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Coco regulates dorsoventral specification of germ layers via inhibition of TGFβ signalling

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SUMMARY

One of the earliest steps in embryonic development is the specification of the germ layers, the subdivision of the blastula embryo into endoderm, mesoderm and ectoderm. Maternally expressed members of the Transforming Growth Factor β (TGFβ) family influence all three germ layers; the ligands are required to induce endoderm and mesoderm, whereas inhibitors are required for formation of the ectoderm. Here, we demonstrate a vital role for maternal Coco, a secreted antagonist of TGFβ signalling, in this process. We show that Coco is required to prevent Activin and Nodal signals in the dorsal marginal side of the embryo from invading the prospective ectoderm, thereby restricting endoderm- and mesoderm-inducing signals to the vegetal and marginal zones of the pre-gastrula *Xenopus laevis* embryo.

KEY WORDS: Coco, Ectoderm, Endoderm, Mesoderm, TGFβ, *Xenopus*

INTRODUCTION

Members of the TGFβ family are required for germ layer specification and for patterning and organisation of endoderm, mesoderm and ectoderm; a vegetal-animal gradient of Nodal-related signals opposes an animal-vegetal gradient of their inhibitors. The secreted TGFβ ligands Vg1, Activin and *Xenopus* Nodal-related (Xnr), promote formation of both the endoderm, which gives rise to the gut and associated organs, and the mesoderm, which gives rise to muscle, blood and skeleton. Vg1 is expressed vegetally before the onset of Nodal and Activin expression, and has been shown to promote these other TGFβ signals (Joseph and Melton, 1998). Nodal and Activin are required for both mesoderm and endoderm formation, with the highest activity level in endoderm and moderate levels in mesoderm (Hudson et al., 1997; Yasuo and Lemaire, 1999; Piepenburg et al., 2004; Kimelman, 2006; Shen, 2007; Luxardi et al., 2010).

Recently, a number of cell-autonomous factors that inhibit TGFβ signalling have been shown to be required for differentiation of the ectoderm. Foxi2, a maternal transcription factor expressed in the animal region of blastula and gastrula stage *Xenopus* embryos is required for ectodermal specification (Cha et al., 2012). Foxi2 induces the expression of Xema (*Xenopus* Ectodermally Expressed Mesendoderm Antagonist), a zygotically expressed member of the Foxi family. Xema acts in the animal pole to inhibit TGFβ mesoderm-inducing signals from the marginal zone (Suri et al., 2005; Mir et al., 2007; Mir et al., 2008). A second maternal gene expressed in the animal pole, Ectodermin, inhibits mesoderm-inducing TGFβ signals from the marginal zone by ubiquitylation of Smad4 (Dupont et al., 2005). Finally, maternal xNorrin, a secreted factor, can inhibit Activin, Nodal and BMP signalling whilst additionally acting as an agonist of Wnt signalling, promoting neurectoderm specification (Xu et al., 2012).

We now identify a novel role for the gene Coco (Bell et al., 2003) in germ layer specification. Coco, an antagonist of TGFβ (Activin, BMP, Nodal, Derrière) and Wnt signalling, is expressed maternally in the animal pole and zygotically in posterior paraxial mesoderm. Previous experiments have shown a role in head formation (Bell et al., 2003) and left/right patterning (Vonica and Brivanlou, 2007; Schweickert et al., 2010). However, it was unclear whether the strong maternal expression of Coco could be vital in germ layer specification. Here, we investigate this early requirement of Coco and demonstrate that Coco is an essential inhibitor of dorsal marginal Activin and Xnr signals and therefore is vital for correct specification of endoderm and mesoderm.

