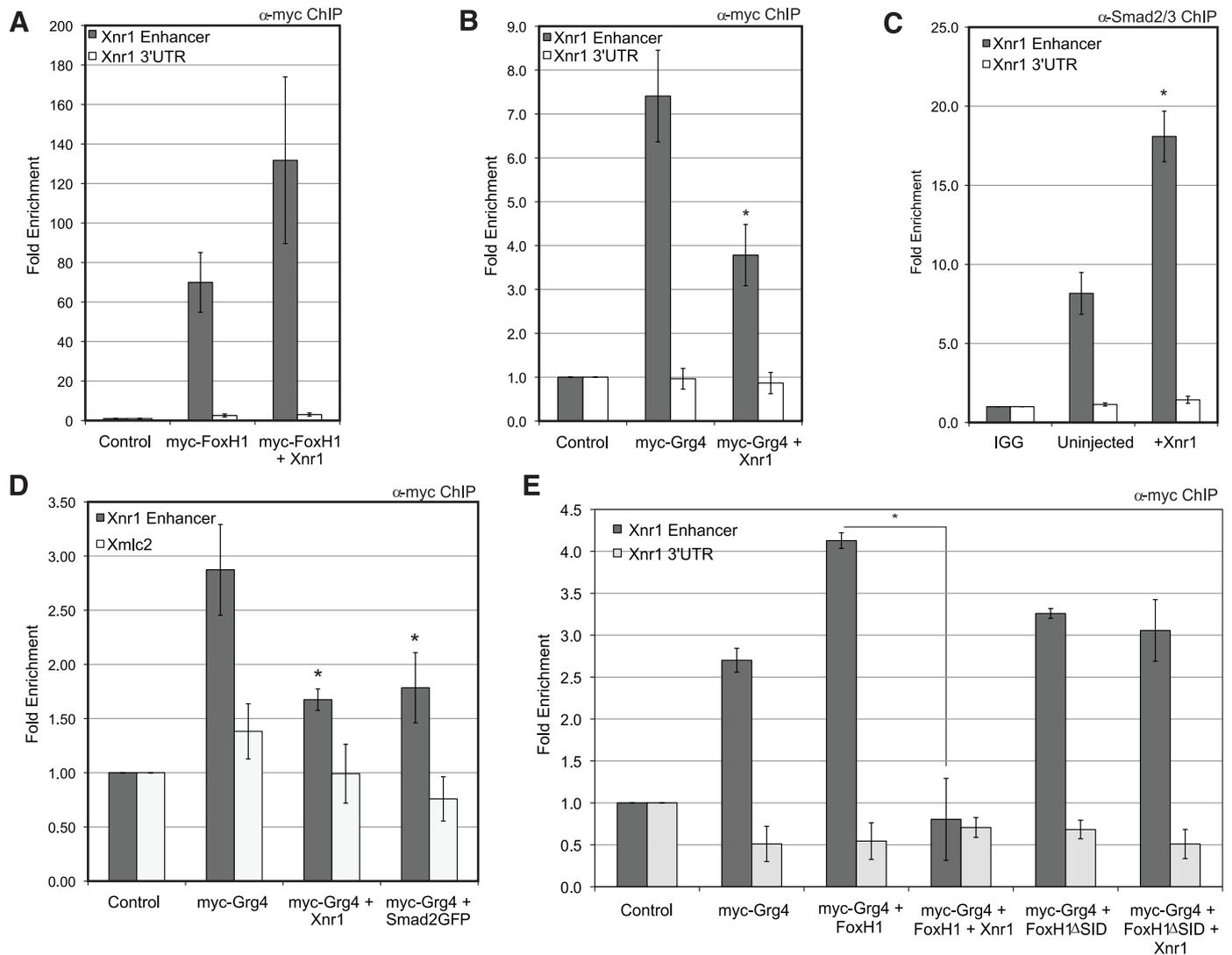


Fig. 5. Grg4 occupies the Xnr1 enhancer in a Nodal-responsive manner. (A) ChIP for myc-FoxH1 alone or coexpressed with Xnr1 (myc-FoxH1 + Xnr1) or (B) ChIP for myc-Grg4 alone or coexpressed with Xnr1 (myc-Grg4 + Xnr1) was evaluated by qPCR for enrichment of the Xnr1 enhancer. (C) ChIP for endogenous Smad2/3 in uninjected embryos or embryos expressing 50 pg Xnr1 mRNA (+Xnr1) was evaluated by qPCR for the Xnr1 enhancer. Rabbit IGG ChIP serves as a negative control (IGG). (D) ChIP for myc-Grg4 alone, or coexpressed with Xnr1 (myc-Grg4 + Xnr1) or Smad2GFP mRNA (myc-Grg4 + Smad2GFP) was evaluated by qPCR for the Xnr1 enhancer. (E) ChIP for myc-Grg4 expressed alone or in combination with the following: FoxH1 (myc-Grg4 + FoxH1), FoxH1 and Xnr1 (myc-Grg4 + FoxH1 + Xnr1), FoxH1 Δ SID (myc-Grg4 + FoxH1 Δ SID) or FoxH1 Δ SID and Xnr1 (myc-Grg4 + FoxH1 Δ SID + Xnr1) were evaluated by qPCR for the Xnr1 enhancer. The data shown represent three independent biological replicates. Immunoprecipitation using anti-myc antibody was performed on uninjected embryos (Control). White bars represent qPCR for genomic *Xmhc2* or genomic *Xnr1* 3'UTR as additional negative controls. * indicates $p < 0.05$.

displace Grg4 from the Xnr1 enhancer, ChIP was performed for myc-Grg4 alone or coexpressed with Xnr1 or Smad2-GFP (Fig. 5D). Myc-Grg4 occupancy at the Xnr1 enhancer was significantly reduced by Smad2-GFP, similar to the reduction observed with exogenous Xnr1 (Fig. 5D). These data suggest that Smad2/3, when activated in response to Nodal signaling, may displace Grg4 by binding to FoxH1.

