Developmental Biology 351 (2011) 297–310

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

A gene regulatory network controlling *hhex* transcription in the anterior endoderm of the organizer

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ARTICLE INFO

Article history:
Received for publication 21 July 2010
Revised 15 November 2010
Accepted 17 November 2010
Available online 4 January 2011

Keywords:
hhex
Xenopus
Endoderm
Organizer
Nodal
Wnt
Siamois
Vent
Transcription

ABSTRACT

The homeobox gene *hhex* is one of the earliest markers of the anterior endoderm, which gives rise to foregut organs such as the liver, ventral pancreas, thyroid, and lungs. The regulatory networks controlling *hhex* transcription are poorly understood. In an extensive cis-regulatory analysis of the *Xenopus hhex* promoter, we determined how the Nodal, Wnt, and BMP pathways and their downstream transcription factors regulate *hhex* expression in the gastrula organizer. We show that Nodal signaling, present throughout the endoderm, directly activates *hhex* transcription via FoxH1/Smad2 binding sites in the proximal −0.44 Kb promoter. This positive action of Nodal is suppressed in the ventral–posterior endoderm by Vent 1 and Vent2, homeodomain repressors that are induced by BMP signaling. Maternal Wnt/β-catenin on the dorsal side of the embryo cooperates with Nodal and indirectly activates *hhex* expression via the homeodomain activators Siamois and Twin. Siamois/Twin stimulate *hhex* transcription through two mechanisms: (1) they induce the expression of Otx2 and Lim1 and together Siamois, Twin, Otx2, and Lim1 appear to promote hhex transcription through homeobox sites in a Wnt-responsive element located between −0.65 to −0.55 Kb of the *hhex* promoter. (2) Siamois/Twin also induce the expression of the BMP-antagonists Chordin and Noggin, which are required to exclude Vents from the organizer allowing *hhex* transcription. This study reveals a complex network regulating anterior endoderm transcription in the early embryo.

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Introduction

The homeodomain (HD) transcription factor Hhex is one of the earliest markers of the foregut progenitor cells that give rise to the liver, ventral pancreas, thyroid, and lungs (Keng et al., 1998; Newman et al., 1997; Thomas et al., 1998). The regulatory networks that control gene expression in the early foregut progenitors, and *hhex* transcription in particular, are poorly understood. A greater understanding of this process could provide insight into congenital foregut organ defects and enhance our ability to direct the differentiation of stem cells into foregut organ lineages.

In *Xenopus*, *hhex* is first expressed at the blastula stage in the dorsal–anterior endoderm of the Spemann organizer, which after gastrulation gives rise to the ventral foregut progenitors (Brickman et al., 2000; Jones et al., 1999; Newman et al., 1997). The organizer and its equivalent in other species is a heterogeneous population of cells that plays an essential role in axial patterning, with sub-regions of the organizer having distinct functions (De Robertis, 2009; Niehrs, 2004). The chordomesoderm component regulates trunk formation, whereas the *hhex*-expressing anterior endoderm regulates head and cardiac induction (Bouwmeester et al., 1996; Foley and Mercola, 2005; Jones et al., 1999; Niehrs, 2004). Hhex function is essential for these activities as *hhex*-deficient mouse and *Xenopus* embryos have head truncations as well as heart and foregut organ defects (Bort et al., 2004; Keng et al., 2000; Martinez Barbera et al., 2000; McLin et al., 2007; Smithers and Jones, 2002).

In *Xenopus*, the organizer is formed in the dorsal margin of the blastula by the intersection of Nodal signaling in the vegetal cells and a maternal Wnt11/β-catenin (mWnt) pathway active on the future dorsal side of the embryo (Heasman, 2006). Activation of the canonical Wnt signaling causes β-catenin to accumulate in the nucleus, where it interacts with Tcf/Lef transcription factors to displace Groucho/Tle co-repressors and directly stimulates the transcription of Wnt-target genes such as the related HD factors Siamois (Sia) and Twin (Twn) (Brannon et al., 1997; Carnac et al., 1996; Fan et al., 1998; Kessler, 1997; Laurent et al., 1997; Lemaire et al., 1995). In addition, β-catenin/Tcf complexes cooperate with the vegetally localized maternal T-box transcription factor VegT to activate transcription of Nodal-related ligands (*xnr1, 5, 6*) resulting in high levels of Nodal signaling in the dorsal–vegetal cells of the blastula (Hilton et al., 2003; Hyde and Old,

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doi:10.1016/j.ydbio.2010.11.037

2000). Nodal-activated receptors phosphorylate Smad2 proteins, which translocate to the nucleus and interact with DNA-binding proteins such as Foxh1, Wbscr11, Mixer, and Bix2 to activate mesendoderm gene transcription (Chen et al., 1997; Germain et al., 2000; Ring et al., 2002).

The combination of Nodal and mWnt signaling promotes the expression of organizer-specific transcription factors including Gsc, Otx2, Lim1/Lhx1, as well as a number of secreted BMP and Wnt antagonists. These include Chordin, Noggin, Sfrp2, Sfrp3/FrzB, Crescent, Dkk1, and Cerberus, which mediate the organizer's inductive activities by inhibiting BMP4 and zygotic Wnt8 (zWnt8) ligands expressed in the ventral marginal zone (De Robertis, 2009; Niehrs, 2004). BMP4 and zWnt8 promote ventral–posterior fates and restrict dorsal–anterior fates, in part by inducing the expression of the HD transcriptional repressors Vent1 and Vent2, which inhibit organizer gene expression (Friedle and Knochel, 2002; Karaulanov et al., 2004; Onichtchouk et al., 1998; Ramel and Lekven, 2004; Rastegar et al., 1999; Sander et al., 2007).

Promoter analyses in *Xenopus* have begun to reveal how interactions between these various signaling pathways and transcription factors are integrated on cis-regulatory elements to control gene expression. One of the most extensively characterized models of organizer transcription is the *gsc* promoter, which is coordinately regulated by Nodal and mWnt signaling through distinct proximal and distal cis-elements (PE and DE respectively) (Koide et al., 2005). Nodal/Activin stimulate *gsc* transcription though Smad–Foxh1 complexes binding to the PE and Smad–Wbscr11 complexes binding to a the DE (Blythe et al., 2009; Labbe et al., 1998; Ring et al., 2002; Watabe et al., 1995). Studies have shown that Sia/Twn also bind to the PE to stimulate transcription in response to mWnt signaling (Kessler, 1997; Laurent et al., 1997; Watabe et al., 1995). After the initial activation of *gsc* transcription, a number of other HD factors including Lim1, Otx2, Bix2, Mix1, and Mixer maintain *gsc* expression by binding to a series of homeobox sites in the PE and DE (Germain et al., 2000; Latinkic and Smith, 1999; Mochizuki et al., 2000). In the ventral–posterior mesendoderm, these same homeobox sites appear to be utilized by the HD repressors Vent1/2, Msx1, and Pou2, which inhibit *gsc* transcription (Danilov et al., 1998; Trindade et al., 1999; Witta and Sato, 1997).

A few other organizer gene promoters (*sia*, *twn*, *lim1*, *foxa4*, *noggin*, and *cerberus*) have also been analyzed (Howell and Hill, 1997; Kaufmann et al., 1996; Tao et al., 1999; Watanabe et al., 2002), but other than *cerberus*, their expression is not restricted to endoderm component of the organizer like *hhex*. An analysis of *cerberus* transcription indicates that it is an indirect target of Nodal and mWnt signaling and suggests that like *gsc* it is cooperatively regulated by Sia, Lim1, Otx2, and Mix1 complexes (Yamamoto et al., 2003). It is unclear to what extent this mode of regulation can explain all anterior endoderm transcription.

In this study, we have examined how the Nodal, Wnt, and BMP pathways and their downstream transcription factors impact cis-regulatory elements to control *hhex* transcription in the dorsal–anterior endoderm of the organizer. By coupling promoter analysis in *Xenopus* transgenics with an extensive series of loss-of-function and rescue experiments, we have elucidated a gene regulatory model linking our understanding of axial patterning to early foregut organ development.

Materials and methods

Embryo manipulations and gene expression assays

Xenopus laevis embryos were cultured as previously described (Zorn et al 1999). Embryos with clear dorsal and ventral pigmentation differences were selected for 32-cell stage injections. In explant experiments, the following were added to the media as indicated: cycloheximide (10 µg/ml; Sigma), dexamethasone (4 µg/ml; Sigma), Recombinant human Activin A (100 ng/ml; R&D systems), LiCl (200 mM; Sigma) or BIO (10 µM; Stemgent).

Generation of the −*6Kb:hhex:gfp* transgenic lines was previously described (McLin et al., 2007). For deletion analysis, *hhex* promoter fragments were PCR amplified (details available upon request), sequence verified, and cloned into either the pGFP3 or the pGL2-Basic (Promega) reporter vectors. Mutations were made using the GeneTailor site-directed mutagenesis kit (Invitrogen). Transient transgenics were generated by nuclear transplantation as previously described (Kroll and Amaya, 1996; Sparrow et al., 2000). To visualize GFP, transgenic embryos were fixed in MEMFA for 2 h, bisected in PBS and fluorescence was directly imaged by microscopy.

For luciferase assays, *hhex:luc* promoter constructs (300 pg) were microinjected along with a pRL-TK:Renilla control vector (25 pg) and activity was determined using standard kits (Promega). In every experiment, each construct was assayed in biological triplicate (three tubes of 5 embryos each) and the mean normalized luciferase/renilla activity and standard deviation were determined. Experiments were repeated at least three separate times. In all cases, the same trends were observed and a representative example is shown.

In situ hybridization (McLin et al., 2007) and RT-PCR analysis (Kofron et al., 2004) were performed as previously described. The cDNA for the maternal FoxH1 depletion experiment was from Kofron et al. (2004). Chromatin immunoprecipitation (ChIP) analysis was performed as described in Blythe et al. (2009) with minor modifications using the PCR primers provided in supplementary Table S1.

Morpholino oligos and synthetic mRNAs

All morpholino oligos (MOs) in this study, with the exception of the Smad2a-MO (20 ng, 5′-ggtgaaaggcaagatggacgacatg-3′) and Smad2b-MO (20 ng, 5′-ggtgaatggcaaaatcgagcacatg-3) have been previously published and shown to generate specific loss-of-functions: β-catenin-MO (Heasman et al., 2000), Tcf3-MO (Liu et al., 2005), Siamois-MO and Twin-MO (Ishibashi et al., 2008), Otx2-MO (Carron et al., 2005), Lim1-MO (Schambony and Wedlich, 2007), Chordin-MOs (Oelgeschlager et al., 2003), Noggin-MO (Kuroda et al., 2004), Gsc-MO, Vent1-MO and Vent2-MO (Sander et al., 2007). For each MO, we reproduced the published phenotypes (Supplementary Fig. S1).

