

Embryonic mesoderm and endoderm induction requires the actions of non-embryonic Nodal-related ligands and Mxtx2

Sung-Kook Hong¹, Moon Kyoo Jang², Jamie L. Brown¹, Alison A. McBride² and Benjamin Feldman^{1,*}

SUMMARY

Vertebrate mesoderm and endoderm formation requires signaling by Nodal-related ligands from the TGF β superfamily. The factors that initiate Nodal-related gene transcription are unknown in most species and the relative contributions of Nodal-related ligands from embryonic, extraembryonic and maternal sources remain uncertain. In zebrafish, signals from the yolk syncytial layer (YSL), an extraembryonic domain, are required for mesoderm and endoderm induction, and YSL expression of *nodal-related 1* (*ndr1*) and *ndr2* accounts for a portion of this activity. A variable requirement of maternally derived Ndr1 for dorsal and anterior axis formation has also been documented. Here we show that Mxtx2 directly activates expression of *ndr2* via binding to its first intron and is required for *ndr2* expression in the YSL. Mxtx2 is also required for the Nodal signaling-independent expression component of the *no tail a* (*ntla*) gene, which is required for posterior (tail) mesoderm formation. Therefore, Mxtx2 defines a new pathway upstream of Nodal signaling and posterior mesoderm formation. We further show that the co-disruption of extraembryonic Ndr2, extraembryonic Ndr1 and maternal Ndr1 eliminates endoderm and anterior (head and trunk) mesoderm, recapitulating the loss of Nodal signaling phenotype. Therefore, non-embryonic sources of Nodal-related ligands account for the complete spectrum of early Nodal signaling requirements. In summary, the induction of mesoderm and endoderm depends upon the combined actions of Mxtx2 and Nodal-related ligands from non-embryonic sources.

KEY WORDS: Mxtx2, Ndr1, Ndr2, Ntla, Mesendoderm, Yolk syncytial layer, Zebrafish

INTRODUCTION

Vertebrate mesoderm and endoderm (mesendoderm) formation requires Nodal proteins, a subclass of TGF β superfamily ligands (Schier and Shen, 2000). Altered *NODAL* expression in humans is associated with heterotaxy (MIM ID: 601265), congenital heart disease and holoprosencephaly (Roessler et al., 2009) and melanoma (Topczewska et al., 2006). The mechanisms underlying the zygotic activation of *Nodal* family gene transcription are incompletely understood. In *Xenopus*, transcriptional initiation of Nodal-related gene expression requires the maternal transcription factor VegT (Kofron et al., 1999), but an analogous mechanism not been found in other species. In zebrafish, maternal β -catenin signaling accounts for Nodal-related (*ndr1/2*) gene activation in the dorsal organizer (Kelly et al., 2000), but the factors initiating zygotic Nodal-related gene transcription at more ventral positions are unknown. Some of these factors appear to reside in an extraembryonic domain termed the yolk syncytial layer (YSL), which harbors mRNA-encoded signals that induce adjacent cells of the embryonic margin to differentiate as mesendoderm (Carvalho and Heisenberg, 2010; Chen and Kimelman, 2000; Mizuno et al., 1996). However, assessing the role of the YSL in initiating Nodal-related gene expression or mesendoderm differentiation is complicated by the presence of Nodal-related gene transcripts in both the YSL and mesendoderm precursors.

Indeed, in zebrafish, as in mice, the relative roles of Nodal-like activities from embryonic, extraembryonic and maternal sources are only partially understood. Whereas positive feedback of Nodal signaling occurs in embryonic cells, this mechanism appears to be excluded from the YSL (Fan et al., 2007; Harvey and Smith, 2009). Expression of the zebrafish Nodal-related genes *ndr1* (*sqt*) and *ndr2* (*cyc*) in the YSL is required for a degree of mesoderm and endoderm induction (Fan et al., 2007), but the complete genetic disruption of *ndr1* and *ndr2* causes more severe mesendoderm deficits, as characterized by the absence of endoderm and anterior (head and trunk) mesoderm in *ndr1;ndr2* mutants (Feldman et al., 1998). Finally, a requirement of maternal Ndr1 for dorsal and anterior axis formation is observed in certain contexts (Gore et al., 2005; Hagos et al., 2007), but not in others (Bennett et al., 2007b; Pei et al., 2007).

We have investigated the function of genes expressed in the YSL and report here that the transcription factor Mxtx2 is required for the extraembryonic expression of *ndr2* and *sox32* (*cas*), a pan-endodermal marker (Kikuchi et al., 2001), as well as for the Nodal signaling-independent expression of the pan-mesodermal marker *no tail a* (*ntla*) (Schulte-Merker et al., 1994) in embryonic cells. We further find that co-disruption of extraembryonic Ndr2, extraembryonic Ndr1 and maternal Ndr1 prevents the differentiation of endoderm and anterior mesoderm, thereby recapitulating the phenotype of embryos completely deficient for Nodal signaling via removal of exclusively non-embryonic factors.

MATERIALS AND METHODS

Zebrafish strains and embryo production

The AB strain was used throughout and the *oep*^{ts57} mutant allele was used to generate the *MZoep* line, as previously described (Gritsman et al., 1999). For *ndr1* mutants, the *ndr1*^{hi975} allele (Amsterdam et al., 2004; Pei et al., 2007), a presumed null allele, was used throughout. Wild-type (WT) males

¹Medical Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD 20892, USA. ²Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA.

* Author for correspondence (bfeldman@mail.nih.gov)

were crossed with *ndr1*^{-/-} females to produce embryos lacking maternal Ndr1 (i.e. lacking maternally derived Ndr1; also termed *Mndr1* embryos). Embryos lacking zygotic Ndr1 (also termed *Zndr1* embryos) and their control siblings were generated in equal proportions by crossing *ndr1*^{-/-} males with *ndr1*^{+/-} females. Embryos lacking maternal and zygotic Ndr1 (also termed *MZndr1* embryos) were generated by crossing *ndr1*^{-/-} males with *ndr1*^{-/-} females. For embryo production from parents of identical genotypes, up to three males and three females were placed together in the morning and embryos were collected immediately after spawning. Incubations were at 28.5°C or room temperature, and staging was according to Kimmel (Kimmel et al., 1995). Animal handling was consistent with international guidelines.

