RESEARCH ARTICLE

Genome-wide view of TGFβ/Foxh1 regulation of the early mesendoderm program

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ABSTRACT

Nodal/TGFβ signaling regulates diverse biological responses. By combining RNA-seq on Foxh1 and Nodal signaling loss-of-function embryos with ChIP-seq of Foxh1 and Smad2/3, we report a comprehensive genome-wide interaction between Foxh1 and Smad2/3 in mediating Nodal signaling during vertebrate mesendoderm development. This study significantly increases the total number of Nodal target genes regulated by Foxh1 and Smad2/3, and reinforces the notion that Foxh1-Smad2/3-mediated Nodal signaling directly coordinates the expression of a cohort of genes involved in the control of gene transcription, signaling pathway modulation and tissue morphogenesis during gastrulation. We also show that Foxh1 may function independently of Nodal signaling, in addition to its role as a transcription factor mediating Nodal signaling via Smad2/3. Finally, we propose an evolutionarily conserved interaction between Foxh1 and PouV, a mechanism observed in Pou5f1-mediated regulation of pluripotency in human embryonic stem and epiblast cells.

KEY WORDS: TGFβ, Foxh1, Smad, Mesendoderm, Morphogenesis, Cell fate, Oct4, Pou5f1, Xenopus tropicalis, Signaling, Genomics, ChIP-seq, RNA-seq

INTRODUCTION

Cell signaling is essential for coordination of the dynamic spatiotemporal expression of genes. Systems level comprehension of the signaling inputs that control cell specification will facilitate decoding the fundamental regulatory mechanisms dictating animal development. Here, we examine Nodal signaling to uncover the notion that Foxh1-Smad2/3-mediated Nodal signaling directly coordinates the expression of a cohort of genes involved in the control of gene transcription, signaling pathway modulation and tissue morphogenesis during gastrulation (Kubo et al., 2004). These diverse activities raise the issue of the underlying mechanism conferring specificity of gene regulation by Nodal in various developmental contexts.

Nodal signaling is triggered by ligand binding to type I and II receptors, which in turn phosphorylate Smad2 or Smad3. Phospho-Smads complex with Smad4, translocate to the nucleus and, together with other transcription factors (TFs) such as Foxh1, regulate target gene expression (Chen et al., 1996, 1997). Currently, the relative contribution of Foxh1 in mediating Nodal signaling has not been systematically examined in vivo. Nodal signaling is crucial for the proper induction of mesoderm and endoderm. The Nodal mutant mouse lacks the primitive streak and fails to form normal mesoderm, leading to developmental arrest shortly after gastrulation (Conlon et al., 1994; Varlet et al., 1997; Zhou et al., 1993). Zebrafish cyclops (cyc)squint (sqt) double mutants fail to form the shield and gastrulation is disrupted (Feldman et al., 1998). In amphibians, these genes are zygotically expressed under the control of the maternal T-box transcription factor Vg1 (Heasman, 2006). Inhibition of Nodal signaling in Xenopus via the overexpression of Nodal antagonists or by treatment with small-molecule Nodal receptor inhibitors leads to gastrulation defects (Agius et al., 2000; Ho et al., 2006; Osada and Wright, 1999; Sun et al., 1999). Three other Smad2/3-activating TGFβ family members are also important for normal early mesendodermal development (Birsoy et al., 2006; Piepenburg et al., 2004; Sun et al., 1999) and probably have overlapping functions with the Nodal proteins; we therefore refer to collective signaling by these ligands as Nodal signaling for simplicity. These results suggest that Nodal ligands are essential for vertebrate mesoderm development.

Foxh1 (Fast1, forkhead activin signal transducer 1), is a winged-helix TF that is maternally expressed in Xenopus (Chen et al., 1996). It was first discovered as a mediator of Activin-like signaling via binding to the activin response element of the mix1 gene in conjunction with Smad2/3 (Chen et al., 1996). Subsequently, goosecoid (gsc) (Labbé et al., 1998), lhx1 (Watanabe et al., 2002), nodal1 (Osada et al., 2000) and pitz2 (Shiratori et al., 2001) were shown to be regulated by Foxh1. In mouse, Foxh1−/− embryos display a spectrum of phenotypes from severe gastrulation defects to milder anterior and midline deficiencies (Hoodless et al., 2001; Yamamoto et al., 2001). In zebrafish, schmalspur (spr) and midway (mwy) mutants, both defective in the foxh1 gene, are deficient in prechordal plate, notochord and some axial mesoderm development (Pogoda et al., 2000; Sirokin et al., 2000; Slagle et al., 2011). Loss of Foxh1 in Xenopus laevis results in reduced expression of mesendodermal markers, including gsc, nodal1 and mix1, but axial mesoderm was still present, albeit obviously abnormal (Howell et al., 2002; Kofron et al., 2004b; Watanabe and Whitman, 1999). The stronger phenotypes from loss of Nodal signaling, compared with foxh1 loss of function (LOF) in frog, fish and mouse, suggests that Nodal signals through both Foxh1-dependent and -independent pathways. Mixer, Foxh1.2, Gtf2rd1, Gtf2i, Tp53, Eomes and Tcf3 (also known as E2a) have been implicated in activation of Nodal targets in Xenopus (Cordenonsi et al., 2003; Germain et al., 2000, 2001; Howell et al., 2002; Ku et al., 2005; Ring et al., 2002; Slagle et al., 2011; Teo et al., 2011; Yoon et al., 2011), although the extent that any of these TFs play in the broader regulation of Nodal signaling gene expression
batteries in developing embryos remains ill defined. Thus, despite advances made in dissecting the Nodal molecular cascade, significant gaps remain in our knowledge.

To comprehend how mutations in Nodal signaling affect transcriptional networks and cause developmental defects, we examined the cohort of genes regulated by Nodal in the context of early vertebrate embryonic development at a genome-wide level. We determined which of these genes are directly bound and regulated by Foxh1 and/or Smad2/3. Foxh1 and Smad2/3 interact with thousands of genomic sites and affect the transcriptional responses of hundreds of Nodal target genes. We uncovered a large set of new targets, and have begun understanding the complexity associated with the Nodal signaling network. Although Foxh1 and Smad2/3 directly bound and regulated some Nodal-activated genes, we found a Nodal signaling-independent function for Foxh1. Additionally, Foxh1- and Smad2/3-binding site analysis revealed the involvement of Pou family TFs in Nodal target gene expression. PouV family genes (pou5f3s) modulate the expression of some Nodal targets, perhaps functioning as regulators of differentiation.

**RESULTS**

**Foxh1 is crucial for mesendoderm formation**

In *Xenopus*, there are two foxh1 genes: foxh1 and foxh1.2 (Chen et al., 1996; Howell et al., 2002). Foxh1 is expressed maternally and plays a central role in mediating Nodal signaling (Chen et al., 1996; Howell et al., 2002; Kofron et al., 2004b; Watanabe and Whitman, 1999; Yeo et al., 1999). foxh1.2 is expressed after the mid-blastula transition (MBT) (Howell et al., 2002), and its function is unclear. Since the initial activation of Nodal signaling is detected at MBT, before foxh1.2 expression, and foxh1 transcripts are more abundant than foxh1.2 in early gastrulae (Howell et al., 2002; Lee et al., 2001), we examined the contribution of Foxh1 in mediating Nodal signaling.

