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A maternally localised Wnt ligand required for axial patterning in the cnidarian *Clytia hemisphaerica*

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Regionalised activation of canonical Wnt signalling via β-catenin stabilisation is a key early step in embryonic patterning in many metazoans, including the basally diverging cnidarians, but the upstream maternal cues appear surprisingly variable. In *Clytia*, regionalised β-catenin stabilisation defining a presumptive 'oral' territory is determined by two maternally coded Frizzled family Wnt receptors of opposite localisation and function. We have identified a maternally coded ligand, CheWnt3, the RNA of which is localised to the animal cortex (future oral side) of the egg. Antisense morpholino oligonucleotide experiments showed that CheWnt3 is required maternally for regionalised oral β-catenin stabilisation in the early embryo, being only the second clear example of a maternally required Wnt ligand after *Xenopus* Xwnt11. In line with the determinant role of the maternally localised Frizzleds, CheWnt3 overexpression by RNA injection initially had little effect on establishing the oral domain. Subsequently, however, overexpression had dramatic consequences for axis development, causing progressive expansion of β-catenin stabilisation to yield spherical 'oralised' larvae. Upregulation of both *CheFz1* and *CheFz3* RNAs in CheWnt3 morpholino embryos indicated that CheWnt3 participates in an active axial patterning system involving reciprocal downregulation of the receptors to maintain oral and aboral territories. Localised introduction of *CheWnt3* RNA induced ectopic oral poles in CheWnt3 morpholino embryos, demonstrating its importance in directing oral fate. These findings suggest that the complete ligand-dependent Wnt signalling cascade is involved in axial patterning in ancestral eumetazoans. In *Clytia*, two variant Frizzled receptors and one Wnt ligand produced from localised RNAs cooperate to initiate regionalised Wnt pathway activation.

KEY WORDS: Wnt pathway, Cnidaria, Localised RNA, Axis specification

INTRODUCTION

Canonical Wnt signalling involves the stabilisation and nuclear localisation of the transcriptional co-factor β-catenin in response to the stimulation of Frizzled and LRP5/6 family receptors by extracellular Wnt ligands (Huelsken and Birchmeier, 2001; Logan and Nusse, 2004). It is well established that maternally driven activation of this pathway on one side of the embryo is a key component of embryonic patterning in a variety of deuterostome species (including sea urchins, ascidians, amphibians and fish), in which it is associated with the development of endomesoderm and/or 'organiser' fates (Heasman, 2006; Huelsken and Birchmeier, 2001; Imai et al., 2000; Logan et al., 1999; Schier and Talbot, 2005; Wikramanayake et al., 1998). More recently, equivalent mechanisms have been described in the basally diverging phylum Cnidaria (Wikramanayake et al., 2003) on the future 'oral' side of embryos, which is derived from the animal pole of the egg. This suggests that early canonical Wnt pathway activation already operated to specify axial polarity and/or endomesoderm fates in a common cnidarian/bilaterian ancestor or in an even earlier metazoan (Lee et al., 2006; Martindale, 2005). Regionalised β-catenin stabilisation in cleavage-stage embryos has been visualised in two phylogenetically distant cnidarians, the anthozoan (sea anemone) *Nematostella vectensis* and the hydrozoan jellyfish *Clytia hemisphaerica*. In *Clytia*, analysis of regionally expressed genes showed that β-catenin stabilisation specifies the oral-aboral axis of the developing embryo by defining a presumptive oral territory (Momose and Houliston, 2007). In both these species, the animal pole of the egg marks the future gastrulation site from which endoderm forms, and later the oral pole of the larva (see Fig. 1A): inhibition of the canonical Wnt pathway blocks gastrulation, whereas its forced activation favours endoderm formation (Wikramanayake et al., 2003; Momose and Houliston, 2007). Wnt signalling has also been shown to be involved in maintaining oral-aboral polarity in cnidarian adult stages, notably in *Hydra* polyps, in which Wnt ligands, as well as the intracellular regulators TCF and β-catenin, are expressed around the hypostome (oral opening) and contribute to the 'head organiser' properties of this region along with other genes implicated in vertebrate organiser function (Bode, 2003; Broun et al., 2005; Hobmayer et al., 2000).

Much effort has been spent in trying to identify localised maternal factors that drive canonical Wnt activation in the early embryo in different experimental species, without a clear picture emerging. In sea urchins, the cytoplasmic regulator Dishevelled (Dsh) localises dynamically to a vegetal cortical layer of the egg, and has been shown to mediate Wnt signalling activation in this region of the embryos; however, its global overexpression does not affect germ layer specification, implying the involvement of additional localised factors that restrict its activity (Leonard and Ettensohn, 2007; Weitzel et al., 2004). Dsh protein is likewise maternally localised and necessary for subsequent patterning events in *Nematostella* (Lee et al., 2007), in which the absence of detectable maternal expression of any of the genomic Wnt repertoire (Lee et al., 2006) has led to the suggestion that Dsh protein might play a primary directive role. In *Xenopus* embryos, in which β-catenin stabilisation is favoured on the prospective 'organiser' side of the cleavage-stage embryo, corresponding enrichments have been reported not only for Dsh but also for β-catenin, whereas an inhibitor of the regulatory kinase GSK3-β moves in the appropriate direction in the fertilised egg. All of these molecules can force organiser development upon overexpression (Larabell et al., 1997; Miller et al., 1999; Weaver et

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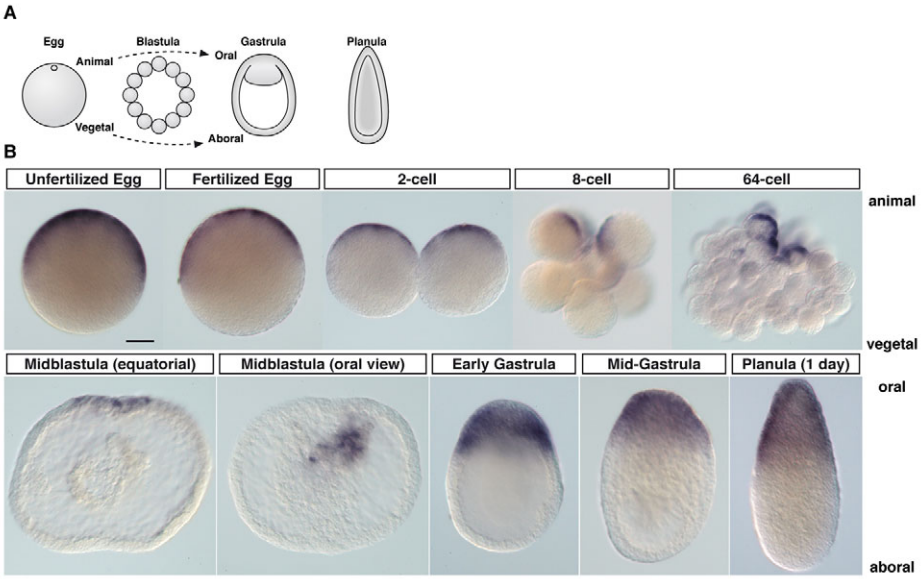

Fig. 1. *CheWnt3* RNA is localised to the animal/oral pole of eggs and embryos. (**A**) The relationship between egg animal-vegetal polarity, gastrulation and embryo oral-aboral polarity in *Clytia*. The animal pole of the egg marks the site of cell ingression at gastrulation and the future oral pole of the embryo and planula larva. (**B**) In situ hybridisation analysis of *Wnt3* expression in embryos from unfertilised eggs to mid-blastula stage (top row) and from early gastrula to 1-day-old planula (bottom row). Embryos are oriented with the animal/oral pole up. Scale bar: 40 μm.