Constructs

For the *mxtx2* mRNA expression construct, cDNA was PCR amplified (*mxtx2*-F, 5'-ATGAAAGACATGTGGACTGACTGC-3'; *mxtx2*-R, 5'-AAGATCCATCTGGCCATTGAAGC-3') from single-stranded cDNA prepared from late blastula stage zebrafish embryos using the SuperScript III First-Strand Synthesis System (Invitrogen), then cloned into the pCS2+ expression vector. The forward primer had an additional 15 bases at the 5' end so as to include a restriction enzyme site followed by a generic Kozak sequence (GCCACC) in the amplified product. To generate Eng-Mxtx2 and VP16-Mxtx2 constructs, the homeobox domain sequence of *mxtx2* (corresponding to amino acids 13-81) was amplified (F, 5'-GGAACTCACAGGCCAGTAAGATTG-3'; R, 5'-GTTGTCCAGCTGTGGGAGCTCTG-3') and subcloned into the *XhoI*-*XbaI* sites of pCS2+-Eng and pCS2+-VP16 vectors. To generate Flag-tagged *mxtx2*, full-length and homeobox domain-deleted *mxtx2* were subcloned into pCS2+-Flag vectors.

Injection of mRNAs and morpholinos (MOs), MO rescue and cycloheximide treatment

All mRNAs were synthesized using the mMESSAGE mMACHINE SP6 Kit (Ambion). Fertilized embryos were injected with 10 pg mRNA for *mxtx2* overexpression, or 2 pg for rescue, 25 pg for *eng-mxtx2* and *vp16-mxtx2* mRNA or 50 pg *gfp* mRNA as a control. The cycloheximide treatment assay shown in Fig. S2 in the supplementary material was performed as previously described (Poulain and Lepage, 2002). Briefly, *eng-mxtx2*, *vp16-mxtx2* mRNA or *gfp* mRNA was injected as above, but a portion of the embryos were then treated with 50 µg/ml cycloheximide (Sigma) beginning at the 64- to 128-cell stage, and all embryos were fixed when untreated embryos reached the 30% epiboly stage and stained for *ndr2* expression.

MOs

All MOs were purchased from Gene Tools (Philomath, OR, USA). Bold indicates nucleotides complementary to ATG start codons and lowercase indicates mismatches. Single or combined MO injections were at the following doses: 2 ng (1 ng for Fig. S1 in the supplementary material) Mxtx2 MO (ZFIN name MO1-mxtx2, 5'-CATTGAGTATTTG-CAGCTCTCTTG-3') (Bruce et al., 2005) or 8 ng Mxtx2 mismatch MO (5'-CATTCAGTAATTTCAGGTCGTGTTG-3'; the Ctrl MO for Fig. 1, Fig. 4E-P, Fig. 6 and Fig. 7), 8 ng standard Gene Tools control MO (5'-CCTCTTACCTCAGTTACAATTATA-3'; the Ctrl MO for Fig. 3, Fig. 4A-D and Fig. S1 in the supplementary material), 10 ng Ndr2 MO (5'-GCGACTCCGAGCGTGTGCATGATG-3') (Karlen and Rebagliati, 2001), 10 ng Ndr1 MO (5'-ATCTGAGAGATTCTTACCTGCATGT-3'; used for Fig. 4N,P, Fig. 6C,F, Fig. 7E-H) (Gore et al., 2005), 10 ng Ndr1 MO (5'-ATGTCAAATCAAGGTAATAATCCAC-3'; used for Fig. 6B,E) (Feldman and Stemple, 2001).

Except for Fig. S1 in the supplementary material and Fig. 4F, in which a standard 1-cell injection was used, all MO injections were into the YSL as follows. Needles were targeted to the region of yolk just below the margin in 512-cell (2.75 hpf) to 1000-cell (3 hpf) embryos and, to assist with targeting, certain MOs had 3' fluorescein tags: the Mxtx2 MO, the Mxtx2 mismatch MO, the Ndr2 MO and the Ndr1 MO in Fig. 6B,E. YSL injected embryos were viewed under a fluorescence dissection scope and only those embryos with fluorescence confined to the YSL and evenly distributed were used (this quality assurance was not employed for Fig. 4F because fluorescent Ndr2 MO was injected throughout the embryo, causing

ubiquitous fluorescence). For the rescue shown in Fig. S2 in the supplementary material, 0.5 pg of *ndr1* RNA was co-injected into the YSL of *Mndr1* embryos along with 10 ng each of the Ndr1 MO (Gore et al., 2005) and the Ndr2 MO.

Whole-mount in situ hybridization and histology

Digoxigenin-labeled RNA probes for *ndr1* (Feldman et al., 1998), *sox32* (Kikuchi et al., 2001), *ndr2* (Rebagliati et al., 1998), *gata5* (Reiter et al., 1999), *ntla* (*ntl*) (Schulte-Merker et al., 1992), *bmp2b* (Nikaido et al., 1997), *foxa2* (*axial*) (Strahle et al., 1993), *desma* (Chen and Tsai, 2002) and *egr2b* (*krox20*) (Nieto et al., 1991) were prepared from linearized template DNAs using an RNA labeling kit (Roche). Whole-mount in situ hybridization was performed as described, except that post-hybridization washing was at 65°C (Toyama et al., 1995). For histology, stained whole-mount embryos were fixed in 4% paraformaldehyde (Sigma) overnight at room temperature, then gradually dehydrated through a methanol series and embedded in JB-4 plastic resin (Polysciences) according to the manufacturer's instructions. Sectioning (7 µm) was performed on a Leica RM2165 microtome (Deerfield, IL, USA). Whole-mount high-resolution double fluorescence in situ hybridizations for Fig. 5 were performed as described (Brend and Holley, 2009), using initial fluorescein labeling and a final fluorescein substrate to visualize the location of *mxtx2* or *sox32* transcripts in green or, alternatively, initial digoxigenin labeling and a final Cy3 substrate to visualize the location of *ntla* or *sox32* transcripts in red.

