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Hex acts with β-catenin to regulate anteroposterior patterning via a Groucho-related co-repressor and Nodal

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In *Xenopus*, the establishment of the anteroposterior axis involves two key signalling pathways, canonical Wnt and Nodal-related TGFβ. There are also a number of transcription factors that feedback upon these pathways. The homeodomain protein Hex, an early marker of anterior positional information, acts as a transcriptional repressor, suppressing induction and propagation of the Spemman organiser while specifying anterior identity. We show that Hex promotes anterior identity by amplifying the activity of canonical Wnt signalling. Hex exerts this activity by inhibiting the expression of *Tle4*, a member of the Groucho family of transcriptional co-repressors that we identified as a Hex target in embryonic stem (ES) cells and *Xenopus* embryos. This Hex-mediated enhancement of Wnt signalling results in the upregulation of the Nieuwkoop centre genes *Siamois* and *Xnr3*, and the subsequent increased expression of the anterior endodermal marker *Cerberus* and other mesendodermal genes downstream of Wnt signalling. We also identified *Nodal* as a Hex target in ES cells. We demonstrate that in *Xenopus*, the Nodal-related genes *Xnr1* and *Xnr2*, but not *Xnr5* and *Xnr6*, are regulated directly by Hex. The identification of Nodal-related genes as Hex targets explains the ability of Hex to suppress induction and propagation of the organiser. Together, these results support a model in which Hex acts early in development to reinforce a Wnt-mediated, Nieuwkoop-like signal to induce anterior endoderm, and later in this tissue to block further propagation of Nodal-related signals. The ability of Hex to regulate the same targets in both *Xenopus* and mouse implies this model is conserved.

KEY WORDS: Endoderm, Mesendoderm, Hex, Hhex, PRH, Anterior, Wnt signalling, Nodal signalling, Organiser, Embryonic stem cells, *Xenopus*

INTRODUCTION

In *Xenopus*, axis specification is initiated maternally. Dorsoventral polarity is established after fertilisation, by a canonical Wnt signal that leads to the accumulation of maternal β-catenin on the prospective dorsal side of the embryo (Heasman et al., 2000; Marikawa et al., 1997). With the onset of zygotic transcription, the vegetally localised maternal transcription factor VegT induces the expression of the Nodal-related genes *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6* (Clements et al., 1999; Takahashi et al., 2000; Xanthos et al., 2001; Yasuo and Lemaire, 1999), which synergise with β-catenin activity to establish the Nieuwkoop centre. Canonical Wnt/β-catenin targets such as *Siamois* (Carnac et al., 1996; Lemaire and Kodjabachian, 1996) and *Xnr3* (McKendry et al., 1997) are expressed in the Nieuwkoop centre region and are important for induction of the Spemann organiser, also referred to as mesendoderm (reviewed by De Robertis et al., 2000; Harland and Gerhart, 1997). Mesendoderm induction is also mediated by Nodal-related molecules, which act in a dose-dependent manner. The highest levels of Nodal signalling induce the most anterior mesendoderm derivative (anterior endoderm) and consequently the dose of Nodal-related activity correlates with anteroposterior positional information (Chen and Schier, 2001; Dyson and Gurdon, 1998; Green and Smith, 1990; Vincent et al., 2003).

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Following mesendoderm induction, the anterior endoderm expresses antagonists of both the canonical Wnt and the Nodal-related pathways, such as Lefty (Meno et al., 1999), Cerberus (Piccolo et al., 1999), Dickkopf (Glinka et al., 1998) and Frzb (Leyns et al., 1997; Wang et al., 1997), which insulate the anterior part of the embryo from the posteriorising influence of continued mesoderm induction (Niehrs, 1999; Piccolo et al., 1999). Accordingly, the anterior endoderm itself may prevent the expansion of trunk into the head field.

A large component of the spatiotemporal patterns of gene expression generated during embryogenesis are mediated by homeodomain transcription factors. However, how these factors integrate their intrinsic activities with specific signalling pathways is largely unknown. Hex, a homeodomain protein required for the specification of anterior mesendoderm (Brickman et al., 2000; Martinez Barbera et al., 2000), has been shown to suppress posterior mesodermal gene expression in the anterior endoderm via the repression of promoters associated with mesendodermal genes such as *Goosecoid* (Brickman et al., 2000). In addition, overexpression of Hex leads to the expansion of anterior endoderm expressing Wnt and Nodal antagonists such as *Cerberus* (Brickman et al., 2000; Jones et al., 1999; Zorn et al., 1999). Thus, Hex plays a pivotal role in anterior specification.

There are two possible explanations for the ability of Hex to induce anterior endoderm. It either suppresses more posterior identities in the mesendoderm or it promotes the inductive events upstream of anterior endoderm formation. To examine these possibilities, we asked whether Hex influenced signalling by the pathways responsible for mesendoderm induction. Here we show that Hex promotes anterior endoderm induction by amplifying early Wnt signalling. To elucidate the mechanism involved in this process, we looked for Hex target genes and identified the Groucho-related

co-repressor/Wnt antagonist *Tle4* as a Hex target in both mouse and *Xenopus*. We also identified four other Hex targets including *Nodal*. Gain and loss-of-function experiments in *Xenopus* suggest that anterior specification by Hex is dependent on *Tle4* and a subset of *Xenopus Nodals*. Together, our work supports a conserved model in which Hex promotes anterior endoderm through both amplification of transduced Wnt signals followed by suppression of Nodal-mediated mesoderm induction.

MATERIALS AND METHODS

Embryo manipulation and injections

Xenopus laevis embryos were obtained by in vitro fertilisation, cultured according to Slack et al. (Slack et al., 1984) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). mRNAs for injection were synthesised from linearised plasmids according to Smith (Smith, 1993). β-catenin, mouse *Hex*, *Hex-λVP2* and nuclear *GFP* were linearised with *Not*I; *Xnr1* was linearised with *Sma*I; *Siamois* was linearised with *Sac*II; *Xtle4* (IC 7011138) was linearised with *Hpa*I. All were transcribed with SP6 polymerase. Activin A (kindly provided by J. C. Smith) was added to explant culture based on units defined by Cooke et al. (Cooke et al., 1987). A morpholino oligonucleotide (GeneTools) was used against the Hex 5′UTR: CCTTAGCTGTACGTCATGGTCGTGG. The control morpholino was the standard provided by GeneTools.

To inhibit protein synthesis, whole embryos were incubated with 10 μg/ml cycloheximide (CHX) from stage 8 to 10.5 and analysed by in situ hybridisation. To monitor protein synthesis inhibition, embryos were cultured in media containing [^{35}S]methionine with and without CHX, and incorporation of radiolabelled methionine into *Xenopus* tissue was measured.

RNA isolation and real-time RT-PCR

Total RNA was isolated using Absolutely RNA RT-PCR (Stratagene). RNA (0.5 μg) was used for cDNA synthesis according to Brickman et al. (Brickman et al., 2000). Real-time RT-PCR was performed using a LightCycler (Roche). For *Xenopus*, cDNA from uninjected DMZ tissue from stage 10.5 embryos was serially diluted and used as template for a standard curve assayed in each run. For ES cells, standard curves were generated using varying amounts of plasmid containing the appropriate gene. Samples were normalised to *Ornithine decarboxylase* (*Odc*) for *Xenopus* embryos and to *β-actin* for ES cells. PCR primers and conditions are listed in Table 1. Melting curve analysis was performed on all PCR runs to ensure that quantification was based on the accumulation of the specific PCR product.

In situ hybridisation

In situ hybridisation was performed according to Harland (Harland, 1991), except that BMpurple (Roche) was used as a substrate. *Xnr3*, *Goosecoid*, *Chordin*, *Cerberus* and *Derrière* were linearised with *Eco*RI; *Siamois* was linearised with *Sty*I; nuclear *GFP* was linearised with *Bam*HI; *Xnr1*, *Xnr2* and X*tle4* (IC 7011138) were linearised with *Sma*I; *Xnr5* and *Xnr6* were linearised with *Not*I; *Xlim1* and *BF1* were linearised with *Xho*I; and *Xbra* was linearised with *Eco*RV. All were transcribed with T7 polymerase, except for *BF1*, which was transcribed with SP6. Double in situ hybridisation was performed based on a protocol developed in N. Papalopulu's laboratory (Hardcastle and Papalopulu, 2000).

Luciferase reporter assays

HEK 293T cells (2×10^5) were plated on a 24-well plate and transfected a day later with 10 ng of reporter plasmids (TOPflash or FOPflash from Upstate Biotechnology) and 90-100 ng of test plasmids according to Brickman et al. (Brickman et al., 2001).

Embryonic stem cell culture and cell lines

ES cells were cultured as described by Li et al. (Li et al., 1995). All cell lines were derived from CGR8s (Nichols et al., 1990). Details for targeting vector construction are available on request. Plasmids are described elsewhere (Brickman et al., 2000; Soriano, 1999; Srinivas et al., 2001). Vectors were electroporated into ES cells (Schmidt et al., 1997), G418-resistant colonies expanded and genotyped by Southern blot (Soriano, 1999). Two independent

Table 1. Primers and cycling conditions used in real-time RT-PCR

PCR primer pair	Sequence	Denat. temp.°C	Annealing temperature/ time*	Extension temperature/ time*	Acquisition temperature/ time*	Reference
Cerberus	U: 5′-GCTTGCAAAACCTTGCCCTT-3′ D: 5′-CTGATGGAACAGAGATCTTG-3′	95	60/5	72/20	81/3	Heasman et al. (2000)
Chordin	U: 5′-AACTGCCAGGACTGGATGGT-3′ D: 5′-GGCAGGATTTAGAGTTGCTTC-3′	95	55/5	72/12	81/3	XMMR*
Goosecoid	U: 5′-CACACACAAGTCGCAGAGTAT-3′ D: 5′-ATGTGTGGGGGAGAAAAATAA-3′	95	60/5	72/6	80/3	Kofron et al. (2001)
Mixer	U: 5′-CACCAGCCCAGCACTTAACC-3′ D: 5′-CAATGTCACATCAACTGAAG-3′	95	55/5	72/12	83/3	Henry and Melton (1998)
Odc	U: 5′-GCCATTGTGAAGACTCTCTCCATTC-3′ D: 5′-TTCGGGTGATTCCTTGCCAC-3′	95	55/5	72/12	83/3	Heasman et al. (2000)
Siamois	U: 5′-CTGTCCTACAAGAGACTCTG-3′ D: 5′-TGTTGACTGCAGACTGTTGA-3′	95	55/5	72/16	81/3	Heasman et al. (2000)
Xbra	U: 5′-TTCTGAAGGTGAGCATGTCG-3′ D: 5′-GTTTGACTTTGCTAAAAGAGACAGG-3′	95	55/5	72/8	75/3	Sun et al. (1999)
Xnr3	U: 5′-CTTCTGCACTAGATTCTG-3′ D: 5′-CAGCTTCTGGCCAAGACT-3′	95	57/5	72/10	79/3	Kofron et al. (1999)
β-actin	U: 5′-GGCCCAGAGCAAGAGAGGTATCC-3′ D: 5′-ACGCACGATTTCCCTCTCAGC-3′	95	58/5	72/20	86/3	Nichols et al. (1998)
Nodal†	U: 5′-GGCGTACATGTTGAGCCTCT-3′ D: 5′-GCCTGGTGGAAAATGTCAAT-3′	95	57/5	72/20	84/3	New
Mouse Tle4†	U: 5′-TGGCAAATGGTTTGTAAGCA-3′ D: 5′-AACAGCGCAAAGTGCTATGA-3′	95	57/5	72/20	86/3	New

Temperature is in °C and time is in seconds.
*XMMR, *Xenopus* Molecular Marker Resource (http://www.xenbase.org/xmmr/Marker_pages/primers.html).
†Primers designed with Primer3 computer program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

clones of Rosa26 HexERT2-λVP2 ES cells and one clone of Rosa26 Hex ES cells were electroporated with pCAGCreIP (gift of A. Smith) and plated at clonal density. Individual clones were picked, expanded and genotyped by PCR (5′-CAAACTCTTCGCGGTCTTTC, with either 3′-GCGTAATCAGGAACGTCATA to detect non-recombined locus or 3′-CCAGAGGCCACTTGTGTAGC for recombined), and analysed by Southern and western blot.