al., 2003). These various examples of localised intracellular canonical Wnt pathway regulators in the absence of detectable localised ligand have encouraged the idea that cytoplasmic determinants can bypass the usual extracellular phase of signalling in the single large cell of the egg. This view was challenged by the discovery in *Xenopus* of a key role for the ligand Xwnt11 (Heasman, 2006; Tao et al., 2005). *Xwnt11* RNA is tightly localised to the vegetal cortex of the oocyte, and becomes enriched on the future dorsal side in cleavage-stage embryos. Depletion of the *Xwnt11* transcript from oocytes prevents organiser formation, whereas overexpression promotes it. Localised Wnt pathway activation by Xwnt11 could contribute, at least in part, to the observed asymmetries of Dsh and β-catenin proteins in the early embryo, by canonical Wnt pathway-directed stabilisation and/or by recruitment to the membrane by the Wnt-planar cell polarity (PCP) pathway (Axelrod et al., 1998). In ascidians, in which neither Dsh nor β-catenin protein is localised maternally, the maternally localised RNA *Wnt5a* provides a good candidate for directing vegetal-specific nuclear localisation of β-catenin, but the consequences of morpholino-mediated inhibition of Wnt5a translation were inconclusive (Kawai et al., 2007).

Apart from Xwnt11, the only maternal molecules to have met the experimental criteria for a Wnt pathway-activating determinant of appropriate endogenous localisation, functional requirement (by knockdown) and functional sufficiency (by over/misexpression) are two Frizzled family Wnt receptors in the new cnidarian model *Clytia hemisphaerica*. Maternal transcripts of *CheFz1* and *CheFz3* are localised to the animal and vegetal sides of the egg, respectively. These receptors were shown to regulate β-catenin stabilisation positively and negatively, respectively, in cells that inherit them, defining prospective oral and aboral territories (Momose and Houliston, 2007), but it was not clear whether a maternal ligand was required upstream (Lee et al., 2007). We have now identified five embryonically expressed Wnt ligands in *Clytia*, including one, CheWnt3, the maternally expressed RNA of which is tightly localised to the animal cortex of the egg. We show using morpholino antisense oligonucleotides that CheWnt3 is necessary for maternally driven canonical Wnt pathway activation on the future oral side of the embryo. Morpholino- and RNA-injection experiments further show that CheWnt3, CheFz1 and CheFz1 then cooperate as part of a dynamic system that defines opposite territories of oral and aboral identity during subsequent development.

MATERIALS AND METHODS

Embryo manipulation and microscopy

Medusae from laboratory-maintained colonies were used for all experiments. Spawning, fertilisation and embryo culture, in situ hybridisation, confocal observations of cell morphology and visualisation of the β-catenin-GFP (Venus variant) fusion protein were all performed as described previously (Chevalier et al., 2006; Momose and Houliston, 2007), except that β-catenin-Venus localisation in early gastrula stage was visualised using a Leica SP2 confocal microscope.

cDNA cloning

Seven Wnt sequences and an Axin sequence were identified from a *Clytia* expressed sequence tag (EST) collection, and full-length cDNA clones retrieved from the corresponding cDNA library (Chevalier et al., 2006). A *Clytia* GSK3 (CheGSK3) cDNA fragment was amplified by PCR using a set of degenerate primers as described previously (Hobmayer et al., 2000), and used to retrieve a full-length CheGSK3 cDNA clone by screening a *Clytia* embryonic-stage phage library.

GenBank accession numbers: CheAxin (EU374716), CheGSK3 (EU374723), CheWntX1A (EU374720), CheWntX1B (EU374722), CheWntX2 (EU374717), CheWntX3 (EU391658), CheWnt3 (EU374721), CheWnt5 (EU374718), CheWnt9 (EU374719).

Morpholino and RNA injection

The Wnt3 coding sequence was amplified by PCR from the EST-identified Express1 clone using primers that introduced five silent mutations in the target sequence of Wnt3-MO and was subcloned into the pRN3 vector (P. Lemaire, Marseille). The coding sequence of CheGSK3 was amplified by PCR and cloned into pRN3 to form pRN3-CheGSK3. The K85R mutation in the kinase domain (Pierce and Kimelman, 1995) was introduced into the plasmid to produce a dominant-negative (dn) form of GSK3 (pRN3-dnGSK3) using the QuickChange Site-directed Mutagenesis Kit (Stratagene).

mRNAs encoding Wnt3 and dnGSK3 were in vitro transcribed from the plasmids using the mMessage mMachine Kit (Ambion). The *Wnt3* RNA was introduced into eggs prior to fertilisation at 0.4 μg/μL, or into single 8-cell stage blastomeres of Wnt3-MO-injected embryos. The RNA encoding dnGSK3 was injected into unfertilised eggs at 2 μg/μL. FITC-labelled dextran was co-injected with *Wnt3* RNA to identify progeny of the injected blastomeres.

Antisense morpholino oligonucleotide (GeneTools) for CheWnt3 (Wnt3-MO, 5′-CCAAAACACACCAGTGTCGAGCCAT-3′) was microinjected at 0.1 μM prior to fertilisation. The in vivo target specificity of the Wnt3-MO-mediated inhibition was confirmed by the opposite phenotype being obtained upon injection of *Wnt3* RNA, and oral pole rescue upon localised injection of *Wnt3* RNA into Wnt3-MO-injected embryos. Other morpholino oligos and RNAs were used as described previously (Momose and Houliston, 2007).

RESULTS

A set of Wnt ligands differentially expressed during *Clytia* development

To identify Wnt ligands acting during *Clytia* early development, we performed degenerate PCR on egg and embryo cDNA and also searched a mixed-stage EST collection. The seven distinct *Clytia* Wnt sequences isolated were named following phylogenetic analyses (see Fig. S1 in the supplementary material). CheWnt3, CheWnt5 and CheWnt9 were clearly members of the corresponding bilaterian orthology groups. CheWntX1A, CheWntX1B, CheWntX2 and CheWntX3 sequences were too divergent to assign orthology confidently, although the two ‘X1’ sequences grouped with the Wnt8 group and CheWntX2 with the Wnt 6 group.

Of the seven *Clytia* Wnt genes identified, expression of five was detected in embryonic or larval stages by in situ hybridisation: *CheWnt3*, *CheWnt5*, *CheWnt9*, *CheWntX1A* and *CheWntX2* (see Fig. S2 in the supplementary material; see also Fig. 2I). Unlike in the anthozoan *Nematostella*, in which no expression of the genomic Wnt repertoire is detected before gastrulation (Lee et al., 2006), one *Clytia* Wnt RNA, *CheWnt3*, was detectable in eggs and

