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Patterning the early *Xenopus* embryo

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Developmental biology teachers use the example of the frog embryo to introduce young scientists to the wonders of vertebrate development, and to pose the crucial question, 'How does a ball of cells become an exquisitely patterned embryo?'. Classical embryologists also recognized the power of the amphibian model and used extirpation and explant studies to explore early embryo polarity and to define signaling centers in blastula and gastrula stage embryos. This review revisits these early stages of *Xenopus* development and summarizes the recent explosion of information on the intrinsic and extrinsic factors that are responsible for the first phases of embryonic patterning.

Introduction

Over the past 5 years, the usefulness of the *Xenopus* model organism has grown considerably as a result of the *Xenopus* Genome Initiative (see www.xenbase.org/). This endeavor has provided a quantum increase in the amount of information available on *Xenopus* genes and the resources with which to study them. The development of loss-of-function technology has also increased our knowledge of individual gene function (Heasman et al., 2000). The result is that many more molecules have been shown to control early *Xenopus* development. The challenge for the modern developmental biologist is to stay abreast of this information. In this review, I summarize these new findings and incorporate them with the old. Inevitably, this survey will be incomplete. [For further information, see De Robertis and Kuroda (De Robertis and Kuroda, 2004), and for a comparison with zebrafish axis patterning, see Schier and Talbot (Schier and Talbot, 2005). For research into germ-line establishment, see also Zhou and King (Zhou and King, 2004).] For example, the nuts and bolts of development, including the cytoskeletal and adhesion machinery, many components of signaling pathways, transcriptional and cell cycle regulators are incompletely covered. The question that drives this review is, 'What insight have recent functional studies given us on the mechanisms that pattern the early *Xenopus* embryo?'.

An overview of early *Xenopus* development

After fertilization, *Xenopus* embryos undergo cell cycles that have characteristic features (Fig. 1). During the first, 90-minute cell cycle, cortical cytoplasmic movements and male and female pronuclear fusion occur. The next eleven divisions occur at 20- to 30-minute intervals with no gap phases, while the embryo forms a ball of 4000 cells, which encloses a fluid-filled blastocoel cavity. This mid-blastula embryo has three regions, the animal cap (which forms the roof of the blastocoel), the equatorial or marginal zone (the walls of the blastocoel) and the vegetal mass (the blastocoel floor) (see Fig. 1B). Although all mid-blastula cells are pluripotent (Heasman et al., 1984), explants of the animal cap form ectodermal derivatives in culture, while equatorial explants form mesoderm and vegetal explants form endoderm. At the end of the twelfth cycle, gap phases reappear, the cell cycle lengthens to 50 minutes and zygotic transcription starts (this is called the mid-blastula transition, MBT). In the 15th cycle, the dorsal lip of the blastopore forms, the cell movements of gastrulation begin and mitosis stops. Gastrulation converts the embryonic ball into three layers, and establishes definitive anteroposterior and dorsoventral axes (Fig. 2, Box 1). In this review, I retrace this developmental pathway and ask how cells become committed to specific fates.

Pre-patterning by maternally stored mRNAs and proteins

To what extent does embryonic patterning rely on mRNAs and proteins inherited from the oocyte, or upon intercellular signaling downstream of zygotic gene transcription? For *Xenopus* development, it was predicted that oocyte stores would be essential for embryonic patterning, because zygotic transcription does not begin until the 4000-cell stage and because newly expressed zygotic genes have localized expression patterns. Recent studies have confirmed this prediction. Included in the essential maternal pool are: genome-wide transcriptional repressors, such as Xkaiso and the LEF/TCF family member Xtcf3 (Houston et al., 2002; Ruzov et al., 2004); transcriptional activators, including forkhead proteins (e.g. FoxH1, Foxi1E) (Kofron et al., 2004a; Suri et al., 2005); the T box protein VegT (Zhang et al., 1998); and cAMP response element-binding protein (CREB) (Sundaram et al., 2003). TATA-binding components of basal transcriptional complexes, TBP and TBP2, are also essential for normal development, and their depletion reduces the transcription of specific zygotic target genes and disrupts gastrulation (Jallow et al., 2004).