MATERIALS AND METHODS

Host transfer of oocytes

Maternal knockdown using host transfer was performed (Heasman et al., 1991). Oocytes were injected with 7.5 ng, 11 ng or 15 ng of a modified antisense oligonucleotide (αs-oligo; designed from the nucleotides TTCATGGACCTGCCGCTA) or with morpholinos (MO) against Coco (Vonica and Brivanlou, 2007). The oocytes were transferred into a host female 24-48 hours later. Once the eggs were laid, they were fertilised *in vitro* and allowed to develop to the required stages. For the rescue experiments, 300 pg of Coco RNA was co-injected with the αs-oligo or MO.

Embryo injections

CocoMO and ControlMO (5′-CTGCTGGCGTCCATCAAGAGCTTGT-3′) were injected animally at the one-cell stage; 50 ng/cell of ActivinMO (5′-CGAGGGTCTCCAAGCGGAGAGGAGA-3′) and 15 ng/cell Xnr5MO (5′-AGATAAAGCCTAGCACAGCCATATC-3′)/Xnr6MO (5′-CAAGACTAAGTTCACTAGGGCCATC-3′) were co-injected with 125 pg β-Gal mRNA into the animal pole of one of the two blastomeres at the two-cell stage. X-Gal staining was performed (Amaya et al., 1993). Coco RNA (5′ mutated version; 250 pg) was co-injected with the MO for the rescue experiments. To analyse head mesoderm, 20 ng CocoMO and 1 ng *lacZ* RNA were injected in one dorsal blastomere at the four-cell stage.

RT-PCR and qPCR

RT-PCRs were performed on oocytes or blastula-stage embryos using standard procedures (25 cycles) (Wilson and Melton, 1994). *Ornithine decarboxylase* (ODC) was used as a loading control. qPCRs were performed from explants cut at stage 9.5 and left until stage 16.

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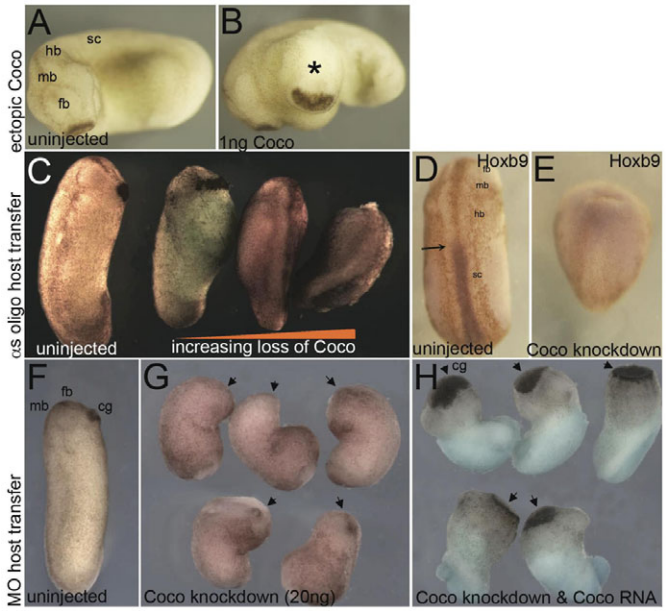

Fig. 1. Knockdown of Coco causes anterior truncations at tadpole stages. (**A-H**) *Xenopus* embryos at stage 28. Overexpression of Coco results in embryos with an ectopic head; compare uninjected embryo (A) with Coco-injected (B, asterisk). Knockdown of Coco causes anterior truncations (C). *Hoxb9* is expressed in the spinal cord (D, arrow); Coco morphant embryos have no expression of *Hoxb9* (E). Host transfers were also performed with MO (F-H). Embryos have a loss of anterior structures (G, arrowheads, compared with control embryo in F). Anterior structures, such as the cement gland (H, arrowheads) could be rescued by injection of Coco RNA. (A,B) Lateral view, anterior to the left. (C-H) Anterior to the top. cg, cement gland; fb, forebrain; hb, hindbrain; mb, midbrain; sc, spinal cord.