Chromatin immunoprecipitation and quantitative (q) PCR

Chromatin immunoprecipitation (ChIP) (Li et al., 2003) experiments were carried out using the protocol provided by NimbleGen with certain modifications, most of which have been described previously (Jang et al., 2009). Twenty-five pg of control (pCS2+-Flag) DNA, *mxtx2-flag* mRNA (full length) and *delHD-mxtx2* mRNA (homeodomain deletion) were injected into 1- to 2-cell stage embryos and harvested at the 30% epiboly stage. Approximately 1000 injected embryos for each injection condition were fixed in 1.85% formaldehyde (Sigma) for 10 minutes at room temperature and the fixation was stopped with glycine. The embryos were then washed and lysed, and the chromatin DNA was harvested and sheared to an average size of 500 bp. A portion of this sheared DNA was kept (the pre-IP DNA) and the rest was subjected to ChIP. For ChIP, sheared DNA was incubated overnight with anti-FLAG M2 bound to Dynabeads protein G (Invitrogen). Negative control ChIPs were performed using standard mouse IgG instead of anti-FLAG M2. The IPs were washed extensively, chromatin DNA was eluted, reverse cross-linked with proteinase K, purified by organic extraction and precipitated with ethanol. Immunoprecipitated DNA was analyzed by real-time PCR using the primers listed in Table S1 in the supplementary material. For real-time PCR, the SYBR Green Master Mix (Roche) was used to monitor amplification of sample cDNA using an ABI Prism 7900HT Sequence Detection System. For each primer pair, cycle thresholds (Ct) were determined in amplifications of two duplicate IP DNA samples and a dilution series of pre-IP DNA. Percentage inputs for each primer pair were calculated by dividing the average IP sample DNA concentration by the pre-IP DNA concentration needed to achieve a Ct value equal to the average Ct value for the two IP samples. Standard deviations are a function of the variation between the Ct values of the two IP samples.

Transfections and luciferase assays

Luciferase constructs were generated in the pGL3-Promoter Vector. A 1.0 kb stretch of the first intron of *ndr2*, including the region enriched in our ChIP qPCR studies, was amplified with the following primers: F, 5'-CCCAACGAAATCTCATGAAATTGTG-3'; R, 5'-GTGTGATGAAAGTCATGTGGTATCG-3'. As a negative control, 1.6 kb spanning the second intron of *ndr2* was also amplified, using the following primers: F, 5'-CCAGATGTGCTCAACAATGACAAC-3'; R, 5'-GTGTCTCAGAAATCATCTCTCCGTTTC-3'. The amplified PCR fragments were inserted into *KpnI*-*MluI* sites of the pGL3-Promoter Vector.

Transient transfections were performed in 6-well dishes. COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum. Cells were transfected with the following DNAs: 0.5 µg firefly luciferase linked to the first or second intron of *ndr2*, as well as 0.5

µg of either *mxtx2*-Flag, delHD-Flag or Flag vector alone, and 1 ng *Renilla* luciferase, using the FuGENE 6 (Roche) transfection reagent. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) after 24 hours of transfection. All assays were performed at least three times.

RESULTS

Mxtx2 induces mesoderm and endoderm

To identify mesendoderm-inducing genes from the YSL, we injected zebrafish embryos with synthetic mRNA for YSL genes that we had previously identified through a microdissection-based screen (Hong et al., 2010) and assessed their ability to induce markers of various cell fates. We uncovered a novel and potent mesendoderm-inducing and ventralizing activity for *mxtx2* (Fig. 1A-L), which encodes a Mix/Bix family transcription factor that is co-expressed in the YSL with the related family member *mxtx1* (Hirata et al., 2000).

To examine the requirement of extraembryonic Mxtx2 for mesendoderm gene expression, we injected the YSL of wild-type (WT) embryos with an antisense morpholino oligonucleotide (MO) designed to disrupt translation of *mxtx2* mRNA. Confirming previous reports (Bruce et al., 2005; Wilkins et al., 2008), Mxtx2-depleted embryos displayed a lethal defect in epiboly, which is a canonical gastrulation movement, that was rescued by co-expressing *mxtx2* mRNA (see Fig. S1 in the supplementary material). Mxtx2-depleted embryos displayed normal mesendoderm gene expression at the onset of gastrulation (data not shown), but earlier, during the time of mesendoderm specification, they showed substantial reductions in mesendoderm gene expression that were not previously reported. Specifically, blastula stage expression of *ndr1*, *ndr2*, *ntl*, *sox32* and *gata5* was reduced, whereas expression of the ventral marker *bmp2b* was unaffected (Fig. 1M-X). Thus, extraembryonic Mxtx2 is essential for early mesendoderm specification, but not for ventral identity.

Mxtx2 positively regulates *ndr2* transcription via binding to its first intron

We noted particularly strong changes in *ndr2* expression in Mxtx2 gain- and loss-of-function studies (Fig. 1F,R), leading us to hypothesize that Mxtx2 endogenously regulates *ndr2* expression. We confirmed this in several ways. First, we verified that *mxtx2* is co-expressed at the time of initial *ndr2* expression. *mxtx2* was initially expressed throughout the margin and vegetal embryo (Fig. 2A) and we observed scattered *ndr2*-expressing cells within the same domain (Fig. 2B). Later, *mxtx2* expression became restricted to the YSL, and the YSL was also the first site of robust *ndr2* transcription (Fig. 2C,D).

Second, to distinguish whether Mxtx2 upregulation of *ndr2* is via repression or activation, we tested the effects of Engrailed (Eng) repressor (Conlon et al., 1996) and VP16 activator (Sadowski et al., 1988) forms of Mxtx2 on *ndr2* expression (Fig. 2E-H). Overexpression of Eng-Mxtx2 quenched *ndr2* expression (Fig. 2G), whereas overexpression of VP16-Mxtx2 increased *ndr2* expression (Fig. 2H), demonstrating that *ndr2* is upregulated via activation.