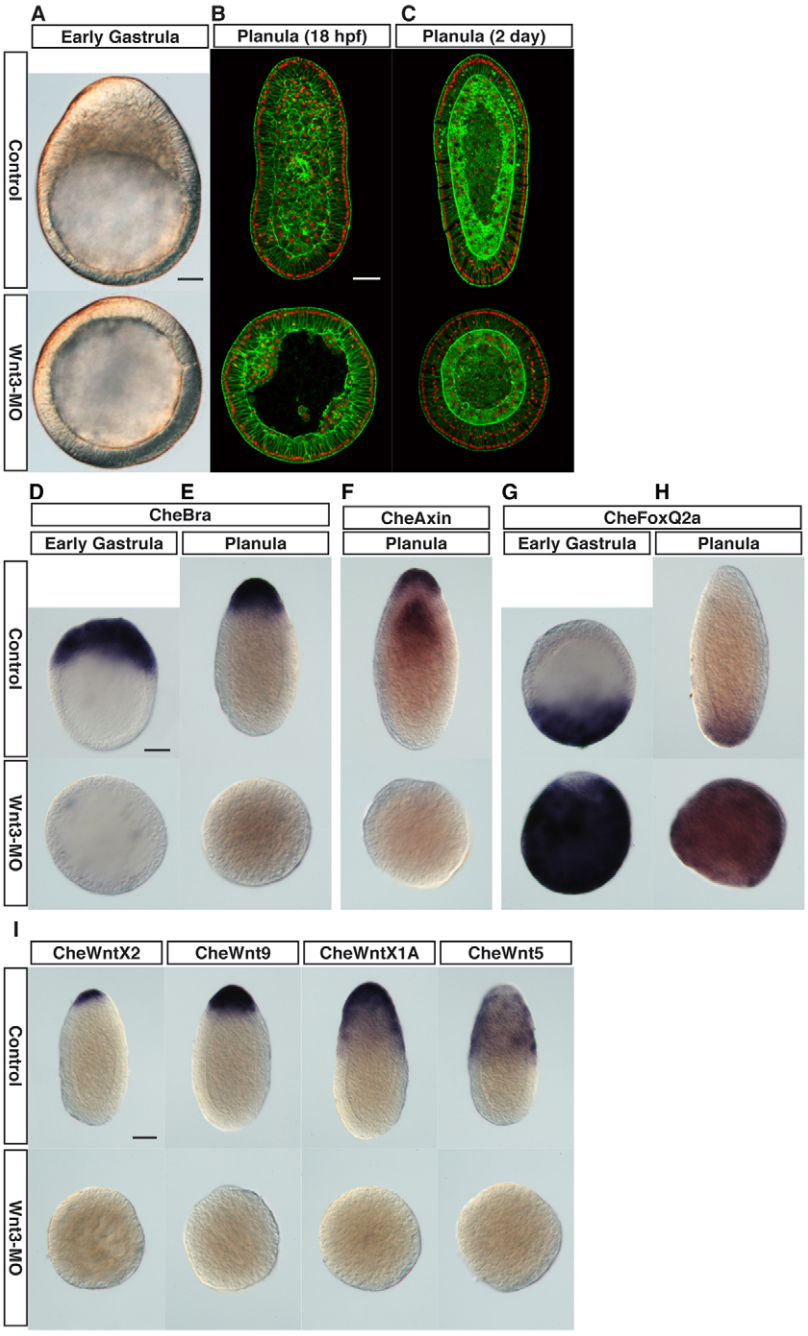

Fig. 2. CheWnt3 is required for the development of axial polarity and oral fates. (**A**) Gastrulation and elongation along the oral-aboral axis in a normal *Clytia* embryo (top row) was completely blocked in Wnt3-MO-injected embryos (bottom row), shown fixed at the early gastrula stage (15 hpf). (**B**) Complete loss of morphological axis in Wnt3-MO-injected embryos at late gastrula (20 hpf) stage. Cell contours were visualised by phalloidin (green) and nuclei by To-Pro3 (red). Gastrulation was severely delayed compared with uninjected embryos. The exact timing and site of residual cell ingression formation varied. (**C**) Equivalent 2-day-old planulae. No morphological oral-aboral axis is discernable in Wnt3-MO-injected embryos; however, endoderm formation has recovered. (**D**) Representative in situ hybridisation image of characteristic oral *CheBra* expression in early gastrulae (15 hpf) that is lost in Wnt3-MO-injected embryos. (**E**,**F**) Loss of *CheBra* expression in oral ectoderm, and of CheAxin in oral endoderm and ectoderm in Wnt3-MO-injected planulae (1.5 day). (**G**,**H**) Expansion of aboral *FoxQ2A* expression to nearly the entire body in Wnt3-MO-injected embryos fixed at early gastrula and planula stages (1.5 day). (**I**) Loss of expression of oral-ectoderm-expressed ligands *CheWntX2*, *CheWnt9*, *CheWntX1A* and *CheWnt5* in Wnt3-MO-injected embryos fixed at the planula stage (1.5 day). Scale bars: 40 μm.

cleavage-stage embryos (see below). Following gastrulation, the five detectable *Clytia* Wnt ligand RNAs defined overlapping domains of the oral ectoderm (see Fig. S2 in the supplementary material). There was no clear correspondence between these domains and those described for the *Nematostella* Wnts; notably, the five *Clytia* Wnts were detected only in the ectoderm, whereas a subset of *Nematostella* Wnt orthologues (including the *CheWnt5* orthologue) is uniquely endodermal. Furthermore, *CheWnt9*, expressed at the oral tip of the *Clytia* larva, has no orthologue in *Nematostella*; indeed, the Wnt9 subfamily previously lacked a cnidarian member (Guder et al., 2006a; Kusserow et al., 2005). These findings demonstrate that the sequences and expression domains of the Wnt genes expressed along the oral-aboral axis in cnidarian embryos have diverged extensively within the cnidarian clade from a set of genes already present in the eumetazoan common ancestor.

CheWnt3: a maternal RNA localised to the animal cortex

CheWnt3 RNA was the only Wnt RNA detectable in the *Clytia* egg. It showed a highly localised distribution both before and after fertilisation, with tight restriction to a cortical layer of the animal hemisphere (Fig. 1B). This distribution is distinct from that of two previously described localised maternal *Clytia* RNAs, *CheFz1* (which forms a cytoplasmic gradient from animal to vegetal pole) and *CheFz3* (localised to the vegetal cortex) (Momose and Houliston, 2007). The maternal localisation of the *CheWnt3* RNA resembles that reported for *HeWnt*, a *Wnt3* orthologue identified in *Hydractinia* (Plickert et al., 2006). *CheWnt3* RNA remained tightly localised to the animal cortex during cleavage divisions, until at least the 64-cell stage. It accumulated in the cleavage furrow at each division, becoming progressively more concentrated around the animal pole. Thus, *CheWnt3* RNA was confined to a small patch inherited by three to four animal blastomeres at the 8-cell stage, and about 8 cells at the 64-cell stage. By the mid-blastula stage, it was detected as one or more small but strongly stained patches in a restricted area at the future oral end, presumed to be maternal RNA inherited from the egg animal pole. From this stage onwards, an additional diffuse in situ hybridisation signal was detectable in a wider oral region, becoming progressively stronger during blastula development. This signal was considered to correspond to the onset of zygotic expression because of its clear distinction from that of residual maternal RNA, the timing coinciding with the onset of transcription of a number of other developmental genes in *Clytia* (our unpublished observations). CheWnt3 expression restricted to the oral ectoderm was maintained through gastrulation and in planula larvae (Fig. 1B and see Fig. S2 in the supplementary material). At the early gastrula stage, expression within this domain appeared slightly reduced at the oral pole region, from where cell ingression was starting.