Whole-mount *in situ* hybridisation/sectioning

Whole-mount *in situ* hybridisation was carried out as described (Harland, 1991). Digoxigenin (DIG)-labelled *in situ* probes were made as described previously: *Chordin* (Sasai et al., 1994), *Gsc* (Cho et al., 1991), *Hoxb9* (Wright et al., 1990), *Sox17β* (Hudson et al., 1997), *Xbra* (Smith et al., 1991). Embryos were embedded and sectioned in 20% gelatin (Kriebitz et al., 2009).

Western blot and reporter gene assay

Dissected and whole embryos were lysed in RIPA buffer (plus proteinase and phosphatase inhibitors), and analysed as described (Dorey and Hill, 2006). Antibodies used were anti-P-Smad2 (Millipore) and anti-Coco (Vonica and Brivanlou, 2007). Transcription assays were performed as described (Vonica and Brivanlou, 2007), with the difference that CocoMO (40 ng/embryo) was injected in the animal pole of one-cell-stage embryos. *ARE* reporter and *Xnr1* RNA were injected in the animal pole of ventral blastomeres at the four-cell stage. Values are normalised to respective control explants.

RESULTS AND DISCUSSION

Coco loss of function causes anterior truncations at tail bud stages

Coco is a secreted bone morphogenetic protein (BMP)/Wnt/TGFβ inhibitor that is expressed maternally in the animal pole and marginal zones of *Xenopus* embryos until gastrulation (Bell et al., 2003). Overexpression induces ectopic heads (Fig. 1A,B) consistent with the known role of BMP and Wnt inhibition in head induction (Glinka et al., 1997). In order to test the endogenous function of Coco, we decided to inhibit the activity of maternal Coco using αs-oligos (Fig. 1C-E) and morpholino oligonucleotides (MOs) (Fig. 1F-H) in *Xenopus* oocytes prior to fertilisation. Efficiency of the αs-oligos and MOs was confirmed by RT-PCR (supplementary