A simple strategy that provides a blueprint for development is the localized positioning of maternal mRNAs in the oocyte so that they are inherited by specific areas of the embryo. Transcripts of the transcription factors *Zic2* and *Xenopus grainyhead 1* (*Xgrhl1*) are localized to the animal hemisphere of the oocyte and early embryo (Houston and Wylie, 2005; Tao et al., 2005a). By contrast, *VegT* transcripts are localized in the oocyte vegetal hemisphere (Zhang and King, 1996), and VegT protein is inherited by only vegetal cells (Stennard et al., 1999).

The list of vegetally localized mRNAs continues to grow and includes transcripts of the signaling molecules Vg1 (Weeks and Melton, 1987) and Wnt11 (Ku and Melton, 1993), of the transcription factor Otx1 (Pannese et al., 2000) and of the RNA-binding protein bicaudal C (Wessely and De Robertis, 2000). The cortical cytokeratin filament network is likely to hold these transcripts in place, as antibodies specific for cytokeratin disruption dislodge localized mRNAs (Kloc et al., 2005). Unexpectedly, the degradation of two of the localized mRNAs themselves, *VegT* mRNA and the non-translated mRNA *Xlsrts*, also dislodges other mRNAs (Heasman et al., 2001; Kloc and Etkin, 1994; Kloc et al., 2005) and disrupts the cytokeratin network. These effects are rescued by *VegT* mRNA, suggesting that it has an architectural role, although the mechanism is unresolved (Kloc et al., 2005).

Vegetally localized mRNAs do not all fall into one spatial group. For example, transcripts of the RNA-binding protein Xdazl (Houston et al., 1998) and *Xpat* mRNAs (Machado et al., 2005)

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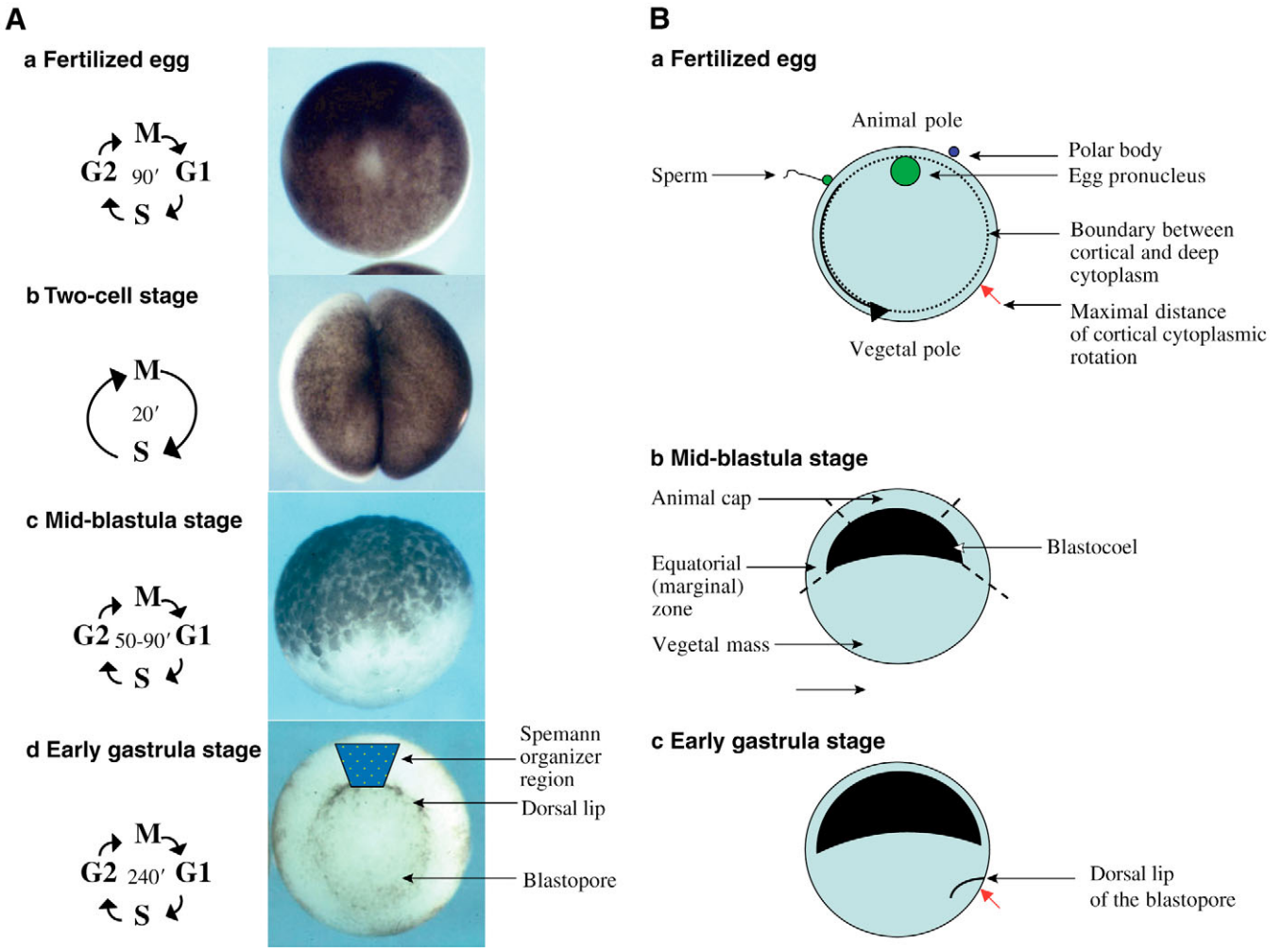