CheWnt3 is essential for axial polarity

We tested the function of CheWnt3 by injection of a specific morpholino antisense oligonucleotide (Wnt3-MO) into eggs prior to fertilisation to block translation. The use of morpholinos in *Clytia* was validated in the previous study of CheFz1 and CheFz3 (Momose and Houliston, 2007), and in the current study the specificity of Wnt3-MO was confirmed by comparison of the phenotypes with those obtained by *Wnt3* RNA overexpression, and by the reversal of Wnt3-MO phenotypes by local introduction of the *Wnt3* RNA (see below). Eggs microinjected with Wnt3-MO underwent regular cleavage divisions but developed without morphological polarity (Fig. 2A). They neither elongated along the oral-aboral axis, nor showed any sign of presumptive endoderm ingression from the oral end during the normal gastrulation period (10-18 hpf at 18°C). The developing embryos remained completely spherical for at least 2 days. In contrast to CheFz1-MO embryos, in which cell ingression leading to endoderm formation was permanently blocked, Wnt3-MO-injected embryos eventually formed morphologically normal endoderm by a multipolar 'recovery' mechanism starting at the late gastrula/early planula stage (~18-20 hpf) (Fig. 2B,C). In addition, the ectoderm of CheWnt3-MO-injected embryos formed highly motile cilia and embryos swum vigorously, albeit without the normal aboral-oral directionality. This contrasts with the defective cilia formation and orientation in CheFz1-MO-injected embryos, a phenotype associated with disruption of PCP (Momose and Houliston, 2007). We conclude that the ligand CheWnt3 is required together with the Frizzled receptors for development of the oral-aboral axis, although, unlike CheFz1, it might not be essential in the acquisition cellular polarity.

To characterise further the role of CheWnt3 in establishing cell fate along the oral-aboral axis, we examined the effect of CheWnt3-MO injection on regional gene expression (Fig. 2D-H). Oral ectoderm expression of the *Brachyury* orthologue *CheBra* was almost completely lost at the early gastrula stage (Fig. 2D) and was no longer detectable in planulae (Fig. 2E). Another oral marker, *CheAxin*, a component of the canonical Wnt signalling pathway that is expressed in oral endoderm and ectoderm, was also abolished in planulae by CheWnt3 knockdown (Fig. 2F). Conversely, the expression domain of the aboral marker *FoxQ2A* (Chevalier et al., 2006) expanded to cover nearly the entire embryo at early gastrula and planula stages, with just a small region of low expression remaining (Fig. 2G,H). Expression of the four orally expressed zygotic Wnt genes (*CheWntX2*, *CheWnt9*, *CheWntX1A* and *CheWnt5*) was also undetectable in Wnt3-Mo embryos at the planula stage (Fig. 2I), indicating that they too are dependent on prior expression of CheWnt3. It thus appears unlikely that these later-expressed Wnt ligands are responsible for the delayed endoderm formation observed in the absence of the early-expressed ligand Wnt3.

Taken together, the morpholino experiments show that the ligand CheWnt3 is essential for the development of axial polarity in *Clytia*. CheWnt3 functions to promote the development of oral fates at the expense of aboral fates.

CheWnt3 is required for early canonical Wnt pathway activation

Regionalised activation of canonical Wnt signalling at as early as the 32-cell stage (3-3.5 hpf) has been demonstrated in *Clytia* and in *Nematostella*, largely preceding morphological and cytological manifestation of axial properties (12-15 hpf). To address whether maternal CheWnt3 participates with CheFz1 and CheFz3 in establishing this initial β-catenin-stabilisation domain, we monitored canonical Wnt signalling activation using a β-catenin-Venus (modified GFP with yellow fluorescence) fusion protein (Momose and Houliston, 2007). At the early- and mid-blastula stages, embryos injected with RNA encoding β-catenin-Venus alone showed a restricted domain of stabilised nuclear and cytoplasmic β-catenin-Venus protein on one side (Fig. 3A). This was almost completely undetectable following co-injection of Wnt3-MO (Fig. 3B), clearly indicating that the ligand CheWnt3, translated from maternal RNA, is necessary for early regionalised activation of the canonical Wnt pathway.

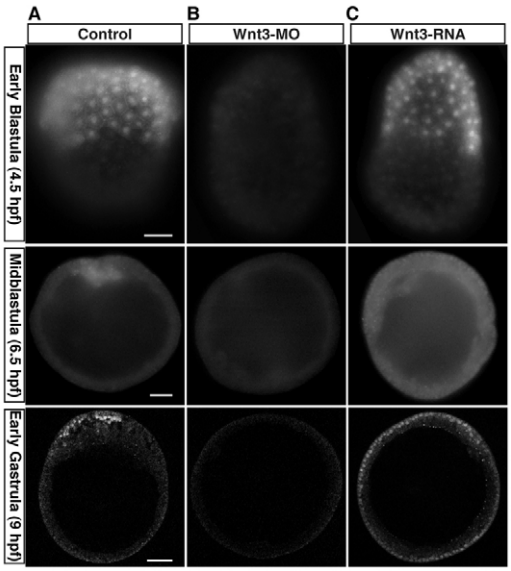

Fig. 3. CheWnt3 is required first to activate the canonical Wnt pathway and then to restrict it to the oral pole. Activation of the canonical Wnt pathway in control *Clytia* embryos (**A**) and embryos derived from eggs injected with Wnt3-MO (**B**) or *Wnt3* RNA (**C**) were visualised by injecting eggs prior to fertilisation with RNA encoding a β-catenin-Venus (GFP) fusion protein and visualised at early blastula (128-cell/4.5 hpf), mid-blastula (6.5 hpf) and early gastrula stages. Scale bars: 40 μm.

To determine the contribution of animally localised *CheWnt3* RNA to the spatial restriction of canonical Wnt pathway activation, we injected synthetic *CheWnt3* mRNA along with the RNA encoding β-catenin-Venus prior to fertilisation to achieve global expression across all regions of the embryo (Fig. 3C). The exogenous CheWnt3 did not prevent formation of a sharply demarcated β-catenin-stabilisation domain at the early blastula stage (4.5 hpf). The only discernable effect of *CheWnt3* RNA at this stage was a slightly elevated nuclear β-catenin-Venus signal observed in the aboral domain of RNA-injected embryos (Fig. 3C). During subsequent development (6.5-9 hpf), however, the domain of β-catenin stabilisation spread progressively across the embryo, such that by the onset of gastrulation, nuclear β-catenin was detectable in nearly all cells (Fig. 3C). In the absence of injected *Wnt3* RNA, nuclear β-catenin at this stage had become tightly restricted to the small group of inwardly migrating presumptive endoderm cells at the gastrulation site (Fig. 3A). The initial failure of injected *CheWnt3* RNA to cause strong Wnt pathway activation in aboral regions is unlikely to be due to slow translation, as β-catenin-Venus protein from co-injected RNA could be detected at least 1 hour earlier.

These analyses show that although CheWnt3 translation is absolutely essential for canonical Wnt pathway activation in the early embryo, the tight localisation of its RNA to the egg animal pole is not the main factor accounting for the initial spatial restriction of this activation, a role attributable essentially to *CheFz1* and *CheFz3* mRNAs. The subsequent strong upregulation of canonical signalling across the embryo probably results from the action of the regulatory feedback system that maintains polarity during the blastula-gastrula period (see below). The final strong activation of canonical Wnt signalling in aboral regions could be initiated by the modest increase in β-catenin stabilisation and/or represent expansion of the oral domain owing to artificially elevated CheWnt3 levels.