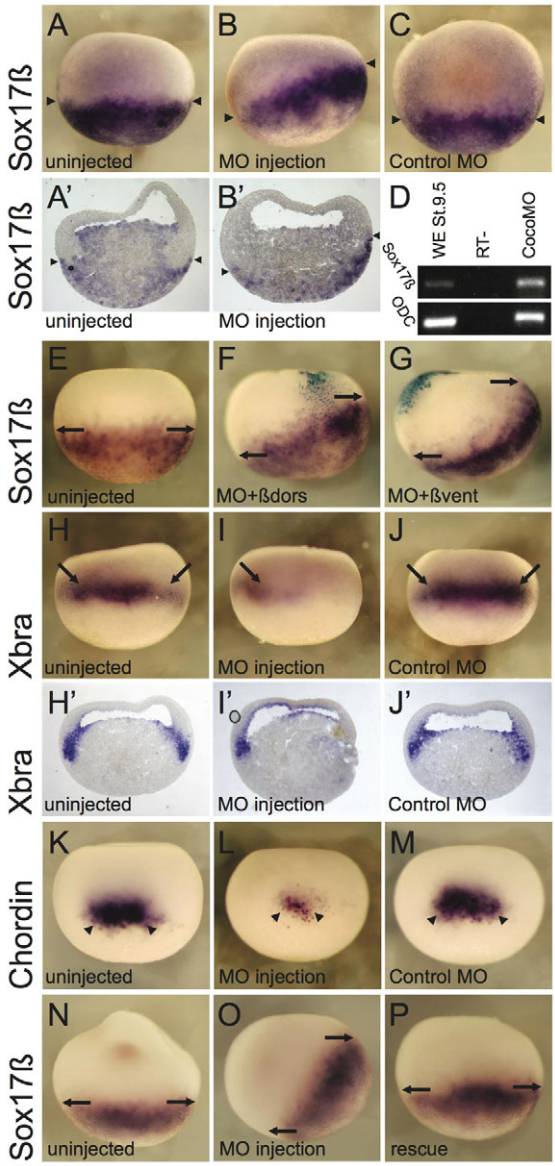

Fig. 2. Knockdown of Coco causes germ layer defects at blastula stage. (**A-M**) *Xenopus* embryos were injected with either CocoMO or ControlMO and analysed at stage 9.5. Whole-mount *in situ* hybridisation was performed to identify endoderm (*Sox17β*), mesoderm (*Xbra*) and presumptive dorsal tissue (*Chordin*). Injection of CocoMO causes both a shift in *Sox17β* expression (A-C, arrowheads; using the blastocoel floor as a reference this shift is clearly seen in sections shown in A′,B′) and an upregulation of expression (D; compare RT-PCR of uninjected embryo and CocoMO-injected embryo). CocoMO-injected embryos were additionally injected with β-Gal in either a dorsal or ventral blastomere at the four-cell stage. Compared with an uninjected control (E, arrows) the shift of *Sox17β* expression is on the same side as dorsally injected β-Gal (F) but on the opposite side as ventrally injected β-Gal (G). Loss of Coco also caused a reduction of both *Xbra* (H-J′, arrows) and *Chordin* (K-M, arrowheads) expression, effects that are not seen following ControlMO injections. The shift of *Sox17β* expression following CocoMO injection (N,O, arrows) is rescued with an injection of Coco mRNA (P), demonstrating specificity. A-C,E-J,K-P are whole-mount lateral views and A′-B′;H′-J′ are sagittal sections.