The following synthetic mRNAs have been previously described: Cer-S (Piccolo et al., 1999), stabilized pt-β-catenin (Yost et al., 1996), ΔNTcf3 (Molenaar et al., 1996), Xnr1 (Zorn et al., 1999), FoxH1 (Kofron et al., 2004), FoxH1-EnR and FoxH1-VP16 (Watanabe and Whitman, 1999), Smad2 (Shimizu et al., 2001), Siamois and Sia-EnR (Kessler, 1997), GR-Siamois (Kodjabachian and Lemaire, 2001), Otx2 (Gammill and Sive, 1997), Lim1 and GR-Lim1/3 m (Yamamoto et al., 2003), Gsc (Yao and Kessler, 2001). To construct pT7Ts-GR-Vent2-VP16, the Vent2–VP16 open reading frame was PCR amplified from the pRN3-Vent2–VP16 vector (Onichtchouk et al., 1998), cloned in-frame into the pT7TS-GR plasmid, and sequence verified.

Results

A −6Kb hhex:gfp transgene recapitulates anterior endoderm expression

To better understand the gene regulatory network controlling early anterior endoderm gene expression, we analyzed the regulation of *hhex* transcription in transgenic *Xenopus laevis* embryos. Previously we generated two independent −*6Kb:hhex:gfp* transgenic lines containing approximately 6 Kb of genomic *laevis* sequence upstream of the *hhex* transcriptional start site (McLin et al., 2007). Here we show that these transgenic lines recapitulate early *hhex* expression in the anterior endoderm (Fig. 1). Transcription of endogenous *hhex* and *gfp* were simultaneously activated in the dorsal–anterior vegetal cells of the late blastula (stage 9.5) and exhibited identical expression in the anterior endoderm and ventral foregut until stages 25–27. By

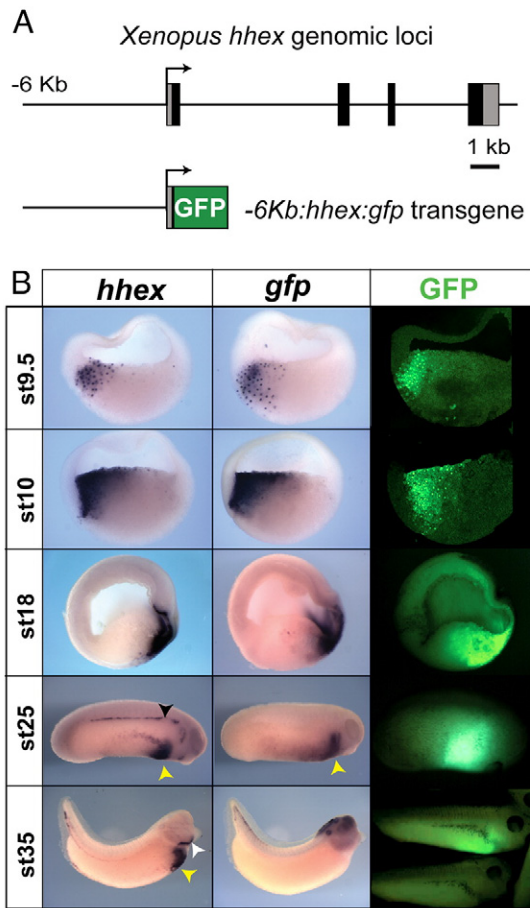

Fig. 1. A −*6Kb:hhex:gfp* transgene recapitulates *hhex* expression. (A) Diagram of the *Xenopus laevis hhex* genomic locus and −*6.0Kb:hhex:gfp* transgene. (B) The transgene recapitulates *hhex* expression in the anterior endoderm from blastula (st9.5) to stages 25. Transgenic embryos assayed by in situ hybridization for *hhex* or *gfp* mRNA and for GFP fluorescence with a confocal (st9.5 and 10) or stereomicroscope (st18–35). Embryos from st9.5 to 18 are bisected; in st9.5–11, dorsal is left; and in st18–35, anterior is right. The GFP image at st35 shows both a transgenic (top) and non-transgenic embryos (bottom). Two independent transgenic lines exhibited identical expression patterns. Arrowheads indicate endothelial cells (black), thyroid (white), and liver bud (yellow).

stage 35 *gfp* mRNA was undetectable in the *hhex*-expressing liver and thyroid primordia (Fig. 1), although persistent GFP fluorescence was still detected. Unlike endogenous *hhex* the transgene was not expressed in developing vasculature and we observed ectopic transgene expression in the head at stage 35. Thus the -6.0 Kb upstream sequence is sufficient to recapitulate early *hhex* expression in the anterior endoderm.

Regulation of hhex transcription by Nodal and Wnt/β-catenin signaling

mWnt and zygotic Nodal signaling are known to regulate *hhex* transcription in the organizer (Xanthos et al., 2002; Zorn et al., 1999). However, it was unclear whether these pathways acted in parallel or if one was epistatic to the other. Moreover, it was not known in any species whether Nodal or Wnt signaling directly activate *hhex* transcription.

We therefore performed a series of loss-of-function and rescue experiments in −*6Kb:hhex:gfp* transgenic embryos at early gastrula. Inhibition of the mWnt pathway either by injection of an antisense β-catenin morpholino oligo (β-cat-MO) (Heasman et al., 2000) or mRNA encoding a constitutive repressor form of Tcf (ΔNTcf3) (Molenaar et al., 1996) resulted in a severe reduction of *hhex* and *gfp* expression (Fig. 2A). Moreover, injection of a Tcf3 morpholino (Tcf3-MO) (Liu et al., 2005) resulted in ectopic *hhex* and *gfp* expression throughout the endoderm (Supplementary Fig. S2). This is consistent with published findings that Tcf3 represses organizer gene expression in ventral cells that lack mWnt signaling, whereas in the dorsal cells where mWnt/β-catenin are active, Tcf3 repression is lifted and partially redundant Tcf1 and Tcf4 activate organizer transcription (Houston et al., 2002; Liu et al., 2005; Standley et al., 2006). In regards to the Nodal pathway, injection of mRNA encoding a secreted Nodal-antagonist Cer-S (Piccolo et al., 1999) abolished *hhex* and *gfp* expression. In rescue experiments, injection of *nodal (xnr1)* mRNA was sufficient to induce *hhex* and *gfp* expression in embryos where mWnt signaling was blocked by either the β-cat-MO or the ΔNTcf3. In contrast, injection of mRNA encoding stabilized β-catenin (Yost et al., 1996) was unable to rescue *hhex* or *gfp* expression in embryos where Nodal signaling was inhibited by Cer-S (Fig. 2A).

These data demonstrate that the −*6.0Kb:hhex* promoter is regulated in an identical fashion to endogenous *hhex*, and that both Nodal and mWnt are required to initiate *hhex* transcription. While these data suggest that mWnt signaling lies upstream of nodal ligand (*xnr*) expression (Supplemental Fig. S3) (Hilton et al., 2003; Xanthos et al., 2002), they do not exclude the possibility that mWnt might also function in parallel with Nodal signals to simulate *hhex* transcription.

To test this possibility, we injected a −*6Kb:hhex:luc* reporter construct (the -6 Kb *hhex* promoter driving luciferase), into either the C1 (dorsal–anterior mesendoderm), C4 (ventral–posterior mesendoderm), or A4 (ectoderm) blastomeres at the 32-cell stage and assayed luciferase activity at stage 10. Similar to endogenous *hhex*, the reporter was highly active in the dorsal–anterior mesendoderm, weakly active in ventral cells, and exhibited little if any expression in ectoderm (Fig. 2B). Injection of either *xnr1* or β-*catenin* mRNAs were sufficient to activate the −*6Kb:hhex:luc* reporter in the ectoderm, with low doses of *xnr1* (5 pg) plus β-*catenin* (20 pg) having an additive effect (Fig. 2B). Importantly β-catenin does not activate *nodal* expression (*xnr1*, *2*, *4*, *5*, *6*) in animal cap ectoderm cells, as it does in vegetal tissue (Sinner et al., 2004; Takahashi et al., 2000). β-Catenin does induce *xnr3*, but this divergent ligand does not signal via the Smad pathway. We conclude that (1) Wnt/β-catenin alone can stimulate *hhex* transcription in the ectoderm independently from promoting *nodal* ligand expression and (2) β-catenin can cooperate with Nodal signaling to induce robust *hhex* expression.

We next tested whether *hhex* is a direct transcriptional target of Nodal or Wnt signaling (Fig. 2C). Animal cap ectoderm tissue was isolated from −*6Kb:hhex:gfp* transgenic blastulae and treated with cycloheximide (CHX) to block the translation of secondary factors. After 30 min, control and CHX-treated explants were further exposed to either Activin to stimulate the Nodal pathway or Gsk3 inhibitors (Bio or LiCl) to stimulate the Wnt pathway. Analysis of explants at stage 11 showed that while both Activin and the GSK3 inhibitors induced *hhex* and *gfp* expression, only Activin induced their expression when translation was blocked by CHX (Fig. 2C; data not shown). As controls we also assayed *xnr3*, a direct transcriptional target of β-catenin/Tcf (McKendry et al., 1997), and *cerberus*, an indirect Nodal target (Yamamoto et al., 2003).

The results from Fig. 2 demonstrate that Nodal signaling is required to directly activate *hhex* transcription. Maternal Wnt/β-catenin is also essential but acts indirectly by promoting *xnr* expression in the dorsal–anterior endoderm, as well as through Nodal-independent mechanisms. We next sought to determine how these signaling pathways impact the *hhex* promoter.