CheWnt3 is required for the cross-regulation between CheFz1 and CheFz3

We showed previously that strong reciprocal downregulation mechanisms between CheFz1 and CheFz3 involving unknown additional components are responsible for maintaining and sharpening the opposite localisation of these RNAs by the early gastrula stage (Momose and Houliston, 2007). Now we were able to show that CheWnt3 is an essential component of this regulatory system. MO-mediated inhibition of CheWnt3 translation was found to cause a dramatic increase in the levels of both *CheFz1* and *CheFz3* transcripts by the early gastrula stage (Fig. 4A), mimicking the phenotype of double knockdown of CheFz1 and CheFz3. There was no detectable effect on *CheFz1* or *CheFz3* RNA distribution at the early blastula stage (4.5 hpf; Fig. 4A), indicating that the regulatory interactions between these molecules operate during the blastula-early gastrula period, and probably involve zygotic transcription.

To test the participation of CheWnt3 expression itself in the feedback system for canonical signalling regulation involving the two Frizzled receptors, we examined its response to manipulating different regulatory components (Fig. 4B). Somewhat surprisingly, neither forced global activation of the canonical Wnt pathway (by injection of an RNA encoding a dominant-negative form of the regulatory kinase GSK3-β, or by MO-mediated inhibition of CheFz3), or global β-catenin degradation (by MO-mediated inhibition of CheFz1), had a significant effect on the extent of the oral *CheWnt3* expression domain, despite the severe loss-of-polarity phenotypes observed following these treatments. These results indicate that zygotic

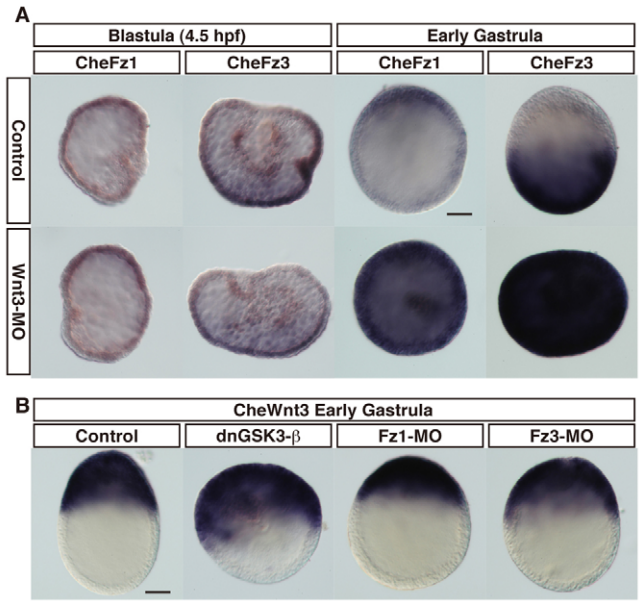

Fig. 4. Participation of CheWnt3 in Wnt pathway regulatory interactions. (**A**) CheWnt3-MO injection into *Clytia* eggs did not affect the graded distribution of *CheFz1* and *CheFz3* RNAs at the early blastula stage (4.5 hpf; essentially maternally derived RNA). However, by the early gastrula stage (~10 hpf), both RNAs were found strongly expressed throughout the embryo. This shows that CheWnt3 is required for mutual negative regulation of the receptors during the intervening period, probably at the transcriptional level. (**B**) The oral territory of zygotic Wnt3 expression was not greatly affected by treatments that upregulate (dnGSK3, Fz3-MO) or downregulate (Fz1-MO) the canonical Wnt pathway.

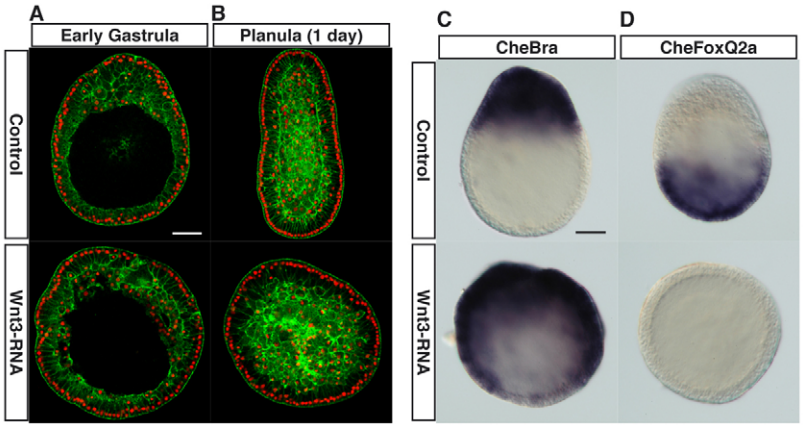

Fig. 5. CheWnt3 drives the development of oral fate. (**A**) Confocal images of early gastrula stage *Clytia* embryos stained with phalloidin (green) and To-Pro3 (red), showing characteristic phenotypes obtained following *Wnt3* RNA injection into eggs before fertilisation. Presumptive endoderm cell ingression took place over a broad area of the embryo, with a graded distribution of *CheBra* apparent. (**B**) Equivalent observation at the 1-day planula stage, showing the lack of a distinguishable morphological oral-aboral axis. (**C**,**D**) Representative in situ hybridisation images of *CheBra* and *FoxQ2A* expression at early blastula stage (15 hpf). In embryos injected with *CheWnt3* RNA, *CheBra* expression has expanded to the entire body with a residual graded distribution, and *FoxQ2A* expression has been lost. Scale bars: 40 μm.

CheWnt3 transcription is not regulated principally by the canonical Wnt pathway, and imply the existence of Frizzled- and β-catenin-independent pattern regulation mechanisms operating along the oral-aboral axis of the developing embryo.

CheWnt3 confers oral identity

In line with the dramatic expansion of canonical Wnt pathway activation during blastula-gastrula stages in embryos overexpressing Wnt3 (Fig. 3), morphological polarity was severely perturbed during gastrulation following *Wnt3* RNA injection (Fig. 5). The effect of forced global *CheWnt3* expression on axis development was essentially opposite to that of Wnt3-MO injection (Fig. 2). The territory of inwardly migrating presumptive endoderm cells at the oral pole of the gastrula was greatly expanded, such that it covered almost the entire embryo (Fig. 5A), and the resultant planulae were spherical (Fig. 5B). Nevertheless, residual polarity could be detected during gastrulation; the ingressing cells showed a graded distribution, and a corresponding broad gradient of *CheBra* expression was detected (Fig. 5C). This presumably is due in part to the initial asymmetry in β-catenin stabilisation along the oral-aboral axis in CheWnt3-overexpressing embryos (see above). The severe 'oralisation' phenotype was confirmed by the absence of expression of the aboral marker *FoxQ2A* at the early gastrula stage (Fig. 5D). Note that even in the vegetal hemisphere of the embryo, exogenous CheWnt3 can promote progressive stabilisation of β-catenin, upregulation of *CheBra* and downregulation of *FoxQ2a*, despite the initial inhibitory influence of CheFz3 on canonical Wnt pathway activation in the vegetal hemisphere prior to the early blastula stage.