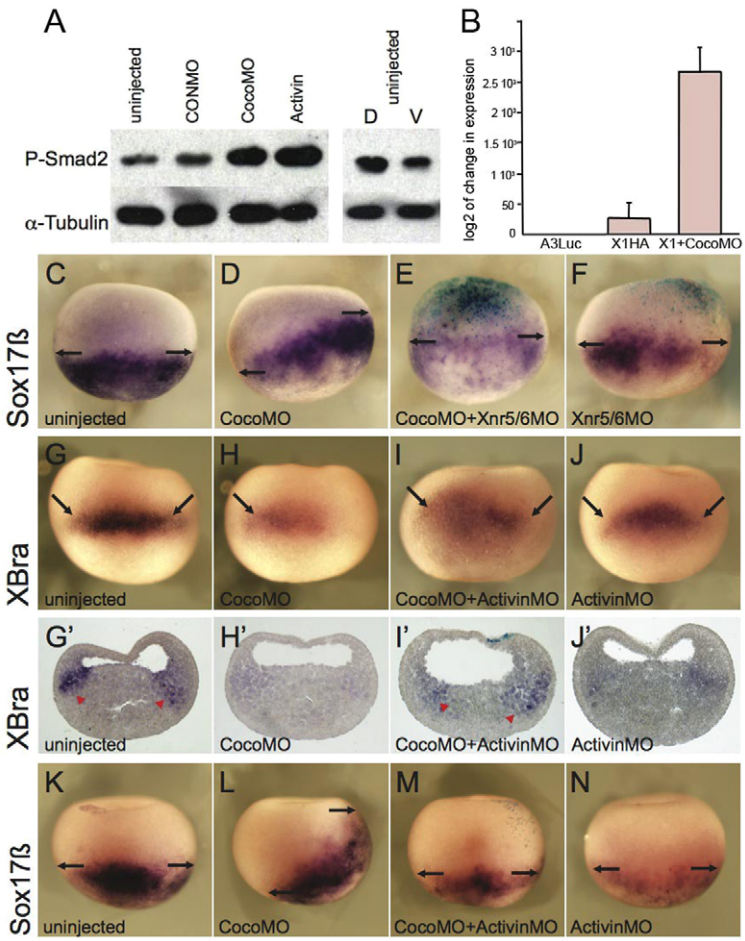

Fig. 3. Germ layer defects in Coco morphant embryos are caused by an increase in Xnr5/6 and Activin signalling. (**A**) Western blot analysis demonstrating an increase of P-Smad2 following CocoMO and Activin overexpression, compared with uninjected and ControlMO-injected embryos. A dorsoventral bias of P-Smad2 is detected in normal development (compare dorsal half with ventral half). (**B**) Coco depletion increases Nodal-induced transcription of a reporter gene. Error bars represent standard errors. Assays were performed in triplicate. (**C-N**) Embryos were injected with CocoMO, CocoMO+TGFβMO or TGFβMO and compared with uninjected control embryos at stage 9.5. (C-F) Injection of CocoMO causes a shift in *Sox17β* expression (D, arrows), which is rescued by co-injection with Xnr5/6MO (E). (F) Injection of Xnr5/6MO alone does not affect the *Sox17β* domain, but reduces the intensity of expression. (G-J′) Injection of CocoMO causes a reduction in *Xbra* staining in the marginal zone (H,H′), which is clearly rescued by co-injection with ActivinMO (I,I′), an effect different to that of ActivinMO alone (J,J′; compare black arrows in G-J and red arrowheads in G′,I′). (K-N) Injection of CocoMO causes a shift in *Sox17β* expression (L, arrows), which is also rescued by co-injection of ActivinMO (M, arrows). (N) ActivinMO alone caused a slight reduction in *Sox17β*. D, dorsal half; V, ventral half.

material Fig. S1A) and western blot (supplementary material Fig. S1B), respectively.

Following knockdown of Coco with an αs-oligo, there was a dose-dependent loss of anterior structures (see Fig. 1C; green embryo, 7.5 ng, *n*=3; pink embryo, 11 ng, *n*=3/4; brown embryo, 15 ng, *n*=4/6). In the most severe cases, embryos were totally ventralised as shown by a loss of the spinal cord marker *Hoxb9* (compare normal *Hoxb9* expression in Fig. 1D with that in 1E), indicating a loss of neural tissue. Host transfers performed with CocoMO had similar anterior truncations to the αs-oligo knockdowns (Fig. 1F,G). To show specificity of the loss of Coco activity, experiments were performed in which the effect was rescued by injection of Coco mRNA. Embryos rescued with Coco RNA regained anterior structures, such as cement glands (Fig. 1H, arrowheads; *n*=5). These results demonstrate that a reduction in Coco activity causes a loss of dorsoanterior structures, and therefore Coco is essential for this aspect of development.

Coco loss of function causes germ layer defects prior to gastrulation

As Coco is an inhibitor of TGFβ signalling and is expressed in the animal pole and marginal zone at a stage when germ layers are specified, it is possible that interference with this process underlies the anterior truncations seen in Coco-depleted embryos. To investigate this, MO knockdowns of Coco were performed at the one-cell stage and the patterning of the germ layers investigated (Fig. 2A-J). To investigate whether endodermal tissue was affected, the expression of the endodermal marker *Sox17β* was analysed (Fig. 2A-G). Coco morphants displayed an animally expanded *Sox17β* expression domain (compare Fig. 2A,A′ and 2B,B′, arrowheads; *n*=103/140) not seen in embryos injected with ControlMO (Fig. 2C; *n*=31/33). The global increase in *Sox17β* expression was confirmed by RT-PCR (Fig. 2D). In addition, we noticed a dorsal bias for the increase in the *Sox17β* expression domain [Fig. 2F: dorsal β-gal injection (*n*=16/22); Fig. 2G: ventral β-gal injection (*n*=14/20)].

Coco morphants also displayed a unilateral loss of the pan mesodermal marker *Xbra* when analysed at blastula [Fig. 2H-J′; compare 2I (*n*=71/98) and 2J (*n*=27/28)] and gastrula (data not shown) stages. In addition, CocoMO injection caused a reduction in the dorsal marker *Chordin* [Fig. 2K-M, arrowheads; compare 2L (*n*=7/10) and 2M (*n*=12/13)] and the head mesoderm marker *Gsc* (Cho et al., 1991) (supplementary material Fig. S2; *n*=12/15). To confirm that the effect on the germ layers was specific to a loss of Coco function, embryos injected with CocoMO (Fig. 2O; *n*=8/14) were rescued with Coco RNA (Fig. 2P; *n*=9/14).