Fig. 1. Characteristics of *Xenopus laevis* early development. (**A**) The different cell cycles and the external appearance of (a) the fertilized egg, and (b) two-cell, (c) mid-blastula and (d) early gastrula stages. Aa and Ab are views from the animal pole, Ac from the side and Ad from the vegetal pole. (Aa) Cycle 1 is approximately 90 minutes in length and has G1 and G2 phases. The next 11 divisions have no gap phases and occur every 20-30 minutes. (Ac) At the mid-blastula stage, the embryo consists of 4000 cells, gap phases reappear, the cycle lengthens to 50 minutes and zygotic gene expression commences. This marks the mid blastula transition (MBT). (Ad) The following cell cycle is longer (90 minutes), and the 15th cycle marks entry into gastrulation, which is a period of mitotic quiescence. (**B**) The features of the (a) fertilized egg, (b) the mid-blastula and (c) early gastrula, which are all viewed in section from the side. (Ba) Sperm entry activates the microtubule polymerization, which drives the rotational movement of the outer shell of cytoplasm (cortical cytoplasm) away from the sperm entry point (black arrow). Maximal rotation away from the sperm entry point occurs at a point at the circumference that subtends a 30° angle from the vegetal pole (red arrow). A dye mark placed on this spot (where ~14 cell cycles later the dorsal lip of the blastopore forms) marks the beginning of gastrulation (red arrow in Bc). Immediately above the dorsal lip is the region of the Spemann Organizer (shown as a blue trapezoid in Ad). (Bb) At the mid-blastula stage, the embryo is described as having three regions, the animal cap, equatorial or marginal zone and vegetal mass, explants of which are dissected along the broken black lines shown.

localize to the germplasm and remain in primordial germ cells, while *VegT* mRNA localizes to presumptive endodermal cells (Stennard et al., 1999). *Vg1* mRNA becomes enriched in the dorsal vegetal quadrant of the early embryo compared with the ventral vegetal quadrant (Birsoy et al., 2006; Tao et al., 2005b). Thus, several distinct mechanisms of partitioning probably exist.

From egg to mid-blastula transition

During the first cell cycle, the movement of the cortical cytoplasm (Fig. 1), has long been known to be essential for establishing the embryonic dorsoventral (DV) axis (Vincent and Gerhart, 1987). Cytoplasmic transfer and ultraviolet (UV) irradiation studies lead to the hypothesis that a vegetally localized 'dorsal determinant' is relocated by cortical rotation (Scharf and Gerhart, 1980; Holwill et al., 1987). Several lines of evidence indicate that the dorsal determinant is a component of a canonical Wnt signaling pathway (Heasman et al., 1994; Kofron et al., 2001). The most likely candidate is *Wnt11* mRNA.