In situ hybridization analyses in *Xenopus laevis* have shown that foxh1 transcripts are preferentially localized to the animal and marginal zones, but are excluded from deep mesodermal cells (Howell et al., 2002). However, these results are inconsistent with the widespread notion that Foxh1 is the key player in mediating Nodal signaling during mesendoderm formation (Chen et al., 1996, 1997; Watanabe and Whitman, 1999; Yeo et al., 1999). RT-qPCR analysis from dissected tissue fragments of *X. tropicalis* expressed throughout the embryo. The ectodermally enriched *foxh1.2* is a Wnt target gene. The ectodermal marker; *tbrachyury*, mesodermal marker; *sox17a*, endoderm marker; *rpl11*, expressed throughout the embryo. (B) Embryonic lysates from control or foxh1-MO injected embryos were subjected to immunoprecipitation followed by western blot using anti-Foxh1 antibody. Foxh1 protein levels in crude embryo lysates are not affected by the MO. (C) Both Foxh1 morphant and SB431542-treated embryos exhibit gastrulation delay (vegetal views). (D) Early tailbud stage Foxh1 morphants displaying anterior defects and incomplete blastopore closure; SB431542-treated embryos lack distinctive A-P or D-V features. (E) Examination of Foxh1 MO effects on different germ-layer markers by RT-qPCR. *gsc*, *chrd*, *nodal1*, *nodal3*, *mix1*, *sox17a*, *ventx2.1*, *siaf*, *sox3*, and *foxh1.2*. (F) Early gastrula cleared lysates were immunoprecipitated using either pan-Smad2 or anti-Smad1 polyclonal antibodies covalently coupled to beads. Bound proteins were subjected to western blot using an anti-foxh1 antibody. *ctrl*: Control; *MO*: Foxh1 MO; *Rescue*: Rescue RNA; *DMSO DMSOSB SB*: DMSO DMSOSB SB. (G) Rescue of foxh1 knockdown by co-injecting MO-insensitive *foxh1* mRNA (Fig. 1C, D). We also observed incomplete gastrulation and a shortened anteroposterior (A-P) axis in tailbud embryos, which were rescued by co-injecting MO-insensitive *foxh1* mRNA (Fig. 1D; supplementary material Fig. S2).

Next, we examined the effective concentrations of the Nodal receptor (ALK4/5/7) inhibitor SB431542 (Inman et al., 2002) during *X. tropicalis* mesendoderm development. Embryos were treated with increasing concentrations of SB431542 and the expression of well-characterized Nodal target genes was effectively inhibited at 100 μM (supplementary material Fig. S3), which is also the effective concentration reported in *X. laevis* (Ho et al., 2006). SB431542-treated embryos displayed varying phenotypic severity. Typically, embryos fail to form any blastopore lip, arrest during gastrulation and die during neurulation. In some clutches, SB431542-treated embryos had a severe delay in blastopore lip formation, but survived to tailbud stages. Generally, the phenotypes of embryos treated with SB431542 (Fig. 1C, D) were more severe than those of Foxh1-depleted embryos. We also examined the specificity of SB431542 treatment on Smad2/3...
activation (Fig. 1F). SB431542 inhibited the phosphorylation of Smad2/3, whereas the phosphorylation of Smad1/5/8 was not diminished. We conclude that 100 μM SB431542 specifically inhibits Nodal signaling in *X. tropicalis* embryos.

Foxh1 morphants showed significant reduction in expression of the dorsal mesendoderm-enriched genes *gsc, chrd, nodal1* and *nodal3* (Fig. 1E). Expression of the early endodermal markers *mix1* and *sox17a* was also reduced, whereas expression of the ventral marker *ventx2.1*, the animaly enriched marker *sox3*, and *sia1* (a Wnt target) were minimally affected. Interestingly, *foxh1* was consistently upregulated 8- to 15-fold in *foxh1* morphants (supplementary material Fig. S4), whereas expression of *foxh1* transcripts was only slightly increased (~1.3-fold; Fig. 1E). Delivery of *foxh1* rescue mRNA to morphants either fully or partially reversed *foxh1* MO effects (Fig. 1E). We conclude that Foxh1 is required in *X. tropicalis* for normal mesendoderm development. Other findings are: (1) *foxh1* expression is negatively autoregulated; (2) Foxh1 antibody specifically recognizes endogenous protein; and (3) *foxh1* MO efficiently knocks down Foxh1 protein and downstream mesendodermal gene expression.

**Significantly different sets of genes are responsive to Foxh1 and Nodal**

To examine the contribution of Foxh1 to Nodal signaling, we compared RNA-seq transcriptome profiles between SB431542-treated embryos and *foxh1* morphants. Sequence reads were mapped to the *X. tropicalis* v.7 genome assembly (supplementary material Table S2) and differential gene expression levels between control and perturbed embryos were calculated. SB431542 treatment and *foxh1* MO injection each affected the mRNA expression of hundreds of genes, both positively and negatively (supplementary material Fig. S5). Affected genes include well-studied Nodal targets such as *gsc, nodal1, cer1* and *hhex* (supplementary material Table S1). Fig. 2A-D show read-mapping densities (tracks 3-6 in each panel) across these four genes.

Using a 1.5-fold cutoff (empirically derived from Kolmogorov-Smirnov analysis below), 259 genes were differentially affected by SB431542, 707 were affected by *foxh1* MO and 87 were affected by both *foxh1* MO injection and SB431542 treatment. These expression changes represent both direct and indirect target genes. That only 34% (87) of the 259 SB431542-sensitive genes were affected by *foxh1* knockdown suggests the involvement of additional TFs in Nodal signaling. In addition to its role in mediating Nodal signaling, Foxh1 also regulates the expression of a set of non-Nodal targets. Expression differences between Nodal and Foxh1 loss of function may explain the phenotypic differences observed (Fig. 1).

**Genome-wide binding patterns of Foxh1 and Smad2/3 identify many potential co-regulated target genes**

Differential expression profiling alone is unable to distinguish between direct and indirect transcriptional regulation by Foxh1 and Smad2/3. Therefore, we correlated gene expression data with physical binding of Foxh1 and Smad2/3. The suitability of Foxh1 and Smad2/3 antibodies was validated by ChIP-qPCR (supplementary material Fig. S6), and ChIP-seq (Fig. 2A-D, tracks 1 and 2) was performed on stage 10.25-10.5 early gastrulae using two biologically independent replicates. MACS2 and SIISSRs (Jothi et al., 2008; Zhang et al., 2008) identified bound regions (‘peaks’) and high-confidence peaks were those defined by the intersection of output of these programs (supplementary material Table S2). ChIP-seq analyses revealed 2266 Foxh1 and 939 Smad2/3 peaks. We assigned nearest genes to peaks located within 10 kb upstream of gene 5’ ends, within 10 kb downstream of 3’ ends and within gene transcription units (supplementary material Table S3). These analyses mapped 70.1% (1588) of Foxh1 peaks to 1321 genes, and 65.5% (615) of Smad2/3 peaks to 501 genes, with 297 genes being bound by both Foxh1 and Smad2/3. Most of the peaks and genes identified in Smad2/3 ChIP-seq by Yoon et al. (2011) are also included in our data analysis (see supplementary material Fig. S12).

We found that 12.9% of Foxh1 peak summits were located within 10 kb of Foxh1 peaks. This is consistent with the idea that Foxh1 is a transcriptional activator. The data presented here provide some of the first genome-wide binding data for Foxh1 and Smad2/3 transcription factors. This will be useful for future studies of Nodal signaling.
Smad2/3 showed that 5.7% of peaks are within this 1 kb upstream interval, representing an ~3-fold enrichment. The highest abundance of Foxh1 binding is located within a narrow window flanking the 5’ end of genes, whereas Smad2/3 did not reveal such localization (Fig. 2F).