To test more precisely the oralising capacity of CheWnt3 and to provide confirmation of the specificity of the Wnt3-MO phenotype, we injected *CheWnt3* RNA into one blastomere of 8-cell stage embryos derived from Wnt3-MO-injected eggs (Fig. 6). Since the injected blastomeres were chosen randomly, they were equally likely to be derived from animal or vegetal halves. The ectopically expressed Wnt3 restored characteristic pointed oral poles to the spherical Wnt3-MO embryos in all cases (n=15), with the introduced RNA and co-injected fluorescent dextran always positioned at the oral pole. Cells descended from the *Wnt3* RNA-

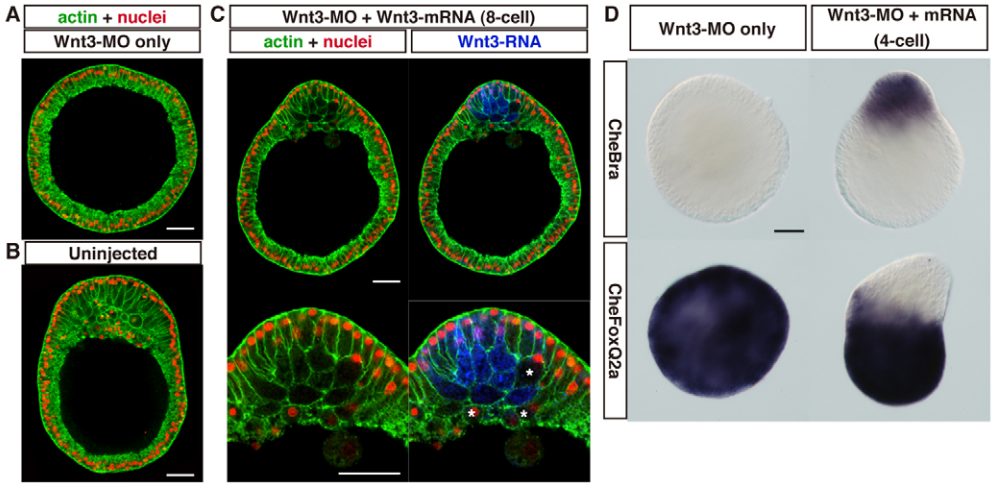

Fig. 6. Restoration of oral poles in Wnt3-MO embryos by ectopic CheWnt3 expression. (**A-C**) Confocal images of early gastrula *Clytia* embryos stained with phalloidin (green) and ToPro3 (red). (B) Uninjected; (A,C) Injected with Wnt3-MO prior to fertilisation; for C, *Wnt3* RNA was further injected into one blastomere at the 8-cell stage (n=15). In C, higher-magnification images of the region derived from the RNA-injected cell oral end, labelled by co-injected fluorescent dextran (blue), are shown beneath. Asterisks indicate the involution of cells neighbouring those containing *Wnt3* RNA. (**D**) Representative in situ hybridisation image of *CheBra* (oral) and *CheFoxQ2a* (aboral) expression in early gastrulae (15 hpf) derived from Wnt3-MO-injected eggs (left). *Wnt3* RNA was injected into a blastomere at the 4-cell stage after Wnt3-MO injection (right). Scale bars: 40 μm.

injected blastomere showed shape changes typical of presumptive endoderm cells: apical constriction, basal migration of the nucleus and inward migration (Fig. 6C). These shape changes were also observed in neighbouring *CheWnt3*-RNA-negative cells (Fig. 6C, asterisks). Thus, CheWnt3 can act at short range in a paracrine manner to direct neighbouring cells to an oral fate. The resultant embryos showed limited elongation along a new axis defined by the position of RNA injection, although Wnt3-MO-containing cells distant from the RNA injection site were not affected. Thus, CheWnt3 protein is able to confer an oral/presumptive endoderm fate at any position in Wnt3-MO embryos, as well as in *Wnt3*-overexpressing wild-type embryos (see above). We further tested oral- and aboral-specific gene expression in Wnt3-MO embryos 'rescued' by *Wnt3* RNA injection at the 4-cell stage (Fig. 6D). The CheBra expression abolished by Wnt3-MO was recovered at the pointed end of RNA-injected embryo (n=15, 87%). Conversely, the expanded CheFoxQ2a expression domain became restricted to the side opposite the newly created oral pole (n=18, 78%).

DISCUSSION

A common mechanism to initiate embryonic patterning with respect to the future body axes is the maternal localisation of 'determinant' molecules that direct cell fate and axial properties in the regions of the embryo that inherit them. Localised RNAs such as those encoding Bicoid, Nanos and Gurken in *Drosophila* oocytes provide classic examples (Lyczak et al., 2002; Riechmann and Ephrussi, 2001). In deuterostomes, the Wnt signalling pathway is commonly involved in maternally directed steps of pattern formation (see Introduction), and the discovery that cnidarian embryos share this feature suggests that it might be evolutionarily ancient (Wikramanayake et al., 2003). We have shown in *Clytia* that a Wnt ligand as well as Frizzled family receptors are crucially involved in axis determination via localised maternal mRNAs, and also in a dynamic regulatory system that maintains and refines the developing oral-aboral axis.

A maternal Wnt ligand required for axial patterning in *Clytia*

Only one of the five Wnt ligands found to be expressed during *Clytia* embryogenesis, CheWnt3, was detectable as maternal RNA. Morpholino-mediated inhibition of CheWnt3 translation completely blocked canonical Wnt pathway activation and the development of oral-aboral polarity. After *Xenopus* Xwnt11, CheWnt3 is only the second example of a Wnt ligand conclusively shown to be required maternally to initiate embryonic patterning.

Our model for the initial phase of axis specification in *Clytia* (Fig. 7A) is that it results from the combined effects of the ligand CheWnt3 and two receptors, all translated from maternal RNA. CheWnt3 protein almost certainly acts directly as a ligand for the classic receptor CheFz1 to activate the canonical pathway in the animal half of the early embryo. The negatively acting Frizzled, CheFz3, plays an important role in establishing a boundary of β-catenin stabilisation by preventing canonical pathway activation in the vegetal half of the embryo, apparently by having a 'dominant-negative' effect (Momose and Houliston, 2007). By contrast, although *CheWnt3* RNA shows tight animal localisation until the 64- to 128-cell stage, and is absolutely required upstream of CheFz1 for canonical Wnt pathway activation at this time, its role in the initial spatial restriction of activation may be relatively minor. Thus, whereas Wnt3-MO completely abolished canonical signalling, ubiquitous CheWnt3 overexpression by RNA injection into the egg had little effect on the initial β-catenin nuclear localisation pattern. Indeed, it appears likely that the endogenous distribution of CheWnt3 protein, although being asymmetric across the embryo, is more widespread than that of its RNA, as β-catenin stabilisation can be promoted throughout the early embryo following knockdown of CheFz3 function (Momose and Houliston, 2007). A broad CheWnt3 protein distribution could be generated from tightly localised RNA by rapid translation within the egg following fertilisation, and from subsequent intracellular and extracellular diffusion. Based on these considerations we propose that canonical Wnt signalling is prevented in the future aboral half of cleavage-stage embryos by a combination of reduced ligand availability and dampening of the cellular response by CheFz3.

