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Evolution of the mechanisms that establish the embryonic axes

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A long-standing debate in developmental biology concerns the extent to which embryos are largely 'mosaic' (cell fates are allocated by localization of maternal determinants that are inherited differentially) or 'regulative' (cell interactions determine cell fates). Generally, it has been thought that amniotes, especially birds and mammals, are at the extreme regulative end of the spectrum, whereas most invertebrates, lower chordates and anamnia are more mosaic. Various studies have identified additional differences, including egg size, the timing of zygotic transcription and the speed of development. However, new research is starting to reveal among the vertebrate classes an astonishing degree of conservation in the intercellular signalling mechanisms that regulate cell fate and embryonic polarity before gastrulation.

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Regulative versus mosaic development

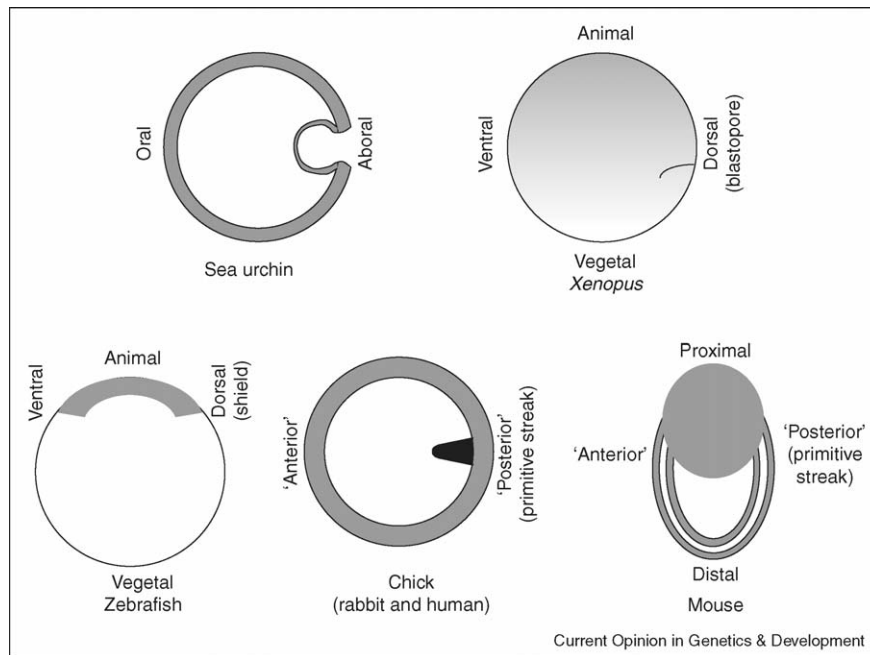
The embryos of all animals are derived from a single cell, the fertilised egg. This divides to form a clone of genetically identical cells — soon, the embryo needs to establish differences that will enable it to define its three major axes (dorsoventral, head–tail and left–right). This is the process that we refer to as 'the establishment of embryonic polarity'. The recent controversy concerning whether mouse embryos establish their polarity as a result of very early differences between blastomeres at early cleavage that bias cell fate [1,2] or much later in development, exclusively through cell interactions [3], has re-awakened the old question: to what extent is early development 'mosaic' (i.e. cell fates are predetermined) and to what extent is it 'regulative' (i.e. cell fates are allocated dynamically and relatively late)? The controversy has not yet been resolved [4]. If it turns out that the fates of early blastomeres in mouse embryos are biased, then this would seriously challenge the long-held belief that

'higher' vertebrates (i.e. amniotes) are largely regulative, whereas 'lower' vertebrates and probably most invertebrates rely much more on maternal determinants.

The chick embryo has also long been thought of as an example of extreme regulative development, ever since the experiments of Lutz [5] and Spratt [6] demonstrated that when an embryo possessing as many as 20 000 cells (i.e. at the mid-blastula stage) is cut into several fragments, each fragment can spontaneously initiate formation of a complete embryonic axis. However this too has been challenged recently by the finding that unknown molecular components (within the so-called γ - and δ -ooplasm, of which the first is peripheral and the second more centrally located) within the acellular yolky material underlying the cleaving embryo can influence cell fate decisions in the early blastoderm, and might bias embryonic polarity [7]. Until we know the molecular nature of these presumed determinants and their mode of action, it will be difficult to understand how they act and whether or not they are similar to the determinants found in other animals.