We performed a de novo motif search of the DNA sequences extending 75 bp to either side of the summits of the 2266 Foxh1 peaks. The Foxh1 motif was the most common sequence found (1436 peaks) (Fig. 3A). Although a similar analysis of Smad2/3 peaks revealed the Smad2/3 motif to be the most abundant, the Foxh1 motif was also frequently found (Fig. 3B, supplementary material Fig. S11), supporting the view that Foxh1 is a major transcriptional partner of Smad2/3.

The primary role of Foxh1 in Nodal signaling is to activate target genes

To examine whether Foxh1 functions as an activator or repressor, or both, we applied the Kolmogorov-Smirnov (KS) test to determine the statistical significance of the correlation between TF binding to genes and changes in gene expression after loss of function (Subramanian et al., 2005). The differential expression values of over 14,000 genes in foxh1 morphants versus those of control embryos were used to rank the genes in ascending order along the y-axis (Fig. 4A,B). This ranking was correlated with the Foxh1 ChIP-seq results by scoring for the presence/absence (green tick marks) of Foxh1 peaks over these genes. A running (cumulative) enrichment score (RES, y-axis) was calculated based on the presence/absence of a Foxh1 peak (blue line). This plot is significantly different from that expected for the null hypothesis (P<5.6e-16), i.e. that peaks are randomly distributed with respect to the foxh1 MO effects on differential expression. Therefore, there is a statistical correlation between Foxh1 binding and changes in gene expression. The density plot (purple) of the genes containing peaks (green tick marks) reveals a tight correlation between the presence of bound Foxh1 and genes downregulated upon Foxh1 depletion, but not with genes upregulated, suggesting that Foxh1 functions primarily as a transcriptional activator.

A strong positive correlation between Foxh1 binding and downregulation of genes in response to SB431542 was also observed in the density plot in Fig. 4B, consistent with the activating role of Foxh1 in Nodal signaling. Finally, a strong correlation was observed between the binding of Smad2/3 and downregulation of genes in response to SB431542 (Fig. 4C). The RESs (blue plots) for both Foxh1-Nodal and Smad2/3-Nodal signaling are significantly different than those expected for their corresponding null hypotheses (P-values in each were <2.2e-16). We conclude that binding of Foxh1 and Smad2/3 are prerequisites for the activation of many Nodal target genes. Interestingly, a comparison between the density plots for Foxh1 and Smad2/3 binding on SB431542-sensitive genes (Fig. 4B,C) shows that Smad2/3 correlates 2.5-fold more closely with Nodal targets than does Foxh1 (see supplementary materials and methods). This is consistent with the RNA-seq results (Fig. 2) that also implicated Foxh1 in Nodal-independent gene regulation.

A large cohort of direct Nodal target genes

Among 259 genes regulated by Nodal signaling, 218 are positively regulated by Nodal and 41 genes are negatively regulated (supplementary material Table S1). Seventy-five genes are putative direct Nodal targets as they are bound by Smad2/3 (blue and green sectors in Fig. 4D).

Among these 75 genes, 60 are co-bound by both Foxh1 and Smad2/3 (Fig. 4D). Fifty-nine of these are activated by Nodal signaling (downregulated by SB431542) (supplementary material Table S1). Seventy-five genes are putative direct Nodal targets as they are bound by Smad2/3 (blue and green sectors in Fig. 4D).

## Table A

<table>
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<th>Motifs logo</th>
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<th>Putative binding protein</th>
<th>Binding motif</th>
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<td>1436</td>
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## Table B

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gsc targets. This list includes the previously identified Nodal targets (Table S4), and thus are potential Foxh1-mediated Nodal direct targets. This list includes the previously identified Nodal targets and thus are potential Foxh1-mediated Nodal direct targets. Wnt8a Nodal targets are signaling. of genes regulating cell fate specification, morphogenesis and cell transduction. These 59 genes were preferentially expressed in genes includes cell surface receptor-linked signal transduction, pattern roles in the early embryo. The remaining 150 SB431542-sensitive genes are not bound by either Foxh1 or Smad2/3, and thus likely to Foxh1 direct targets, SB sensitive Foxh1 direct targets, SB insensitive Foxh1 is a dual regulator and functions independent of Nodal signaling. We investigated the role of Foxh1 in early embryogenesis by comparing foxh1 MO knockdown and SB431542 RNA-seq together with ChIP-seq. Among the 1321 Foxh1-bound genes (Fig. 2D), the expression of 109 is affected (>1.5-fold) following foxh1 MO injection. We consider these to be Foxh1 direct targets. Thirty-seven of these genes are SB431542 sensitive (Fig. 4E), suggesting that they are either regulated by Nodal directly or indirectly. The Smad2/3 ChIP-seq data show that 26 (70%) of these genes are bound (supplementary material Table S8) and therefore likely to be direct Nodal targets. Among the 109 Foxh1 direct target genes, 37 are Nodal-dependent targets and all except one (fos) are positively regulated by Foxh1 (Fig. 4E). Conversely, 72 lack Smad2/3 binding and are insensitive to SB431542 (Fig. 4F), suggesting that Foxh1 regulates these genes in a Nodal-independent fashion. Of these 72 genes, 45 are downregulated in the absence of Foxh1, whereas 27 are upregulated, suggesting that Foxh1 functions as a dual-acting TF to activate or repress target genes in a Nodal-independent manner. RT-qPCR analysis independently confirmed both activator and repressor behavior of Foxh1 (Fig. 4G and supplementary material Fig. S8). The apparent Nodal-independent Foxh1 gene regulation is indeed a specific effect of foxh1 MO knockdown as normal expression of these genes was rescued via Foxh1 expression using a MO-resistant foxh1 mRNA (supplementary material Fig. S9). All 72 Nodal-independent Foxh1 regulated genes appear to be
ubiquitously expressed in the early gastrulae, based on public expression data (Xenbase) and RNA-seq results from dissected early gastrulae (W.T.C., I.L.B. and K.W.Y.C., unpublished), in contrast to Nodal-dependent gene expression, which is confined to the mesendoderm. GO term analysis failed to reveal any term enrichment, and the developmental significance of these genes will require further investigation.

**Xenopus PouV class transcription factors interact with Foxh1 to regulate Nodal targets**

To identify additional TFs that interact with Foxh1 to regulate target genes, we searched for over-represented sequence motifs in the vicinity of Foxh1 peak summits. The five most statistically significant motifs (based on E-values) ranked by abundance are shown in Fig. 3A, with the Foxh1 motif being the most frequent. Although searches of various motif databases (UniPROBE, JASPAR and TRANSFAC) failed to identify candidate factors that matched the second and third motifs, the fourth and fifth resemble Pou and Heb/Zic motifs. Because Heb, also known as Tcf12, was previously implicated as a Foxh1/Smad partner (Yoon et al., 2011), we investigated the role of Pou TFs that participate in Nodal signaling regulation.