Wnt11 mRNA localizes to the vegetal cortex during oogenesis (Ku and Melton, 1993), and loss-of-function experiments show that maternal Wnt11 is necessary and sufficient for specification of the embryonic DV axis (Tao et al., 2005b). It acts as a canonical Wnt in this regard, as depletion of the transcriptional co-activator of Wnt target genes, β catenin, blocks the dorsalization caused by *Wnt11* mRNA overexpression. Furthermore, β catenin overexpression rescues Wnt11-ventralized embryos (Tao et al., 2005b). In addition, UV-irradiation of the vegetal pole of the fertilized egg causes a reduction in the amount of *Wnt11* mRNA (Schroeder et al., 1999).

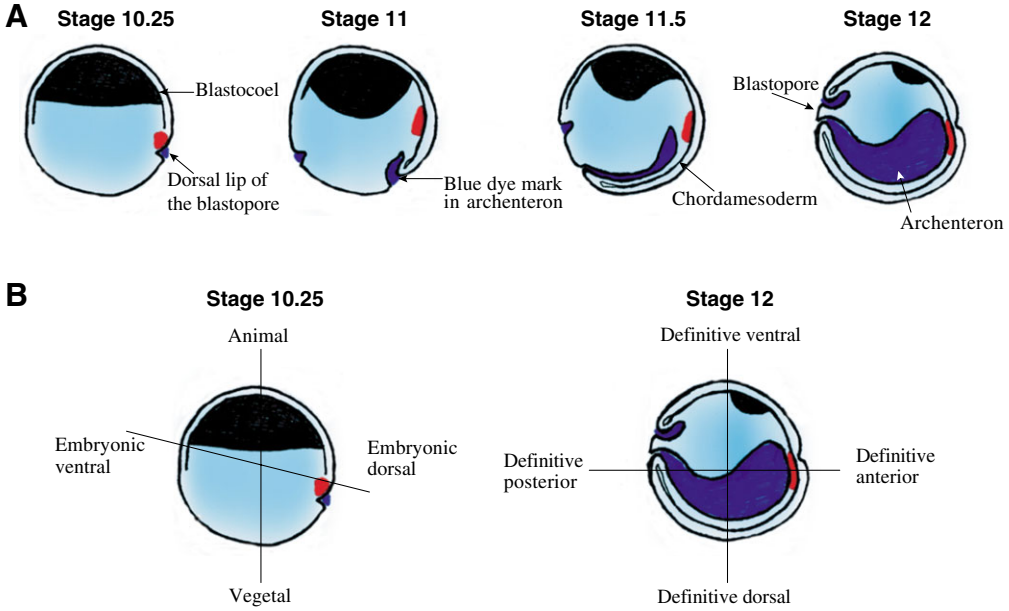

Fig. 2. The relationship of the embryonic dorsal axis to the definitive dorsal axis. (**A**) The cell movements of gastrulation obliterate the blastocoel, enclose a new cavity (the archenteron) and move cells into new positions. A patch of cells (red) in the region of the prechordal mesoderm lie above the dorsal lip of the blastopore at stage 10.25 (early gastrula stage). These cells move animalwards during gastrulation, away from the site of the blastopore lip, along the blastocoel roof to become part of the anterior (head) mesoderm by the end of gastrulation (stage 12). Meanwhile, the convergence extension of the chordamesoderm encloses the vegetal mass, and by the end of gastrulation, the definitive dorsoventral axis can be defined by a line at right-angles to the definitive anteroposterior axis. The obliterating blastocoel (black) lies on the ventral surface and the closing blastopore marks the definitive posterior end. (**B**) A comparison of the embryonic axes, at the early gastrula stage (stage 10.25), and the definitive axes, at the end of gastrulation (stage 12).

However, the movement of cortical *Wnt11* mRNA during the first cell cycle has not been directly visualized. Indirect evidence comes from the greater abundance of both *Wnt11* mRNA and protein on the dorsal side compared with the ventral side of the embryo at the 32-cell stage (Schroeder et al., 1999; Tao et al., 2005b). Once asymmetrically concentrated, and because there is no new synthesis of *Wnt11* mRNA, the translation and secretion of Wnt11 might be enhanced in dorsal vegetal cells compared with ventral vegetal cells. *Wnt11* mRNA may not be the only 'dorsal determinant'. For example, *Vg1* mRNA is also localized and enriched dorsally at the 32-cell stage and is important in early patterning events (Birsoy et al., 2006).