Regardless of the answers to these controversies, research in the past few years has started to reveal an unexpected level of conservation of the mechanisms that establish the earliest cell fate differences, and hence embryonic axial polarity, among vertebrates and even in non-chordate Metazoa. Within the vertebrates, the earliest strides were made in the amphibian *Xenopus laevis*. The large egg size of this species, and the fact that it does not activate zygotic gene expression until after the 10th cleavage — at the mid-blastula transition — have been a significant advantage in that they enable injection of mRNA (for gain-of-function experiments) or morpholinos (for loss-of-function experiments), the phenotypic consequences of which could be examined at later stages. Pioneering studies revealed that the vegetal part of the egg (Figure 1) was enriched with mRNAs encoding the TGF β (transforming growth factor beta) superfamily member Vg1 [8] and the transcription factor VegT [9–12], defining the animal–vegetal axis. At right angles to this axis, the 'dorsal' side of the embryo — where gastrulation will later be initiated — is characterised by the localisation of β -catenin in the nucleus of cells, implicating activation of the canonical Wnt-pathway [13,14]. The intersection of Wnt and TGF β signalling would then specify the first signalling centre, or Nieuwkoop centre, which in turn is responsible for inducing the Spemann's organiser and dorsal mesoderm [15]. Mesoderm and endoderm induction were found to be mediated by activins and, in particular, Nodal (first discovered in mice

Figure 1



Schematic diagram comparing different species to clarify the terminology used to define the 'gastrula-anti-gastrula' axis — the former pole refers to the site where gastrulation begins. Sea urchin, *Xenopus*, zebrafish, mouse, and chick, rabbit and human embryos are all shown oriented so that the site of initiation of gastrulation appears on the right, along with the terminology usually applied to each in the current literature.

[16]), the effects of which are potentiated by FGFs (fibroblast growth factors) [17]. In turn, dorsoventral polarity is also reinforced by the activity of bone morphogenetic proteins (BMPs), which act as ventralising factors, antagonised at the dorsal side mainly by Chordin [18,19].

The finding that early localisation of maternal determinants is required for polarity to be established in *Xenopus* was immediately reminiscent of the then better studied *Drosophila* system, in which anterior-posterior polarity is established by initial localisation of determinants such as *bicoid* mRNA and protein and of several gene products that define the 'terminal' ends of the embryo at the syncytial blastoderm stage [20]. Likewise, in the embryos of ascidians, which are non-vertebrate chordates, it has long been known that maternal determinants specify cell fates and that these animals follow a largely mosaic mode of early development. A recent study [21] revealed that *macho-1* [22], a muscle determinant, and *PEM1* (*posterior end mark RNA1*) mRNA gradients define the animal-vegetal axis, whereas cortical endoplasmic reticulum and a mitochondria-rich sub-cortical cytoplasm become localised posteriorly at first cleavage and are distributed unevenly in the first two blastomeres. A comparison of these molecular determinants in *Xenopus*, *Drosophila* and *Ciona* does not immediately reveal a conserved mechanism for establishing polarity among all Metazoa. However, there are some extraordinarily strong parallels,

among which the BMP-Dpp-Chordin-Sog pathway for specifying dorsoventral polarity in *Xenopus* and *Drosophila* stands out. Indeed, a very recent study by the De Robertis group demonstrated convincingly that morpholino-mediated knockdown of three of the BMPs (BMP2, BMP4 and BMP7) together with the BMP-related but dorsally expressed ADMP (anti-dorsalising morphogenetic protein), causes a complete radialisation and dorsalisation of the entire embryo [23^{••}], a convincing demonstration that BMP signalling is absolutely required to specify the ventral ('anti-gastrular') side of the embryo.

Recent studies in chick have unexpectedly revealed a high level of conservation of the mechanisms first identified in *Xenopus*, despite the fact that the chick retains regulative ability until advanced stages of development (see above). In particular, mRNA encoding Vg1 is first localised in the chick posterior marginal zone — a tissue with extra-embryonic fate, topologically equivalent to the dorsovegetal region of *Xenopus* — and cooperation between Vg1 and canonical Wnt-signalling is required for initiation of the embryonic axis [24]. An early target of Vg1 + Wnt is *Nodal* transcription, which is activated at the posterior ('dorsal', using *Xenopus* terminology; Figure 1) edge of the embryonic epiblast [25,26]. Formation of a single embryo from a disc capable of initiating gastrulation at multiple sites is regulated by Nodal antagonists, including Cerberus expressed by the hypoblast

underlying the embryonic disc — and probably Lefty [25,27,28]. Other evidence [27] has revealed the existence of at least one additional inhibitor that is distinct from these two and which travels across the embryo at an amazing speed (at least 500 $\mu\text{m}/\text{hour}$). Also, as in *Xenopus*, the role of Nodal in the initiation of mesendoderm formation is enhanced by FGFs (particularly FGF8 and possibly FGF3 [27]) and counteracted by BMPs (particularly BMP4 [29]). The pathway now believed to control the location of the initial gastrulation site ('posterior') in the chick is summarised in Figure 2.