*Xenopus* and zebrafish Pou family TFs have been associated with the regulation of early mesendoderm development (Burgess et al., 2002; Cao et al., 2007, 2006, 2008; Lunde et al., 2004; Morrison and Brickman, 2006; Reim and Brand, 2006; Reim et al., 2004), and are evolutionarily closely related to mammalian Pou5f1/Oct4 (Frankenberg et al., 2010; Frankenberg and Renfree, 2013). *Xenopus* PouV genes (*pou5f3.1, pou5f3.2 and pou5f3.3; previously oct91, oct25 and oct60, respectively) are locally triplicated in the genome (Hellsten et al., 2010) and differentially expressed in the early embryo. *pou5f3.2* and *pou5f3.3* are expressed maternally, whereas *pou5f3.1* is zygotic (Fig. 5A) (Hinkley et al., 1992). The three PouV genes are expressed in all three primary germ layers (Fig. 5B).

We examined whether PouV proteins directly regulate Foxh1-Smad2/3-mediated Nodal target genes as a general mechanism. We bioinformatically mined the Foxh1 and Smad2/3 peak sequences on the 60 direct Nodal targets (SB-sensitive) bound by these TFs (Fig. 4D, supplementary material Table S4) for the presence of Pou motifs. Among these, 22 contain Pou motifs within these regions (supplementary material Table S4), but only 11 are affected in Foxh1 MO-injected embryos (based on RNA-seq). After PouV knockdown, three out of these 11 genes (gsc, nodal2 and mespb), by conservative criteria, were consistently affected at both stages examined (>2-fold, Fig. 5C), and expression of these genes was rescued after co-injecting a cocktail containing MO-insensitive versions of all three PouV mRNAs (supplementary material Fig. S10). Expression of many of the other genes was affected to varying degrees in a stage-dependent manner that makes both molecular phenotypes and rescue rather difficult to assess (see also Livigni et al., 2013). gsc and nodal2 are upregulated in response to the PouV MO, suggesting that these genes are negatively regulated by PouV proteins, whereas mespb expression is reduced in

![Fig. 5. Functional analysis of PouV genes in regulating Nodal targets.](image-url)

(A) RT-qPCR analysis of *pou5f3.1, pou5f3.2 and pou5f3.3* transcript levels at egg, 128-cell, blastula (stage 9), early (stage 10) and mid-gastrula (stage 10.5) stages. Transcript levels were normalized to the *pou5f3.1* level in egg RNA. (B) RT-qPCR analysis of *pou5f3.1, pou5f3.2 and pou5f3.3* in animal, marginal and vegetal fragments of the gastrula (stage 10-10.5) stage embryo. (C) RT-qPCR of mesendodermal targets in PouV-depleted embryos at early gastrula (stage 10.5) and mid-gastrula (stage 11). (D) ChIP-qPCR strategy to show FLAG-Pou5f3.2 binding to Pou motif-containing regions within Foxh1 peaks. (E) Sequential ChIP-qPCR analyses for Foxh1 and PouV co-binding on Nodal targets. Chromatin from embryos expressing FLAG-Pou5f3.2 was immunoprecipitated using anti-Foxh1 antibody, followed by a second immunoprecipitation using anti-FLAG antibody or anti-IgG antibody (negative control).
MO-injected embryos suggesting it may instead be positively regulated by PouV. Such a dual role for mammalian Pou5f1/Oct4 has been well documented (Pan et al., 2002; Hammachi et al., 2012). These results suggest that PouV proteins regulate the expression of a subset of Nodal target genes that are crucial to early mesendodermal development.

We also examined whether PouV proteins directly interact with the Pou motif regions found in these genes, which would imply direct PouV regulation. Because antibodies against Xenopus PouVs are not available, we microinjected mRNA encoding FLAG-tagged Pou5f3.2 into embryos and performed ChIP-qPCR to interrogate binding to the identified regions containing the Pou motif. FLAG-Pou5f3.2 protein preferentially bound to the cis-regulatory regions of all genes (Fig. 5D) that respond to PouV knockdown (Fig. 5C), but not to two genes (ins and rpl11) that were used as negative controls. We next investigated whether both PouV and Foxh1 proteins can co-occupy the cis-regulatory regions of a subset of these genes. A sequential ChIP-qPCR analysis (Geisberg and Struhl, 2004) showed that six of the eight genes tested were co-bound by Foxh1 and PouV proteins (>2-fold compared with an IgG control; Fig. 5E). These results suggest that the combinatorial action of inputs both from Foxh1-Smad3/2 (Nodal) signaling and PouV proteins is required for the establishment of proper expression levels of some of these genes during mesendodermal specification and patterning. Interestingly, Mullen et al. (2011) reported that Pou5f1 and Smad3 co-occupy target genes in mouse ESCs, suggesting that PouV proteins may have a more general role in modulating Activin/Nodal signaling.

DISCUSSION

We report the genome-wide examination of a Smad ‘partner’ in a developing vertebrate embryo. This study greatly expands the number of bona fide Nodal direct target genes operating in early gastrulation. Our analyses strongly support the notion that the coordinated action of Foxh1-Smad2/3 is a major in vivo driver of the mesendoderm transcriptional program. We also uncovered a new dual function for Foxh1 that is Nodal independent. PouV TFs were found to interact with Foxh1 to regulate Nodal target genes. And, finally, a cohort of Nodal target genes was identified that likely function in morphogenesis. Therefore, this study significantly broadens our view of the mechanisms that underlie TGFβ signaling control over the coordination of cell fate and behavior in early embryos.

Mesendodermal specification and patterning

Xenopus mesendodermal GRNs containing numerous connections between genes have been published (Koide et al., 2005; Loose and Patient, 2004). Furthermore, an endodermal core GRN based on studies from a variety of vertebrate systems suggests the involvement of Mix, Gata, Foxa, Hnf1b and Sox17 family members (Zorn and Wells, 2007). The molecular details of the interactions between Nodal signaling and these TFs are only partially understood. Here, we have confirmed six out of 11 suggested connections for Foxh1 direct regulation (gsc, chrd, otx2, mix1, gata4, bix1) and nine out of 16 connections for direct Nodal regulation (nodal1, nodal2, pitx2, bix1, cer1, mix1, gata4, gata6 and lefty). Additionally, numerous new direct connections were found for Foxh1 and Nodal regulation, respectively. Thus, this study has not only validated many interactions that were previously inferred, but also increased the total number of direct Nodal targets, providing a more sophisticated blueprint of the vertebrate mesendoderm GRN.

We found a number of genes encoding secreted signaling ligands and extracellular modifiers of signaling are direct Nodal targets. nodal1, nodal2 and wnt5a are directly upregulated by Nodal, whereas fgf16 and wnt11b are indirectly regulated. These Nodal genes and wnt5a are also direct Foxh1 targets. Secreted antagonists of Nodal, BMP and Wnt signaling, including cer1, lefty, frzb and dkk1, are direct targets of Nodal, implying that cells responding to Nodal signaling secrete these antagonists to coordinate the integration of multiple signaling activities. Others have attempted to identify direct Foxh1 and Smad2/3 targets. Microarray experiments by Pei et al. (2007) using zebrafish Foxh1 morphants identified cytokeratins 1 and 2, and keratins 4, 8 and 18 to be possible Foxh1 targets. These genes are not found in our list of direct Foxh1 targets and may be indirect targets of Foxh1 depletion in zebrafish. Silvestri et al. (2008) searched for human, mouse and rat genes with conserved Foxh1-Smad2/3 DNA motifs to identify potential targets of these factors. Among 21 candidates, excluding five well-known Nodal target genes (gsc, mix1, lefty, pitx2 and nodal), the remaining 16 genes are not found in our list of Nodal or Foxh1 target genes. Perhaps these genes are under Nodal regulation at later stages in development, but not gastrulation.