Other components of the Wnt signaling pathway, the intracellular dishevelled protein Xdsh and kinesin-binding protein GBP, also move in cortical cytoplasm. Xdsh-GFP- and GBP-GFP-containing vesicles move with cortical rotation towards the dorsal side of the embryo (Miller et al., 1999; Weaver et al., 2003). GBP depletion causes a loss of dorsal structures (Yost et al., 1998), but Xdsh has not yet been directly shown to be required for dorsal axis formation. Unexpectedly, tagged Xdsh protein is localized in *Xenopus* nuclei, suggesting Xdsh nuclear localization is required for canonical Wnt signaling (Itoh et al., 2005).

Until recently, no maternal factors were known to localize along the embryonic left/right axis. However, the tryptophan derivative, 5 hydroxytryptamine (5-HT or serotonin) was shown to be distributed equally in the vegetal hemisphere at the two-cell stage, but then to accumulate specifically in the daughters of the right ventral blastomere from the four-cell stage onwards, in a gap junction-dependent process (Fukumoto et al., 2005). Inhibition of serotonin signaling with receptor blockers shows that it is required for the later left-sided expression of the nodal-related *Xnr1* mRNA, as well as for correct gut and heart looping. This raises intriguing questions about how serotonin is localized in this fashion and how it interacts with canonical signaling pathways.

After the 90-minute marathon of the first cell cycle, the following eleven division cycles are more rapid (Fig. 1). This is a period of apparent quiescence in terms of cell signaling and transcriptional events. Signaling through the TGFβ and FGF pathway is low until MBT, as shown by immunostaining for activated forms of Smad1, Smad2 and MAP kinase (Faure et al., 2000; Lee et al., 2001; Schohl and Fagotto, 2002). Heterochronic co-culture assays using pre-MBT

Box 1. Defining the axes of *Xenopus* embryos

The first axis of the *Xenopus* embryo is the animal-vegetal axis, which passes through the animally localized egg pronucleus, the center of the egg and the vegetal pole (Fig. 1B, Fig. 2B). The second axis is defined by the sperm-entry point and by the position of maximal movement of the cortical cytoplasm away from the sperm-entry point (Fig. 1B, Fig. 2B). At gastrulation, the dorsal lip of the blastopore forms opposite the sperm-entry point. Although described as 'dorsal', this Spemann Organizer region (Fig. 1A) contains anterior precursors, including prechordal mesoderm (Dale and Slack, 1987; Keller, 1975; Lane and Sheets, 2000; Moody, 2000; Shook et al., 2004).

As the term 'dorsal lip of the blastopore' is established in the literature, it continues to be used here, accepting the fact that the region includes non-dorsal precursors. As shown in Fig. 2, the embryonic dorsoventral axis describes the position of cells relative to the sperm-entry point and to the site of future formation of the dorsal lip blastopore (Fig, 2B). The definitive dorsoventral axis refers to the axis at right angles to the anteroposterior axis, which is established at the end of gastrulation (see Fig. 2B).

and post-MBT vegetal masses with mid-blastula animal caps also show that no mesoderm induction can be detected (using the expression of the somite marker MyoD) from pre-MBT vegetal masses (Wylie et al., 1996). Furthermore, zygotic expression levels for most genes are low or undetectable before MBT.

By contrast, there is accumulating evidence that pre-MBT maternal Wnt signaling occurs. First, Wnt11-induced dorsalization occurs most effectively if the mRNA is introduced into the oocyte rather than the fertilized egg (Tao et al., 2005b), suggesting that it is required soon after fertilization. Second, nuclear localization of β catenin on the dorsal side of the embryo happens before MBT (Larabell et al., 1997; Schneider et al., 1996). Third, depletion of maternal β-catenin protein (Heasman et al., 2000) or activation of a dominant-negative Xtcf3 [the transcription factor activated by maternal β catenin (Yang et al., 2002)] blocks dorsal axis formation at the two- or four-cell stage, but not later. This indicates that the signaling pathway cannot be inactivated by the late cleavage stage. Finally, Xtcf3 activity in pre-MBT embryos is sensitive to the transcription inhibitor actinomycin D, and two of its target genes, the TGFβ nodal-family members *Xnr5* and *Xnr6*, are expressed from the 256-cell stage onwards (Yang et al., 2002). These experiments provide strong circumstantial evidence that the earliest phase of β-catenin/Xtcf3 interaction happens during the cleavage to early blastula stages.