Microarray analysis

ES cells (2×10^6) were plated on 10 cm^2 gelatinised dishes and cultured for 8-12 hours. Cells were then cultured with or without 600 nM 4-hydroxytamoxifen (4-OHT) for 7.5 hours. RNA was prepared using RNeasy and QIAshredder (Qiagen). RNA quality was tested with an Agilent Bioanalyzer and a minimum of three samples applied to Affymetrix MGU74Av2 GeneChip Oligonucleotide Arrays as described in Frye et al. (Frye et al., 2003). Data were normalised using the RMA algorithm in Genespring 7.2. Expression measurements for each gene were represented as average fold-change between each treatment condition group. The groups used for comparison were Rosa-Hex compared with Cre-recombined Rosa-Hex (RRosa-Hex); non-recombined cell lines Rosa-HexERT2-λVP2 (clone1) and Rosa-HexERT2-λVP2 (clone2) with and without 4-OHT; and the recombined versions of these lines (RRosa-HexERT2-λVP2 clones 1 and 2) with and without 4-OHT (Fig. 4A,B). Fold filtering was performed to select for profiles that differed in both RRosa-HexERT2-λVP2 clones upon 4-OHT treatment and did not change in the non-recombined controls. The list of genes obtained was further filtered to remove candidates with low absolute expression levels. During the analysis, some individual samples expressed lower levels of the pluripotency marker Oct4. The candidate list was therefore re-examined excluding these samples for an added level of refinement. All microarray data are available from GEO (series accession number GSE5141).

RESULTS

Hex induces anterior identity in combination with a Wnt signal

Hex is normally expressed in embryonic regions where signalling pathways responsible for mesendoderm induction are active. One of the functions of a transcriptional repressor such as Hex may be to ensure that specific targets of these pathways are activated only in the appropriate cells. We tested this hypothesis with respect to Wnt signalling by co-expressing Hex alongside β-catenin in regions where neither Hex nor this pathway is active, i.e. ventral-vegetally. Although ectopic expression of β-catenin induced a complete secondary axis with both head and trunk structures, the combination of Hex and β-catenin induced only an ectopic head and in some cases dorsalised the primary axis. Embryos were scored both morphologically and by expression of the neural marker *BF1* (forebrain/otic vesicle area) (Fig. 1A, see Table S1 in the supplementary material). Injection of Hex on its own produced a small ventral outgrowth of the gut (Fig. 1A, inset).

The ability of Hex to anteriorise β-catenin activity can be interpreted in two ways; Hex suppresses the induction of trunk by β-catenin or Hex anteriorises β-catenin signal. To distinguish between these possibilities, we examined whether Hex could anteriorise β-catenin activity in ventral marginal zone (VMZ) explants. Although modest doses of β-catenin (500 pg) induced dorsal morphology, it never induced key anterior features such as cement glands or eyes (Fig. 1B, see Table S2 in the supplementary material). However, when this dose of β-catenin was combined with Hex, VMZ explants were anteriorised and appeared indistinguishable from uninjected dorsal marginal zone (DMZ) explants. Interestingly, a similar phenotype was observed in response to higher doses of β-catenin (1000 pg), suggesting that Hex increased the ability of low levels of β-catenin to transduce the canonical Wnt signal. Together, these results support a role for Hex in anteriorising a β-catenin signal.

Hex amplifies Wnt target activation

To better understand the level at which Hex regulated Wnt signalling, we looked at Wnt-responsive gene expression. We quantified the expression of the β-catenin direct targets *Siamois* and *Xnr3* in VMZ explants by real-time RT-PCR (Fig. 1C,D). We included in the analysis Hex-λVP2, a VP16 fusion protein that turns Hex into a transcriptional activator (Brickman et al., 2000). Hex and Hex-λVP2 showed very little or no effect in the absence of β-catenin. However, the levels of *Siamois* and *Xnr3* induced by β-catenin were significantly increased by co-injection of Hex (Fig. 1C) and suppressed by co-injection of Hex-λVP2 (Fig. 1D). Similar results were obtained by in situ hybridisation where the upregulation of Xnr3 expression was particularly pronounced in the animal hemisphere (Fig. 1E, insets and see Table S3B in the supplementary material).

To reveal the consequence of activating β-catenin and Hex locally, we injected Hex, Hex-λVP2 and/or β-catenin mRNA into specific blastomeres at the four-cell stage, alongside nuclear GFP (nucGFP) used as a lineage tracer and examined by double in situ hybridisation (light blue, Fig. 1E). When embryos were injected on the dorsal side, where β-catenin is normally active, Hex strongly increased endogenous *Xnr3* expression, whereas Hex-λVP2 inhibited it within the injected cells (Fig. 1E, see Table S3A in the supplementary material). Moreover, co-injection of Hex and β-catenin into a single-ventral blastomere led to a pronounced enhancement of the ability of β-catenin to induce ectopic *Xnr3* (Fig. 1E, see Table S4A in the supplementary material).

To determine whether endogenous Hex was required for β-catenin/Nieuwkoop centre activity, Hex was knocked-down by injection of an antisense morpholino oligonucleotide (MO). We used a previously described Hex MO known to induce an anterior phenotype in *Xenopus laevis* (Foley and Mercola, 2005; Smithers and Jones, 2002). *Siamois* and *Xnr3* expression was strongly reduced by Hex depletion, and this depletion also led to a reduction in the ability of β-catenin to induce ectopic expression of both *Siamois* and *Xnr3* (Fig. 1F, see Table S7 in the supplementary material). The phenotypes obtained were rescued by mouse Hex mRNA (Fig. 1F), which differs with *Xenopus* Hex in DNA sequence around the ATG initiation codon, but is indistinguishable in terms of activity (Brickman et al., 2000; Smithers and Jones, 2002).

Hex, Wnts and mesendoderm induction

Although the immediate early response to β-catenin signalling is the induction of direct targets such as *Siamois* and *Xnr3*, these regulators have complex activities and induce a range of different cell types, including both the anterior endoderm expressing *Cerberus* and *Hex* itself, but also more posterior axial mesoderm expressing genes such as *Goosecoid*. Interestingly, anterior gene products such as *Hex* and *Cerberus* then block the induction of more posterior cell types, creating a complex network of antagonistic interactions (Brickman et al., 2000; Piccolo et al., 1999). Based on the anteriorised phenotypes observed in Fig. 1, we hypothesised that amplification of Wnt signalling by Hex should preferentially induce anterior endoderm, suggesting a role for Hex in specifying regional identities within the mesendoderm. To test whether Hex-amplified Wnt signalling preferentially induced anterior endoderm and determine how this early activity of Hex relates to its later known role in the suppression of posterior mesendodermal identity, we looked at the expression of a number of mesendodermal markers in response to a Hex-amplified β-catenin signal. Fig. 2 shows the response of

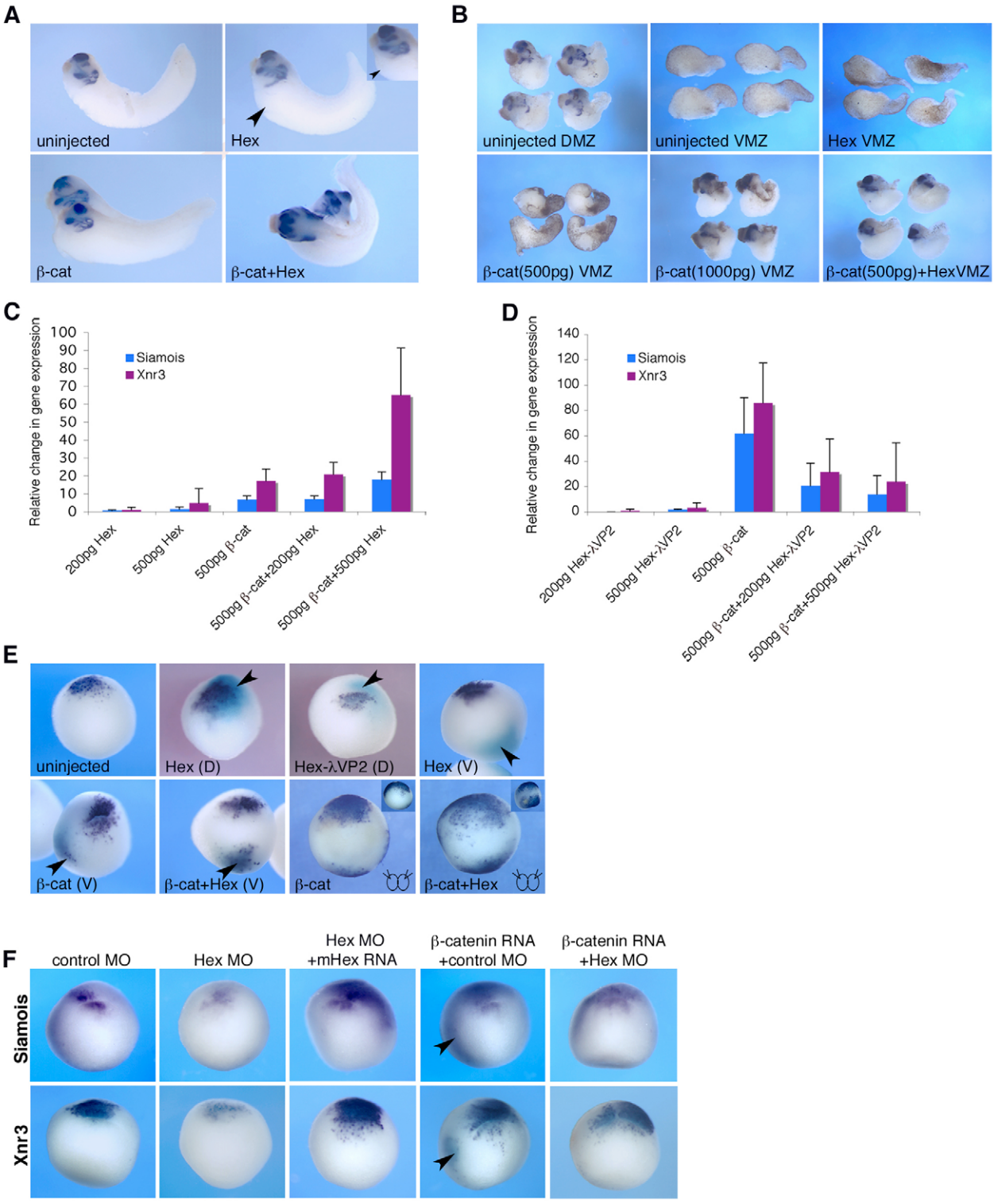


Fig. 1. See next page for legend.

the anterior endodermal marker *Cerberus*, alongside the axial mesendodermal markers *Goosecoid* and *Chordin* to a Hex-amplified Wnt signalling.