TGFβ signaling and Smad partner proteins

Foxh1 is a major Smad2/3 co-factor acting in early development, and several other Smad2/3 partners (e.g. Mixer, Gfi2i, Eomes) have also been implicated. The broader roles of these additional TFs in Nodal target regulation are unknown. We performed a motif analysis on Smad2/3-bound regions to determine the relative contribution of putative Smad2/3 partners. We confirmed that a large percentage of Smad2/3-bound Nodal-responsive genes contain Smad motifs. This analysis identified 5′-CAGAC-3′ in only 26 out of 75 direct Nodal targets. However, when we searched for the minimal Smad2/3-binding motif (5′-AGAC-3′) (Shi et al., 1998), this was found on 88% (66/75) of direct Nodal target genes. Sequences matching the Foxh1 motif 5′-AATMCACA-3′ were found on 66% (50/75) of direct Nodal targets, consistent with the view that Foxh1 is a major regulator of this signaling pathway.

The motif 5′-TAATYNRATTA-3′ is also enriched under Smad peaks and contains an inverted repeat (underlined) recognized by paired-family homeodomains (Wilson et al., 1993). This motif was found on 37% (28/75) of direct Nodal targets. The paired homeodomain proteins Mixer and Bix2 were shown to physically interact with Smad2 to regulate gsc expression (Germain et al., 2000) and finding this element on over one-third of direct Nodal target genes suggests that these proteins play an important role in Nodal signaling.

Another enriched motif we identified is the Heb (bHLH family) motif (supplementary material Fig. S11), also previously reported by Yoon et al. (2011) as being over-represented under Foxh1 and Smad peaks in human ESCs. The Heb sequence was also associated with 18 out of 75 (24%) Foxh1-bound regions in our ChIP-seq data, consistent with evidence from Yoon et al. that Heb/Tcf12 and E2a/Tcf3 may also regulate Nodal targets in Xenopus.

A T-box motif is enriched under Smad2/3 peaks on 20% (15/75) of direct Nodal target genes (supplementary material Fig. S11). Eomes, a T-box protein, binds Smad2 and has been implicated in Nodal-mediated mesendoderm induction in Xenopus, zebralsh and mammalian epiblast stem cells (Arnold et al., 2008; Picozzi et al., 2009; Slagle et al., 2011; Teo et al., 2011). Recently, ChIP-seq analysis of Eomes has been reported in Xenopus (Gentsch et al., 2013), allowing for a direct test of whether both Smad2/3 and Eomes are bound to the aforementioned 15 genes. Our analysis confirms that 12 out of 15 (80%) genes indeed have Eomes bound to the T-box motifs found under Smad2/3 peaks.
We found an HMG/Sox motif under Smad2/3 peaks on 27% (21/78) of direct Nodal target genes. Although there has been no demonstration that HMG/Sox family TFs interact with Smads, two SoxF genes, Sox7 and Sox17, are both expressed vegetally and have been implicated in mesendoderm specification. We hypothesize that these SoxF proteins might cooperate with Smad2/3. Finally, the Pou motif is enriched in both our Foxh1 and Smad2/3 ChIP-seq datasets (see below). We did not find enrichment for Gf2ird1, Gf2ir2 or Tp53 motifs.

**PouV in mesendoderm and pluripotency**

Among 60 SB431542-sensitive Foxh1-Smad2/3 bound genes, 31 genes (Fig. 4D blue sector, e.g. cxcr4 and hes7.2) were unaffected by Foxh1 MO injection. We hypothesize that these genes are Foxh1-Smad2/3 targets that receive strong inputs from other TFs that may act together with Foxh1, or in parallel. TFs predicted by motif analyses of Foxh1 peaks may be involved in this regulation.

Motif enrichment analysis found 47% (37/75) of direct Nodal targets contain a Pou motif under Smad2/3 peaks (supplementary material Tables S4 and S6). The involvement of PouV TFs in targets containing a Pou motif under Smad2/3 peaks (currently known as mix1), has previously been suggested (Cao et al., 2008; Levigni et al., 2013). Our analysis of PouV MO-injected embryos further extends these observations by identifying two other Nodal targets (nodal2, mespb) that are subjected to regulation by PouV (no Pou motif is present under Smad2/3 peaks in the X. tropicalis mix1 gene). Interestingly, 37% (13/35) of the direct Nodal targets containing Pou motifs are not co-bound by Foxh1, suggesting that the Foxh1-PouV interaction may not be the only mechanism for regulation of these genes.

Human ESCs (hESCs) are likely of epiblast origin, which is comparable to late blastula in *Xenopus*. Frog embryonic cells at this stage are pluripotent, can be directed into all three germ layers by appropriate signals and thus have characteristics that mirror ESCs. Furthermore, although hESCs require Activin/Nodal signaling for maintenance of pluripotency, high doses of Activin drive these cells into mesendodermal lineages. Perhaps PouV-mediated regulation of Nodal targets in *Xenopus* is analogous to Pou5f1 regulation of pluripotency in epiblast and hESCs. Experiments differentiating hESCs into definitive endoderm have indeed shown that Pou5f1 represses a number of Nodal-induced endodermal markers, including gsc (Teo et al., 2011), which we showed are similarly repressed in *Xenopus*. These observations support a model for evolutionarily conserved PouV-mediated repression of Nodal-dependent mesendodermal differentiation.

**Nodal-independent Foxh1 functions and repression**

Our KS tests confirm the view that Foxh1 generally functions as a transcriptional activator. The nodal3, nodal8 and fkh1 genes were previously implicated to be repressed by Foxh1 in either *X. laevis* or zebrafish (Choi et al., 2007; Kofron et al., 2004b), but in *X. tropicalis*, neither Foxh1 nor Smad2/3 binding to these genes was detected. Thus, the observed repression by Foxh1 may be indirect or occurs at stages other than the early gastrula. Our analysis also suggests that foxh1 is negatively autoregulated as foxh1 MO injection increases the expression of foxh1 itself. However, ChIP-seq fails to find Foxh1 binding to the foxh1 gene, implying that negative autorepression is indirect.

Our analysis revealed 72 Foxh1 direct targets that are regulated independently of Nodal signaling (supplementary material Table S8). Knockdown of foxh1 reveals that it acts as both an activator and repressor, independent of Nodal-Smad2/3. The spatial expression patterns of Nodal-independent Foxh1 target genes were variable in gastrula stage embryos (W.T.C., I.L.B. and K.W.Y.C., unpublished), unlike the Foxh1-Smad2/3 targets, which are either mesendoderm-specific or significantly enriched in this region. Although GO term analysis for the 71 Nodal-independent Foxh1 targets did not reveal useful functional information, the list includes a noteworthy mix of secreted growth factors, TFs, GTPase modifiers and adhesion molecules.

**Regulation of morphogenesis**

Nodal signaling regulates mesodermal morphogenesis by affecting cellular activities involved in tissue dynamics. Among the 60 direct Nodal targets, many were previously shown to affect cell behaviors. One Nodal-regulated TF, snail1, is involved in epithelial-mesenchymal transitions (EMTs) in numerous contexts, and has been implicated in the morphogenesis of gastrulation (Spring et al., 2002).