Two phases of Wnt-dependent axis formation in *Clytia*

In situ analyses revealed two phases of CheWnt3 expression. Maternally deposited RNA is localised to the animal cortex and is inherited by a small group of animal blastomeres during cleavage stages. Zygotic expression is first detectable at the mid-blastula stage, overlapping with the maternal expression. The transition from

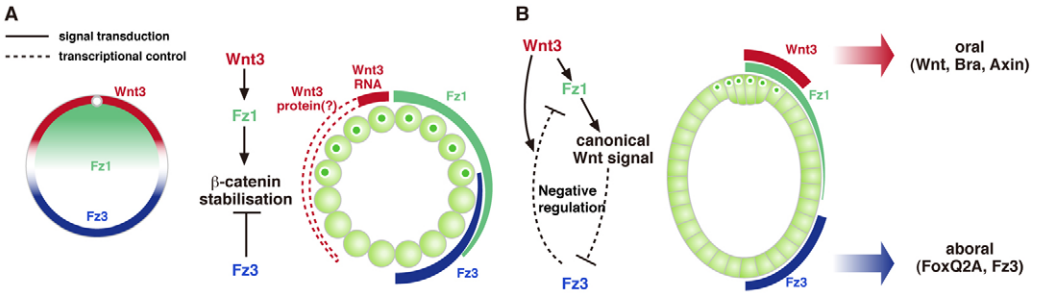

Fig. 7. A current model for axis determination in *Clytia*. (**A**) In early stages (from egg to early blastula), the ligand CheWnt3 and its receptor CheFz1, produced from animally concentrated RNAs, are required for canonical Wnt signalling in the the animal hemisphere. *CheFz1* RNA is distributed as an animal-vegetal gradient in the cytoplasm, whereas *CheWnt3* RNA is tightly localised to the animal cortex. CheWnt3 protein probably adopts a wider distribution upon translation (see text). The negatively acting receptor CheFz3, produced from a vegetal cortical RNA, is likely to be the major factor restricting canonical Wnt signalling activation to the future oral end. (**B**) In later stages, tightly localised zygotic *CheWnt3* expression at the oral pole becomes a major factor restricting canonical Wnt pathway activation. Wnt3 is also involved in the reciprocal negative regulation between the receptors CheFz1 and CheFz3 by unknown mechansims. Arrows and dotted lines represent signalling pathways and transcriptional regulation pathways, respectively. The coloured boxes represent RNA distribution, the area with a red dashed outline the predicted Wnt3 protein distribution, and green circles β-catenin stabilisation and nuclear localisation.

maternal to zygotic *CheWnt3* transcription coincides with a change in its role from being a necessary but non-directive factor in early β-catenin activation, to defining the oral pole within a regulative oral-aboral patterning system operating in blastula and early gastrula stages (Fig. 7B). The importance of this second role was dramatically demonstrated by the strong oralised phenotype upon *CheWnt3* RNA injection, despite the initial refractiveness of the β-catenin-stabilisation domain to this treatment, and by the ability of ectopic *Wnt3* mRNA injection to restore a morphologically normal oral pole to morpholino-injected embryos. There is an interesting similarity between the two phases of Wnt-dependent signalling in *Clytia* and axial patterning in vertebrates. In vertebrates, the classic 'dorsal organiser' signalling centres responsible for anterior-posterior patterning are also established within previously established domains of canonical Wnt pathway activation, specified maternally in the case of amphibian and fish and by Wnt-ligand-dependent self-organising mechanisms in mouse and chick (see Meinhardt, 2006; Stern et al., 2006).

Wnt-based feedback regulatory systems are well placed to account for the well-characterised regulative abilities of hydrozoans, including the blastulae of *Clytia* and *Podocoryne* (Bode, 2003). Axis regulation can be explained by the presence of a 'reaction-diffusion' system (Meinhardt, 2008), in which the role of a locally produced activator could be filled by Wnts expressed at the oral pole, and that of fast-diffusing inhibitors perhaps by antagonists of the sFRP and Dickkopf families, gene sequences for which are found in the *Nematostella* and *Hydra* genomes (Guder et al., 2006a; Guder et al., 2006b; Lee et al., 2006) and in our *Clytia* EST collections. Consistent with this hypothesis, *Hydra* polyps rapidly upregulate the expression of Wnt3 and of downstream activators in the 'head organiser' (hypostome) region during budding and regeneration to define oral fate (Broun et al., 2005; Hobmayer et al., 2000). The axial regulatory system in the *Clytia* blastula remains largely uncharacterised; however, negative regulation between CheFz1 and CheFz3 to define mutually exclusive oral and aboral domains is clearly an important component. Massive RNA accumulation of each receptor is observed when translation of the other is prevented (Momose and Houliston, 2007), a reciprocal regulation now shown to require CheWnt3. The oral downregulation of *CheFz3* is most easily explained by negative regulation by canonical Wnt signalling, because lithium treatment, which upregulates the canonical pathway, clearly reduces *CheFz3* RNA levels. By contrast, aboral downregulation of *CheFz1* might involve a non-canonical mechanism, as LiCl treatment does not have a strong effect on *CheFz1* RNA levels (Momose and Houliston, 2007). The dependence of *CheFz1* downregulation on Wnt3 could involve direct interaction of the ligand with CheFz3 in the aboral region, or be effected indirectly, for instance via factors secreted from the oral region. Signalling systems other than the canonical Wnt pathway clearly operate during oral-aboral patterning in the *Clytia* embryo because zygotic *Wnt3* transcription was initiated correctly when the canonical Wnt pathway was blocked.

Evolution of axis determination

The requirement for maternal Wnt ligands in both *Xenopus* and *Clytia* strongly suggests that the entire ligand receptor-cytoplasmic signalling module was used to regionalise the embryo in the common ancestor of bilatarians and cnidarians. One attractive scenario is that an intercellular Wnt signalling system originally functioned within groups of equivalent cells to create stable asymmetries of canonical activity. There is evidence for systems with such properties in reconstituted cell aggregates from *Hydra*, which can be accounted for theoretically using the reaction-diffusion system evoked to explain pattern regulation (Gierer et al., 1972; Meinhardt, 2008; Technau et al., 2000). An alternative possibility is that Wnt ligand-Fz receptor signalling between the oocyte and somatic cells in the gonad was used ancestrally in directing the acquisition of oocyte polarity during oogenesis.

From an ancestral usage of the whole Wnt pathway, maternal concentrations of one or several regulatory components might have been adopted during evolution in different species to provide a reliable maternal cue. In *Clytia*, at least three localised maternally coded Wnt pathway regulators, comprising one ligand and two antagonistically acting receptors, exhibit localised distributions. All three are required for proper axis formation and are separately localised in the eggs and early embryos. Additional localised regulators might also contribute: in *Hydractinia*, RNA for the transcriptional regulator Tcf is localised along with that of a Wnt3 orthologue in the egg (Plickert et al., 2006). Furthermore, we have found animal pole localisation of a *Clytia Dsh* RNA in the egg, localisation of Dsh protein being detectable from the 8-cell stage (T.M., unpublished). The parallel localisation of more than one maternal factor might help to ensure a robust mechanism to establish a reliable Wnt activity asymmetry in the face of intrinsic developmental variability and extrinsic disturbances. In *Xenopus*, Dsh, GBP and β-catenin proteins as well as *Xwnt11* RNA show localised maternal distributions (Heasman, 2006). In *Nematostella*, requirement for the ligand might have been lost or diminished, with Dsh protein localisation assuming a more important role (Lee et al., 2007; Lee et al., 2006). In sea urchin, maternal Dsh concentrations also appear to be important, although additional localised factors are required to account for vegetally restricted β-catenin nuclear localisation (Leonard and Ettensohn, 2007; Logan et al., 1999).