Unexpected conservation of the basic pathway

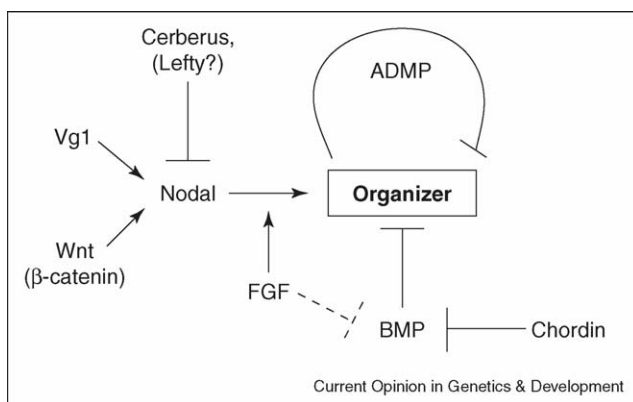
Several recent studies have started to uncover various conserved features of this pathway in a wide variety of organisms. A particularly striking example is the finding that the involvement of nuclear β -catenin in the canonical Wnt-pathway has very ancient roots, dating back to pre-bilaterians such as the Cnidarian *Nematostella vectensis*. In this animal, there is differential stabilisation of β -catenin along the oral–aboral axis, the protein moves to the nucleus at the future gastrulation site and is then used to specify endoderm [30]. In non-chordate deuterostomes such as the sea urchin *Strongylocentrotus purpuratus*, in which Wnt signalling has long been known to be involved in specifying the 'primary' (animal–vegetal) axis, a new study [31] now reveals that Nodal–Activin (also known as SpNodal) is both sufficient and necessary to establish oral–aboral polarity (i.e. a 'secondary' axis, equivalent to the dorsoventral axis of *Xenopus*). A different mechanism to regulate this axis was uncovered by Coffman *et al.* [32], who showed that the oral–aboral axis in the same sea urchin species is regulated by differential distribution of mitochondria, and a resulting redox state; this is rather reminiscent of many studies from the 1960s to the early 1970s suggesting that redox state is a crucial regulator of gastrulation and early embryo polarity, but these studies

were never followed up fully at the time. The zygote inherits maternal mitochondrial polarity, and centrifugation to redistribute mitochondria causes severe defects. Interestingly these defects can be rescued by overexpression of Nodal, suggesting that Nodal functions downstream of the proposed redox gradient and of maternal determinants; this would have been expected from *Xenopus* and chick data implying that Nodal is a downstream effector of gastrulation but not of the initial symmetry-breaking event.

Despite the fact that Vg1 was first discovered in *Xenopus*, evidence for it being crucial for polarity establishment in this organism remained elusive. This is partly because *Xenopus* Vg1 contains an unusual cleavage site — required for producing mature protein — and because morpholino-mediated depletion of Vg1 mRNA never gave clear results. Surprisingly, perhaps, Beckham *et al.* [33] recently reported that in the direct developing frog *Eleutherodactylus coqui*, which in some ways develops more like an amniote than like a typical anuran, both Vg1 and VegT are localised in the animal part of the embryo rather than in the vegetal hemisphere as they are in *Xenopus*. This observation prompted the authors to suggest that new mechanisms could have evolved in direct-developing species that do not rely as much on vegetally located molecules. This might be expected because the vegetal pole does not exist as such in amniotes. Moreover, Vg1 homologues with appropriate localisation and activity in mice had not been discovered. Apart from the striking localisation of the mRNA and the activity of a chimeric Vg1–Dorsalin construct in *Xenopus*, the best evidence for direct involvement of Vg1 in axis formation came from chick experiments.

Three recent studies suggest that Vg1 does indeed play a crucial role in the determination of early embryonic polarity. The most striking of these is a study by Birsoy *et al.* [34^{••}], who found that *Xenopus* embryos treated with Vg1-morpholinos so as to deplete both maternal and zygotic levels of Vg1 mRNA do have very severe axial defects and fail to form normal mesoderm. Importantly, these defects cannot be rescued by the original Vg1, but they can be rescued impressively by a newly discovered allele of Vg1 that contains a single amino acid change to its cleavage site, rendering it processable by endogenous convertases. The same group [35^{••}] also reveal a novel pro-protein convertase, XPACE4, which is capable of cleaving Vg1 and three Nodal-related proteins (Xnr1, Xnr 2 and Xnr 3) but not the related TGF β s Activin or Derriere. XPACE4 mRNA is localised initially in the mitochondrial cloud and vegetal hemisphere of the oocyte and is required for the endogenous mesoderm-inducing activity of vegetal cells in the classic Nieuwkoop-assay [15]. Together, these findings suggest not only that the localisation of the TGF β -related molecule itself is important but also that the localisation and

Figure 2



A basic pathway of signalling molecules contributing to specify the site of initiation of gastrulation (which also corresponds to where Spemann's organizer will form) in vertebrates.

activity of the maturing enzyme contributes to refine its action both spatially and temporally.