Third, to determine whether activation and repression of *ndr2* expression by the Mxtx2 constructs is direct, we tested whether they could function when protein synthesis is blocked after 2 hours post-fertilization (hpf) with cycloheximide. This inhibition of protein synthesis during the time that the presynthesized constructs presumably act upon their targets did not block the respective induction or repression of *ndr2* expression, indicating that Mxtx2

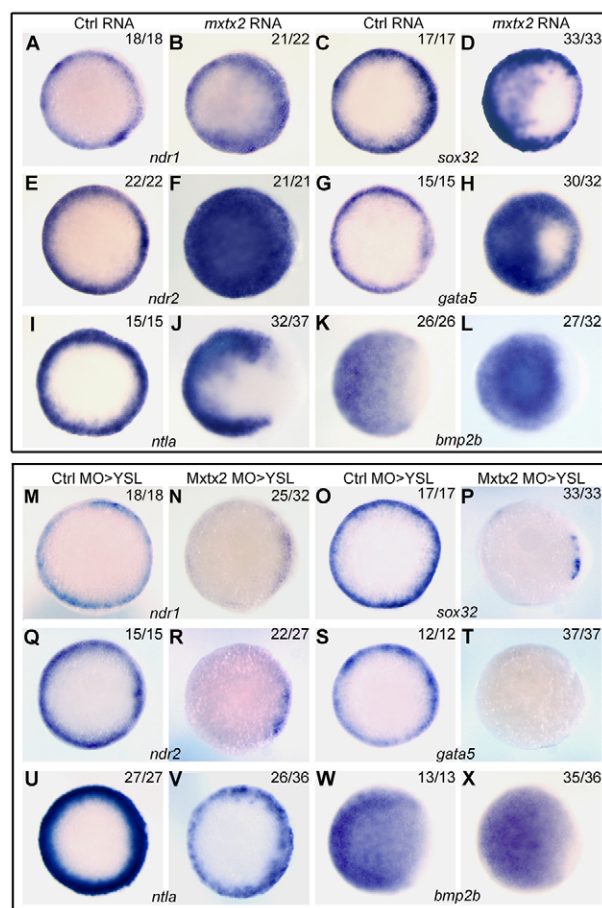
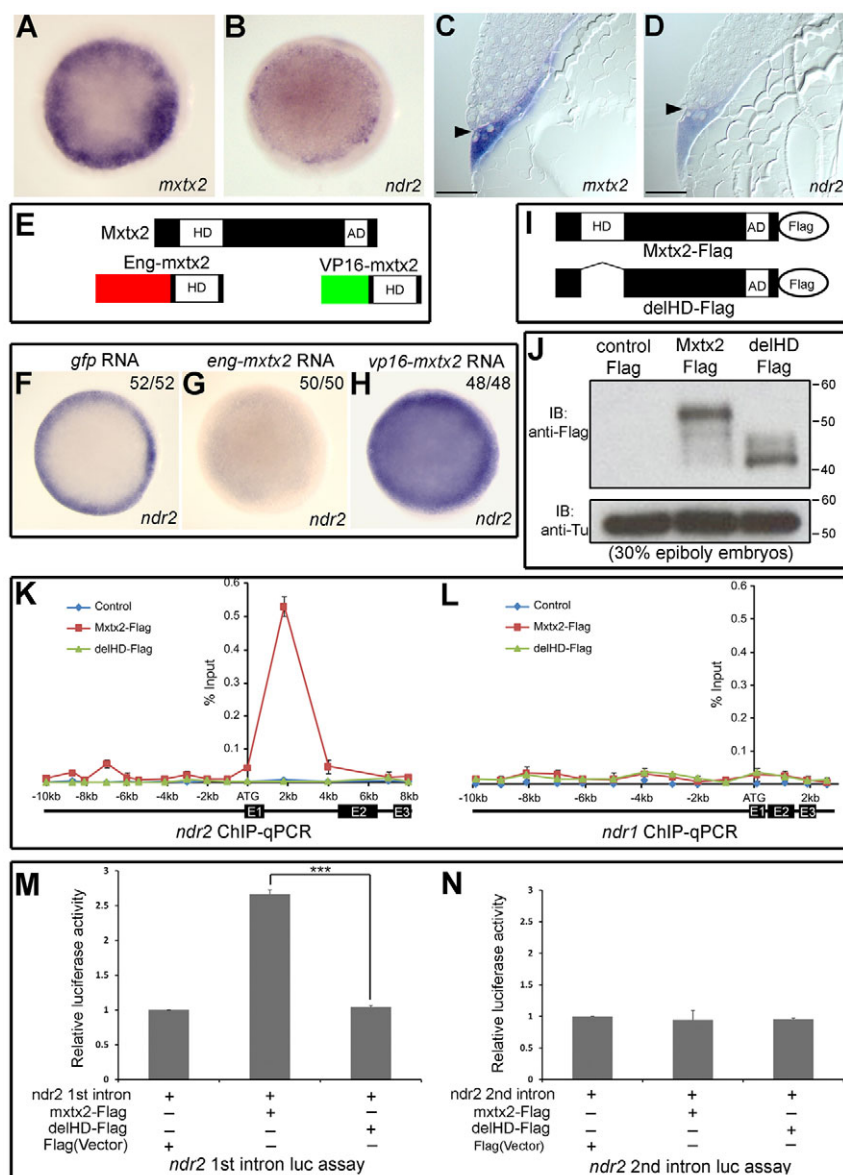


Fig. 1. Mesendoderm induction by Mxtx2. (A-X) Control *gfp* mRNA (50 pg) or *mxtx2* mRNA (10 pg) was injected into whole zebrafish embryos (A-L), or Ctrl MO (8 ng) or Mxtx2 MO (2 ng) was injected into the yolk syncytial layer (YSL) (M-X), and embryos were fixed at 30% epiboly (4.7 hpf) and stained by whole-mount in situ hybridization (WISH) for the indicated markers. Animal pole views are shown. Fractions indicate the number of equivalent outcomes/number of embryos observed. See also Fig. S1 in the supplementary material.

activation of *ndr2* is independent of additional protein synthesis (see Fig. S2 in the supplementary material).

Fourth, to determine whether and where Mxtx2 binds to the *ndr2* locus, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR). Embryos were injected with mRNA encoding Flag-tagged Mxtx2 or controls (Fig. 2I) and harvested at the 30% epiboly stage (Fig. 2J). Analysis of 18 kb of the *ndr2* locus revealed enriched binding to the first intron of *ndr2* (Fig. 2K). The first intron is a common location for transcriptional enhancers, as demonstrated for other Nodal-related genes including *ndr1* (Fan et al., 2007; Osada et al., 2000). However, ChIP-qPCR across a similar span of the *ndr1* locus, including its first intron, did not demonstrate Mxtx2 binding (Fig. 2L). Consistent with this, we observed no changes in *ndr1* expression after injection of the Eng-Mxtx2 or VP16-Mxtx2 constructs (see Fig. S2 in the supplementary material).

