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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.

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 mesendoderm 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 Mixl1 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-FoxH1ΔSID (this study). For HDAC-treated ectoderm, explants were prepared at the blastula stage and cultured in 0.5× 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-FoxH1ΔSID, 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 digoxygenin-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 EF1α, 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