These results demonstrate that a loss of Coco activity causes a shift of endoderm into the dorsal marginal zone, at the expense of dorsal mesoderm. This suggests that the anterior truncations seen following host transfer knockdown are a consequence of the reduction in dorsal mesoderm and organiser activity.

TGFβ ligands are inhibited by Coco to ensure correct specification of the germ layers

Coco has been shown to inhibit the TGFβ ligands Xnr and Activin (Bell et al., 2003), factors that are essential for mesoderm and

endoderm specification (Thomsen et al., 1990; Green et al., 1992; Jones et al., 1993; Jones et al., 1995; Yasuo and Lemaire, 1999; Ninomiya et al., 1999; Kofron et al., 1999; Takahashi et al., 2000; Piepenburg et al., 2004; Luxardi et al., 2010). We propose that Coco is required in normal development to ensure correct specification of the germ layers by limiting TGFβ signals in the marginal zone to allow mesoderm specification, and inhibiting them in animal poles to allow ectoderm specification. To analyse the behaviour of individual layers from Coco-depleted embryos, we dissected and cultured in isolation animal caps and marginal zones of wild-type, depleted and Coco RNA-rescued embryos (supplementary material Fig. S3A,B). At stage 16, both types of explants from Coco-depleted embryos showed increased expression of mesodermal markers (*Xbra* in animal caps, supplementary material Fig. S3A; and *MyoD* in marginal zones, supplementary material Fig. S3B). Equatorial explants develop in the absence of strong TGFβ signals originating in vegetal cells, which could explain the difference in *Xbra* expression between explants (increased) and whole embryos (decreased). Therefore, knockdown of Coco in both whole embryo (Fig. 2) and explants (supplementary material Fig. S3) results in a long-lasting increase in TGFβ activity (manifested by an increase in endoderm or mesoderm depending on experimental conditions and stages).

We directly tested the effect of endogenous Coco depletion on TGFβ signalling using biochemical and transcription assays. An antibody specific to C-terminal phosphorylated, active Smad2 demonstrated biochemically that CocoMO causes an upregulation of endogenous TGFβ signals (Fig. 3A). In addition, Xnr1-induced transcriptional activation of a specific reporter gene (ARE) was strongly increased in animal poles of Coco-depleted embryos (Fig. 3B), demonstrating the inhibitory effect of endogenous Coco on nodal signalling.

To identify specific Coco-regulated TGFβ ligands, CocoMO injections were coupled with injection of MOs targeting specific TGFβ ligands (ActivinMO or Xnr5/6MO + β-Gal tracer) in order to test whether we could inhibit this overactivation and restore correct specification of the germ layers (Fig. 3C-N). Co-injection of CocoMO and Xnr5/6MO could partially rescue the loss of Coco defects (Fig. 3C-F). CocoMO-injected embryos have a clear shift in endoderm (Fig. 3D; n=18/22). However, after injection of CocoMO with Xnr5/6MO (Fig. 3E; n=12/16) the shift was suppressed. The domain of *Sox17β* seemed slightly higher in these embryos compared with control embryos; however, Xnr5/6MO on its own caused *Sox17β* to be expressed more marginally (Fig. 3F, arrows; n=16/22). In contrast to the suppression of the endoderm phenotype, the loss of mesoderm could not be rescued (data not shown). This result suggests that Coco is required to prevent Xnr5/6 signals from working more animally. However, the effects of Coco loss were not completely suppressed, suggesting that Coco is required to inhibit other vegetal/marginal signals.