As Hex has been previously found to induce *Cerberus* within dorsal endoderm (Fig. 2B) (Jones et al., 1999; Brickman et al., 2000; Zorn et al., 1999), we were not surprised to find that β-catenin and Hex synergised to induce ectopic *Cerberus* expression (Fig. 2A,B, see Table S4 in the supplementary material). Consistently, morpholino-mediated depletion of Hex resulted in both a reduction in endogenous *Cerberus* and in the ability of β-catenin to induce ectopic *Cerberus* (Fig. 2F). However, in contrast to the results obtained with *Cerberus*, co-expression of Hex alongside β-catenin led to a context-dependent enhancement of Wnt-stimulated ectopic *Goosecoid* and *Chordin*. Thus, in an explant culture that gives an

Fig. 1. Hex anteriorises the phenotypes induced by β-catenin and regulates its downstream targets. (**A**) Axis duplication phenotypes induced by Hex and β-catenin. Embryos were injected with 500 pg Hex, β-catenin, or both RNAs into a single-ventral blastomere at the four-cell stage. In situ hybridisation was performed for the anterior neural marker *BF1* at stage 35. The inset and arrowheads indicate a small ventral outgrowth produced by Hex injection. (**B**) Phenotypes induced by Hex and β-catenin in ventral marginal zone explants. Embryos were injected with the indicated RNA into both blastomeres at the two-cell stage, cultured to gastrulation, VMZ explants dissected and cultured until staging control embryos reached stage 35. *BF1* in situ hybridisation was performed to highlight the phenotypes obtained. Hex RNA was injected at 500 pg. (**C**,**D**) Real-time RT-PCR analysis of *Siamois* and *Xnr3* expression in VMZ explants analysed at stage 10.5. Embryos were injected as in B with the indicated RNA. Values were normalised to the expression level of *Odc* and the relative change in gene expression for the genes analysed was calculated by dividing the values from injected samples by the values from the uninjected. Data are based on three independent experiments. (**E**) Cell automonous induction of β-catenin targets *Siamois* and *Xnr3*. RNA was injected into a single blastomere at the four-cell stage with the indicated RNA alongside nucGFP RNA. Dorsal injections are indicated with 'D' where either 250 pg Hex or 100 pg Hex-λVP2 were used. Ventral injections are indicated with 'V' where 500 pg Hex and 500 pg β-catenin were used. Embryos were processed by double in situ hybridisation and stained for both nucGFP to indicate the injected cells (light blue) and Xnr3 (dark blue). The schematic diagram in the lower right-hand corners of the lower panels indicates that the injection was carried out in both blastomeres at the two-cell stage. The insets in β-catenin and β-catenin co-injection with Hex show Xnr3 staining in the animal hemisphere. Arrowheads indicate the site of injection. (**F**) Expression of β-catenin targets *Siamois* and *Xnr3* in embryos depleted of endogenous Hex. Embryos were injected with a total of 40 ng Hex MO or control MO at the two-cell stage, either alone or in combination with 500 pg β-catenin. 500 pg mouse Hex (mHex) was used to rescue the phenotypes. *Siamois* (upper panel) and *Xnr3* (lower panel) expression was analysed by in situ hybridisation at stage 10.5. Arrowheads indicate the ectopic expression induced by β-catenin.

indication of the net effect in a population of cells, the ability of Hex to stimulate Wnt signalling appeared to compete with the ability of Hex to inhibit axial mesoderm (Fig. 2C,D). Modest levels of Hex (200 pg) inhibited β-catenin-mediated stimulation of these genes, whereas at higher doses of Hex (500 pg), β-catenin-stimulated gene expression recovered somewhat more pronouncedly in VMZ explants (Fig. 2D). However, when this effect is examined at a cellular level, by in situ hybridisation on an injected clone of cells, Hex-stimulated levels of Wnt signalling appeared to predominate (Fig. 2E, see Tables S5 and S6 in the supplementary material). This observation was particularly striking with respect to *Goosecoid*, a defined Hex target, where Hex and β-catenin synergise to induce *Goosecoid*, but the effect is predominately cell non-autonomous (Fig. 2E, rightmost panels). As with *Goosecoid*, *Chordin* is also synergistically induced by β-catenin and Hex, but in this case the induction is primarily cell-autonomous (Fig. 2E, rightmost panels), implying that the inhibition of *Chordin* expression observed in explant culture (Fig. 2C,D) was probably an indirect effect arising from the ability of Hex to suppress axial mesoderm.

These observations are consistent with the view that Hex is required for the maximal level of early β-catenin signalling to induce anterior endoderm and then in the mesendoderm to specify/maintain anterior endoderm by suppressing axial mesoderm. Remarkably, we found that the ability of β-catenin to induce all three markers was impaired in Hex-depleted embryos (Fig. 2F, see Table S7 in the supplementary material). Moreover, in Hex-depleted embryos where β-catenin was not overexpressed, *Cerberus* and *Chordin* expression was lost, whereas that of *Goosecoid* was expanded (Fig. 2F, see Table S7 in the supplementary material). These phenotypes were rescued with mouse Hex RNA and presumably reflect the ability of Hex to both regulate early Wnt signalling and suppress axial mesoderm. The similar behaviour of both *Chordin* and *Cerberus* may reflect an absolute dependence on Wnt signalling for the expression of both markers at blastula stages (Agius et al., 2000). However, blastula stage expression of *Goosecoid* and the majority of mesendoderm, is induced by a combination of both Wnt and Nodal-related signalling and thus may be less dependent on the ability of Hex to amplify early Wnt signalling and more sensitive to Hex-mediated suppression of axial mesoderm. Consistent with this interpretation, we found that other mesendodermal markers that are dependent on both Nodal-related and Wnt signalling for their initial induction such as *Xlim1* and *Brachyury* (*Xbra*) were increased in Hex-depleted embryos (see Fig. S1A and Table S7 in the supplementary material). Moreover, the ability of Hex to synergise with β-catenin to preferentially induce anterior endoderm and suppress more posterior mesendoderm was also evident in their synergistic ability to suppress *Derrière*, a gene involved in posterior axial patterning (see Fig. S1B in the supplementary material).

Amplification of Wnt signalling by Hex is mediated by a nuclear antagonist

As the amplification of Wnt signalling by Hex occurs at the level of immediate-early targets of the Wnt pathway, we asked if it could be recapitulated in vitro. We tested the ability of Hex to regulate the activation of TOPflash, a minimal Wnt reporter in HEK 293T cells (Fig. 3A). Co-transfection of Hex with β-catenin enhanced the ability of β-catenin to activate TOPflash transcription (Fig. 3B). As Hex is known to act as a transcriptional repressor and the activator Hex-λVP2 suppressed β-catenin activity in vivo, we addressed whether in vitro activation of TOPflash was indirect. Consistent with an indirect mechanism, co-transfection of Hex-λVP2 dramatically reduced the ability of β-catenin to induce the TOPflash reporter (Fig. 3B). In no case did Hex, Hex-λVP2 or β-catenin significantly affect the levels of FOPflash, a mutant version of TOPflash used as a negative control.

Normally, induction of the Wnt signal suppresses the ability of GSK3 to phosphorylate β-catenin (Yost et al., 1996). The unphosphorylated form of β-catenin is then stabilised and enters the nucleus to interact with the Tcf/Lef DNA-binding proteins and induces Wnt targets. To determine at which level Hex regulates this pathway, we asked whether Hex or Hex-λVP2 could modulate the activity of: (1) stabilised β-catenin, a constitutively activated variant of β-catenin that had been rendered insensitive to GSK3 regulation through mutations of GSK3 phosphorylation sites (Munemitsu et al., 1996); (2) LefdnβCTA, a nuclear localised fusion of the activation domain of β-catenin with Lef1 that is still dependent on the ability of the activation domain of β-catenin to stimulate transcription (Vleminckx et al., 1999); and (3) Xtcf3-λVP2, a fusion of Xtcf3 to two copies of the VP16 activation domain that acts independently of any β-catenin specific activation function. Activation of TOPflash by all three molecules was potentiated by Hex and efficiently suppressed by Hex-λVP2 (Fig. 3C), strongly suggesting that Hex modulates the expression of a Wnt nuclear antagonist downstream of β-catenin that can be recruited by the Xtcf/Lef complex.