Identification of cis-elements controlling hhex spatial expression

To identify the cis-regulatory elements controlling *hhex* transcription, we generated a series of deletion constructs and tested these in *hhex:gfp* transient transgenics or by injecting *hhex:luc* constructs into either the dorsal-C1 or the ventral-C4 blastomeres. We then assayed

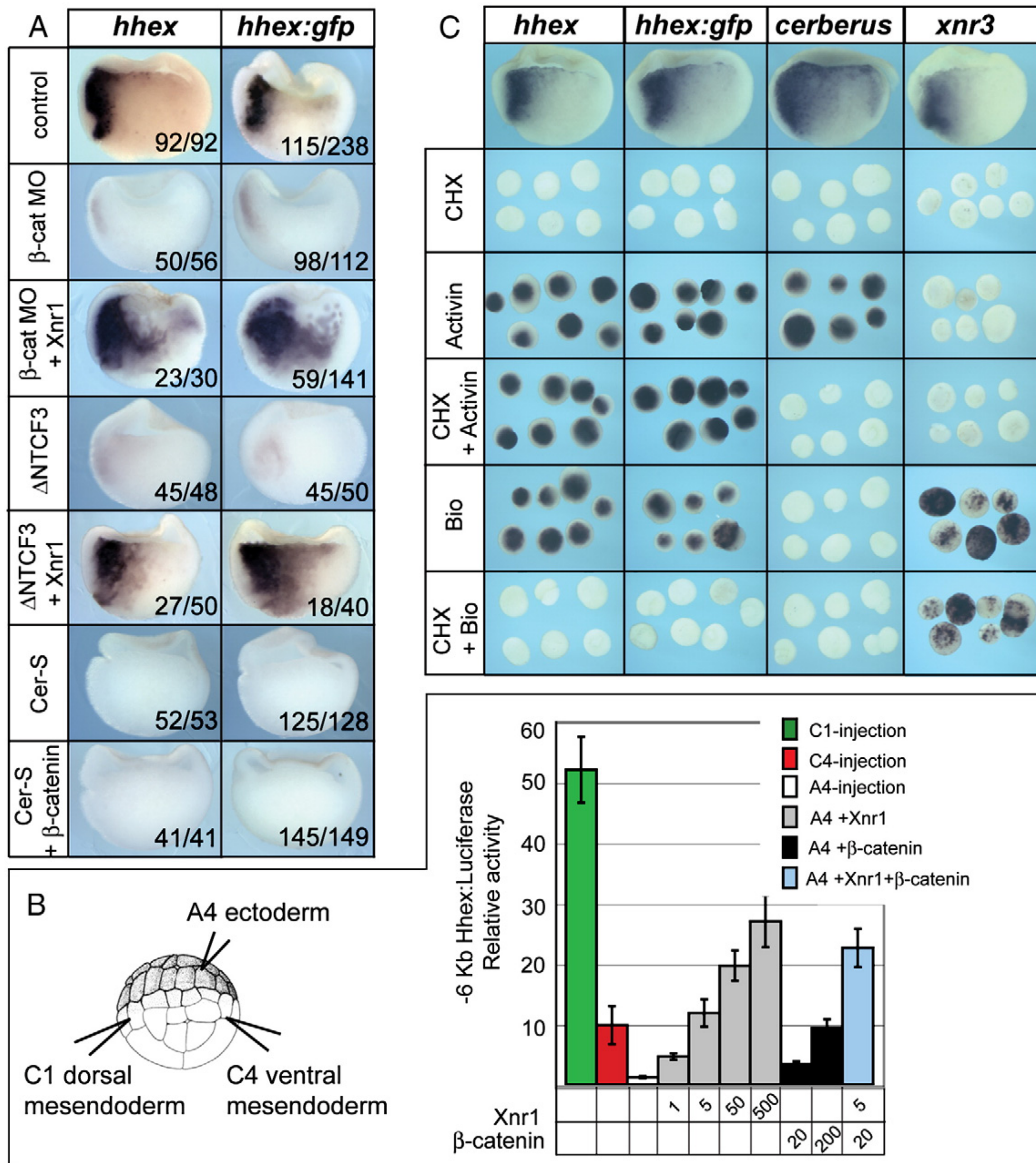

Fig. 2. Regulation of *hhex* expression by Nodal and Wnt/β-catenin signaling. (A) Nodal and Wnt signaling are required for *hhex* expression. In situ hybridization of *hhex* and *gfp* in bisected −*6.0Kb:hhex:gfp* transgenic gastrulae injected with β-catenin-MO (20 ng), Δ*Ntcf3* RNA (500 pg), stabilized pt-β-*catenin* RNA (250 pg), *xnr1 RNA* (50 pg), and/or *cer-s* RNA (750 pg). The number of embryos exhibiting the depicted phenotype is indicated. Transgenic F1 males are heterozygous, and approximately 50% of the embryos are expected to express *gfp*. (B) The schematic indicates the cells injected with the *hhex:luc* reporter (300 pg) plus pRL-TK:Renilla (25 pg). Co-injection with *xnr1* RNA (1–500 pg) and/or β-*catenin* RNA (20–200 pg) resulted in a dose-dependent activation the *hhex:luc* reporter in the ectoderm. The histogram shows the average normalized luciferase activity and standard deviation from a single injection experiment performed in biological triplicate. A representative example of 4 independent experiments is shown. (C) *hhex* is a direct Nodal-target and an indirect Wnt-target. Blastula stage animal cap tissue was cultured either untreated, with Activin or with BIO. Some explants were incubated in CHX for 30 min prior to and during culture to block protein synthesis. At stage 11, the animal caps were assayed by in situ for *hhex*, *gfp*, *cerb*, and *xnr3*. This experiment was repeated 3 times, with identical results and a total of 12–15 caps per treatment. A representative example is shown.

GFP or luciferase activity at stage 10.5. Transgenic expression of the −6.0, −3.2, and −1.56 Kb constructs were indistinguishable from endogenous *hhex* (Fig. 3A). Robust anterior endoderm expression was observed in all deletion constructs from −6.0 to −0.44 Kb, whereas the −0.38 Kb deletion was not expressed above background. Together, the transgenics and the luciferase assays indicated that deletion of sequences between −2.3 and −0.55 Kb resulted in a progressive increase in ectopic GFP and luciferase in the central and ventral endoderm (Fig. 3A, B; Table S2), suggesting the loss of repressor elements. This ectopic expression was more obvious in sensitive luciferase assays (compare the ratio of C1 to C4 activity) than in transgenics (Table S2), consistent with previous reports that GFP fluorescence under-reports in the opaque yolk-rich endoderm (Ahmed et al., 2004).

Mapping Nodal- and Wnt-responsive elements

To define Nodal- and Wnt-responsive cis-elements, we injected the *hhex:luc* deletion constructs with or without *xnr1* or β-*catenin* RNA into the A4 ectoderm cells (Fig. 3C). This analysis indicated that a Nodal-responsive element (NRE) was contained within the proximal −0.44 Kb, which coincides with the minimal region required for endoderm expression (Fig. 3A). A separate Wnt/β-catenin-responsive element (WRE) localized between −0.65 and −0.55 Kb (Fig. 3C),

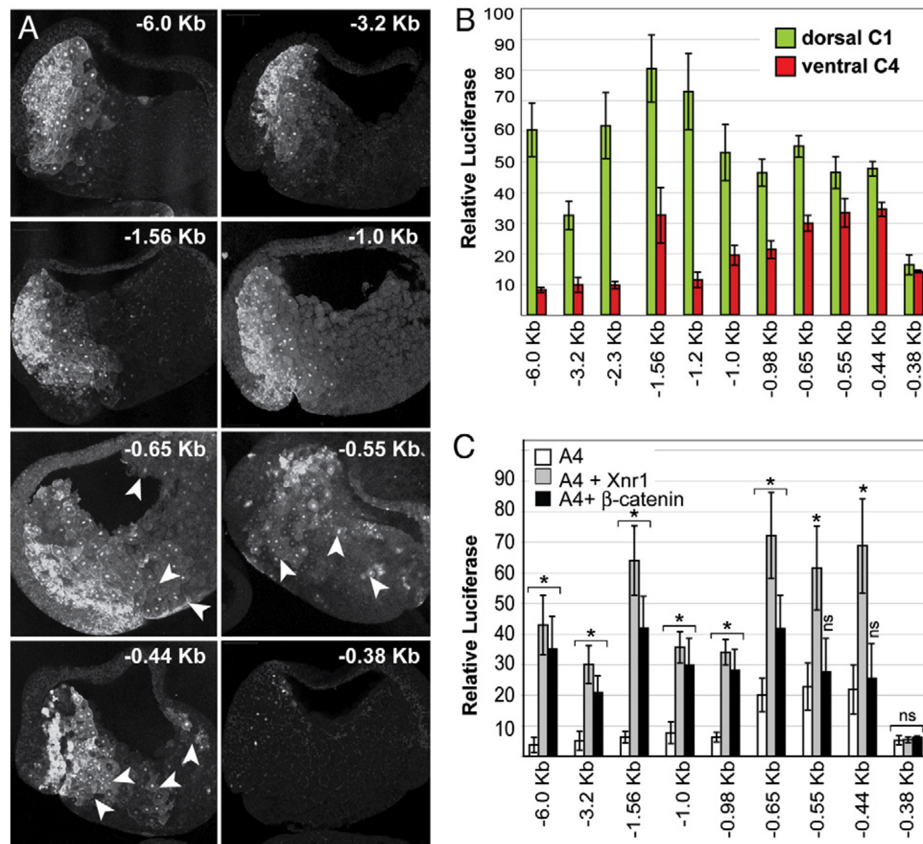

Fig. 3. Mapping Nodal and Wnt-responsive cis-elements. (A) Representative examples of GFP fluorescence from *hhex:gfp* deletion constructs in bisected transgenic gastrulae. Arrowheads show ectopic GFP. (B) Relative luciferase activity of early gastrulae injected with *hhex:luc* deletion constructs into either dorsal C1 (green), the ventral C4 cells (red) or (C) into the A4 ectoderm cells with *xnr1* RNA (50 pg; gray) or stabilized *pt-β-catenin* RNA (250 pg; black). (B–C) Histograms show the average normalized luciferase activity and standard deviation from injections performed in biological triplicate. Representative examples from 3 independent experiments are shown. *$p<0.05$ in Student *T*-tests when compared to injection of the same reporter alone; ns = no statistical difference. The NRE localized between −0.44 and −0.38 Kb, and the WRE between −0.65 and −0.55 Kb.

confirming that mWnt signaling can stimulate *hhex* transcription by mechanisms other than just promoting *xnr* expression. This arrangement of distinct Nodal- and Wnt-responsive elements (Fig. 4A) is similar to the cis-regulation of *gsc* described by Cho and colleagues (Koide et al., 2005; Watabe et al., 1995). We next sought to determine how Nodal and Wnt signaling regulated *hhex* transcription through these cis-elements.