Finally, to determine whether the first intron of *ndr2* contains a genuine Mxtx2-response element, luciferase plasmids carrying either *ndr2* intron 1, or *ndr2* intron 2 as a control, were transfected into COS-7 cells along with an *mxtx2* expression vector. An *ndr2*



intron 1-dependent enhancement of luciferase activity was observed (Fig. 2M,N). Thus, Mxtx2 activates *ndr2* transcription via direct binding to its first intron. We presume that the modest alterations in *ndr1* expression that we observed in response to Mxtx2 alterations (Fig. 1B,N) reflect an increased or decreased contribution of Ndr2 to the Ndr positive-feedback loop.

Yolk syncytial layer expression of *ndr2* and *sox32* requires Mxtx2

To better understand the recovery of downregulated genes in Mxtx2-depleted embryos, we focused on the spatiotemporal recovery of *ndr2* and *sox32* expression. Between 4.7 hpf (30% epiboly stage, Fig. 1) and 5.3 hpf (50% epiboly stage, Fig. 3), expression of these two genes had substantially recovered (compare Fig. 3F with Fig. 1P and Fig. 3J with Fig. 1R). Sections of *sox32*- and *ndr2*-stained embryos revealed that this recovery occurs in the embryonic margin, but not the YSL (Fig. 3A-D). Disruption of Nodal signaling causes a reciprocal effect on *sox32* expression, eliminating it from the margin but not the YSL

(Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). We confirmed this by visualizing persistent *sox32* staining in the YSL of *MZoepe* embryos (Fig. 3G), which lack both maternal and zygotic sources of an essential Nodal co-receptor (Gritsman et al., 1999), and we observed the same YSL persistence for *ndr2* (Fig. 3K). Consistent with the above, we were able to entirely eliminate *ndr2* and *sox32* expression by injecting Mxtx2 MOs into the YSL of *MZoepe* embryos (Fig. 3H,L). Thus, extraembryonic Mxtx2 is essential for the extraembryonic expression of *ndr2* and *sox32*.

Embryonic expression of *ntla* depends on non-embryonic Mxtx2 and Ndr1

We found that combining Mxtx2 and Nodal disruptions also affects the expression of *ntla*, the human ortholog of which, *BRACHURY* (*T*), is implicated in spina bifida and chordomas (MIM ID: 601397) (Fig. 4). Unlike the expression of most margin-specific genes, expression of *ntla* persists in the absence of Nodal signaling in all but the dorsal-most aspect (Fig. 4C, arrowhead)

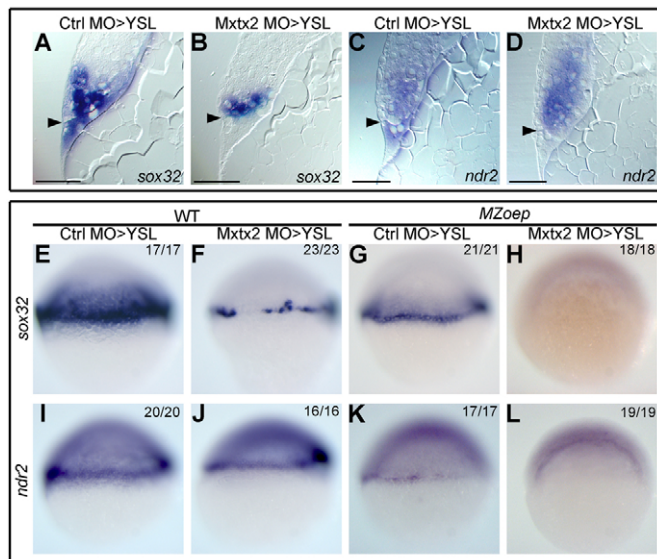


Fig. 3. YSL expression of *sox32* and *ndr2* depends on Mxtx2.

(A–L) Expression of the indicated marker genes at the 50% epiboly stage (5.3 hpf) in wild-type (WT) or *MZoepr* zebrafish embryos after YSL injection of 8 ng Ctrl MO (A,C,E,G,I,K) or 2 ng Mxtx2 MO (B,D,F,H,J,L). (A–D) Sections through the margin and YSL of stained embryos. Arrowheads indicate the margin–YSL boundary. Fractions indicate the number of equivalent outcomes/number of embryos observed. Scale bars: 50 μ m.

(Gritsman et al., 1999). Disruption of Mxtx2 in the YSL of WT embryos had no lasting effect on *ntla* expression (compare Fig. 4B with Fig. 1V), but co-disruption of Oep and extraembryonic Mxtx2 nearly eliminated expression (Fig. 4D). Thus, extraembryonic Mxtx2 and Nodal signaling are collectively required, but individually sufficient, to activate ventrolateral *ntla* expression in the margin, explaining the resilience of *ntla* expression. Persistence of *ntla* expression in *MZoepr* embryos accounts for their residual posterior mesoderm formation (Harvey et al., 2010); therefore, our findings identify a combinatorial requirement for Mxtx2 and Nodal signaling that underlies the specification of posterior mesoderm.

To examine the possibility that Mxtx2 directly activates *ntla* transcription, we searched for a domain of *mxtx2* and *ntla* co-expression. We were able to visualize the co-expression of *sox32* with *mxtx2* (Fig. 5C,G) or *ntla* (Fig. 5D,H) using double fluorescence in situ hybridization, but *mxtx2* and *ntla* transcripts were respectively confined to the YSL and embryonic margin, with no overlap in expression (Fig. 5B,F). This, and the theoretical inability of a non-secreted transcription factor to cross the cellular membranes that separate the YSL cytoplasm from the cytoplasm of neighboring embryonic cells, argue that Mxtx2 influences *ntla* via activation of an intercellular signaling intermediary. Candidate intermediary factors include BMP and Wnt ligands, based on the demonstrated synergy of these pathways with the Nodal pathway in activating *ntla* expression (Harvey et al., 2010).