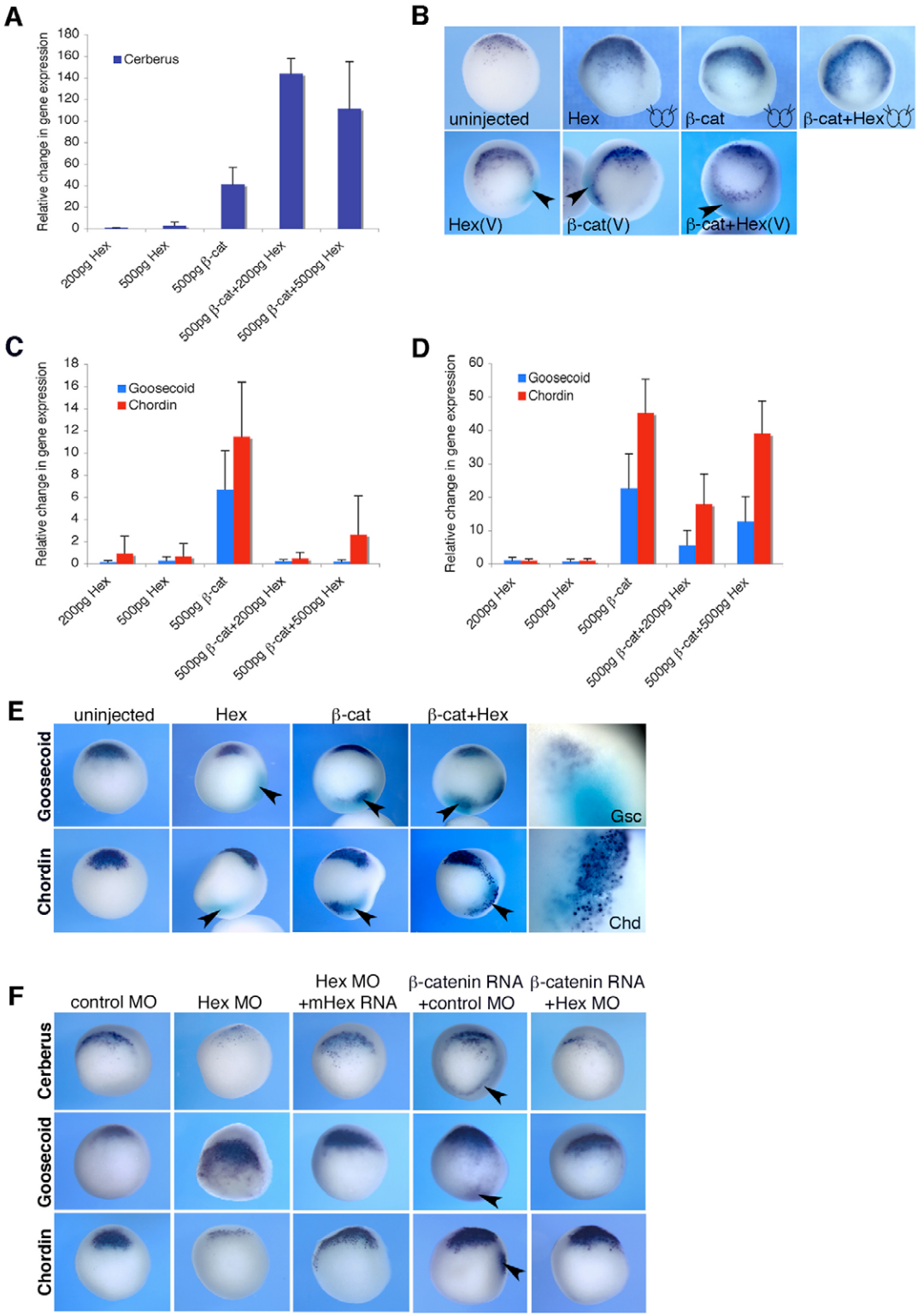


Fig. 2. See next page for legend.

The Groucho co-repressor Tle4 and Nodal are novel Hex targets

To gain insight into the mechanism by which Hex positively acts on Wnt signalling, we sought to identify Hex transcriptional targets. To facilitate this process, we used a mouse ES cell-based model, as it enabled us to work with large numbers of cells that reflect an undifferentiated early embryonic phenotype that can be genetically manipulated in vitro.

ES cells expressing Hex and HexERT2-λVP2, an inducible version of Hex-λVP2 that responds to 4-hydroxytamoxifen (4-OHT) (Indra et al., 1999) were generated alongside clonally related control

Fig. 2. Downstream response to the induction of early embryonic signalling by Hex. (**A**) Real-time RT-PCR of *Cerberus* expression in VMZ explants analysed at stage 10.5. Embryos were injected with the indicated RNA and *Cerberus* expression quantified as in Fig. 1C. Data are based on three independent experiments. (**B**) In situ hybridisation for *Cerberus* expression and double in situ for *Cerberus* (purple) and *nucGFP* (light blue) expression at stage 10.5. Embryos were injected with 500 pg Hex, β-catenin or both at the two-cell stage into both blastomeres (indicated with a schematic diagram in the lower right-hand corner) or into a single-ventral blastomere at the four-cell stage (indicated by 'V') alongside a nucGFP RNA. Arrowheads indicate the site of injection. (**C**,**D**) Real-time RT-PCR analysis of *Goosecoid* and *Chordin*. Embryos were injected as in A and RNA from either animal caps (C) or VMZ (D) explants extracted and analysed at stage 10.5. Values are normalised to the expression level of *Odc* and the relative change in gene expression for the genes analysed was calculated by dividing the values from injected samples by the values from the uninjected. Data is based on three independent experiments. (**E**) In situ hybridisation for *Goosecoid* and *Chordin* expression at stage 10.5. Embryos were injected with 500 pg Hex, β-catenin, or both, at the four-cell stage into a single-ventral blastomere alongside nucGFP. Double staining was performed. Arrowheads indicate the injected cells. High-magnification views of embryos co-injected with Hex and β-catenin are shown for both *Goosecoid* (indicated as *Gsc*) and *Chordin* (indicated as *Chd*) expression. (**F**) Depletion of Hex by Hex MO. Embryos were injected as in Fig. 1F and in situ hybridisation for *Cerberus*, *Goosecoid* and *Chordin* performed at stage 10.5. Arrowheads indicate the ectopic domain induced by β-catenin injection.

cell lines, by targeting Hex or HexERT2-λVP2 to the Rosa26 locus by homologous recombination (Soriano, 1999; Srinivas et al., 2001) (Fig. 4A). These cell lines contain a stop cassette flanked by LoxP sites that can be removed by transfection with Cre-recombinase, allowing recombination-mediated induction of gene expression. Following excision of the stop cassette, Hex or HexERT2-λVP2 were expressed from the Rosa26 promoter and detected by western blot (see Fig. S2A in the supplementary material). HexERT2-λVP2 activity was confirmed using a *Goosecoid* reporter gene (see Fig. S2B in the supplementary material).

We used affymetrix MGU74Av2 murine gene chips to profile gene expression in the different Rosa26 ES cell cultures as outlined in Fig. 4B. We reasoned that Hex targets should obey three criteria: (1) induction by 4-OHT in the two independent lines expressing HexERT2-λVP2; (2) regulation by Hex; and (3) invariant expression in parental lines that do not express a Hex derivative. Although the third criterion may exclude some Hex targets, it was an essential noise filter for analysis of heterogeneous ES cell cultures (Fig. 4C). When all three criteria were applied, a list of five candidates presented in Fig. 4D was obtained, and we conclude they represent the most likely Hex targets from this analysis.

Two of the identified genes directly impact on early embryonic signalling: the Wnt antagonist/Groucho-related co-repressor *Tle4* and the mesoderm inducer *Nodal*. Induction of these genes by HexERT2-λVP2 in ES cells in the presence of 4-OHT was confirmed by real-time RT-PCR (Fig. 4E). Based on the sequence of previously identified Hex-binding sites (Brickman et al., 2000; Crompton et al., 1992), we searched the mouse genome for putative sites in regions around *Nodal* and *Tle4*. In *Tle4*, we found several clusters of putative Hex-binding sites located within highly conserved blocks in introns 6, 12, 14 and 16 (see Fig. S3C in the supplementary material; data not shown). The majority of these sequences are conserved in *Xenopus* in addition to other mammalian genomes. In *Nodal*, Hex-binding sites were found both upstream and within intron 1 (see Fig. S3A in the supplementary material). This binding site pattern appears to be conserved in the *Xenopus* Nodal homologues Xnr1 and Xnr2 (see Fig. S3B in the supplementary material).

The *Xenopus* homologue of *Tle4*, *Xtle4*, has been shown to be a potent antagonist of Wnt/β-catenin signalling at the level of Xtcf in early *Xenopus* embryos (Roose et al., 1998). Based on the phenotypes obtained in Fig. 1, we reasoned that Hex-mediated repression of *Tle4* could provide the molecular basis for the regulation of Wnt signalling by Hex in *Xenopus* embryos.

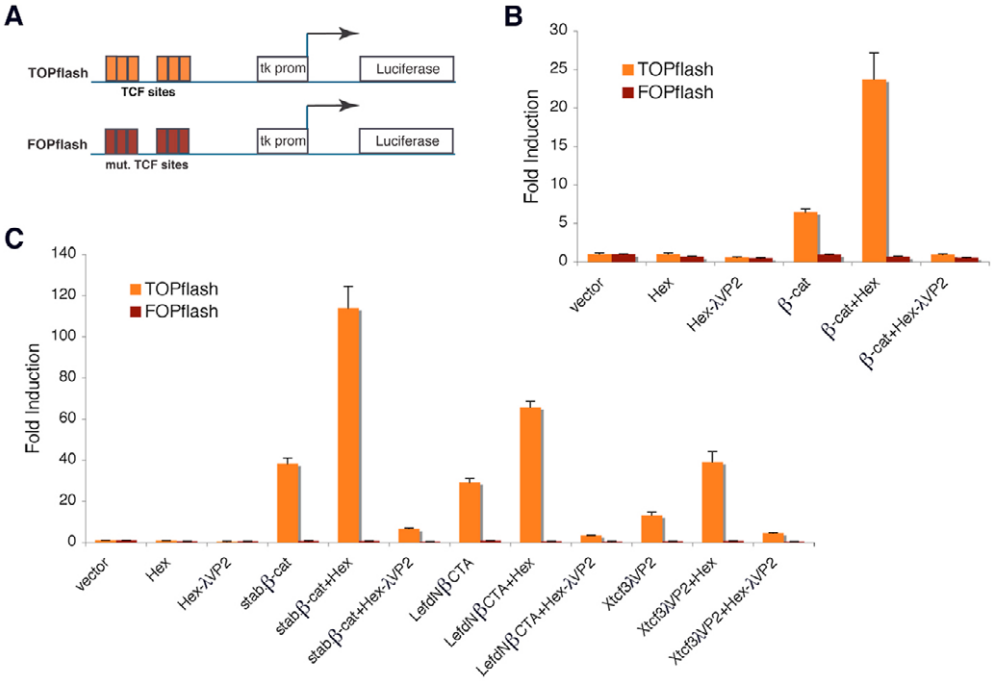
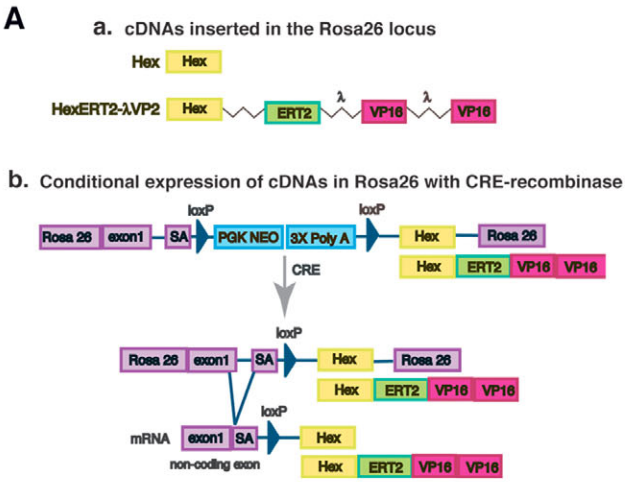
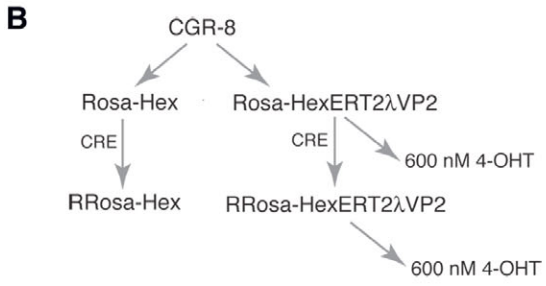


Fig. 3. Hex amplifies the transcriptional activity of β-catenin in HEK 293T cells. (**A**) Reporter plasmids used in the transfections: TOPflash, a reporter gene containing reiterated Tcf-binding sites upstream of the tk promoter and FOPflash, a mutated version of TOPflash used as a negative control. (**B**,**C**) Wnt reporter gene activity in response to transfection of Hex and Wnt pathway components. The indicated DNA was co-transfected with either TOPflash or FOPflash. Hex, Hex-λVP2 and β-catenin were used at 100 ng. Stabilised β-catenin (stab. β-catenin), LefdNβ-CTA and Xtcf3-λVP2 at 90 ng. Values are represented as fold induction of the indicated reporters. Experiments were carried out in triplicate.