Nodal directly activates hhex transcription via Foxh1 and Smad2

In *Xenopus,* Nodal-responsive transcription can be mediated by Foxh1, Wbscr11, or the HD proteins Mixer and Bix2 (Chen et al., 1997; Germain et al., 2000; Ring et al., 2002). There are no obvious Wbscr11 DNA-binding sites in the proximal −0.44 Kb promoter and although there is one putative homeobox site (Fig. 4), it is not predicted to be bound by Mix-family proteins (Germain et al., 2000; Latinkic et al., 1997; Noyes et al., 2008). However, the NRE contains three potential Foxh1 DNA-binding sites, two of which are flanked by putative Smad-binding sites (Fig. 4). We mutated the two Foxh1/Smad DNA-binding sequences in the context of the *−6Kb:hhex:luc* reporter (Fig. 5A) and assayed their activity in the dorsal–anterior mesendoderm at early gastrula. Mutation of individual Smad sites (ΔS1 or ΔS2) resulted in a modest but significant reduction in luciferase activity, whereas mutation of the either Foxh1 site (ΔF1 or ΔF2) severely compromised expression (Fig. 5B) and mutation of both Smad sites (ΔS1 + S2) or both Foxh1 sites (ΔF1 + F2) largely abolished expression (Fig. 5B). Moreover, the Foxh1 and Smad sites were required to mediate robust Nodal-stimulated transcription in ectoderm injections (Fig. 5C).

To determine whether Mix-like factors might also contribute to *hhex* activation downstream of Nodal, we tested whether over-expression of Mix1, Mixer, Bix1, Bix2, or Bix4 stimulated *hhex* transcription in animal cap assays. Only Bix1 and Bix4 (and not the Smad-interacting Mixer or Bix2) robustly activated the *−6Kb:hhex:luc* reporter but deletion analyses indicated that they act through sequences between −1.0 and −0.65 Kb and not via the NRE (Supplementary Fig. S4).

To confirm that Foxh1 and Smad2 regulated endogenous *hhex*, we performed a series of loss- and gain-of-function experiments. Injection of morpholino oligos to knockdown Smad2 or mRNA encoding a Foxh1-Engrailed (Foxh1-EnR) constitutive repressor construct (Watanabe and Whitman, 1999) abolished *hhex* expression, whereas ventral injection of constitutively active Foxh1:VP16 or Smad2:VP16 fusion constructs induced ectopic *hhex* (Fig. 5D). Ventral over-expression of wild-type Foxh1 or Smad2 individually had no effect, but together Foxh1 + Smad2 were sufficient to induce ectopic *hhex* (Fig. 5D). Finally, we examined embryos where maternal *foxh1* mRNA had been depleted using the host transfer method (Kofron et al., 2004) and found that *hhex* expression was severely reduced. This was partially rescued by adding back synthetic *foxh1* mRNA (Fig. 5E). Expression of the *foxh1*-related gene *fast3* was not affected in these experiments.

We next used chromatin immunoprecipitation (ChIP) to determine whether Foxh1 associated with the NRE in vivo. As there are no *Xenopus* anti-Foxh1 antibodies available, we injected a low level of myc-tagged Foxh1 mRNA (50 pg) into embryos and performed ChIP with anti-myc. This level of Foxh1-myc had no detectable effect on development or endogenous *hhex* expression (Fig. 5D). QPCR of immunoprecipitated chromatin amplified DNA fragments containing the F1 and F2 Foxh1-binding sites in the *hhex* NRE from both the dorsal and ventral mesendoderm at levels equivalent to the positive

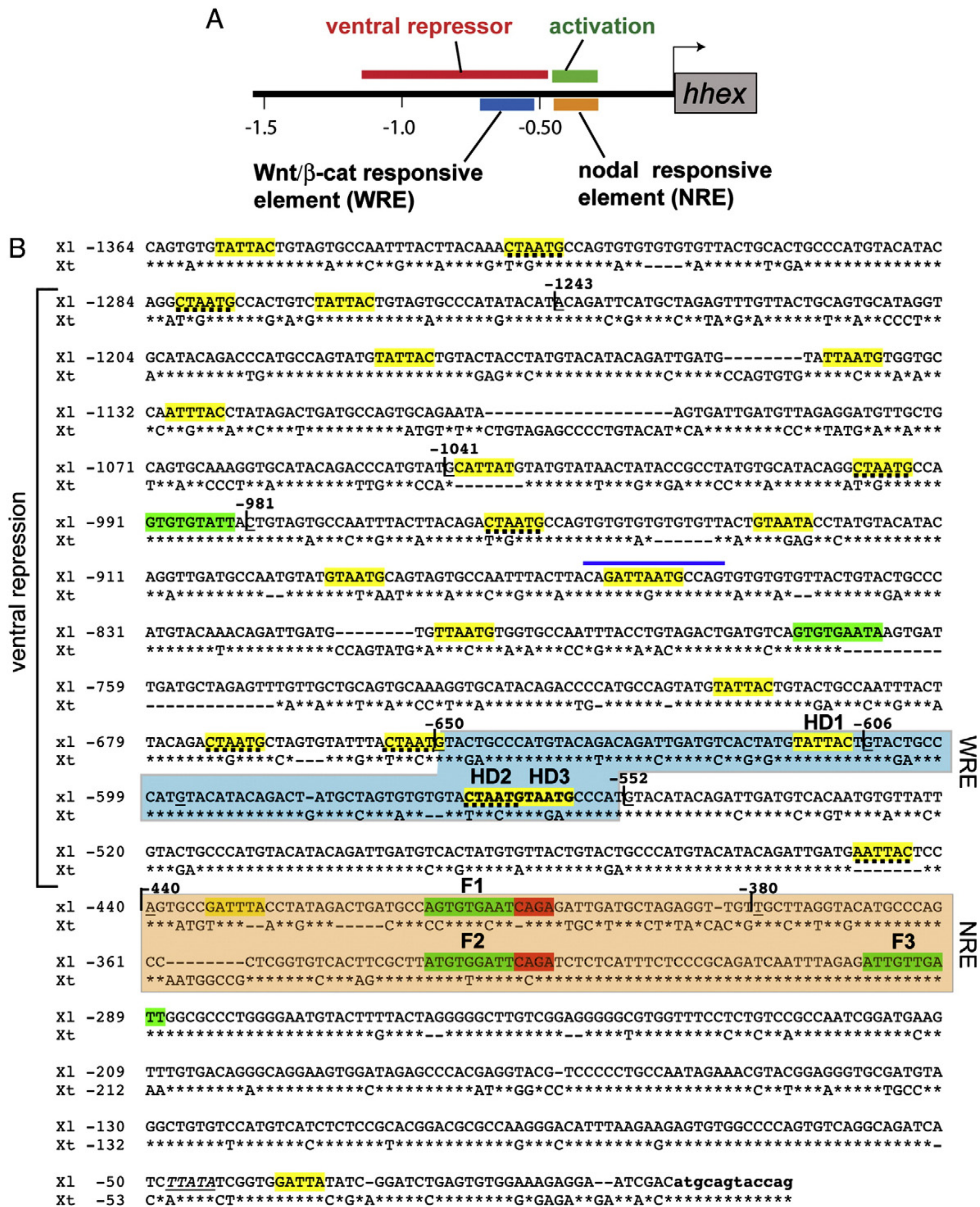

Fig. 4. Sequence analysis of the *hhex* promoter. (A) Summary of the cis-regulatory elements controlling *hhex* expression. (B) Alignment of the −1.4 Kb upstream *hhex* sequence from *Xenopus laevis* (Xl; top row, accession no. EF059707) and *Xenopus tropicalis* (Xt; bottom row, JGI genome assembly v4); identical nucleotides are indicated by a "*" in the *Xt* sequence. The 5′ ends of the various deletion constructs are indicated with vertical lines. The putative DNA-binding sites for homedomain (yellow), Vent1/2 (yellow with dash underline), Foxh1 (green), Smad (red), and Wbscr11 (blue line) factors are indicated including the three homeobox sites (HD1, HD2, and HD3) in the WRE and the three Foxh1-binding sites (F1, F2, and F3) in the NRE. Note that the F1 site is divergent from an optimal Foxh1-binding sequence.

control *mix2* Activin response element (*mix2*-ARE) (Chen et al., 1997). The negative control gene *mlc2* was not amplified (Fig. 5F). We conclude that Nodal signaling directly activates *hhex* transcription through Foxh1/Smad-binding sites in the proximal −0.44 Kb NRE.

Siamois and Twin promote hhex expression downstream of mWnt

We next examined the Wnt-responsive element in more detail. Consistent with Wnt/β-catenin acting indirectly, the WRE does not contain Tcf/Lef-binding sites. It does, however, contain three homeobox sites including two tandem sites with the sequence 5′-TAATGTAAT-3′ (Figs. 4, 6; HD2 and HD3); this is identical to the sequence found in the Wnt-responsive proximal enhancer of *gsc*, that can be bound by the HD factor Twin (Laurent et al., 1997; Watabe et al., 1995). Direct transcriptional targets of mWnt, Sia, and Twn are transiently expressed in the dorsal–anterior endoderm of the blastula similar to *hhex* (Fig. 6A).

To test whether Sia/Twn mediate the mWnt activation of *hhex*, we performed a series of loss-of-function and rescue experiments in