Both Ndr1 and Ndr2 are expressed during pre-gastrula stages and Oep mediates the signaling of both ligands. To determine whether the Mxtx2/Nodal synergy driving *ntla* expression is specific to Ndr1 or Ndr2, we tested several combinatorial disruptions. Co-depletion of Mxtx2 and Ndr2 from the YSL did not recapitulate the Mxtx2/Oep synergy (Fig. 4F), as might have been predicted because *ndr2* expression in the YSL should also be

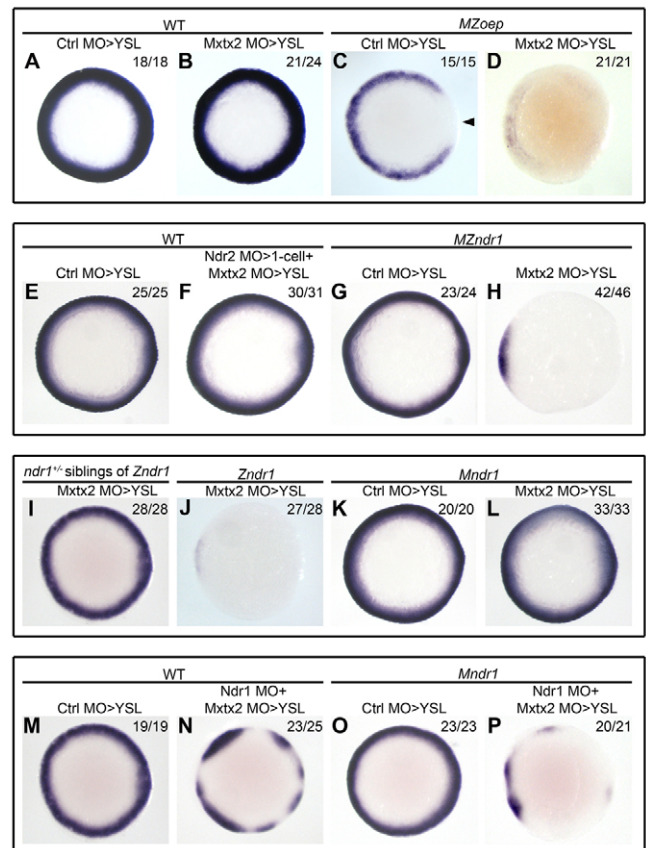


Fig. 4. Mxtx2/Ndr1 synergy underlies posterior mesoderm specification.

(A–P) Ctrl MO (8 ng), Mxtx2 MO (2 ng) or Ndr1 MO (10 ng) were injected singly or in combination, as indicated, into the YSL of the following zebrafish embryos: WT (A,B,E,M,N), WT pre-injected with Ndr2 MO (F), *MZoepr* (C,D), *MZndr1* (G,H), *Zndr1* (I) and their *ndr1*^{+/−} siblings (J), and *Mndr1* (K,L,O,P). Embryos in A–D were fixed at the 50% epiboly stage (5.3 hpf), whereas all others were fixed at the shield stage (6 hpf), and all were stained for *ntla*. Animal pole views are shown, with dorsal to the right. Fractions indicate the number of equivalent outcomes/number of embryos observed.

eliminated in embryos injected with Mxtx2 MO (Fig. 4B). However, disruption of maternal and zygotic sources of Ndr1 synergized with Mxtx2 disruption to nearly eliminate *ntla* expression in the same manner as Oep disruptions (Fig. 4H). Thus, *ntla* expression, and therefore posterior mesoderm specification, depends on the combined actions of Mxtx2 and Ndr1.

To further dissect the genetic interaction of Mxtx2 and Ndr1, we looked at the effects of Mxtx2 removal on *ntla* expression in embryos lacking zygotic, extraembryonic and maternal sources of Ndr1. We uncovered two scenarios of combinatorial disruption with strong effects on *ntla* expression. First, *ntla* expression was disrupted by removal of Mxtx2 from the YSL of embryos that lack zygotic sources of Ndr1 (Fig. 4J). In the second scenario, *ntla* expression was disrupted by simultaneous removal of Mxtx2 from the YSL, Ndr1 from the YSL and maternal Ndr1 (Fig. 4P). This latter scenario was achieved by co-injecting MOs for Mxtx2 and Ndr1 into the YSL of *Mndr1* embryos generated by crossing an *ndr1*^{−/−} female and a WT male. Thus, a combination of non-embryonic factors, namely maternal Ndr1, extraembryonic Ndr1 and extraembryonic Mxtx2, is synergistically required and individually sufficient for posterior mesoderm specification.

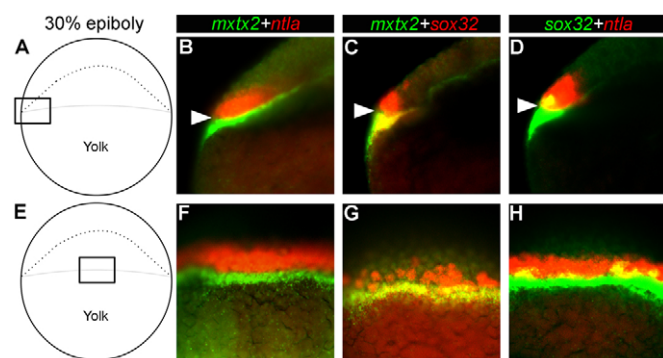


Fig. 5. Confinement of *ntlA* transcripts to the embryonic margin and *mxTx2* transcripts to the YSL. (B-D,F-H) Double fluorescence WISH was performed on WT zebrafish embryos at the 30% epiboly stage, with *mxTx2* labeled in green, *ntlA* in red and *sox32* in red or green (as indicated by the text color). Overlapping expression appears in yellow. (A,E) Schematics indicating the embryonic regions (boxed) visualized in B-D and F-H, respectively. *sox32* expression completely overlaps with *mxTx2* in the YSL (C,G) and partially overlaps with *ntlA* in the margin (D,H). By contrast, no overlap is seen between *mxTx2* in the YSL and *ntlA* in the margin (B,F). The thin yellow line in B and F, which is far narrower than a single cell, is an optical bleeding artifact. Arrowheads indicate the YSL-margin boundary.

Endoderm and anterior mesoderm formation depends on non-embryonic sources of Ndr2 and Ndr1

Although there is a clear function for embryonic expression of *ntlA* in posterior mesoderm specification, the developmental function of extraembryonic Ndr2 downstream of MxTx2 is less apparent. We found that extraembryonic Ndr2 synergizes with Ndr1 in an

analogous fashion to MxTx2 with Ndr1. Here we were able to examine the effects of our perturbations on the second day of embryonic development, during the so-called pharyngula stage when zebrafish embryos acquire the phylotypic vertebrate body plan (Ballard, 1981). This had not been possible for the MxTx2-depletion studies owing to early lethality.