Fig. 5 shows expression of *Xtle4* in response to expression of Hex-λVP2 and Hex depletion by MO. At stage 10.5, *Xtle4* is normally expressed throughout the animal hemisphere and in the superficial regions of the marginal zone (Fig. 5A,B). Hex-λVP2 injection in deep dorsal tissue resulted in ectopic induction of *Xtle4* (Fig. 5B, see Table S8 in the supplementary material). Moreover, Hex MO injection led to a significant upregulation of *Xtle4* in the marginal zone, including the superficial dorsal





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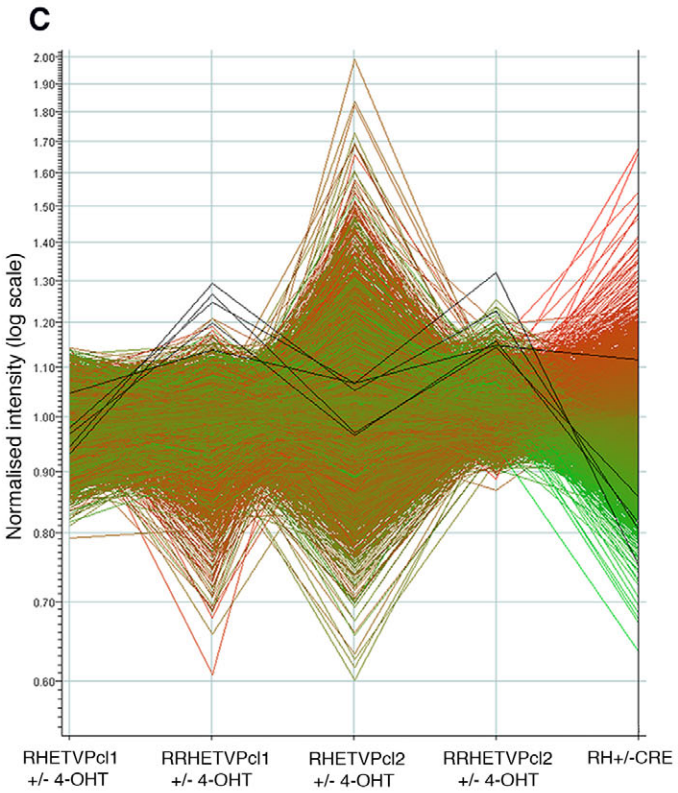
Affymetrix ID	Common name	Accession number	Gene description
102959_at	Tle4	U61363	transducin-like enhancer of split 4, homolog of Drosophila E(spl)
93141_at	Nr0b1	U41568	nuclear receptor subfamily 0, group B, member 1
94020_at	Ptk9l	Y17808	protein tyrosine kinase 9-like (A6-related protein)
98843_at	Zic2	D70848	Zic family member 2 (odd-paired homolog, Drosophila)
99801_at	Nodal	X70514	nodal



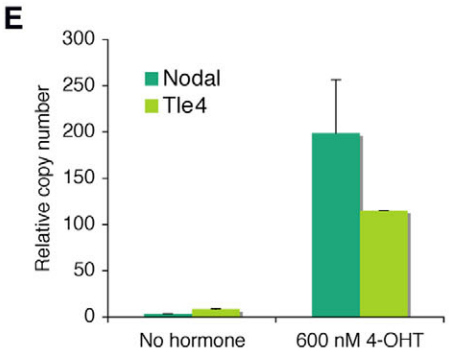


Fig. 4. Identification of Hex targets in ES cells. (**A**) Schematic representation of the ES cell lines used in the Hex target screen. (a) cDNAs inserted into the Rosa26 locus. (b) Strategy for the generation of clonally related cell lines. Top line illustrates the two Rosa26 targeting constructs used to generate parental control cell lines that do not express the transgene because of the triple PolyA stop cassette. Transfection of these lines with CRE-recombinase induces recombination between the two LoxP sites, removing the stop cassette and allowing expression of the indicated cDNA. This strategy ensures that all the transgenic lines are derived from a clonally related control. SA refers to Rosa26 splice acceptor. (**B**) Outline of the Hex target screen. RRosa is recombined Rosa. (**C**) Profile of gene expression across a series of treatment comparisons. RHETVPcl1+/–4-OHT refers to the average ratio of gene expression levels in Rosa HexERT2-λVP2 in the presence and absence of 4-OHT for clone 1. RRHETVPcl1+/–4-OHT refers to the average ratio of gene expression levels in the recombined Rosa HexERT2-λVP2 in the presence or absence 4-OHT for clone 1. RHETVPcl2+/–4-OHT refers to the average ratio of gene expression levels in Rosa HexERT2-λVP2 in the presence or absence of 4-OHT for clone 2. RRHETVPcl2+/–4-OHT refers to the average ratio of gene expression levels in the recombined Rosa HexERT2-λVP2 in the presence or absence of 4-OHT for clone 2. RH+/–CRE refers to the average ratio of gene expression levels in Rosa Hex in the presence or absence of CRE. The five candidate genes obtained are shown in black against the background of all gene expression profiles. The gene expression averages in this plot are based on samples considered to represent undifferentiated cultures as judged by expression of key markers such as Oct4. (**D**) List of candidate Hex target genes. (**E**) Tle4 and Nodal expression is induced by the addition of 4-OHT to the cultured HexERT2-λVP2-expressing cells. Cells were cultured and treated with 600 nM 4-OHT in serum-free media and expression of the genes analysed by real-time RT-PCR. Removal of serum from ES cell cultures appears to remove a suppressor of HexERT2-λVP2 activity and consequently we observe more robust target gene induction. Values are normalised to the expression level of β-actin and presented as relative copy number.

lip mesendoderm, while injection of mouse Hex RNA rescued this phenotype (Fig. 5A, see Table S7 in the supplementary material).

While there is a single *Xenopus* homologue of *Tle4*, there are multiple *Xenopus* Nodals. Fig. 5 shows the expression of the *Xenopus* Nodal-related genes *Xnr1*, *Xnr2*, *Xnr5* and *Xnr6* at stage 10.5. As previously described, Xnr5 and Xnr6 are expressed earlier than Xnr1 and Xnr2; however, Xnr1 and Xnr2, but not Xnr5 and Xnr6 are autoregulatory (Adachi et al., 1999; Hyde and Old, 2000; Osada et al., 2000; Vincent et al., 2003). Interestingly, Hex depletion by Hex MO injection resulted in a very pronounced upregulation of Xnr1 and Xnr2, but not of Xnr5 and Xnr6. Moreover, this ectopic expression of Xnr1 and 2 was rescued by mouse Hex, which had no effect on Xnr5 and Xnr6 (Fig. 5A, see Table S7 in the supplementary material). The autoregulatory activity of Xnr1 and Xnr2 also explains why Hex depletion results in ectopic Xnr expression in cells that do not normally express Hex. Although we were unable to detect a role for Hex in the regulation of *Xnr5* and *Xnr6* by morpholino depletion, Hex-λVP2 mRNA injection induced all the Xnrs tested (Fig. 5B, see Table S8 in the supplementary material). Differential regulation between these subsets of Xnrs may be due to Xnr-specific co-factors required for Hex repression.

To address whether Hex repression of candidate genes was direct, we performed experiments in the presence of cycloheximide (CHX) to block zygotic translation. In *Xenopus*, the majority of zygotic transcription does not begin until mid-blastula transition (MBT); thus, we were able to transfer Hex-depleted embryos rescued with mouse Hex RNA to media containing CHX prior to MBT to block protein synthesis. As mouse Hex RNA was injected into embryos at the two-cell stage, it was translated before protein synthesis was blocked. Thus, the ability of mouse Hex RNA to inhibit transcription of its targets in the presence of CHX suggests that these targets are direct, and independent of any intermediate molecule. Fig. 5A shows a reduction in *Xtle4*, *Xnr1* and *Xnr2* expression in the presence of CHX as a consequence of mouse Hex injection, indicating that these genes are direct targets of Hex. We also observed that CHX alone had some effect on embryonic transcription of these genes in addition to developmental timing. Similar observations have been reported previously (Clements and Woodland, 2003; Sinner et al., 2004) and these factors can complicate interpretation. However, taken together with the in silico identification of conserved Hex binding sites and the fact that these genes are induced after a short period of treatment of 4-OHT in the array data set, these findings support the notion that *Xtle4*, *Xnr1* and *Xnr2* are direct Hex targets.

Tle4 overexpression antagonises Hex-mediated anterior induction

To determine whether Hex regulation of Xtle4 was relevant to Hex-mediated amplification of Wnt signalling, we overexpressed Xtle4 and asked how this impacted on the ability of β-catenin and Hex to stimulate the Wnt targets *Xnr3* and *Cerberus* in *Xenopus* embryos. Fig. 6A shows that overexpression of Xtle4 blocked the ability of Hex to potentiate β-catenin induction of both *Xnr3* and *Cerberus* (see Table S3B and Table S4B in the supplementary material).