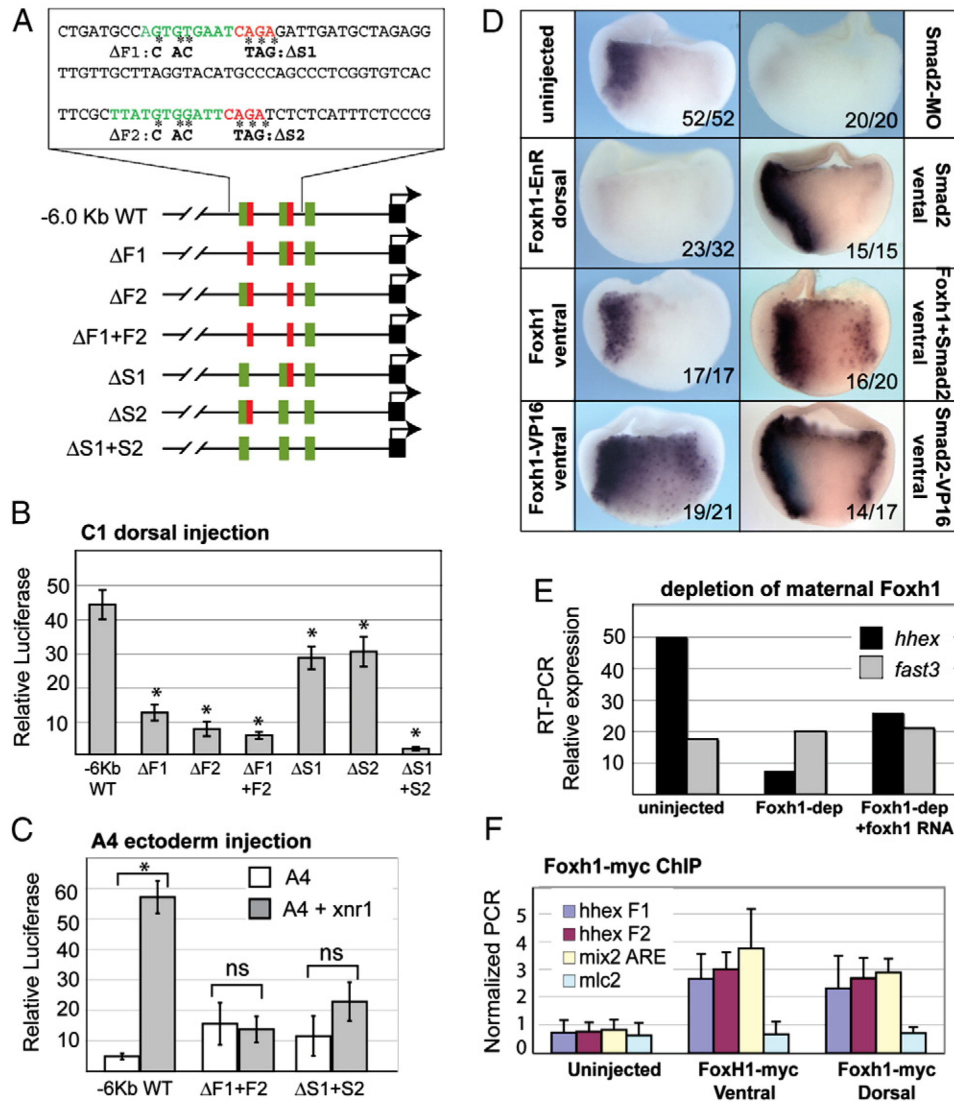

Fig. 5. Nodal-signaling directly activates *hhex* transcription through Foxh1/Smad2 binding in the proximal −0.44 Kb promoter. (A) Schematic and sequence of the NRE indicating the putative Foxh1 (green) and Smad (red) DNA-binding sites and corresponding ΔF and ΔS mutations. (B–C) Relative luciferase activity in gastrulae injected with the indicated reporter constructs into (B) the C1 dorsal mesendoderm or (C) the A4 ectoderm along with or without *xnr1* RNA (50 pg). Histograms show the average normalized luciferase activity and standard deviation from a single injection experiment performed in biological triplicate. A representative from 3 independent experiments is shown. *$p<0.01$ in Student *T*-test in (B) compared to the wt reporter or in (C) compared to injection of the same reporter alone; ns = no statistical difference. (D) Foxh1 and Smad2 regulate endogenous *hhex* expression. *Hhex* in situ of gastrulae injected into either dorsal or ventral cells with: *foxh1-EnR* RNA (500 pg), *foxh1-VP16* RNA (200 pg), Smad2a/b -MOs (20 ng each), *Smad2-VP16* RNA (200 pg) *foxh1* RNA (250 pg), *smad2* RNA (250 pg), or *foxh1* + *smad2* RNAs (125 pg each). (E) Normalized QRT-PCR of *hhex* and *fast3* mRNA levels in gastrulae depleted of maternal Foxh1 and rescued by co-injection of *foxH1* RNA (100 pg). (F) Normalized QPCR analysis of chromatin immunoprecipitated (in triplicate) from gastrulae injected dorsally or ventrally with Myc-Foxh1 RNA (50 pg). Primers amplified genomic DNA fragments containing the F1 or F2 Foxh1-sited in the hhex NRE, the *mix2* ARE as a positive control and *mlc2* promoter as a negative control.

−*6.0Kb:hhex:gfp* transgenic embryos (Fig. 6C). Knockdown of Sia and Twn by antisense MOs (Ishibashi et al., 2008) caused a dramatic reduction in *hhex* and *gfp* expression (Fig. 6C). In addition *sia* mRNA injection rescued *hhex* expression in β-catenin-depleted embryos, consistent with Sia/Twn acting downstream of mWnt. Although endogenous *xnr* mRNA levels were largely unchanged in Sia/Twn-MO embryos (Supplementary Fig. S3), we found that Xnr1 over-expression restored *hhex* and *gfp* expression in Sia/Twn-depleted embryos. In contrast Sia over-expression did not rescue *hhex* or *gfp* when Nodal signaling was blocked (Fig.6C), consistent with reports that Sia needs to cooperates with Nodal to induce some organizer genes (Engleka and Kessler, 2001).

Using the *hhex:luc* deletion constructs, we confirmed that Sia stimulates *hhex* transcription via the WRE (Fig. 6D) and that homeobox sites were required for Sia-induced activation of the reporter in animal caps (Fig. 6B, E). Mutation of the HD1 site alone had no effect on Sia responsiveness, the ΔHD23 construct exhibited reduced activation, and the ΔHD123 construct with all three sites mutated was not activated above reporter-alone levels. In addition, we observed that Sia cooperated with Xnr1 to activate the *hhex:luc* reporter, and this cooperation required both the WRE and the Foxh1 sites in the NRE (Supplementary Fig. S5).

Mechanisms of Sia/Twn regulation

These results, together with published reports that Twn can bind to the HD23 sequence from the *gsc* promoter, suggested that Sia/Twn directly activate *hhex* transcription. Since there are no ChIP antibodies available to assay Sia/Twn's association with chromatin in vivo, we tested whether a dexamethasone (DEX)-inducible form of Sia (GR-Sia) (Kodjabachian and Lemaire, 2001) could directly activate *hhex* transcription in ventral mesendoderm or animal cap explants when

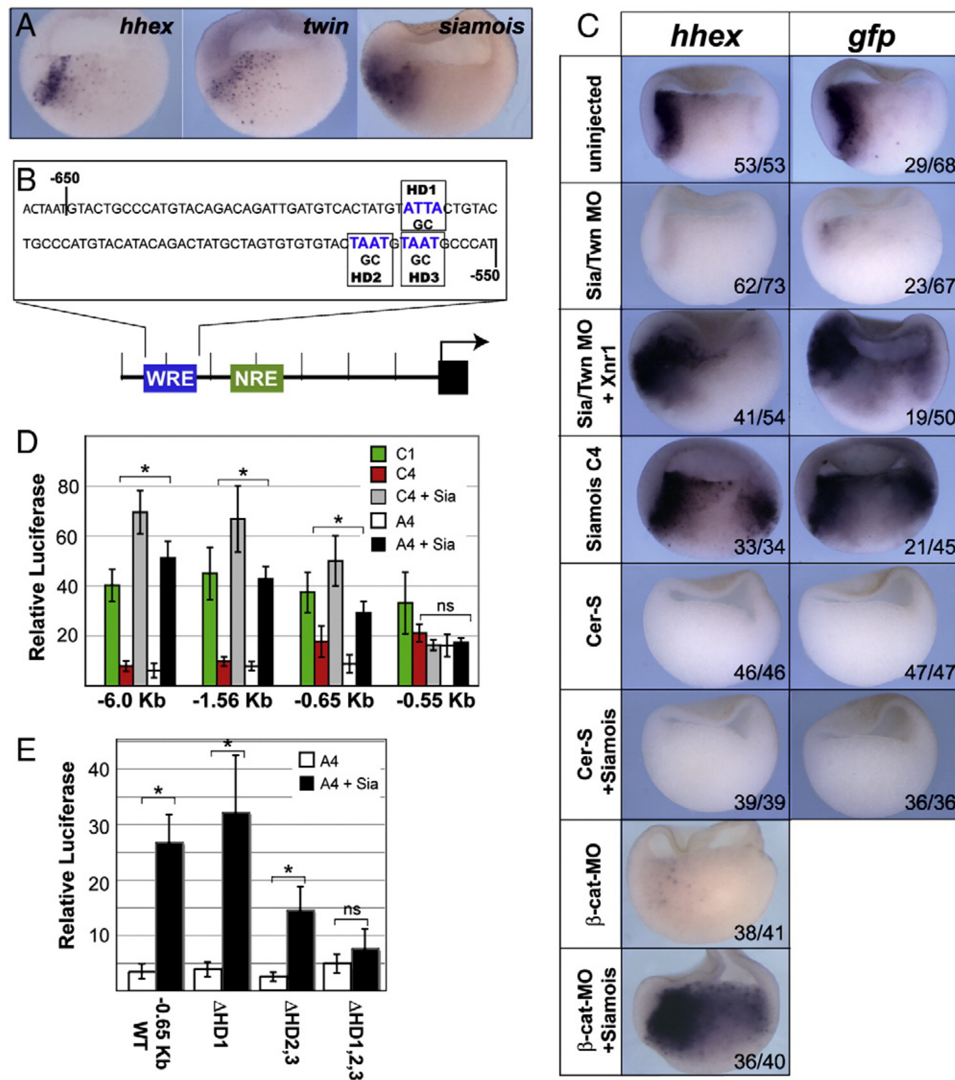

Fig. 6. Sia/Twn act downstream of Wnt/β-catenin to activate the WRE. (A) In situ hybridization of *hhex*, *twin*, and *siamois* in bisected blastula. (B) Schematic and sequence of the WRE indicating the homeobox sites (HD) and the ΔHD mutations. (C) In situ hybridization of *hhex* or *gfp* in bisected −*6.0Kb:hhex:gfp* transgenic gastrulae injected as follows: a combination of Sia-MO and Twn-MO (20 ng each; dorsal at 4-cell), Sia/Twn-MOs (20 ng each; dorsal at 4-cell) + *xnr1* RNA (50 pg; dorsal–vegetal at 8-cell), *sia* RNA (25 pg; ventral 4-cell), *cer-S* RNA (500 pg; dorsal 4-cell) to block Nodal signaling, *cer-S* + *sia* RNA (500 pg + 25 pg; dorsal 4-cell), β-catenin-MO (20 ng; 2-cell) and β-catenin-MO (20 ng; 2-cell) + *sia* RNA (50 pg; dorsal–vegetal at 8-cell). (D–E) Sia activates *hhex* transcription via HD sites in the WRE. Embryos were injected with the indicated *hhex:luc* constructs into either the C1 dorsal mesendoderm, the C4 ventral mesenoderm or the A4 ectoderm, with or without *sia* RNA (25 pg). Histograms show the average normalized luciferase activity and standard deviation at stage 10.5 from a single injection experiment performed in biological triplicate. A representative from 3 independent experiments is shown. *$p<0.01$ in Student *T*-test compared to injection of the same reporter alone and ns = no statistical difference.

translation was blocked by CHX. Surprisingly, DEX-activated GR-Sia could not induce *hhex* expression in either CHX-treated caps or ventral explants (Fig. 7A; data not shown). We considered two mechanisms to explain this result (Fig. 7B).