It was previously shown, by injecting the YSL with MOs that block Ndr1 and Ndr2 translation, that endoderm specification and a degree of dorsal mesoderm specification require an essential synergy of extraembryonic Ndr2 and extraembryonic Ndr1 (Fan et al., 2007). We revisited this experiment, obtaining very similar results (Fig. 6B,E), but found that additional mesendoderm deficits arose when we used an alternate Ndr1 MO (Gore et al., 2005) (Fig. 6C,F). Thus, the synergistic requirements for extraembryonic Ndr1 and Ndr2 in mesendoderm induction are stronger than previously believed.

Working with embryos carrying *ndr1* mutations, we uncovered an even more dramatic requirement for extraembryonic Ndr2. Specifically, removal of extraembryonic Ndr2 from *ndr1* mutant embryos (Fig. 6H,K) recapitulated the Ndr signaling-deficient phenotype of *ndr1;ndr2* double-mutant embryos (Feldman et al., 1998), *MZoep* mutant embryos (Gritsman et al., 1999) and embryos in which *lefty1* (*antivin*) is overexpressed (Thisse and Thisse, 1999). This phenotype is characterized by a complete lack of endoderm and anterior (head and trunk) mesoderm, with persistence of some mesoderm in the tail. This recapitulation was evidenced by the indistinguishable morphology of these embryos from *MZoep* embryos (compare Fig. 6H and 6I) and was further supported by the loss of heart and trunk somite labeling in both classes of embryo (compare Fig. 6K and 6L). Thus, in the absence of all sources of zygotic Ndr1, extraembryonic expression of *ndr2* is required to support endoderm and anterior mesoderm formation.

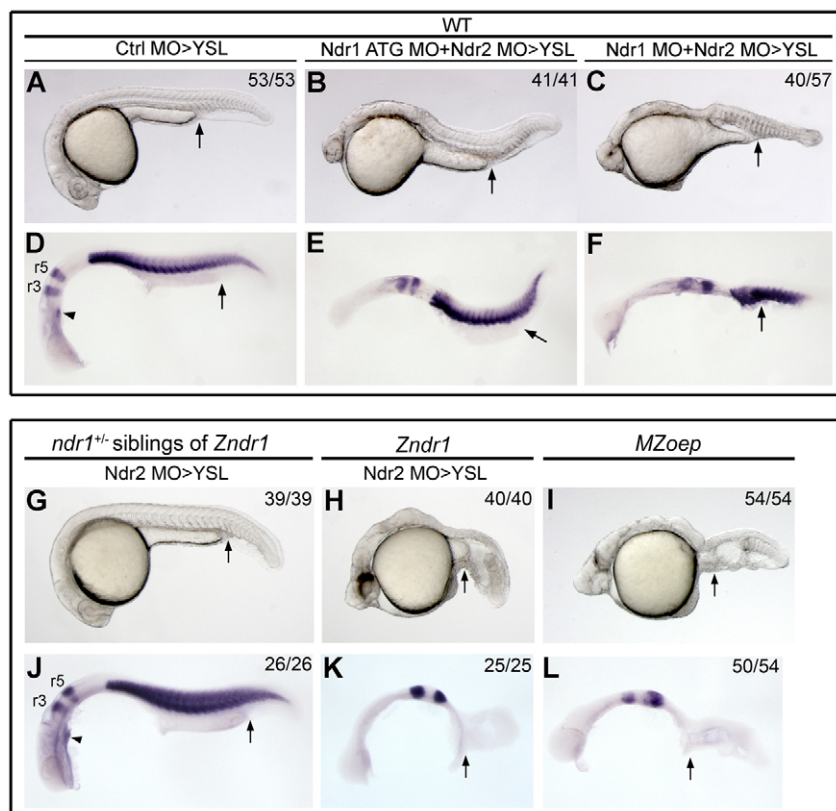


Fig. 6. Unexpected roles for extraembryonic Ndr2 and Ndr1 in mesoderm induction. (A-L) Live (A-C,G-I) and RNA-stained (D-F,J-L) phenotypes of pharyngula stage (24 hpf) WT zebrafish embryos, *Zndr1* embryos and their sibling controls and *MZoep* embryos after YSL injection of Ctrl MO (A,D), Ndr2 MO (G,H,I,K), Ndr2 MO combined with the Ndr1 ATG MO reported by Fan et al. (Fan et al., 2007; Feldman and Stemple, 2001) (ZFIN ID: MO4-ndr1) (B,E), or Ndr2 MO combined with the Ndr1 MO reported by Gore et al. (Gore et al., 2005) (ZFIN ID: MO2-ndr1) (C,F). Embryos in J-L were fixed and stained with two antisense probes: *egr2b*, which encodes a zinc-finger transcription factor and labels hindbrain rhombomeres 3 and 5; and *desma*, which encodes the intermediate filament protein Desmin A and labels muscles in the heart, trunk somites and tail somites. Arrows point to the anterior limit of the tail; arrowheads point to heart muscle staining by *desma* and r3 and r5 indicate rhombomere 3 and 5 staining by *egr2b*. Fractions indicate the number of equivalent outcomes/number of embryos observed. Siblings of the embryos in C and F are also shown in Fig. 7E and 7G, respectively.

Finally, similar to the second scenario that we delineated for Mtxt2-Ndr1 interaction, we found that losses of extraembryonic Ndr2 and extraembryonic Ndr1 synergized with a loss of maternal Ndr1 (Fig. 7). Embryos lacking maternal Ndr1 displayed normal morphologies and normal labeling of heart muscle, somite muscles and hindbrain rhombomeres 3 and 5 on the second day of development (Fig. 7B). At the gastrula stage, these embryos exhibited normal endoderm and dorsal mesoderm labeling (Fig. 7D). By contrast, MO-based perturbation of extraembryonic Ndr2 and extraembryonic Ndr1 in embryos lacking maternal Ndr1 recapitulated the mesoderm and endoderm deficiencies of Ndr signaling-deficient embryos. This was apparent from their morphology, from their lack of heart and trunk somite marker expression at the pharyngula stage (Fig. 7F) and from their lack of dorsal mesoderm and endoderm marker expression at the gastrula stage (Fig. 7H). In support of the specificity of this effect, we were able to substantially rescue this Ndr deficiency-like phenotype by co-injecting *ndr1* RNA into the YSL (see Fig. S3 in the supplementary material) and milder morphological and molecular marker expression alterations were also seen in WT embryos in which extraembryonic Ndr2 and extraembryonic Ndr1 were depleted (Fig. 7E,G). Thus, the specification of endoderm and anterior mesoderm is determined by the combined actions of Nodal-related ligands from non-embryonic (extraembryonic and maternal) sources.