The direct Nodal induction of flrt3, a transmembrane protein that regulates cell adhesion, is necessary for proper morphogenesis of the organizer cells (Ogata et al., 2007). We found rnd1, pdgfra and pcdh8.2, confirming previous reports that they were either responsive to Nodal signaling or implicated as direct targets (Luxardi et al., 2010; Ogata et al., 2007). We also identified plekha5, prickle2, ctnmt8, efnb2, cxcr4, ntn3 and cass4 as direct Nodal targets that likely participate in morphogenetic movements during *Xenopus* gastrulation. Plekha5 regulates Rnd1 protein activity (Goh and Manser, 2012) and thus might function in an adhesion pathway with rnd1 and flrt3. Prickle2 regulates cell polarity in response to non-canonical Wnt signaling (Antic et al., 2010; Tao et al., 2012, 2011). Cxcr4 regulates endodermal cell migration during zebrafish gastrulation (Nair and Schilling, 2008) but its function in *Xenopus* gastrulation is unknown. Ctnmt8, efnb2, ntn3 and cass4 have all been implicated in EMT, cell migration and/or differential cell adhesion in various cell types. Here, we have identified a gene battery that is directly regulated by Nodal and likely functions in coordinating complex cellular behaviors that affect morphogenesis during gastrulation. Our future goal is to further examine the mesendoderm network of *Xenopus tropicalis* and better elucidate the conserved GRN architecture involved in vertebrate gastrulation.

**MATERIALS AND METHODS**

**Embryo handling**

*X. tropicalis* embryos were obtained by *in vitro* fertilization. Four-cell stage embryos were immersed in 100 μM SB431542 (Tocris Bioscience) in 1/9×MMR and cultured at 25°C until mock (solvent)-treated siblings reached gastrula stage. MO injections were performed at the 2-cell stage.

**Polyclonal antibody generation**

A GST fusion protein containing amino acids 14-113 of *X. tropicalis* Foxh1 was produced in BL21 cells and purified using glutathione-agarose chromatography. Rabbit polyclonal antisera were produced by Covance and affinity purified.

**MO knockdowns and rescue constructs**

The foxh1 MO sequence is 5′-TCATCTGAGGCTCCGGCCCTCTCTA-3′. A 5′UTR deletion was created by BstEII/BsaSI digestion of *X. tropicalis* foxh1 cDNA TGas103n06 (Gilchrist et al., 2004) followed by self-ligation to generate a MO-resistant foxh1. PouV MO sequences are: pou5f3.1, 5′-CCGGTTGTGGTATACTAGTTGGTCC-3′; pou5f3.2, 5′-GCTGTTG-GCGTATCTAGTTGGTCC-3′; pou5f3.3, 5′-TGCAGAGCCGGTTGGTCC-ATGGTTC-3′. Full details of the construction of PouV rescue mRNA plasmid templates can be found in the methods in the supplementary material.
Immunoprecipitation and western blot
Embryonic extracts from either control or Foxh1 MO-injected mid-gastrula embryos were immunoprecipitated followed by western blot analysis. Anti-Foxh1 antibody coupled to CNBr-activated sepharose (GE Healthcare) was used for immunoprecipitation. Beads were washed, boiled in SDS sample buffer, and eluent was subjected to western blot analysis. The blot was probed with anti-Foxh1 and then HRP-coupled anti-protein A antibodies. Anti-β-catenin antibody was used to control for morpholino specificity. For Smad IP-westerns, extracts were incubated with either Smad2/3 or Smad1/5/8 antibodies coupled to CNBr-activated beads. After washing, eluted Smad protein was subjected to western blotting and probed with either anti-pSmad2 or anti-pSmad1/5/8. Bands were visualized using HRP-coupled secondary antibody using an ECL Prime kit. Membranes were re-probed with anti-Smad2/3 or anti-Smad1/5/8. Details of antibodies are given in supplementary material Table S11.

Quantitative RT-PCR analysis
RNA samples from morphant or control were reverse transcribed using MMLV reverse transcriptase (Invitrogen). Quantitative RT-PCR of cDNA samples was performed using a Roche LightCycler 480. Details of primers are given in supplementary material Table S9.

RNA-seq analyses
Total RNAs were extracted from control and experimental early gastrulae as described previously (Chomczynski and Sacchi, 1987). Total RNA was oligo(dT) selected and fragmented, and libraries were prepared for single-end sequencing using a TruSeq RNA-seq sample preparation kit (Illumina). More details can be found in the methods in the supplementary material.

ChIP
Anti-Smad2/3 (1.5 μg) and custom anti-Foxh1 (4 μg) antibodies were used per 100-embryo equivalents of chromatin for ChIP. Two thousand early gastrulae were subjected to ChIP for each antibody. Formaldehyde fixation, sonication, immunoprecipitation and washing steps were performed as previously described (Lee et al., 2006, Stewart et al., 2006) with modifications. Immunoprecipitated chromatin was purified and resuspended in Qiagen EB solution for subsequent library generation using NEXTflex ChIP-Seq Kit (Bio Scientific). More details can be found in the methods in the supplementary material. Details of primers are given in supplementary material Table S10.

For sequential ChIP, embryos microinjected with FLAG-tagged Pou5f3.2 mRNA were fixed at late blastula stage 9. Crosslinked chromatin was first immunoprecipitated using anti-Foxh1 antibody, followed by the regular ChIP washes. Immunocomplexes were eluted and subjected to a second immunoprecipitation with mouse anti-FLAG M2, anti-Foxh1 or rabbit anti-IgG antibodies, then washed and eluted. DNA's were extracted, purified and subjected to qPCR.

Bioinformatic analysis
Differential gene expression analysis of RNA-seq data was performed using TopHat (v. 1.3.3) (Trapnell et al., 2009) and Cuffdiff (v. 1.3.0) (Roberts et al., 2011). ChIP-seq reads were mapped using Bowtie (v.0.12.9) (Langmead et al., 2009); peaks were called using MACS (v.2.0.10) (Zhang et al., 2008) and SISSRs (v. 1.4) (Jothi et al., 2008). Differentially expressed genes were identified using the fDR package (v.1.4) (Grant et al., 2011). The Broad database was used to identify enriched GO terms. GO analysis was performed with DAVID Bioinformatic Resources 6.7 (Huang et al., 2009). More details can be found in the methods in the supplementary material. The Gene Expression Omnibus accession number for high-throughput sequencing data reported in this paper is GSE53654.

Kolmogorov-Smirnov analysis
ChIP peak calls were compared with RNA-seq differential expression data (Foxh1 MO or SB-431542) using a Kolmogorov-Smirnov (KS) plot, similar to Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005). More details can be found in the methods in the supplementary material.

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Competing interests
The authors declare no competing financial interests.

Author contributions
W.T.C., R.C.L., M.B.F. and I.L.B. performed wet bench experiments. W.T.C., Y.L., J.B. and X.X. performed bioinformatics analyses. W.T.C., I.L.B., R.C.L., M.B.F. and K.W.Y.C. drafted the manuscript. K.W.Y.C. conceived and coordinated the study. All authors read and approved the final manuscript.