In the first model, Sia/Twn activate the expression of other HD factors, which in turn stimulate *hhex* transcription via the WRE (either by themselves or in a complex with Sia/Twn). Candidates include Otx2, Lim1, and Gsc because they are all regulated by Sia/Twn and their expression overlaps with *hhex* (Fig. 7C) (Blitz and Cho, 1995; Cho et al., 1991; Kodjabachian et al., 2001; Laurent et al., 1997; Taira et al., 1994; Xanthos et al., 2002).

In the second model, Sia/Twn indirectly promote *hhex* transcription via inhibition of BMP signaling (Fig. 7B). Sia/Twn are known to activate the expression of the secreted Bmp antagonists Chordin and Noggin in the organizer (Collart et al., 2005; Ishibashi et al., 2008; Kessler, 1997), which inhibit expression of the BMP targets *vent1* and *vent2*. In this model Vents repress *hhex* transcription in the ventral endoderm, but not in the dorsal–anterior endoderm as a result of Sia/Twn activity. In support of this model, there are at least eight potential Vent DNA-binding sites (5′-CTAAT-3′) (Friedle et al., 1998; Trindade et al., 1999) in the −1.4 Kb *hhex* promoter (Fig. 4) and ectopic overexpression of Vent2 can inhibit *hhex* expression in the foregut during later somite stages of development (McLin et al., 2007).

Finally it was possible that Sia/Twn promote *hhex* expression via both mechanisms. Since we observed that GR-Sia directly activated the transcription of *otx2*, *lim1*, and *chordin* in ventral explants treated with CHX + DEX, and that GR-Sia indirectly suppressed *vent1/2* expression (Fig. 7A), we therefore tested both models.

Regulation of hhex transcription by Otx2, Lim1, and Gsc

Consistent with the first model, *gsc*, *otx2*, and *lim1* were dramatically down-regulated in Sia/Twn-depleted embryos and were ectopically induced in the ventral mesendoderm by Sia injection (Fig. 7C). We then tested if Gsc mediated the effects of Sia/Twn and found that injection of *gsc* mRNA was unable to rescue *hhex* expression in Sia/Twn-depleted embryos (data not shown). Moreover, injection of a Gsc-MO had no obvious effect on *hhex* expression

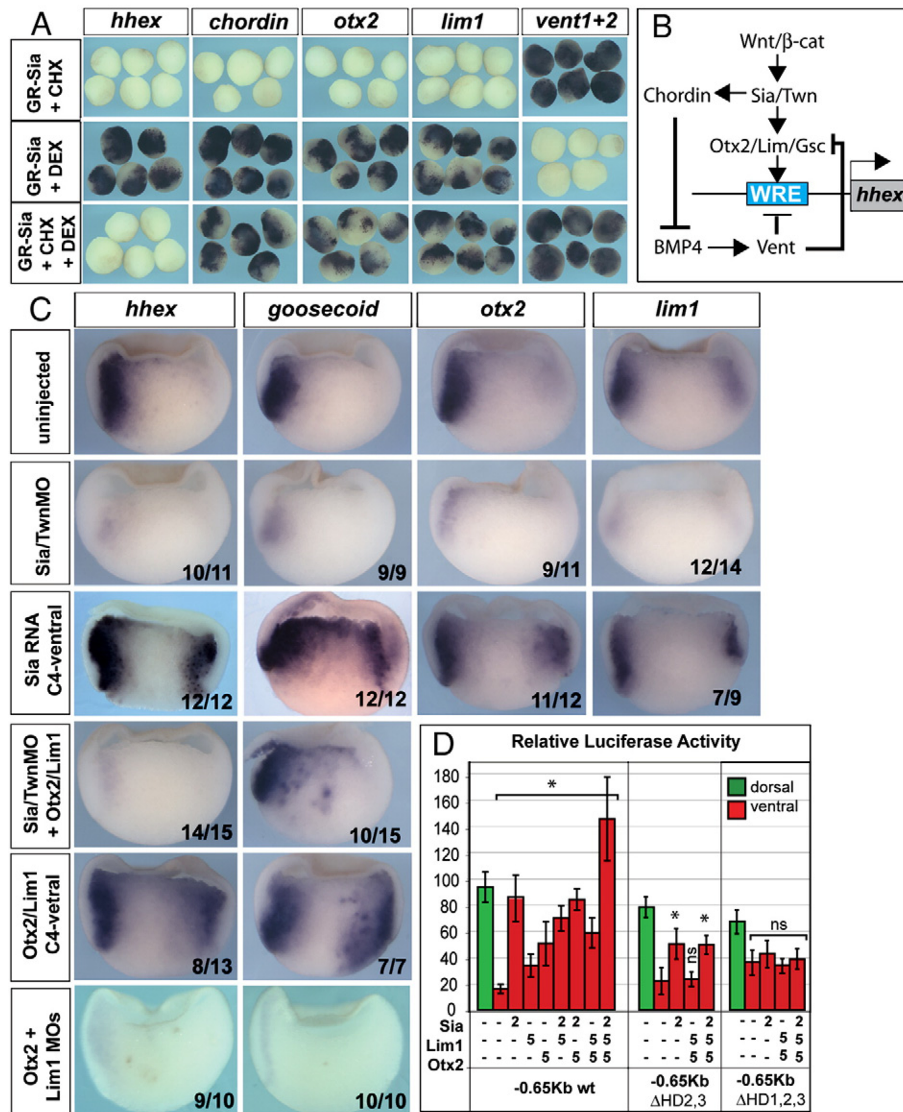

Fig. 7. Otx2 and Lim1 promote *hhex* transcription downstream of Sia/Twn. (A) *GR-sia* RNA (30 pg) + *gfp* RNA (200 pg) was injected into ventral–vegetal cells and explants were isolated at blastula stage based on GFP fluorescence. Explants were cultured with or without CHX for 30 min, and then DEX was added to activate the GR-Sia as indicated. In situ hybridization at stage 11 shows that *chordin*, *otx2*, *and lim1*, but not *hhex* are directly induced by GR-Sia in CHX treated explants. This experiment was repeated 3 independent times with identical results and >10 explants for each condition. (B) A model showing two mechanisms by which Sia/Twn could promote *hhex* transcription. (C) Induction of *hhex* by Sia/Twn is partially mediated by Otx2 and Lim1. In situ hybridization of *hhex*, *gsc*, *otx2*, and *lim1* in gastrulae injected as follows: Sia/Twn-MOs (20 ng each, dorsal 4-cell), *sia* RNA (50 pg, ventral–vegetal 8-cell), Sia/Twn-MOs (20 ng each, dorsal 4-cell) + *otx2* and *lim1* RNA (50 pg each dorsal–vegetal 8-cell), *otx2* and *lim1* RNA (50 pg each ventral–vegetal 8-cell), Otx2-MO + Lim-MO1 (20 ng each, dorsal 4-cell) or Gsc-MO (60 ng, dorsal 4-cell). (D) WT or ΔHD mutant −0.65 Kb *hhex:luc* reporters were injected into either C1 dorsal (green) or C4 ventral cells (red), with or without the indicated mRNAs (pg). Histograms show the average normalized luciferase activity and standard deviation from a single injection experiment performed in biological triplicate. A representative example from 3 independent experiments is shown. $^{*}p<0.05$ in Student *T*-test when compared to injection of reporter alone and ns = no statistical difference.

at stage 10.5 (Supplementary Fig. S6), even though control genes *vent1*, *vent2*, and *wnt8,* which are known to be repressed by Gsc (Sander et al., 2007), were up-regulated. However, by stage 12 *hhex* was severely reduced in Gsc-depleted embryos (Supplementary Fig. S6). This demonstrates that while Gsc is not required to initiate *hhex* transcription, it participates in maintaining *hhex* expression, possibly by suppressing Vents and Wnt8.

We next examined the role of Otx2 and Lim1. Injection of a Lim1-MO (Schambony and Wedlich, 2007) or an Otx2-MO (Carron et al., 2005) resulted in a modest reduction of *hhex* expression (Supplementary Fig. S7). However, depletion of both Otx2 and Lim1 resulted in a dramatic loss of *hhex* transcripts, comparable to Sia/Twn depletion (Fig. 7C). Otx2/Lim1-depleted embryos also exhibited reduced *gsc*, *chordin*, and expanded *vent1/2* expression (Supplementary Fig. S7). Ectopic over-expression of either Otx2 or Lim1 alone was not sufficient to induce *hhex* (data not shown), but when co-injected together, they did induce ectopic *hhex* and *gsc*. However, this combination of Otx2 + Lim1 did not rescue *hhex* expression in Sia/Twn-MO embryos (but did rescue *gsc*) (Fig. 7C). One possible explanation for this result is that Otx2 and Lim1 might require other interacting partners, possibly Sia/Twn themselves.

Since *otx2* + *lim1* mRNA injection was sufficient to induce *hhex* in the ventral mesendoderm, we tested whether they acted via the homeobox sites in the WRE and if they could cooperate with Sia. We found that Otx2 plus Lim1 activated the −0.65 Kb reporter in an additive fashion that was enhanced by Sia co-injection, and that their activity required the HD DNA-binding sites (Fig. 7D). Together these data suggest that Otx2 and Lim1 act downstream of, or in combination with, Sia/Twn to promote *hhex* transcription via the WRE. We next tested whether the co-injection of GR-Otx2 + GR-Lim1 (with or without GR-Sia) could directly induce *hhex* expression in ventral explants treated with CHX and DEX. They could not directly induce

hhex, but GR-Oxt2 + GR-Lim1 did directly induce *chordin* transcription (data not shown). This, along with the observation that Otx2 and Lim1 are required for *chordin* expression (Fig. S7), suggests that while Oxt2 and Lim1 may act positively through the WRE, they also indirectly promote *hhex* transcription by inhibiting BMP and Vent.

Sia/Twn promote hhex expression by inducing Chordin and inhibiting Vent

We next tested the second model where Sia/Twn indirectly promote *hhex* expression by inhibiting BMP activity (Fig. 7B). As predicted, *chordin* was dramatically reduced in Sia/Twn-depleted embryos whereas *vent1* and *vent2* were ectopically expanded into the dorsal–anterior endoderm (Fig. 8). Consistent with this, ventral injection of *sia* mRNA induced ectopic *chordin* and caused a dramatic reduction in *vent1/2* transcripts (Fig. 8). The Sia/Twn-MO phenotype was partially rescued by inhibiting BMP signaling in the dorsal–anterior mesendoderm with a dominant BMP receptor (tBR), confirming that the ectopic *vent* and repressed *hhex* was due in part to elevated BMP signaling.