Next, CocoMO and ActivinMO were co-injected and the patterning of the endoderm and mesoderm investigated to see whether the effect of loss of Coco was caused by an increase in Activin activity (Fig. 3G-N). CocoMO embryos had a unilateral loss of mesoderm (compare uninjected in Fig. 3G,G′ with CocoMO-injected in 3H,H′; n=12/14); however, after co-injection with ActivinMO, the mesoderm patterning was restored (Fig. 3I,I′; n=12/16). By contrast, ActivinMO on its own caused a reduction of the mesoderm (Fig. 3J,J′; n=14/17). ActivinMO was also able to suppress the shift of endoderm (Fig. 3K,L, shift in endoderm seen in 3L; n=12/15). Expression following CocoMO/ActivinMO co-injection (Fig. 3M; n=16/26) resembled the normal expression of *Sox17β* in uninjected embryos (Fig. 3K). Injection of ActivinMO alone did not alter the domain of *Sox17β* but expression was slightly weaker (Fig. 3N; n=11/13). Depletion of neither Vg1, a maternal TGFβ ligand, nor Xnr1 was able to rescue the germ layer defects (data not shown), further demonstrating Coco specificity for Xnr5/6 and Activin.

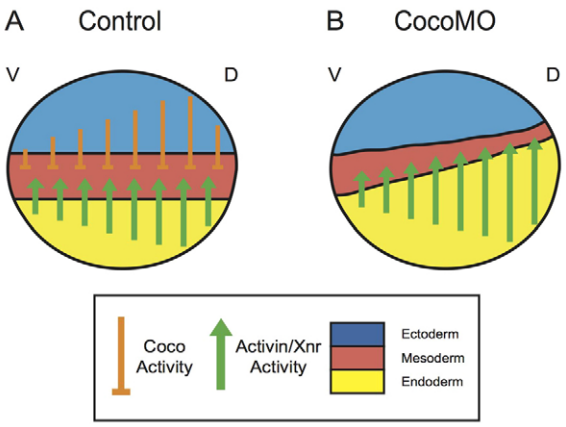

Fig. 4. Coco controls germ layer specification via an inhibition of both Activin and Nodal signals. (**A**) A dorsoventral gradient of Coco activity (brown) restricts Activin and Xnr signals (green) from acting in the animal pole, ensuring correct spatial organization of the germ layers. (**B**) Knockdown of Coco allows dorsal marginal Activin and Nodal signals to become active in a more animal domain, disrupting mesoderm and endoderm formation resulting in a loss of anterior structures. D, dorsal; V, ventral.

These results show that Coco is an essential inhibitor of animal Activin and Xnr5/6 signalling on the dorsal side of the pre-gastrula embryo. Activin and Xnr5/6 have previously been shown to have stronger activity in the dorsal half of the embryo (Fig. 3A) (Agius et al., 2000; Faure et al., 2000; Schohl and Fagotto, 2002). Based on our results, we propose that the loss of Coco unmasks this dorsoventral regulation, resulting in the dorsal shift of endoderm and loss of mesoderm. Coco therefore acts to inhibit dorsal marginal Activin and Xnr signals (Fig. 4A) from becoming active in the animal region of the embryo, and limits their activity in the marginal zone, allowing expression of *Xbra* to occur. When Coco activity is reduced, these signals become overactive (Fig. 4B) causing both mesodermal and endodermal germ layer disruptions. These results provide an explanation for the observation that Coco morphant embryos lack anterior structures: the reduction of dorsal mesoderm causes a decrease in the size of the organiser region that is essential for head formation. In addition, the inhibition of both BMP and Wnt by Coco might play a further role in preventing ventralising and posteriorising signals from acting in the ectoderm, whereas its role on the ventral side of the blastula embryo remains to be understood. For the first time, our data emphasise a dorsal maternal requirement for Coco. In conclusion, Coco is an essential inhibitory molecule that ensures correct spatial localisation of germ layer induction.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
A.V., A.H.B. and E.B. designed the experiments; T.J.D.B., A.V., J.H. and E.B. performed the experiments and analysed the data. T.J.D.B. and E.B. wrote the paper with critical input from A.V. and A.H.B.

Supplementary material
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