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Supplementary material
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SUPPLEMENTARY MATERIALS AND METHODS

Construction of PouV rescue mRNA plasmid templates

Rescue constructs all contained the PouV ORFs cloned downstream, and in frame, of a 3XFlag epitope coding sequence in pCS2+. This results in loss of their MO complementary sequences. The Pou5f3.2 rescue construct was created by cloning a RsaI-XbaI fragment from pCS2+Pou5f3.2 between the Stul and XbaI sites in pCS2+3XFlag. Rescue constructs for Pou5f3.1 and Pou5f3.3 were created using the Gibson isothermal assembly cloning strategy (Gibson et al., 2009). pCS2+Pou5f3.3 and pCS2+Pou5f3.1, containing wild-type cDNAs were PCR-amplified to create linear products using the following primer pairs:

Pou5f3.3 F, GACCAACCCTTTCTGTACAACC
Pou5f3.3 R, CATGGTGCCGGATCTCTG
Pou5f3.1 F, TATAACCAACAGGCCTACCCTTC
Pou5f3.1 R, CATGGCATCGATGGGATC

Primers used to amplify 3XFLAG insert w/ ends for vector homology were:

Pou5f3.3 3XFLAG F
CTTGTTCTTTTTGCAGGATCCGCCACCATGGACTACAAAGACCATGACGGTGAT
Pou5f3.3 3XFLAG R
GGCAGGTTGTTGTCAGAAGCGGTTGGCCTTTGTCATCGTCATCCTTTGTAGTC
Pou5f3.1 3XFLAG F
TTCTTTTTGCAGGATCCCATCGATGCCATGGACTACAAAGACCATGACGGTGAT
Pou5f3.1 3XFLAG R
GGCAAAGGAAGGGTAGGCCTGTGGTATTACTTTGTCATCGTCATCCTTTGTAGTC

Chromatin Immunoprecipitation Analysis

Two thousand embryos were cultured until early gastrula (stage 10.5). Embryos were crosslinked in batches of 200 embryos with 1ml of 1% formaldehyde in 1/9XMMR at room temperature for 45 minutes with gentle rocking. Crosslinking reactions were
quenched by removal of the formaldehyde solution and incubation in 1ml of 125mM glycine for 5 minutes at room temperature. Fixed embryos were then washed twice with ice-cold RIPA buffer (50 mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1% NP40, 0.1% SDS, 0.5 mM DTT, and Roche cOmplete protease inhibitor cocktail). The embryos were subsequently homogenized with an Eppendorf tube pestle and microfuged at 14,000 rpm, 4°C for 15 minutes. The pellets, which contain chromatin, were re-suspended in 750µl RIPA buffer and sonicated on ice using a Branson Digital Sonifier 450 (18% power output, 20 sec per pulse, 1 minute “rest”, 10 pulses) resulting in DNA fragmentation between 200-500bp long. The resulting samples were microfuged at 14,000 rpm for 20 minutes at 4°C to remove insoluble cellular debris. Supernatant containing fragmented chromatin was “pre-cleared” by incubating with 20µl Protein A-coated Dynabeads (Invitrogen) for 2 hour at 4°C. 50 embryo equivalents (eeq) volume of the chromatin supernatant was frozen for use as “input control”. Antibodies were pre-bound to 20µl of Protein A Dynabeads (per 200 eeq) by incubating at 4°C for 30 minutes. 200 eeq of chromatin was then added to antibody-bound Dynabeads and incubated overnight at 4°C on an end-over-end rotator. The next day, supernatants were discarded, the recovered beads were washed once each for 15 minutes with ice-cold: ChIP wash solution I (50mM HEPES-KOH pH7.5, 2mM EDTA, 150mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1mM DTT, and 0.4mM PMSF), ChIP wash solution II (50mM HEPES-KOH pH7.5, 2mM EDTA, 500mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1mM DTT, and 0.4mM PMSF), ChIP wash solution III (0.25 M LiCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, and 0.4 mM PMSF), and finally TE (10mM Tris, 1mM EDTA) supplemented with 1 mM DTT, and 0.4 mM PMSF. The DNA was then eluted with TE buffer containing 1% SDS, and reverse-crosslinked at 65°C for 6 hours to overnight. Frozen sonicated input control chromatin was diluted 3-fold with elution buffer and also
incubated at 65°C. On the following day the samples were treated with 0.2µg RNase A/µl for 2 hours at 37°C, followed by 0.2µg Proteinase K/µl for 2 hours at 55°C, followed by phenol/chloroform extraction and ethanol precipitation. DNA pellets were resuspended in Qiagen EB solution. For ChIP-qPCR analyses on enriched chromatin fragments, immunoprecipitated DNA was amplified using specific primers to detect enrichment in the denoted genomic regions. Results were normalized against input DNA. Following qPCR validation, total DNA recovery was estimated by Qubit fluorimetry using a Quant-iT™ dsDNA High Sensitivity Assay Kit (Invitrogen). Twenty to thirty nanograms of immunoprecipitated DNA was processed to construct sequencing libraries using ChIP-seq library kits from Illumina, NEB and Bioo Scientific and single-end 40bp or 50bp single-end sequencing runs were performed on Illumina GAIIx and HiSeq2000 instruments at UC Riverside’s Genomics Core Facility and UC Irvine’s Genomic High Throughput Facility.

For sequential ChIP experiments, 150 embryos microinjected with 150pg FLAG-tagged Pou5f3.2 mRNA were crosslinked as described above. Crosslinked chromatin was immunoprecipitated by incubating with anti-Foxh1 antibody-coated Dynabeads overnight. Subsequently, the chromatin precipitates were washed with ChIP I, II, III and TE buffers containing protease inhibitors and 1mM DTT. The immunocomplexes were eluted in a solution of 10mM DTT, 500mM NaCl, and 0.1% SDS at 37°C for 30 minutes. Eluted protein-chromatin complexes were diluted 10-fold with RIPA buffer, and subjected to a second immunoprecipitation with mouse anti-FLAG M2 antibody (Sigma), anti-Foxh1 or rabbit IgG control, then washed and eluted, as described above. The isolated DNAs were extracted, purified, and then subjected to qPCR analyses.
**RNA-seq Analyses**

Total RNAs were extracted from ~25-30 control and experimental early gastrula embryos using the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi 1987), followed by selective precipitation of RNA using 2.5M LiCl. The quality of the RNA was examined using an Agilent BioAnalyzer 2100 instrument. One microgram of total RNA was subjected to oligo(dT) selection to extract polyadenylated RNA, which was then chemically fragmented and libraries were generated for single-end sequencing according to Illumina’s RNA-seq sample preparation kit.

**Bioinformatics Analyses**

**Differential Gene Expression Analysis for RNA-seq**

Raw RNA-seq reads from Illumina sequencing were mapped to the most recent *Xenopus tropicalis* genome assembly v7.1 using TopHat (v. 1.3.3) (Trapnell et al. 2009), with an option “-G <Xentr7_2_Stable.gff3>”. The differential expression of genes in control versus perturbed samples were calculated using Cuffdiff (v. 1.3.0) (Roberts et al. 2011) with default settings. Genes were determined to be significantly regulated by Nodal signaling/Foxh1 based on the following criteria: 1) Cuffdiff must designate a gene’s analysis as “OK” in the column labeled “status”; 2) “yes” assigned in the column labeled “significant” and 3) its log2 fold change was less than -0.583 or was greater than 0.583 in the Cuffdiff output file (see Supplemental Table 1 for details).