To formally test whether Chordin and Noggin are required for *hhex* expression, we injected antisense MOs to knockdown these factors (Kuroda et al., 2004; Oelgeschlager et al., 2003) in *hhex:gfp* transgenic embryos. As predicted, Chd/Nog-depleted embryos exhibited a striking reduction in *hhex*, *gfp*, *chordin*, *gsc*, *otx2*, and *lim1* levels and ectopic *vent1/2* (Fig. 8; data not shown). Conversely, when we injected antisense MOs targeting both Vent1 and Vent2 (Sander et al., 2007), we observed ectopic *hhex* and *gfp* expression throughout the endoderm. Vent1/2 depletion also resulted in increased *gsc*, *otx2*, and *chordin* expression but did not alter *xnr1*, *2*, *4*, *5*, *6* or *sia*, mRNA levels (Supp Fig. S8). Finally, we tested whether the loss of *hhex* caused by Sia/Twn-MOs could be rescued by knockdown of the ectopic Vent1/2. Co-injection of Sia/Twn-MOs plus Vent1/2-MOs into the dorsal–anterior mesendoderm strikingly restored *hhex*, *gfp*, and *chordin* levels (Fig. 8), although their expression boundaries were not as defined as in control embryos. These data indicate that Vent1/2 repress *hhex* expression in the ventral–posterior endoderm and that Sia/Twn exclude *vent1/2* from the organizer through the action of BMP antagonists, thereby creating a permissive environment for *hhex* transcription.

Vents repress hhex transcription

To test if Vents can act directly on the *hhex* promoter, we generated an inducible GR-Vent-2-VP16 construct, which converts Vent2 from a transcriptional repressor into a potent activator (Onichtchouk et al 1998). In animal cap assays Dex-activated GR-Vent2–VP16 directly induced *hhex* and *otx2* transcription (but not *chordin*) when translation was inhibited by CHX (Fig. 9A). This suggests that during normal development, Vent2 directly represses the *hhex* and *otx2* promoters.

There are 8 potential Vent DNA-binding sites in the *hhex* upstream region (Fig. 4). To map where Vent1/2 act we injected

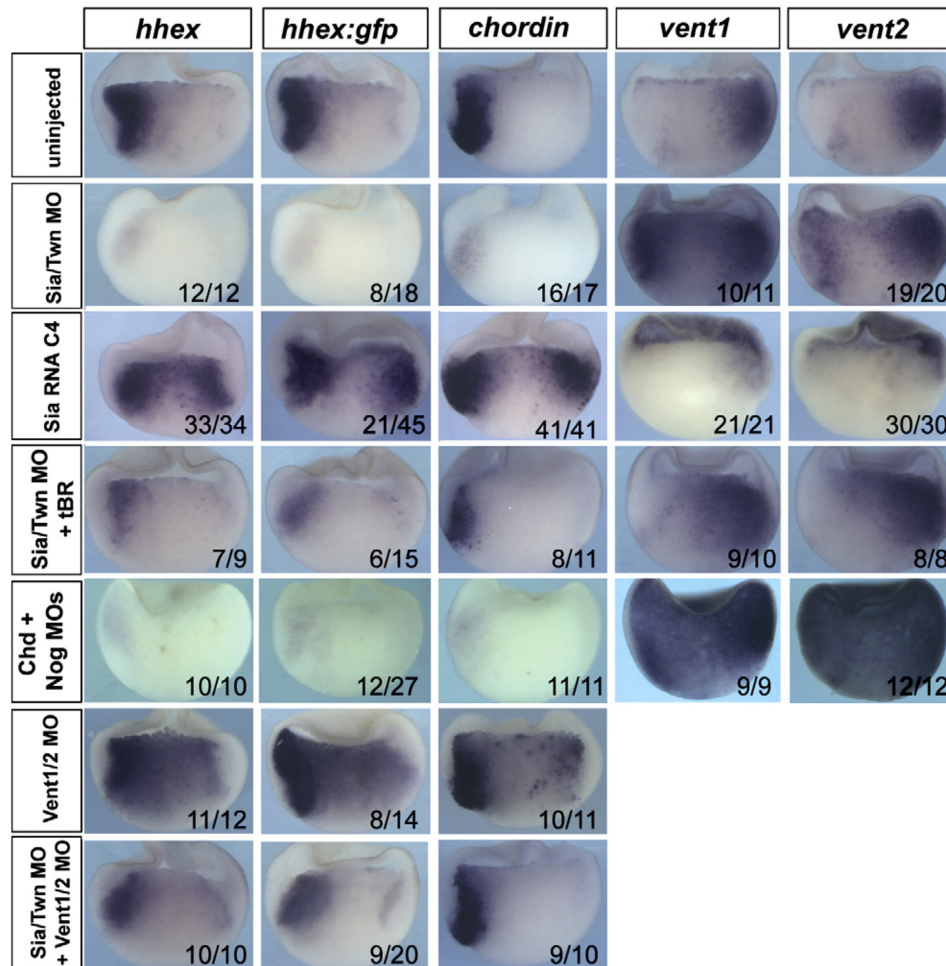

Fig. 8. Sia/Twn promote *hhex* transcription by inhibiting BMP and Vent. In situ hybridization of −*6.0Kb hhex:gfp* transgenic embryos injected as follows: Sia/Twn-MOs (20 ng each, 4-cell dorsal), Sia/Twn-MOs (20 ng each, 4-cell dorsal) + *tBR* mRNA (400 pg, 8-cell dorsal–vegetal), Vent1-MO + Vent2-MO (30 ng each; 4-cell ventral), Sia/Twn-MOs (20 ng each, 4-cell dorsal) + Vent1/2-MOs (30 ng each, 8-cell dorsal–vegetal), Chordin-MO + Noggin-MO (10 ng each, 4-cell dorsal) or *sia* mRNA (50 pg, 8-cell ventral–vegetal).

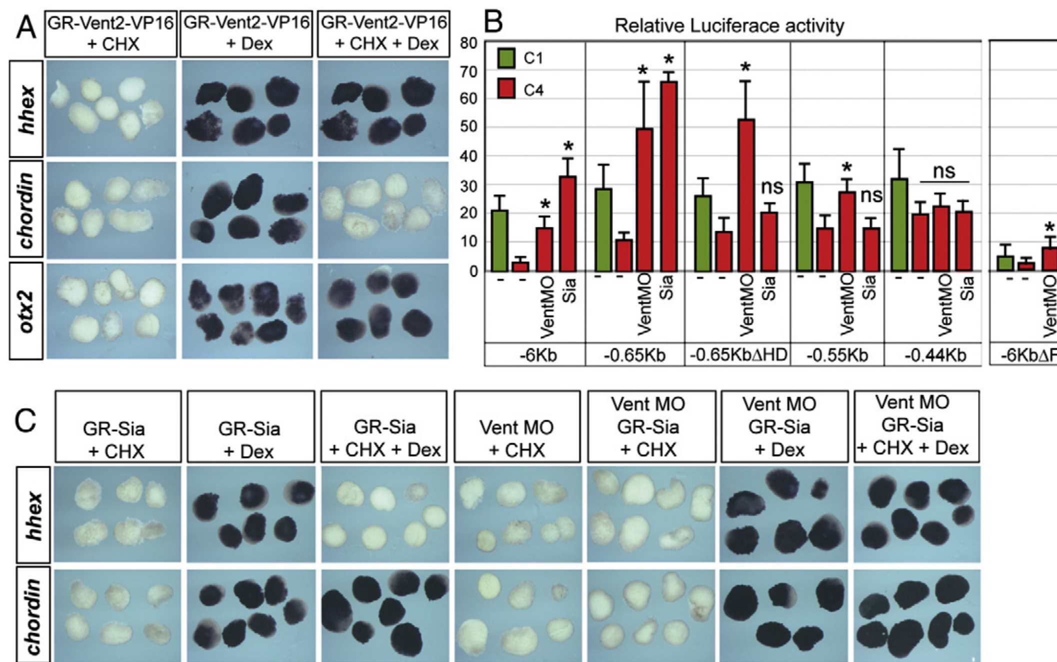

Fig. 9. Vent and Sia directly act on the hhex promoter. (A) Animal cap explants injected with *GR-Vent2–VP16* RNA (100 pg) were cultured with or without CHX for 30 min and then treated with DEX. In situ hybridization at stage 11 shows that *hhex* and *otx2*, but not *chordin*, were directly induced by GR-Vent2–VP16 in CHX-treated explants. (B) The indicated *hhex:luc* reporters were injected into either C1 dorsal (green) or C4 ventral cells (red), with either Vent1/2-MOs (30 ng each) or Sia mRNA (50 pg). The histogram shows the average normalized luciferase activity at stage 10.5 and standard deviation from a single representative injection experiment performed in biological triplicate. *$p<0.05$ in Student *T*-test when compared to injection of reporter alone and ns = no statistical difference. A representative example of 3 independent injection experiments is shown. (C) GR-Sia can directly activate hhex transcription in Vent-depleted animal caps. Animal caps injected with *GR-sia* RNA (30 pg) with or without Vent1/2-MOs (30 ng each) were cultured with CHX, DEX, or CHX + DEX followed by in situ hybridization at stage 11 for *hhex* or *chordin* mRNA. (A, C) Animal cap experiments were repeated twice with identical results and a total of ~10 explants/condition. One experiment is shown.

hhex:luc deletion constructs into the ventral–posterior mesendoderm along with the Vent1/2-MOs (Fig. 9B). Vent depletion resulted in a robust activation (de-repression) of the −6.0 Kb, −0.65 Kb, and −0.65Kb:ΔHD reporter constructs. The −0.55 Kb *hhex:luc* construct was also significantly activated over background by Vent depletion, albeit to lower levels than the −0.65 Kb construct. This suggests that the WRE mediates some but not all of Vent's repressive activity. In contrast Vent-MO injection did not stimulate the −0.44 Kb nodal-responsive proximal promoter and mutation of the Foxh1 sites dramatically impaired Vent-MO mediated activation of the −6 Kb reporter (Fig. 9B), arguing that the ectopic *hhex* expression in Vent-depleted embryos was due to Nodal signaling. These data suggest that Vent1/2 act at several locations on the *hhex* promoter, including sequences between −0.55 and −0.44 Kb, which contain one consensus Vent DNA-binding site (Fig. 4).