DISCUSSION

We propose the following model of mesendoderm specification (Fig. 7I). Induction of endoderm and anterior mesoderm is executed by a failsafe combination of three non-embryonic sources of Nodal-related ligands with overlapping activities. Nodal signaling is then amplified in the embryonic margin through autoregulatory positive feedback requiring embryonic Ndr1 and Ndr2 (Feldman et al., 2002; Osada et al., 2000), with a particular reliance on Ndr1 when the stimulus of non-embryonic Nodal factors is low. Recent reports indicate that positive Nodal feedback cannot occur in the YSL (Fan et al., 2007; Harvey and Smith, 2009), and expression of *mtxt2* in the YSL is Nodal independent (Hirata et al., 2000), suggesting that the extraembryonic to embryonic signaling that we have uncovered is unidirectional and that Mtxt2 has a primary role upstream of Nodal signaling. This is distinct from other Mix/Bix factors, including the zebrafish family members Bon (Mixer) and Mezzo (Og9x – Zebrafish Information Network), the expression of which is downstream to, and dependent upon, Nodal signaling (Kikuchi et al., 2000; Poulain and Lepage, 2002). Mtxt2 also activates a Nodal-independent signal that is sufficient for the establishment of posterior mesoderm.

Our demonstration of a functional requirement for maternal Ndr1 is significant because the function of maternal Ndr1 is controversial (Bennett et al., 2007a; Gore et al., 2005). This is the first demonstration of an invariant requirement for maternal *ndr1* using a mutant maternal allele. Our findings support the proposal that maternal Ndr1 functions in dorsal mesoderm specification (Gore et al., 2005), but we find that it is only essential in a combinatorial context and we did not recapitulate the requirement for maternal Ndr1 in anterior axis formation (Gore et al., 2005; Hagos et al., 2007).

The signaling mechanisms that we have discovered in zebrafish might prove to be relevant to higher vertebrates. Low levels of *Nodal* mRNA are detected throughout the preimplantation inner cell mass of mouse embryos (Takaoka et al., 2006) and no

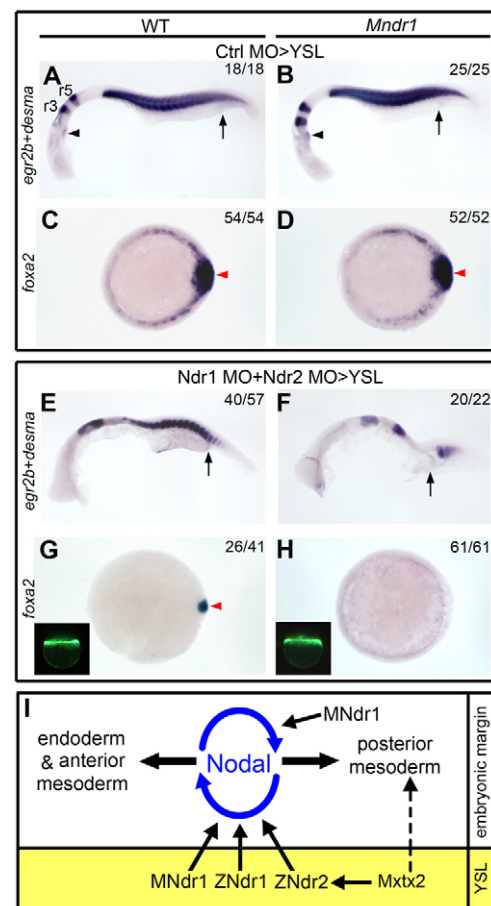


Fig. 7. Endoderm and anterior mesoderm formation requires non-embryonic sources of nodal-related ligands. (A-H) Pharyngula stage (24 hpf; A,B,E,F, lateral views) and gastrula stage (7 hpf; C,D,G,H, animal pole views) phenotypes of WT and *Mndr1* zebrafish embryos after YSL injection of 8 ng Ctrl MO or a combination of 10 ng Ndr2 MO and 10 ng Ndr1 MO. Embryos were fixed and stained with antisense probes against *egr2b* and *desma* RNAs (A,B,E,F; see Fig. 6 legend) and against RNA encoding the forkhead box transcription factor *Foxa2*, which labels gastrula stage endoderm and dorsal mesoderm (C,D,G,H). Black arrowheads indicate heart muscle staining by *desma*. Red arrowheads indicate dorsal mesoderm; the remaining *foxa2* stain, where present, marks endoderm. Arrows indicate the anterior limit of the tail. Fractions indicate the number of equivalent outcomes/number of embryos observed. Siblings of the embryos in E and G are also shown in Fig. 6E and 6F, respectively. Insets in G and H show the distribution of fluorescently labeled MO in representative YSL injections. (I) Model of the developmental requirements for non-embryonic sources of Mtxt2, Ndr2 and Ndr1. The dashed line indicates a Nodal-independent pathway downstream of Mtxt2. Anterior mesoderm refers to the totality of head and trunk mesoderm.

transcription factor driving this expression has been identified. In light of our finding that Mtxt2 regulates *ndr2*, it will be interesting to learn whether early *Nodal* expression in mouse is regulated by any of several co-expressed paired homeodomain proteins similar to Mtxt2.

Our evidence for a failsafe switch comprising diversely sourced Nodal-related ligands acting upstream of the Nodal autoregulatory loop might also be relevant to the early mouse embryo, in which other ligands with Nodal-like activities, particularly Gdf3 (Chen et

al., 2006) and maternally derived activins (Jones et al., 2006), are detected alongside Nodal. Perhaps a combination of these TGF β family ligands acts in an analogous fashion to ensure the initiation of Nodal autoregulation in mice.

In conclusion, this study elucidates essential molecular inputs underlying three central embryonic patterning events: the early activation of a Nodal-related gene (*ndr2*), which requires Mxtx2 binding to the first intron of *ndr2*; posterior mesoderm specification, which depends on the combined actions of extraembryonic Mxtx2, maternal Ndr1 and extraembryonic zygotic Ndr1; and the induction of endoderm and anterior mesoderm, which is implemented by an essential trio of non-embryonic sources of Nodal-related ligands.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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