In mice, a TGF β -related factor called Gdf3 is now shown to be the closest relative of Vg1. It acts at pre-gastrula stages and has Nodal-like activity, which includes regulating the formation and/or positioning of the anterior visceral endoderm [36]. Although these findings could suggest that Gdf3 is a Nodal-like molecule, the authors find that Nodal expression is defective in *Gdf3*^{-/-} mutants, suggesting that Gdf3 acts upstream of Nodal and regulates its expression, properties consistent with it having Vg1-like functions and suggesting that the pathway proposed in chick embryos is conserved in mammals.

The involvement of Wnt signalling in early axis determination is well established in many organisms. This signalling includes both canonical (β -catenin-dependent) signalling, which regulates dorsoventral polarity in *Xenopus* (see above), and the planar cell polarity (PCP) pathway, which is important for regulating convergence-extension movements in the ectoderm, required for axis elongation, as first demonstrated in both zebrafish and *Xenopus* [37–39]. However, research in mice lagged behind in this respect because little or no evidence existed to suggest that similar mechanisms — along either pathway — were involved in determining polarity at pre-gastrulation stages or indeed in regulating cell movements. Now, Kimura-Yoshida *et al.* [40^{*}] have revealed unexpectedly that canonical, rather than PCP-dependent, Wnt signalling, together with the Wnt-antagonist Dkk1 (Dickkopf1), regulate the ‘anterior–posterior’ axis (equivalent to the gastrular–anti-gastrular axis or, in *Xenopus*, the dorsoventral axis) by guiding migration of the anterior visceral endoderm. Importantly, Dkk1 rescues the effects of Otx2 (orthodenticle-related homeobox 2) deficiency, as does removal of just one copy of *β -catenin*. The authors also show that canonical Wnt is repulsive, whereas Dkk1 is attractive, for anterior visceral endoderm. These findings suggest that Wnt signalling can direct cell movements through both pathways, and they reveal an important role in polarity of the very early mouse embryo. In support of this, Kemler *et al.* [41] show that, in the mouse epiblast, β -catenin stabilisation — by making it resistant to GSK β (glycogen synthase kinase beta)-mediated proteasome degradation — leads to premature epithelial–mesenchymal transition, the equivalent of mesoderm formation.

Some progress has also been made in elucidating the important relationships between ‘dorsoventral’ patterning — determining the site of gastrulation — and convergent-extension movements in different germ layers, a problem that had previously been difficult to overcome experimentally because convergent-extension and axial elongation are so intimately linked with gastrulation and

dorsal fate determination. In zebrafish, Formstone and Mason [42] show that the Flamingo-related protein Fmi1 functions with Wnt11 and Strabismus through the PCP pathway to promote convergence–extension movements at the gastrula stage in zebrafish, without altering dorsoventral patterning. These results might have been expected from the original studies of Heisenberg *et al.* [37] and Tada *et al.* [38], but it is striking that until recently the mechanisms responsible for regulating the extension of mesodermal structures (e.g. the notochord) had not been explicitly separated from those that regulate elongation in the ectoderm. Now, a new study [43] shows that Wnt signalling through the PCP pathway regulates convergent-extension in the ectoderm, whereas the chordamesoderm undergoes convergence–extension independently of this and is instead dependent on a graded activin-like signal, which is regulated autonomously within the chordamesoderm cells.

Analysis of both the directionality of cell movements and the relative sites of expression of very early genes that might act as predictors of embryonic polarity is dependent on being able to know which side of the early embryo will correspond to the site where gastrulation will be initiated. Although in the frog and chick there are good morphological landmarks (e.g. grey crescent and other differences in pigmentation in the frog; Koller’s sickle and the gradual spreading of the hypoblast layer in the chick), this has been particularly hard in mice because of the absence of molecular landmarks. The early mouse embryo (called ‘egg cylinder’) at day 5.5–6.0 has the shape of a short cylinder, slightly flattened along one axis. It had been generally thought, although never directly investigated, that the future head–tail axis would develop along the ‘fold’ (i.e. the edge of the longer side) of the flattened cylinder. Recent studies [44^{*},45^{*},46] provide very convincing evidence that the future mouse-axis develops at right angles to this, with the future tail-site in the middle of one of the flattened sides, and the future head-site at the opposite side. No doubt these findings will now make it much easier to identify markers whose early expression predicts embryonic polarity.

Conclusions

Rapidly growing evidence now suggests that a fundamental pathway involving Vg1, Wnt, Nodal, FGF and BMP signals is strongly conserved throughout the vertebrate classes, irrespective of the degree to which different vertebrate species turn out to be largely regulative or mosaic in terms of the mechanisms that establish cell fate asymmetries in the early embryo.

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