Siamois can directly activate hhex transcription in the absence of Vent1/2

Altogether our data suggest that Sia/Twn promote *hhex* transcription by simultaneously preventing Vent repression (via Chordin and Noggin) and activating the HD sites in the WRE. We therefore repeated the CHX using the GR-Sia construct, but also injected Vent1/2-MOs to deplete animal caps of endogenous Vent1/2. The depletion of Vent1/2 from the cap would negate the need for Sia to induce BMP antagonists and allow us to ask whether Sia can directly act on the *hhex* promoter. It is important to note that the Vent1/2-MOs do not induce ectopic *hhex* in animal cap ectoderm (Fig. 9) as they do in ventral–posterior mesendoderm (Fig. 8) because animal caps lack Nodal signaling. We found that when Vent1/2 were depleted from animal caps, DEX-activated GR-Sia was now able to directly induce *hhex* expression in the presence of CHX. We conclude that Sia/Twn promote *hhex* transcription by both relieving Vent repression and by activating the WRE.

Discussion

A gene regulatory network controlling hhex transcription

We have uncovered a complex gene regulatory network controlling *hhex* expression in the early embryo. This study, combined with the work of others, links our understanding of axis specification with foregut organogenesis. Our data suggest a model (Fig. 10) to explain how the three major signaling pathways in distinct spatial domains of the *Xenopus* blastula: (1) Nodal signaling active throughout the mesendoderm, (2) maternal Wnt11/β-catenin on the dorsal side, and (3) repressive BMP/Vent activity in the ventral–posterior region, all converge on DNA cis-regulatory elements to control *hhex* transcription in the dorsal–anterior endoderm of the organizer.

Nodal signaling is absolutely required to directly activate *hhex* transcription via Foxh1/Smad2 complexes binding to DNA sites in the −0.44 Kb proximal NRE. Bix1 and Bix4 further maintain *hhex* expression downstream of Nodal through cis-elements between −1.0 and −0.65 Kb. Activation of *hhex* transcription by Nodal is repressed in the ventral mesendoderm by Vent1/2, which are targets of BMP and zygotic Wnt8 signals. Our data suggest that Vent1/2 directly repress *hhex* transcription through multiple DNA sites located between −1.5 and −0.44 Kb of the *hhex* promoter, although further work is needed to precisely define these. Our data suggest that the balance between stimulation by Foxh1/Smad2 and repression by Vent results in the *hhex* promoter being poised, but not actively transcribed.

Maternal Wnt signaling on the dorsal side of the blastula cooperates with Nodals to indirectly promote *hhex* transcription by a number of means. First mWnt promotes *xnr1*, *5*, *6* transcription resulting in higher Nodal activity in the dorsal–anterior endoderm. mWnt also directly induces the expression of Sia and Twn, which activate *hhex* transcription via two complementary mechanisms: (1) Sia/Twn activate the *hhex* transcription (possibly in a complex with Otx2 and Lim1) via homeobox sites in the −0.65 to −0.55 Kb WRE. (2) Sia/Twn (as well as Otx2 and

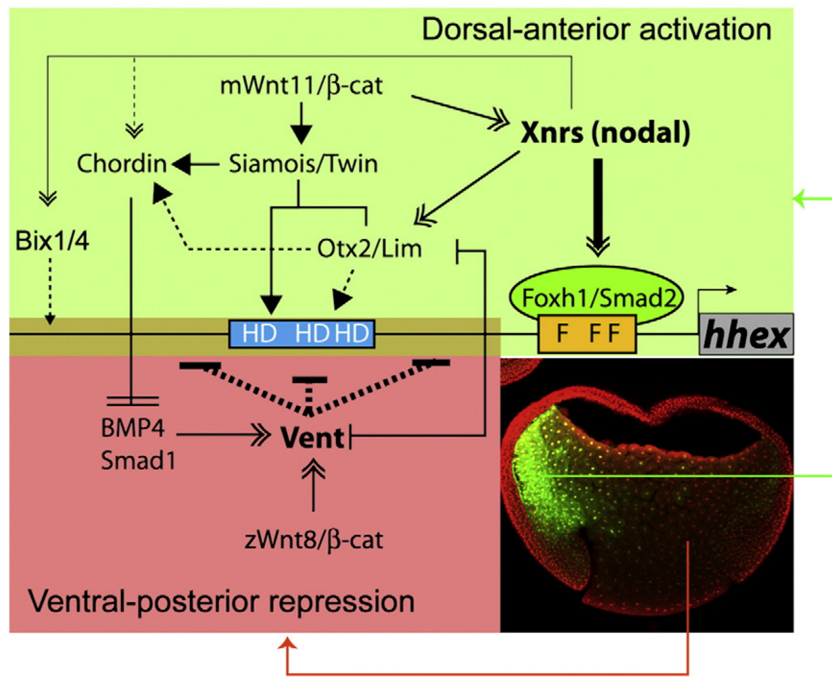

Fig. 10. A model of the regulatory network controlling *hhex* transcription. The Nodal and mWnt pathways leading to activation of *hhex* transcription in the dorsal–anterior endoderm are shown in the green box. Repression of *hhex* transcription by BMP, zygotic Wnt8, and Vents are shown in the red box. Interactions mediated by secreted factors are indicated by hatched arrows, and interactions not yet known to be direct are indicated by dashed lines. The Nodal- and Wnt-responsive cis-elements are indicated in orange and blue boxes, respectively.

Lim1) induce the expression of Chordin and Noggin, which inhibit BMP activity and exclude *vent1/2* expression from the organizer. Thus *hhex* is not repressed in these cells. Our data indicate that both activation and preventing Vent-repression are essential for *hhex* transcription with the interaction between positively acting Sia/Chordin and negatively acting BMP/Vent defining the *hhex* expression domain.

Comparison to other organizer genes

The dual activation and de-repression mechanism that we describe may be broadly applicable to the regulation of many Sia-target genes. Cis-regulatory analyses suggest that *gsc* and *cerberus* transcription are also regulated by the combination of positively acting Sia/Otx2/Lim-containing complexes and negatively acting Vent-containing complexes that interact with clusters of overlapping homeobox sites (Koide et al., 2005; Mochizuki et al., 2000; Yamamoto et al., 2003). In the future, it will be important to determine how endogenous HD complexes are assembled on chromatin in vivo and to test, for example, whether Sia- and Vent-containing complexes compete for the same cis-regulatory elements.

Another striking parallel between *hhex* and *gsc* is the functional interaction between distinct Sia-associated WREs and Smad-associated NREs (Koide et al., 2005). Interestingly we, and others, have found that Sia requires Nodal signaling to induce certain organizer genes (Engleka and Kessler, 2001). Sia over-expression could not activate *hhex* transcription in the endoderm where Nodal signaling was blocked and mutation of the NRE impaired the ability of Sia to activate the *hhex:luc* reporter. Although the mechanisms of this Nodal dependency are unknown, one possibility is that in order for Sia–WRE interactions to stimulate transcription the NRE must also be bound by Smad2. Indeed, recent studies indicate that Smad2 DNA binding can cause epigenetic modifications that make chromatin transcriptionally permissive (Dahle et al., 2010).

Regulation of hhex transcription in mammals

There is ample genetic evidence that the signaling pathways regulating anterior endoderm gene expression are conserved in mammals. For example, in mice Wnt3a signaling in the primitive streak promotes *Nodal* expression and Nodal, Smad2, Foxh1, Otx2, and Lim1 are all required for anterior mesendoderm development (Zorn and Wells, 2009). In addition a combination of Wnt3a and Activin are commonly used to induce anterior endoderm lineages in human and mouse ES cells. Finally there is evidence that BMP antagonism protects Nodal signaling to promote anterior development in the mouse gastrula (Yang et al., 2010). Thus the overall signaling crosstalk that we describe here is likely to be broadly applicable to all vertebrates.

There are, however, some distinctions between *hhex* regulation in Xenopus and mice. For example, mice lack Sia/Twn and Vent orthologs, although there is a Ventx in humans (Moretti et al., 2001). We speculate that in mice Otx2 and Lim1 might substitute for Sia/Twn, while Msx factors might play the role of Vents. In addition, deletion analysis of the mouse *hhex* locus concluded that gastrula endoderm expression was controlled by elements in the 3rd intron and not the upstream region as we have found in *Xenopus* (Rodriguez et al., 2001). A cross-species blast revealed no obvious homology between the *Xenopus* −6 Kb upstream region and mammalian *hhex* genomic loci (Supplementary Fig. S9), although the mouse 3rd intron does contain putative Smad, HD and Fox DNA-binding sites (Rodriguez et al., 2001). While the functional importance of these sites has not been tested, it is possible that *Xenopus* and mouse share similar cis-regulatory cassettes located in different genomic regions. It is formally possible that the 3rd intron of the *Xenopus* hhex gene might also contribute to anterior endoderm expression.

Regulation of hhex transcription by temporally distinct Wnt signaling

In this study, we show that maternal Wnt promotes *hhex* transcription; however, during gastrula and early somite stages zygotic Wnt/β-catenin signaling has the opposite effect and represses *hhex* expression (McLin et al., 2007). We propose that these temporally distinct Wnt activities can be explained by a common regulatory cassette: repression by Vents. The *vent2* promoter contains an essential BMP-responsive element, as well as TCF/Lef DNA-binding sites that modulate the strength of *vent2* expression (Friedle and

Knochel, 2002; Karaulanov et al., 2004). In the blastula BMP signaling directly induces *vent2* in the ventral mesendoderm (Onichtchouk et al., 1996; Rastegar et al., 1999), while mWnt, through the action of Sia and Chordin, inhibits BMP and *vents* in the organizer, thus permitting *hhex* transcription. In contrast, during gastrula and somite stages, zygotic Wnts in the posterior ventral–lateral mesoderm cooperate with BMP4 and act on the TCF sites in the *vent2* promoter to maintain its expression (Karaulanov et al., 2004; Li et al., 2008; McLin et al., 2007) in the posterior endoderm. At this time secreted Wnt antagonists such as sFRP5 are required to suppress high levels of Wnt signaling and *vent* expression from the foregut, thus maintaining *hhex*. This provides a paradigm for how crosstalk between signaling pathways can have temporally distinct effects on the same target genes.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.11.037.

Acknowledgments

We are grateful to Drs. Cho, Dawid, De Robertis, Heasman, Hoppler, Kessler, Kodjabachian, Niehrs, Sive, Taira, and Whitman for providing reagents. This work was supported by NIH grants DK70858 to A.M.Z. and P30 DK078392 (DHC bioinformatics core). We are grateful to members of the Zorn and Wells laboratories for helpful suggestions throughout this study and to Ira Blitz and Shelby Blythe for advice on ChIP.

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