To conclude, localised maternal Wnt ligands contributing to axial patterning have been clearly demonstrated in *Xenopus* and *Clytia*, and cannot be discounted in other models in which intracellular regulators may play dominant roles to localise Wnt pathway activation. Different determination factors might have been favoured during evolution, depending on egg structure and reproduction pattern. The absence of maternal Wnt3 but not of Frizzled in *Hydra* (Frobius et al., 2003), whereas both elements are present maternally in *Hydractinia* (Plickert et al., 2006; Teo et al., 2006) and *Clytia*, could represent an example of ongoing developmental flexibility within the hydrozoan clade associated with very different strategies for early development. Currently, insufficient information is available to support clear evolutionary scenarios, but our study suggests that ligand-dependent Wnt signalling was already available in a eumetazoan common ancestor to determine the body axis. A recent report of polarised zygotic Wnt expression in a sponge embryo pushes this usage back even further in evolution (Adamska et al., 2007). It will be of great interest to re-examine the involvement of Wnt ligands in canonical Wnt signalling activation and axis determination in other deuterostome, lophotrochozoan and cnidarian models.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/12/2105/DC1

References

Adamska, M., Degnan, S. M., Green, K. M., Adamski, M., Craigie, A., Larroux, C. and Degnan, B. M. (2007). Wnt and TGF-beta expression in the sponge amphimedon queenslandica and the origin of metazoan embryonic patterning. *PLoS ONE* **2**, e1031.

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Bode, H. R. (2003). Head regeneration in Hydra. *Dev. Dyn.* **226**, 225-236.

Broun, M., Gee, L., Reinhardt, B. and Bode, H. R. (2005). Formation of the head organizer in hydra involves the canonical Wnt pathway. *Development* **132**, 2907-2916.

Chevalier, S., Martin, A., Leclère, L., Amiel, A. and Houliston, E. (2006). Polarised expression of FoxB and FoxQ2 genes during development of the hydrozoan Clytia hemisphaerica. *Dev. Genes Evol.* **216**, 709-720.

Frobius, A. C., Genikhovich, G., Kurn, U., Anton-Erxleben, F. and Bosch, T. C. (2003). Expression of developmental genes during early embryogenesis of Hydra. *Dev. Genes Evol.* **213**, 445-455.

Gierer, A., Berking, S., Bode, H., David, C. N., Flick, K., Hansmann, G., Schaller, H. and Trenkner, E. (1972). Regeneration of hydra from reaggregated cells. *Nat. New Biol.* **239**, 98-101.

Guder, C., Philipp, I., Lengfeld, T., Watanabe, H., Hobmayer, B. and Holstein, T. W. (2006a). The Wnt code: cnidarians signal the way. *Oncogene* **25**, 7450-7460.

Guder, C., Pinho, S., Nacak, T. G., Schmidt, H. A., Hobmayer, B., Niehrs, C. and Holstein, T. W. (2006b). An ancient Wnt-Dickkopf antagonism in Hydra. *Development* **133**, 901-911.

Heasman, J. (2006). Patterning the early Xenopus embryo. *Development* **133**, 1205-1217.

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., von Laue, C. C., Snyder, P., Rothbacher, U. and Holstein, T. W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. *Nature* **407**, 186-189.

Huelsken, J. and Birchmeier, W. (2001). New aspects of Wnt signaling pathways in higher vertebrates. *Curr. Opin. Genet. Dev.* **11**, 547-553.

Imai, K., Takada, N., Satoh, N. and Satou, Y. (2000). (beta)-catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127**, 3009-3020.

Kawai, N., Iida, Y., Kumano, G. and Nishida, H. (2007). Nuclear accumulation of beta-catenin and transcription of downstream genes are regulated by zygotic Wnt5alpha and maternal Dsh in ascidian embryos. *Dev. Dyn.* **236**, 1570-1582.

Kusserow, A., Pang, K., Sturm, C., Hrouda, M., Lentfer, J., Schmidt, H. A., Technau, U., von Haeseler, A., Hobmayer, B., Martindale, M. Q. et al. (2005). Unexpected complexity of the Wnt gene family in a sea anemone. *Nature* **433**, 156-160.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* **136**, 1123-1136.

Lee, P. N., Pang, K., Matus, D. Q. and Martindale, M. Q. (2006). A WNT of things to come: Evolution of Wnt signaling and polarity in cnidarians. *Semin. Cell Dev. Biol.* **17**, 157-167.

Lee, P. N., Kumburegama, S., Marlow, H. Q., Martindale, M. Q. and Wikramanayake, A. H. (2007). Asymmetric developmental potential along the animal-vegetal axis in the anthozoan cnidarian, Nematostella vectensis, is mediated by Dishevelled. *Dev. Biol.* **310**, 169-186.

Leonard, J. D. and Ettensohn, C. A. (2007). Analysis of dishevelled localization and function in the early sea urchin embryo. *Dev. Biol.* **306**, 50-65.

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.

Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.

Lyczak, R., Gomes, J. E. and Bowerman, B. (2002). Heads or tails: cell polarity and axis formation in the early Caenorhabditis elegans embryo. *Dev. Cell* **3**, 157-166.

Martindale, M. Q. (2005). The evolution of metazoan axial properties. *Nat. Rev. Genet.* **6**, 917-927.

Meinhardt, H. (2006). Primary body axes of vertebrates: generation of a near-Cartesian coordinate system and the role of Spemann-type organizer. *Dev. Dyn.* **235**, 2907-2919.

Meinhardt, H. (2008). Models of biological pattern formation: from elementary steps to the organization of embryonic axes. *Curr. Top. Dev. Biol.* **81**, 1-63.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L. and Moon, R. T. (1999). Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol.* **146**, 427-437.

Momose, T. and Houliston, E. (2007). Two oppositely localised frizzled RNAs as axis determinants in a cnidarian embryo. *PLoS Biol.* **5**, e70.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* **121**, 755-765.

Plickert, G., Jacoby, V., Frank, U., Muller, W. A. and Mokady, O. (2006). Wnt signaling in hydroid development: formation of the primary body axis in embryogenesis and its subsequent patterning. *Dev. Biol.* **298**, 368-378.

Riechmann, V. and Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **11**, 374-383.

Schier, A. F. and Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. *Annu. Rev. Genet.* **39**, 561-613.

Stern, C. D., Charite, J., Deschamps, J., Duboule, D., Durston, A. J., Kmita, M., Nicolas, J. F., Palmeirim, I., Smith, J. C. and Wolpert, L. (2006). Head-tail patterning of the vertebrate embryo: one, two or many unresolved problems? *Int. J. Dev. Biol.* **50**, 3-15.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell* **120**, 857-871.

Technau, U., Cramer von Laue, C., Rentzsch, F., Luft, S., Hobmayer, B., Bode, H. R. and Holstein, T. W. (2000). Parameters of self-organization in Hydra aggregates. *Proc. Natl. Acad. Sci. USA* **97**, 12127-12131.

Teo, R., Mohrlen, F., Plickert, G., Muller, W. A. and Frank, U. (2006). An evolutionary conserved role of Wnt signaling in stem cell fate decision. *Dev. Biol.* **289**, 91-99.

Weaver, C., Farr, G. H., 3rd, Pan, W., Rowning, B. A., Wang, J., Mao, J., Wu, D., Li, L., Larabell, C. A. and Kimelman, D. (2003). GBP binds kinesin light chain and translocates during cortical rotation in Xenopus eggs. *Development* **130**, 5425-5436.

Weitzel, H. E., Illies, M. R., Byrum, C. A., Xu, R., Wikramanayake, A. H. and Ettensohn, C. A. (2004). Differential stability of beta-catenin along the animal-vegetal axis of the sea urchin embryo mediated by dishevelled. *Development* **131**, 2947-2956.

Wikramanayake, A. H., Huang, L. and Klein, W. H. (1998). beta-Catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9343-9348.

Wikramanayake, A. H., Hong, M., Lee, P. N., Pang, K., Byrum, C. A., Bince, J. M., Xu, R. and Martindale, M. Q. (2003). An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. *Nature* **426**, 446-450.