**Peak Calling for ChIP-seq**

For each of the ChIP experiments (Foxh1 and Smad2/3), two biological replicates at the early gastrula stage were collected and sequenced. Raw ChIP-seq reads from the replicates were pooled and mapped to an indexed *Xenopus tropicalis* genome (version 7.1) using Bowtie (v. 0.12.9) (Langmead et al. 2009), only uniquely mapped
reads were retained (Supplemental Table 2). An input DNA library was sequenced and used as control for subsequent peak prediction. Two peak-calling software MACS (v. 2.0.10) (Zhang et al. 2008) and SISSRs (v. 1.4) (Jothi et al. 2008) were used to identify genomic regions with enriched reads indicating Foxh1 or Smad2/3 binding. MACS was run using default settings except for modifications to account for the X. tropicalis effective genome size of 1.16e+09. SISSRs was run with the option “-a”, so that only one read is retained when multiple reads align to the same genomic coordinate. The peaks called by the two peak calling software were further compared, and only overlapping peaks were retained for further analyses. Peaks were assigned to the nearest genes using BEDTools (v 2.16.2) (Quinlan and Hall 2010) based on the following criteria: peak summits need to lie within 10 kb upstream of the 5’ end, within the gene body (5’ UTRs, exons, introns, and 3’UTRs), or 10 kb downstream of the 3’ end of the gene.

Motif Analyses
Motif Analyses were performed using DNA sequences consisting of 150bp centered on the summits of the MACS-predicted peaks. These peak sequences were inputted into MEME (v. 4.7) (Bailey and Elkan 1994) with default settings for motif discovery. The discovered motifs were subsequently used in searches of the JASPAR CORE 2009 and UniPROBE databases using the TOMTOM Motif Comparison Tool (Gupta et al. 2007), and TRANSFAC 2011 using STAMP *(Mahony and Benos 2007). FIMO (v. 4.9.0) (Grant et al. 2011) was used to link identified motifs to individual peak locations containing the motif.

Kolmogorov-Smirnov Analysis
The ChIP peak calls were compared with RNA-seq differential expression data (Foxh1 MO or SB-431542) using a Kolmogorov-Smirnov (KS) plot, similar to Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005). The KS test's null
hypothesis is that even with the genes ranked by differential expression, ChIP-bound genes have random ranking. In particular, the KS test compares the cumulative distribution of ranked presence/absence of peaks with a uniform cumulative distribution; the difference between these two distributions at each x-value is plotted as the “Running Enrichment Score” and its maximum value is the $d$ statistic used in the KS test. Using the RNA-seq data as a guide, the KS plot allows us to observe enrichment of Foxh1 bound (or Smad2/3 bound) genes in any region of the differential expression-ranked list.

The comparison between correlations of Smad2/3 binding and Foxh1 binding to SB431542-sensitive genes referred to in the text relevant to Figure 4B,C was performed as follows. Of 239 Smad2/3-bound genes, 77 (32.2%) were down-regulated by SB431542 at least 1.5-fold (log2 fold change threshold as -0.583). Similarly, of 689 Foxh1-bound genes, 90 (13.1%) were down-regulated by SB431542 at least 1.5-fold. Therefore, Smad2/3 binding to SB431542-sensitive genes is enriched ~2.5 fold (32.2%/13.1%) over Foxh1 binding to SB431542-sensitive genes.
References


Figure S1. Generation Of Anti-Foxh1 Antibody. Localization of conserved domains in Foxh1 orthologs from Xenopus tropicalis, human and mouse. Blue boxes represent the Forkhead (FH) DNA binding domains; red boxes represent Fast/Foxh1 motifs (FM); purple boxes represent Smad-Interacting Motifs (SIM) (Randall et al., 2004). Green line (amino acids 14-113) indicates N-terminal region of X. tropicalis Foxh1 used for polyclonal antibody generation.
**Figure S2.** Phenotypic rescue of Foxh1 Morphant (left panel) by co-injecting Foxh1 specific morpholino together with Foxh1 RNA (right panel).
**Figure S3.** Dosage-dependent effect of SB431542 on Nodal-regulated target genes. gsc and chrd showed stronger repression by SB from 10 μM to 100 μM. Whereas ventx2.1 (BMP target), mex3c (nodal-independent Foxh1 direct target, see main text), and ef1a1 (house keeping gene), were not affected.
**Figure S4.** foxh1 shows negative autoregulation. foxh1 transcript levels are significantly up-regulated (~14 fold) upon foxh1 MO injection, while other Foxh1 targets, chrd, gsc, and otx2, were consistently down-regulated.
Figure S5. Differential responses of genes in SB431542 treatment and foxh1-MO injection.  
A. Scatter plot showing expression levels (FPKM) of all genes at early gastrula stage in SB431542-treated compared to control embryos.  
B. Scatter plot for foxh1-MO injected embryo versus controls. Gray and black lines demarcate expression differences greater than 1.5 fold or 2 fold, respectively. Blue; down-regulated, red; up-regulated genes.
Figure S6. ChIP-qPCR assays of several genomic regions validating the efficiency of the ChIP protocol. A. ChIP using anti-Foxh1 antibody was able to enrich cis-regulatory regions of the genes gsc, mix1, cer1 and otx2, as opposed to negative control regions in the odc1 and ventx2.1 promoters. B. ChIP using anti-Smad2/3 antibody was able to enrich mix1 and cer1 promoters and pitx2 intron1 regions, as opposed to odc1 and ventx2.1 promoters. PE, proximal element; ARE, activin response element.
Figure S7. Plot of the distribution of a randomized set of peaks along the lengths of gene bodies, and 10kb upstream and downstream of the genes shows no obvious peak enrichment along these window, as compared to Foxh1’s in Fig. 2F.
Figure S8. RT-qPCR validation of 6 more genes that are Foxh1 direct targets and are independent of Nodal regulation. **A-C.** Foxh1-activated targets. **D-F.** Foxh1 repressed targets.
Figure S9. Morpholino rescue of Nodal independent Foxh1 direct target genes.
**Figure S10.** *gsc, nodal2* and *mespb* expressions were perturbed by PouV MOs injection (also see Fig. 5C), and could be significantly rescued by co-injection of RNAs encoding PouV proteins at both st 10.5 and st 11.
**Figure S11.** A list of top 10 motifs discovered under Smad2/3-bound regions by MEME.
Yoon et al. (2011) published a Smad2/3 ChIP-seq analysis on Xenopus tropicalis early gastrula stage embryos. A comparison between our Smad2/3 ChIP-seq data with these results has been performed using our own analysis pipeline. A. Two hundred one peaks were identified from Yoon et al.’s dataset, whereas 939 peaks were identified from our current data. This disparity may be caused by a number of factors including differences in total reads recovered, read mapping efficiency, ChIP efficiency, etc. Using our analysis pipeline, from a total of ~70M reads, we obtained ~39M uniquely mapped reads. From the Yoon et al.’s dataset of ~15M total reads, ~7M reads mapped uniquely. We used stringent criteria to assign peaks, which included a q-value < 0.05 and the requirement that bound regions are independently identified by two different peak callers, MACS2 and SISSRS. Despite these differences, ~70% (137/201) of peaks identified from Yoon et al.’s data overlapped with our peaks, suggesting high reproducibility and reliability of both datasets.

B. Assigning peaks to genes and comparison of these to differential gene expression data from RNA-seq on control versus SB431542-treated embryos reveals only 4 additional direct Nodal target genes (pdkcc.2, pnhd, wnt11b, and Xetro.A00154 [an unannotated gene that by blast and synteny appears to be mcidas, which is related to geminin]) in the Yoon et al. dataset that are missing from our analysis.
### SUPPLEMENTARY TABLES

**Table S1**
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**Table S2**
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**Table S3**
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**Table S5**
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**Table S6**
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**Table S7**
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**Table S8**
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Table S9. Primers for RT-qPCR

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<th>gene</th>
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Table S10. Primers for ChIP-qPCR

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Table S11. Antibodies used

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