To further assess the role of Smad2/3 in displacing Grg4 from FoxH1 and the Xnr1 enhancer, Nodal regulation of Grg4 occupancy was examined in the presence of FoxH1 Δ SID, which does not bind Smad2/3 (Germain et al., 2000). Myc-Grg4 was coexpressed with untagged FoxH1 or FoxH1 Δ SID in the presence of exogenous Nodal signals, and Grg4 occupancy of the Xnr1 enhancer was examined by ChIP (Fig. 5E). Consistent with the results above, exogenous Nodal signals reduced Grg4 occupancy in the presence of FoxH1. However, Grg4 occupancy was unaffected by Nodal in the presence of FoxH1 Δ SID, indicating a requirement for Smad2/3 interaction with FoxH1 to displace Grg4 from the Xnr1 enhancer. We conclude that FoxH1 acts as a transcriptional switch in the

regulation of Nodal target gene expression, recruiting Grg4 in the absence of Nodal activity and mediating Smad2/3 displacement of Grg4 upon Nodal activation.

4. Discussion

In this study, we define a novel function for FoxH1 as a repressor of Nodal-dependent transcription. FoxH1 physically interacts with Grg4 via a conserved EH1 motif and acts as both a repressor and an activator of Nodal target genes during early *Xenopus* development. Mutation of the EH1 domain of FoxH1 enhances the expression of Nodal target genes *in vivo*, indicating that FoxH1 utilizes the EH1 motif and recruitment of the corepressor Grg4 to restrict Nodal target gene expression. Consistent with this model, Grg4 overexpression blocks Nodal-induced gene transcription while inhibition of Groucho function relieves repression and leads to ectopic mesoendodermal gene expression. Although FoxH1, Grg4, and Smad2/3 can all occupy the Xnr1 enhancer

occupancy decreases in response to Nodal signals, whereas Smad2/3 occupancy increases. In support of the hypothesis that Smad2/3 displaces Grg4 from FoxH1 at the enhancer, we find that the FoxH1 SID is necessary for Grg4 displacement in response to Nodal. Our findings demonstrate a requirement for a FoxH1–Grg4 complex to limit Nodal gene expression and subsequent mesendoderm induction, revealing a novel mechanism for limiting Nodal pathway activity in the embryo.