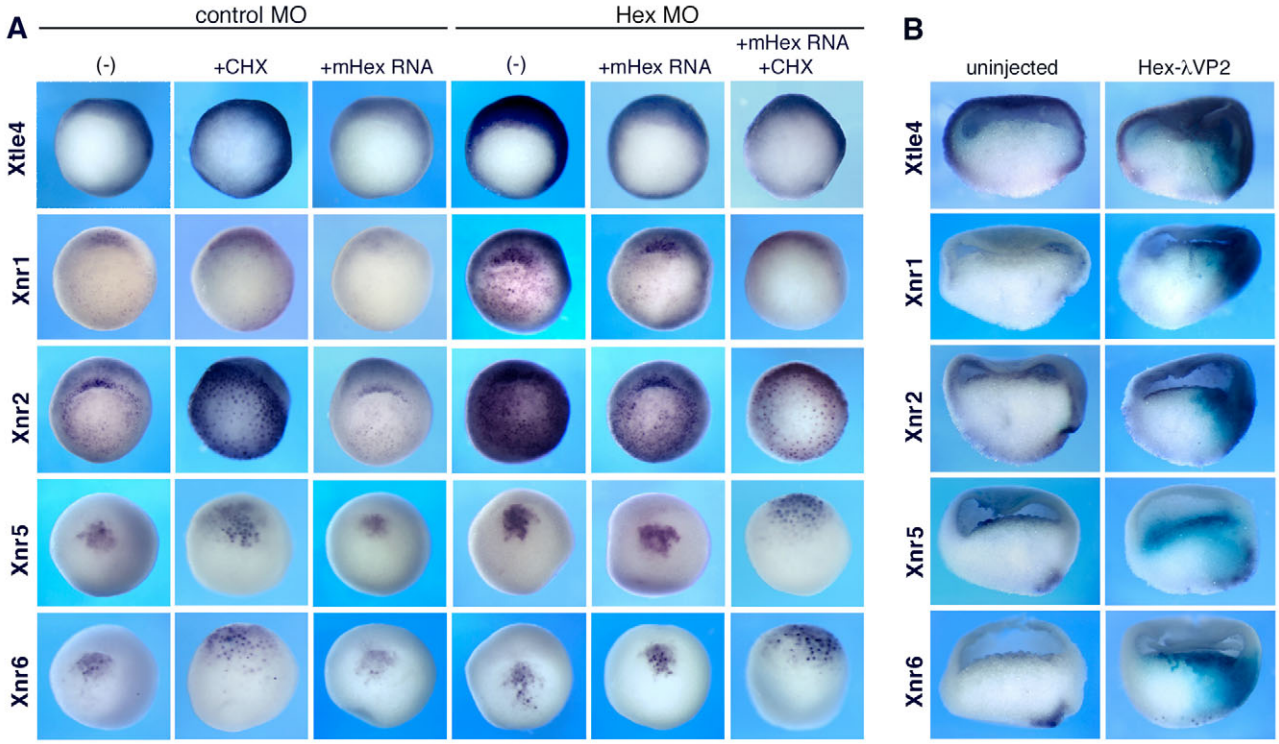


Fig. 5. Validation of Hex targets in *Xenopus* embryos. (**A**) Expression of candidate Hex target genes in response to depleting endogenous Hex. In situ hybridisation for *Xtle4*, *Xnr1, Xnr2, Xnr5* and *Xnr6* in Hex MO depleted and rescued embryos. Embryos were injected as in Fig. 1F with the indicated MO/RNA combination and analysed by in situ hybridisation at stage 10.5. Where CHX is indicated, embryos were transferred to media containing CHX before MBT and collected 90 minutes later for fixation. As CHX slightly delays development, some of these embryos do not present evident dorsal lips. (**B**) Expression of candidate Hex target genes in response to Hex-λVP2. In situ hybridisation of embryos injected with Hex-λVP2 and assayed for the expression of the same set of candidate genes as in A. Embryos were injected at the four-cell stage on the dorsal side alongside nucGFP RNA. Embryos were bisected for better observation of the internal expression. Dorsal is towards the right.

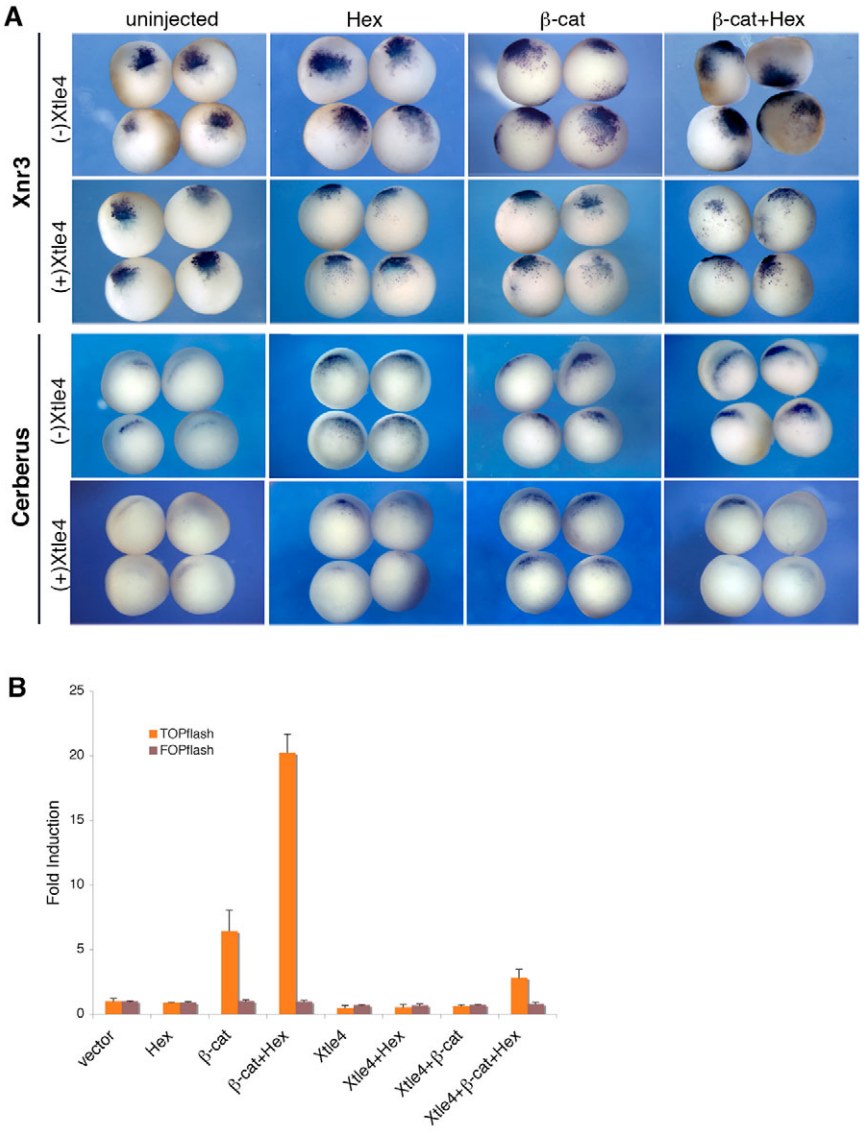


Fig. 6. Xtle4 overexpression antagonises Hex-amplification of Wnt signalling. (**A**) In situ hybridisation for the expression of *Xnr3* and *Cerberus* at stage 10.5. Embryos were injected at the two-cell stage in both blastomeres with 500 pg Hex, β-catenin and/or 3000 pg Xtle4. (**B**) Activity of Hex and β-catenin from the TOPflash Wnt reporter gene in the presence of Xtle4. The indicated DNA was co-transfected with either TOPflash or FOPflash in HEK 293T cells. Hex, Hex-λVP2 and β-catenin were used at 100 ng, Xtle4 at 90 ng. Values are represented as fold induction of the indicated reporters. Experiments were carried out in triplicate.

A similar effect was observed when Xtle4 was transfected in the context of the TOPflash reporter in HEK 293T cells (Fig. 6B). Co-transfection of Xtle4 significantly reduced the stimulated expression levels obtained with β-catenin and Hex co-transfection. Interestingly, while co-transfection of Xtle4 alongside β-catenin led to a complete suppression of the reporter, the presence of Hex allowed this activity to recover slightly, although in no case to the level of β-catenin on its own. Together, these data suggest that Xtle4 has a quantitative effect on Wnt-responsive transcription.

Hex inhibits Nodal signalling

Nodal autoregulation is important for the sustained, high-level stimulation of the pathway itself (Norris and Robertson, 1999; Osada et al., 2000). Moreover, conserved Hex-binding sites are located in intron 1 alongside the Nodal autoregulatory element (see Fig. S3 in the supplementary material) (Norris et al., 2002; Osada et al., 2000). Thus, we reasoned that Hex should interfere with the relay, behaving as a Nodal antagonist. We next tested whether Hex misexpression reduced Xnr1 activity. Thus, while ectopic expression of Xnr1 induced a partial secondary axis marked by a small region of *BF1* expression in *Xenopus* embryos, this axis was completely suppressed by co-injection of Hex (Fig. 7A, see Table S9 in the supplementary material). Similarly, the expression of increasing amounts of Xnr1 in VMZ explants led to a degree of dorsalisation and concomitant neural induction as evidenced by patchy *BF1* staining, while co-expression of Hex alongside Xnr1 inhibited these phenotypes over a range of concentrations tested (Fig. 7B, see Table S10 in the supplementary material).

As the induction of *Cerberus* is not only dependent on β-catenin but also on Nodal signalling, we asked whether Hex stimulates induction of *Cerberus* by Xnr1. As previously reported and also shown here, Xnr1 is a potent inducer of *Cerberus* expression (Fig. 7C, see Table S11 in the supplementary material) (Agius et al., 2000; Niederlander et al., 2001). However, when Hex was co-injected with Xnr1, this induction was dramatically reduced (Fig. 7C). This result is particularly surprising, as Hex did induce *Cerberus* expression on its own and combinatorially with β-catenin. Thus, high levels of Nodal signalling presumably rely on the ability of the Nodal pathway to stimulate Xnr1 and Xnr2 transcription and Hex might interfere with this process. In support of this notion, we observed a general increase in the activity of ectopic Xnr1 in Hex-depleted embryos (Fig. 7D, see Table S7 in the supplementary material).

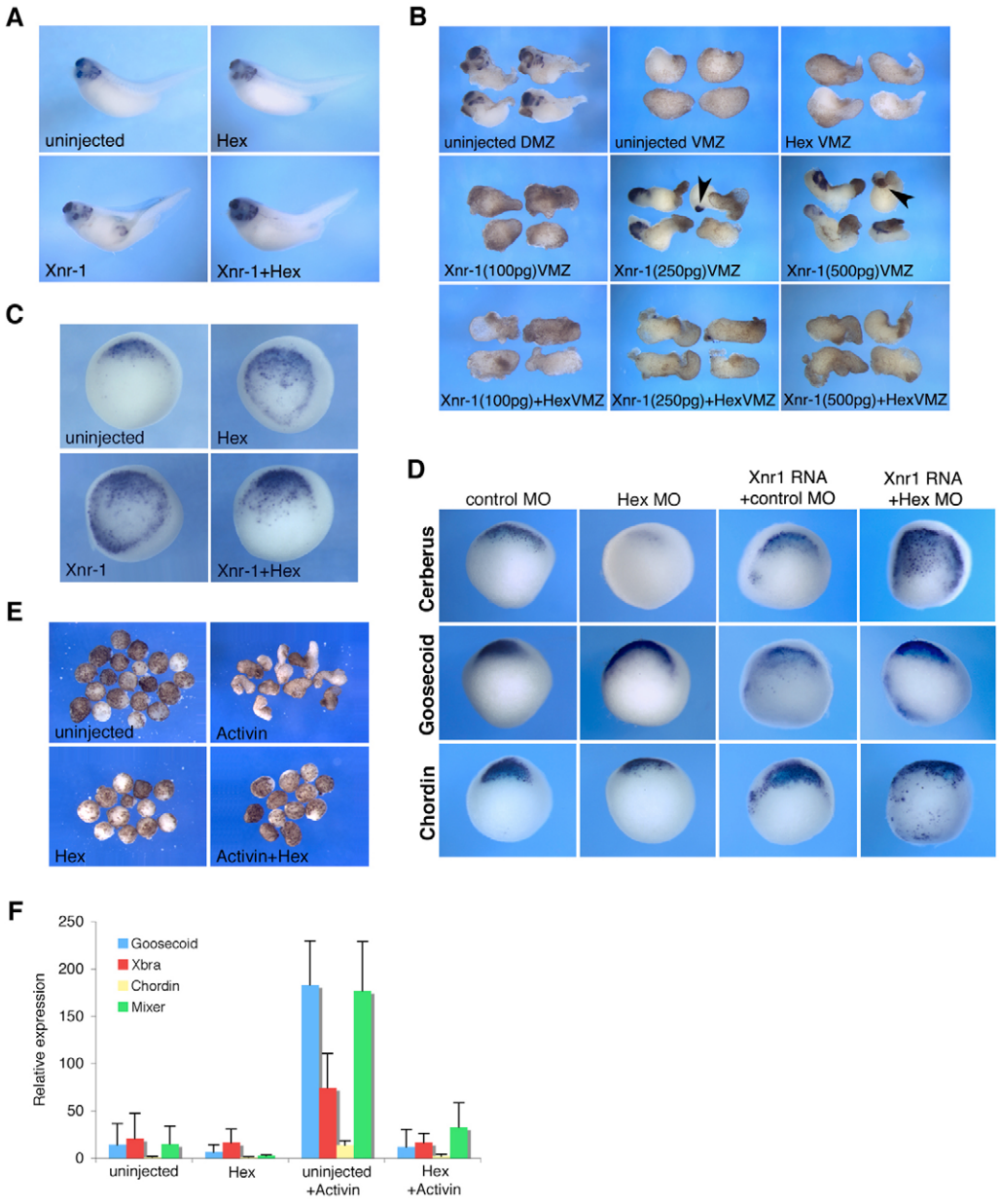


Fig. 7. Hex suppresses the activity of Nodal-related TGFβ proteins. (**A**) Axis duplication phenotypes induced by Hex and Xnr1. Embryos were injected into a single ventral-blastomere at the four-cell stage with 500 pg Hex, 250 pg Xnr1 or both and in situ hybridisation carried out at stage 35 using *BF1*. (**B**) Phenotypes induced by Hex and Xnr1 in ventral marginal zone explants. Embryos were injected with the indicated RNA at the two-cell stage, explants dissected at stage 10.5 and in situ hybridisation for *BF1* carried out when staging control embryos reached stage 35. Hex was injected at 500 pg. Arrowheads indicate patches of *BF1* expression. (**C**) In situ hybridisation for the expression of *Cerberus* at stage 10.5. Embryos were injected at the two-cell stage in both blastomeres with 500 pg Hex and/or 250 pg Xnr1 mRNA. (**D**) In situ hybridisation of Hex-depleted embryos for the mesendodermal markers *Cerberus*, *Goosecoid* and *Chordin*. Embryos were injected as in Fig. 1F. (**E**) Hex suppresses the induction of mesoderm in animal cap explants. Embryos were injected as in B with 500 pg Hex. Animal caps were dissected at blastula stage and cultured in 8 U/ml Activin protein until control embryos reached stage 18. (**F**) Molecular marker analysis in Hex-injected and Activin-treated animal cap explants. Animal caps were isolated from embryos injected as in B, cultured to stage 10.5 in 12 U/ml Activin, and RNA extracted for real-time RT-PCR analysis for *Goosecoid*, *Chordin*, *Xbra* and *Mixer*. Values are normalised to the expression level of *Odc*. Experiments were carried out in triplicate.

The ability of Hex to suppress Nodal-related activity was also tested with Activin, a related TGFβ. Hex inhibited the ability of Activin protein to induce convergent extension phenotypes in animal cap explants (Fig. 7E, see Table S12 in the supplementary material). A quantitative reduction in the ability of Activin to induce *Goosecoid*, *Chordin*, *Xbra* and the endodermal marker *Mixer* was also observed in these explants (Fig. 7F).

DISCUSSION

In this paper, we have shown that Hex promotes anterior identity at two levels. Hex quantitatively enhances Wnt signalling by regulating the expression of the Wnt antagonist/Groucho co-repressor Tle4. By elevating levels of Wnt signalling, Hex induces anterior character. We have also shown that Hex suppresses the activity of a subset of Nodal-related ligands. The suppression of Nodal-related

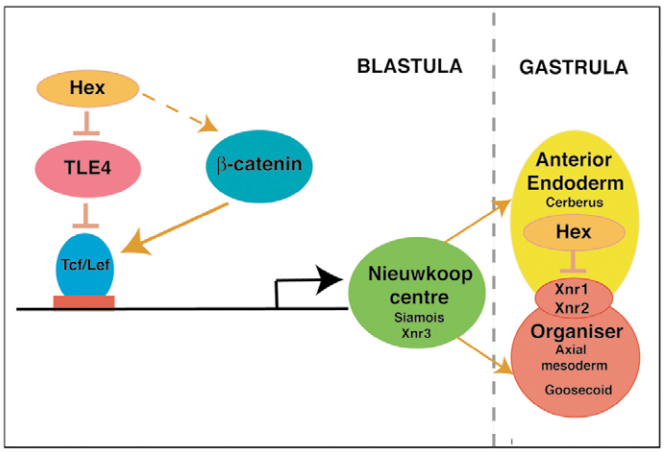


Fig. 8. Schematic representation of Hex interactions with the Wnt and Nodal-related pathways. At blastula stage, Hex amplifies Wnt signalling through the repression of the Wnt antagonist Tle4. The Nieuwkoop centre genes *Siamois* and *Xnr3* are markers of this process and this signalling centre is responsible for the induction of both anterior endoderm and axial mesoderm. Following mesendoderm induction, Hex is expressed in the anterior endoderm where it antagonises the propagation of the Nodal signal to prevent mesoderm formation in the head field. Broken lines indicate the induction of defined domains in the mesendoderm.

transcription by Hex would interfere with the continued expression of Xnr1 and Xnr2 in response to activation of the Nodal pathway and may also contribute to the segregation of anterior endoderm from the continual process of mesendoderm induction. This activity of Hex would also be an important component of the ability of anterior endoderm to insulate the anterior neural plate from the posteriorising influence of continued Nodal signalling (Meno et al., 1999; Perea-Gomez et al., 2002; Piccolo et al., 1999). The model in Fig. 8 illustrates the juxtaposition of these two activities. As Tle4 and Nodal are targets of Hex in both mouse and frog, these regulatory interactions may be conserved.

Our findings that levels of a Groucho-related co-repressor such as Tle4 are specifically regulated by Hex represent a novel regulatory point for the canonical Wnt pathway. Both gain- and loss-of-function experiments demonstrated that *Xtle4* is a potent antagonist of Wnt signalling in early *Xenopus* embryos and represses both *Siamois* and *Xnr3* (Roose et al., 1998). Although *Xtle4* is expressed in the animal hemisphere and superficial regions of the marginal zone at gastrula stage (Molenaar et al., 2000), Hex is expressed in the deep and early involuting endoderm. These complementary expression domains support the idea that Hex represses *Xtle4* to establish an embryonic region that is responsive to Wnt signalling.

Evidence from a number of organisms suggest that high levels of Nodal signalling are required for the induction of endoderm, particularly anterior endoderm (Aoki et al., 2002; Vincent et al., 2003). However although Nodal signalling is seen as required early for anterior endoderm and later for the continued process of more posterior mesoderm induction, Wnt/β-catenin signalling is generally thought of as a posteriorising signal. Yet, the role of nuclear β-catenin in establishing the Nieuwkoop centre makes it one of the earliest determinants of dorsoanterior identity in both *Xenopus* and zebrafish (Kelly et al., 2000; Xanthos et al., 2002) and our data support an early, dose-dependent role for Wnt signalling in anterior endoderm specification.

A role for early Wnt signalling in anterior specification is also consistent with recent revisions to the *Xenopus* fate map that demonstrate an anterior fate for the progeny of cells experiencing high levels of Wnt signalling (Lane and Sheets, 2000). Moreover, in *Xenopus,* both β-catenin (Sinner et al., 2004) and *Siamois* (Zorn et al., 1999) have been implicated in the induction of anterior endoderm and early *Cerberus* induction has been shown to be dependent on β-catenin (Agius et al., 2000; Xanthos et al., 2002). In the mouse, the anterior endoderm is divided into two populations, the anterior definitive endoderm (ADE), formed during gastrulation and the anterior visceral endoderm (AVE), an extra-embryonic tissue formed at peri-implantation stages (Beddington and Robertson, 1999). Hex is initially expressed throughout primitive endoderm at the hatched-blastocyst stage and then becomes restricted to the distal region of the visceral endoderm lineage. These Hex-positive visceral endoderm cells migrate anteriorly to become the AVE a day prior to gastrulation. Hex is later expressed in the ADE (Thomas et al., 1998). Genetic evidence links the induction of these endodermal tissues to Wnt signalling in both the ADE (Hsieh et al., 2003; Kelly et al., 2004) and AVE (Huelsken et al., 2000). In zebrafish, Hex is initially expressed asymmetrically as part of a Wnt-mediated signalling centre in the extra-embryonic yolk syncytial layer (Ho et al., 1999). This region is related to the *Xenopus* Nieuwkoop centre as it induces the earliest mesendoderm (Fekany et al., 1999; Kelly et al., 2000; Yamanaka et al., 1998) and Hex has also been implicated in this activity (Ho et al., 1999). Our data create a molecular framework for these observations and imply that early expression of Hex may represent a conserved component of the Nieuwkoop centre.