4.1. FoxH1 as a transcriptional switch

A transcriptional switch that converts from an actively repressive “off” state to a transcriptionally active “on” state offers a stringent mechanism for signal-dependent gene regulation (Barolo and Posakony, 2002). A single DNA-bound factor that recruits a corepressor in the absence of a signal and a coactivator upon pathway activation constitutes a rapid and robust switch between two transcriptional states, ensuring precise gene activation only in the proper cellular context. In embryonic development, such control is necessary to restrict localized gene expression and regulate cell fate determination.

Many developmentally important pathways rely on the function of transcriptional switches (Barolo and Posakony, 2002). In Notch signaling, the transcription factor Suppressor of Hairless (Su(H)) binds Groucho to inhibit transcription of target genes. Upon pathway stimulation via a Notch–ligand interaction, the cleaved Notch intracellular domain translocates to the nucleus, displaces Groucho, and cooperates with Su(H) to activate target genes [reviewed in Turki-Judeh and Courey (2012)]. Likewise TCF, a DNA-binding factor that regulates Wnt-responsive genes, interacts with Groucho corepressors in the absence of a Wnt signal to maintain target genes in a transcriptionally inactive state. Active Wnt signaling results in displacement of Groucho by β -catenin, which binds TCF and activates transcription (Chodaparambil et al., 2014). The results presented here suggest that activated Smad2/3 facilitates Grg4 displacement from FoxH1 and induction of target gene expression. A detailed understanding of the protein interactions underlying the FoxH1 transcriptional switch remains to be elucidated.

4.2. Spatial restriction of the Nodal pathway

The Nodal pathway is essential for the formation of endodermal and mesodermal germ layers and plays a critical role in the formation of the organizer domain during early embryogenesis. However, Nodal signaling initiates a positive feedback loop that must be limited by signaling antagonists (Osada et al., 2000). Excessive Nodal signaling in ventral tissues induces a partial secondary axis, while Nodal pathway activation in the animal pole results in the conversion of ectoderm to mesendoderm [reviewed in Shen (2007)]. Thus, Nodal activity must be limited within the animal and ventral domains to allow normal embryonic development. Multiple negative regulators of the Nodal pathway have been identified, acting both intracellularly and extracellularly to moderate Nodal activity. A number of these factors are localized to the ectoderm in the *Xenopus* embryo, and thus are likely to be important for maintaining ectodermal identity by counteracting Nodal activity. Among these are the secreted factors Coco and Tomoregulin (TMEFF1), the intracellular Smad inhibitors Ectodermin and PIASy, and the transcription factors Sox3 and Xema (Bell et al., 2003; Chang et al., 2003; Daniels et al., 2004; Dupont et al., 2005; Sachdev et al., 2001; Suri et al., 2005; Zhang et al., 2004). Similarly, the transcriptional corepressor DRP1 is essential for limiting Nodal signaling within the mouse embryo (Iratni et al., 2002). Given that FoxH1 and Grg4 are supplied maternally throughout the embryo, we propose that FoxH1–Grg4 plays a

predominantly repressive role during the blastula and gastrula stages, after which other regulators limit Nodal pathway activity. In support of this idea, transcriptional repression by Groucho corepressors and HDACs is required in the animal pole to prevent ectopic Nodal ligand expression (Fig. 3). It is likely that multiple negative regulators of the Nodal pathway serve both unique and overlapping functions during embryonic development.

The FoxH1–Grg4 repressive complex described here most likely acts on other Nodal target genes in addition to *Xnr1*. Our preliminary data suggests that Grg4 occupies the *Gsc* promoter, an interaction that is also disrupted by Nodal signaling (data not shown). FoxH1–Grg4 would likely contribute to *Gsc* repression in the marginal zone prior to the early gastrula stage, and in the animal domain throughout the blastula and gastrula stages. With the onset of active Nodal signaling in the blastula vegetal domain and gastrula marginal domain, FoxH1–Smad2/3 would mediate the activation of transcriptional targets in those territories. The complex regulation of the Nodal pathway and its signaling output in the control of gastrula gene expression remains an active area of research (Inui et al., 2012) [reviewed in Shen (2007)].

4.3. FoxH1 as a repressor in the Nodal pathway

FoxH1 loss-of-function experiments in mouse, zebrafish and *Xenopus* have yielded differing results that are difficult to reconcile. In the mouse, deletion of FoxH1 causes variable defects in the node and anterior primitive streak (Hoodless et al., 2001; Yamamoto et al., 2001). Only the most severely affected embryos fail to gastrulate; the majority of embryos form a primitive streak and mesoderm, exhibiting defects only in anterior and midline structures (Hoodless et al., 2001; Yamamoto et al., 2001). This is in contrast to Nodal mutants, which fail to form a primitive streak and lack all mesoderm (Conlon et al., 1994). Similarly, point mutations in the zebrafish FoxH1 gene (*schmalspur* or *midway*) result in defects in anterior and axial structures (Pogoda et al., 2000; Slagle et al., 2011), but do not recapitulate the severe loss of head and trunk mesoderm associated with a mutation in the Nodal coreceptor *one-eyed pinhead* (Gritsman et al., 2000). On the other hand, morpholino knockdown of FoxH1 in zebrafish embryos causes a much more severe phenotype, with embryos failing to gastrulate and exhibiting reduced expression of mesodermal and endodermal genes (Pei et al., 2007). This range of results suggests that FoxH1 function is more complex than its well-described role as a positive regulator of Nodal-induced transcription.

We propose that a previously unrecognized repressor function for FoxH1 contributes to these complex phenotypes. Overexpression of FoxH1A6 upregulates Nodal target gene expression, consistent with the ability of FoxH1 to recruit Groucho and actively repress target genes in the early embryo (Fig. 2). The ability of FoxH1 to mediate both repression and activation of Nodal gene expression may account for the relative weakness of FoxH1 loss-of-function phenotypes when compared to Nodal loss-of-function.

In a recent study, Halstead and Wright demonstrated that mouse FoxH1, like the *Xenopus* protein, is interacts with Groucho corepressors through a conserved EH1 motif (Halstead and Wright, 2015). Despite this result, mutation of the EH1 motif of the endogenous FoxH1 locus in the mouse causes no observable phenotype. Experimental methodology may account for the differing results, with point mutagenesis of the endogenous FoxH1 locus in the mouse studies versus injection of wild-type and mutant FoxH1 mRNAs in the *Xenopus* studies. The mouse FoxH1 mutant is not designed as a true loss-of-function allele, but rather for expression of “wild-type” levels of an altered FoxH1 protein. The mutant mouse FoxH1 protein contains a single residue change (F198E) in the absolutely conserved first position of the EH1 motif. While this residue is critical for Groucho binding to EH1-

containing proteins, other conserved positions of the EH1 motif contribute to Groucho binding. For the present studies, Alanine mutagenesis of the FoxH1 EH1 motif, replacing all conserved residues, is predicted to completely disrupt binding of Grg4. Therefore, the differing severity of the EH1 mutations for the mouse and *Xenopus* FoxH1 proteins may account in part for the conflicting phenotypic results.

Halstead and Wright propose an alternative explanation for the absence of a Nodal pathway-related phenotype in the FoxH1 mutant mouse. The Nodal pathway is under the control of multiple positive and negative regulators that they suggest confer a robustness on the Nodal pathway, allowing normal development despite disruption of the FoxH1-Grg4 interaction. This idea is supported by the partial penetrance of FoxH1 loss-of-function deletion mutations (Hoodless et al., 2001; Yamamoto et al., 2001). The feedback antagonist Lefty2 is a likely regulatory component that confers robustness and may mask phenotypic effects of disrupting the FoxH1-Groucho interaction. Further genetic experiments that inactivate potentially redundant Nodal signaling inhibitors, such as Lefty2, in the presence of the FoxH1 EH1 mutant may sensitize the mouse and reveal an essential role for Nodal repression by FoxH1-Grg4.