The anterior endoderm will continue to express Hex, whereas the axial mesoderm will not. The continued expression of Hex in the anterior endoderm represses both *Goosecoid* and Nodal-related gene expression. Repression of Nodal transcription by Hex would add to the inhibitory effects of Nodal antagonists such Cerberus and Lefty. A major component of Nodal-related activity occurs via an autoregulatory loop (Adachi et al., 1999; Osada et al., 2000; Vincent et al., 2003) and we have shown that Hex inhibits Nodal signalling by interfering with Nodal transcription. Moreover, the two *Xenopus* Nodal-related genes that are Hex targets are also the two genes that exhibit autoregulation (Hyde and Old, 2000). Thus, Hex may inhibit Nodal signalling by blocking Nodal production without interfering with Nodal-mediated signal transduction. The implications of this idea are that a Hex-expressing cell could receive the high levels of Nodal signalling required to induce anterior endoderm, but would then no longer transmit Nodal signals to its neighbours. By blocking the continued Nodal autoregulation while still leaving cells competent to respond to the signal, Hex may be in effect defining the end of the anterior axis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/18/3709/DC1

References

Adachi, H., Saijoh, Y., Mochida, K., Ohishi, S., Hashiguchi, H., Hirao, A. and Hamada, H. (1999). Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. *Genes Dev.* **13**, 1589-1600.

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C. and De Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development* **127**, 1173-1183.

Aoki, T. O., David, N. B., Minchiotti, G., Saint-Etienne, L., Dickmeis, T., Persico, G. M., Strahle, U., Mourrain, P. and Rosa, F. M. (2002). Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. *Development* **129**, 275-286.

Beddington, R. S. P. and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.

Brickman, J. M., Jones, C. M., Clements, M., Smith, J. C. and Beddington, R. S. P. (2000). Hex is a transcriptional repressor that contributes to anterior identity and suppresses Spemann organiser function. *Development* **127**, 2303-2315.

Brickman, J. M., Clements, M., Tyrell, R., McNay, D., Woods, K., Warner, J., Stewart, A., Beddington, R. S. and Dattani, M. (2001). Molecular effects of novel mutations in Hesx1/HESX1 associated with human pituitary disorders. *Development* **128**, 5189-5199.

Carnac, G., Kodjabachian, L., Gurdon, J. B. and Lemaire, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* **122**, 3055-3065.

Chen, Y. and Schier, A. F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. *Nature* **411**, 607-610.

Clements, D. and Woodland, H. R. (2003). VegT induces endoderm by a self-limiting mechanism and by changing the competence of cells to respond to TGF-beta signals. *Dev. Biol.* **258**, 454-463.

Clements, D., Friday, R. V. and Woodland, H. R. (1999). Mode of action of VegT in mesoderm and endoderm formation. *Development* **126**, 4903-4911.

Cooke, J., Smith, J. C., Smith, E. J. and Yaqoob, M. (1987). The organization of mesodermal pattern in Xenopus laevis: experiments using a Xenopus mesoderm-inducing factor. *Development* **101**, 893-908.

Crompton, M. R., Bartlett, T. J., MacGregor, A. D., Manfioletti, G., Buratti, E., Giancotti, V. and Goodwin, G. H. (1992). Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. *Nucleic Acids Res.* **20**, 5661-5667.

De Robertis, E. M., Larrain, J., Oelgeschlager, M. and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet.* **1**, 171-181.

Dyson, S. and Gurdon, J. B. (1998). The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell* **93**, 557-568.

Fekany, K., Yamanaka, Y., Leung, T., Sirotkin, H. I., Topczewski, J., Gates, M. A., Hibi, M., Renucci, A., Stemple, D., Radbill, A. et al. (1999). The zebrafish bozozok locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development* **126**, 1427-1438.

Foley, A. C. and Mercola, M. (2005). Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. *Genes Dev.* **19**, 387-396.

Frye, M., Gardner, C., Li, E. R., Arnold, I. and Watt, F. M. (2003). Evidence that Myc activation depletes the epidermal stem cell compartment by modulating adhesive interactions with the local microenvironment. *Development* **130**, 2793-2808.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Green, J. B. and Smith, J. C. (1990). Graded changes in dose of a Xenopus activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.

Hardcastle, Z. and Papalopulu, N. (2000). Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. *Development* **127**, 1303-1314.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611-667.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Henry, G. L. and Melton, D. A. (1998). Mixer, a homeobox gene required for endoderm development. *Science* **281**, 91-96.

Ho, C. Y., Houart, C., Wilson, S. W. and Stainier, D. Y. (1999). A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr. Biol.* **9**, 1131-1134.

Hsieh, J. C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M. E., Rosenquist, T. and Holdener, B. C. (2003). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. *Cell* **112**, 355-367.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C. and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* **148**, 567-578.

Hyde, C. E. and Old, R. W. (2000). Regulation of the early expression of the Xenopus nodal-related 1 gene, Xnr1. *Development* **127**, 1221-1229.

Indra, A. K., Warot, X., Brocard, J., Bornert, J. M., Xiao, J. H., Chambon, P. and Metzger, D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res.* **27**, 4324-4327.

Jones, C. M., Thomas, P. Q., Smith, J. C. and Beddington, R. S. P. (1999). An anterior signalling centre in Xenopus revealed by expression of the homeobox gene XHex. *Curr. Biol.* **9**, 946-954.

Kelly, C., Chin, A. J., Leatherman, J. L., Kozlowski, D. J. and Weinberg, E. S. (2000). Maternally controlled (beta)-catenin-mediated signaling is required for organizer formation in the zebrafish. *Development* **127**, 3899-3911.

Kelly, O. G., Pinson, K. I. and Skarnes, W. C. (2004). The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* **131**, 2803-2815.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. (1999). Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development* **126**, 5759-5770.

Kofron, M., Klein, P., Zhang, F., Houston, D. W., Schaible, K., Wylie, C. and Heasman, J. (2001). The role of maternal axin in patterning the Xenopus embryo. *Dev. Biol.* **237**, 183-201.

Lane, M. C. and Sheets, M. D. (2000). Designation of the anterior/posterior axis in pregastrula Xenopus laevis. *Dev. Biol.* **225**, 37-58.

Lemaire, P. and Kodjabachian, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet.* **12**, 525-531.

Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S. and De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* **88**, 747-756.

Li, M., Sendtner, M. and Smith, A. (1995). Essential function of LIF receptor in motor neurons. *Nature* **378**, 724-727.

Marikawa, Y., Li, Y. and Elinson, R. P. (1997). Dorsal determinants in the Xenopus egg are firmly associated with the vegetal cortex and behave like activators of the Wnt pathway. *Dev. Biol.* **191**, 69-79.

Martinez Barbera, J. P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D. and Beddington, R. S. (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433-2445.

McKendry, R., Hsu, S. C., Harland, R. M. and Grosschedl, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter. *Dev. Biol.* **192**, 420-431.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W. S., Robertson, E. J. et al. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol. Cell* **4**, 287-298.

Molenaar, M., Brian, E., Roose, J., Clevers, H. and Destree, O. (2000). Differential expression of the Groucho-related genes 4 and 5 during early development of Xenopus laevis. *Mech. Dev.* **91**, 311-315.

Munemitsu, S., Albert, I., Rubinfeld, B. and Polakis, P. (1996). Deletion of an amino-terminal sequence beta-catenin in vivo and promotes hyperphosporylation of the adenomatous polyposis coli tumor suppressor protein. *Mol. Cell. Biol.* **16**, 4088-4094.

Nichols, J., Evans, E. P. and Smith, A. G. (1990). Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* **110**, 1341-1348.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379-391.

Niederlander, C., Walsh, J. J., Episkopou, V. and Jones, C. M. (2001). Arkadia enhances nodal-related signalling to induce mesendoderm. *Nature* **410**, 830-834.

Niehrs, C. (1999). Head in the WNT: the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.

Nieuwkoop, P. D. and Faber, J. (1994). *Normal Table of Xenopus laevis (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the end of Metamorphosis*. New York: Garland.

Norris, D. P. and Robertson, E. J. (1999). Asymmetric and node-specific nodal expression patterns are controlled by two distinct cis-acting regulatory elements. *Genes Dev.* **13**, 1575-1588.

Norris, D. P., Brennan, J., Bikoff, E. K. and Robertson, E. J. (2002). The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo. *Development* **129**, 3455-3468.

Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M.,

Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. *Development* **127**, 2503-2514.
Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H. et al. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev. Cell* **3**, 745-756.
Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.
Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608-612.
Schmidt, C., Wilson, V., Stott, D. and Beddington, R. S. (1997). T promoter activity in the absence of functional T protein during axis formation and elongation in the mouse. *Dev. Biol.* **189**, 161-173.
Sinner, D., Rankin, S., Lee, M. and Zorn, A. M. (2004). Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. *Development* **131**, 3069-3080.
Slack, J. M., Dale, L. and Smith, J. C. (1984). Analysis of embryonic induction by using cell lineage markers. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **307**, 331-336.
Smith, J. C. (1993). Purifying and assaying mesoderm-inducing factors from vertebrate embryos. In *Cellular Interactions in Development: A Practical Approach* (ed. D. A. Hartley), pp. xviii, 213. Oxford, New York: IRL Press at Oxford University Press.
Smithers, L. E. and Jones, C. M. (2002). Xhex-expressing endodermal tissues are essential for anterior patterning in Xenopus. *Mech. Dev.* **119**, 191-200.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4.
Sun, B. I., Bush, S. M., Collins-Racie, L. A., LaVallie, E. R., DiBlasio-Smith, E. A., Wolfman, N. M., McCoy, J. M. and Sive, H. L. (1999). derriere: a TGF-beta family member required for posterior development in Xenopus. *Development* **126**, 1467-1482.
Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J. and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. *Development* **127**, 5319-5329.
Thomas, P. Q., Brown, A. and Beddington, R. S. P. (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**, 85-94.
Vincent, S. D., Dunn, N. R., Hayashi, S., Norris, D. P. and Robertson, E. J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev.* **17**, 1646-1662.
Vleminckx, K., Kemler, R. and Hecht, A. (1999). The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis. *Mech. Dev.* **81**, 65-74.
Wang, S., Krinks, M., Lin, K., Luyten, F. P. and Moos, M., Jr (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* **88**, 757-766.
Xanthos, J. B., Kofron, M., Wylie, C. and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. *Development* **128**, 167-180.
Xanthos, J. B., Kofron, M., Tao, Q., Schaible, K., Wylie, C. and Heasman, J. (2002). The roles of three signaling pathways in the formation and function of the Spemann Organizer. *Development* **129**, 4027-4043.
Yamanaka, Y., Mizuno, T., Sasai, Y., Kishi, M., Takeda, H., Kim, C. H., Hibi, M. and Hirano, T. (1998). A novel homeobox gene, dharma, can induce the organizer in a non-cell-autonomous manner. *Genes Dev.* **12**, 2345-2353.
Yasuo, H. and Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in Xenopus embryos. *Curr. Biol.* **9**, 869-879.
Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D. and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.
Zorn, A. M., Butler, K. and Gurdon, J. B. (1999). Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways. *Dev. Biol.* **209**, 282-297.