Another intriguing possibility is that FoxH1 acts primarily as a repressor of Nodal signaling in the pre-gastrula *Xenopus* embryo and as an activator thereafter, whereas early mouse development is less dependent on FoxH1 as a transcriptional repressor. This model is supported by recent ChIP-Seq and RNA-seq studies in which FoxH1 was found to primarily function as a transcriptional activator in the *Xenopus* gastrula (Chiu et al., 2014). This model could also explain why Nodal signaling initiates normally in FoxH1 knockdown embryos (Kofron et al., 1999). In *Xenopus*, the maternal factors VegT and β -catenin initiate expression of the Nodal ligands *Xnr5* and *Xnr6*—which are not controlled by the positive-feedback loop that maintains expression of other Nodal-related genes—prior to the start of widespread zygotic transcription at the mid-blastula transition (MBT) (Yang, 2002; Hilton et al., 2003; Houston et al., 2002; Takahashi et al., 2000). Depletion of maternal FoxH1 increases *Xnr5* and *Xnr6* expression in the vegetal hemisphere, whereas other Nodal genes are downregulated (Kofron et al., 1999). Our results suggest that prior to the MBT, FoxH1 and Grg4, which are maternally supplied like VegT and β -catenin, may limit *Xnr5* and *Xnr6* expression in the vegetal hemisphere prior to the expression of other Nodals. Following MBT, VegT and β -catenin cooperate with *Xnr5* and *Xnr6* to activate zygotic Nodals (Hyde and Old, 2000), which could in turn relieve FoxH1-Grg4-dependent repression as active Smad2/3 draws FoxH1 protein into the Nodal autoregulatory loop. Thus, FoxH1 may regulate *Xnr5* and *Xnr6* expression as a repressor only, independent of Smad2/3 activation. The absence of a comparable early phase of Nodal expression (*Xnr5/6*-like) in the mouse may account for the differing requirement for FoxH1 repressor function in the mouse as compared to *Xenopus*.

4.4. Mechanism of repression by FoxH1-Grg4

The identification of FoxH1 as both a repressor and an activator raises interesting questions as to how FoxH1 may selectively repress some genes and activate others. FoxH1 plays multiple roles throughout development, and its activator/repressor roles are likely dependent on the availability of coactivators, histone modifiers, and other chromatin remodelers in differing contexts. Both the corepressor Grg4 and the coactivators Smad2/3 are expressed maternally and are ubiquitous throughout the blastula and gastrula stages, making it unlikely that expression of either cofactor is solely responsible for switching between activator and repressor functions (Faure et al., 2000; Molenaar et al., 2000).

Groucho proteins recruit HDACs to repress target gene expression (Chodaparambil et al., 2014), and our results demonstrate that HDAC inhibition is sufficient to derepress Nodal target genes in the ectodermal germ layer. The repression state of FoxH1 target genes may be determined by the presence and/or activity of specific HDAC cofactors. Grg4 has also been shown to recruit other chromatin-remodeling factors such as PRMT5, an arginine methyltransferase, to repress transcription (Patel et al., 2012). Conversely, Smad2 and Smad3 are known to recruit the histone acetyltransferase p300 to promote target gene transcription (Inoue et al., 2006; Tu and Luo, 2007). It remains unclear how the expression or activity of specific corepressors and coactivators that mediate the activities of Grg4 and Smad2/3 may modulate the final transcriptional output of the FoxH1 transcriptional switch.

5. Conclusions

The studies presented here demonstrate a previously unappreciated role for a FoxH1-Grg4 complex in negatively regulating Nodal gene expression. Our findings also place FoxH1 on the growing list of key developmental transcription factors that mediate transcriptional switches governing cell fate. We propose that FoxH1 and Grg4 function together to spatially restrict Nodal gene transcription in the blastula and early gastrula embryo, ensuring proper formation of the primary germ layers. The interaction of FoxH1 with Grg4 may also have implications for transcriptional regulation in other Nodal-signaling contexts. This work expands the functional repertoire of FoxH1, and provides a foundation for future mechanistic studies of the Nodal signaling pathway and its transcriptional effectors.

Author contributions

D.S.K., C.D.R., and A.B.S. designed experiments. C.D.R., A.B.S., S.Y., Q.L., S.W., and M.H. performed the experiments. C.D.R., A.B.S., and D.S.K. analyzed the data and wrote the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2016.04.006>.

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