Despite these exceptions, zygotic genes are generally repressed until the 13th cell cycle, and are associated with condensed, hypo-acetylated and H3-methylated chromatin (Meehan et al., 2005). Several recent studies have shed light on the mechanism of the transcriptional activation of zygotic genes. Xkaiso, a transcriptional repressor that binds to specific DNA-binding sequences in a methylation-dependent manner, maintains pre-MBT repression of an estimated 10% of zygotic genes (Ruzov et al., 2004). When Xkaiso is depleted, zygotic transcription starts two cycles earlier than normal (Ruzov et al., 2004). The activation of one Xkaiso target, zygotic *Wnt11*, depends on the binding of the catenin-family member, p120-catenin, which causes Xkaiso to dissociate from the *Wnt11* promoter (Kim et al., 2004).

Maternal Xtcf3 acts in a similar way to Xkaiso, by repressing the expression of Wnt target genes (Brannon et al., 1997; Houston et al., 2002; Roose et al., 1998). New work shows that Xkaiso and Xtcf3 act together to prevent the transcription of the homeobox transcription factor, *siamois* repression (Park et al., 2005). Whether complexes of Xkaiso and Xtcf3 regulate all Xtcf3 target genes is not known. For many zygotic genes, transcriptional activators may also be required. For example, the expression of the nodal-related gene *Xnr5* is repressed ventrally by maternal FoxH1 and Sox3, as well as by β-catenin/Xtcf3, and is activated dorsally by VegT (Hilton et al., 2003; Kofron et al., 2004a; Zhang et al., 2003). In addition, depletion experiments suggest that maternal Xtcf4 acts as an activator of organizer genes, while Xtcf1 has context-dependent activating and repressing roles (Standley et al., 2006).

Another aspect of the re-activation process is the availability of transcriptional co-activators. Until MBT, the transducers of the TGFβ and FGF signaling cascades, Smad1, Smad2 and MAP kinase, are inactive. In addition, a little explored mechanism of regulation of transcriptional activation involves the nuclear matrix, which may be required for the formation of stable transcriptional complexes. Before MBT, transcription factors may be able to bind to DNA but not able to form stable complexes or to recruit the basal transcriptional machinery. When chromatin domains are in an active state, they have a defined, rather than a random, attachment to the nuclear matrix. Activation of the basic helix-loop-helix (bHLH) transcription factor *Myc* has been correlated to specific anchorage sites after MBT, when compared with its random nuclear matrix attachment before MBT (Vassetzky et al., 2000). Whether this mechanism plays a widespread role in transcriptional activation at MBT remains to be resolved.

From MBT to the beginning of gastrulation

As soon as zygotic transcription starts, the instructive events that set up the framework of the three germ layers rapidly become complex. At least four major signaling pathways are essential that activate the signal transducers Smad2, Smad1, β catenin and MAP kinase. Earlier reviews have suggested that gradients of the ligands that activate these pathways (Xnr proteins, activin, Vg1, BMP2, BMP4, BMP7, Wnt11, Wnt8, FGF3, FGF4 and FGF8) pattern the blastula in the embryonic animal-vegetal (AV) or DV axis, but such gradients are hard to demonstrate for endogenous ligands. In addition, the number of potential intracellular and extracellular regulators of these pathways continues to grow, including modulators of transcription, translation, processing, cleavage, co-receptors and antagonists, and of the signal transduction intermediaries, many of which are themselves specifically localized in the embryo. Although gradients may be the outcome of these regulations, the challenge at the moment is to understand the signaling context of each location in the embryo, and the results of such signaling in terms of gene expression and embryonic patterning.

The activin-type TGFβ pathway

VegT is inherited equally by all vegetal cells and that activates the expression of an endodermal-determination network of genes. It also has roles in mesoderm induction and gastrulation (Kofron et al., 1999; Xanthos et al., 2001) (Fig. 3). VegT regulates the transcription

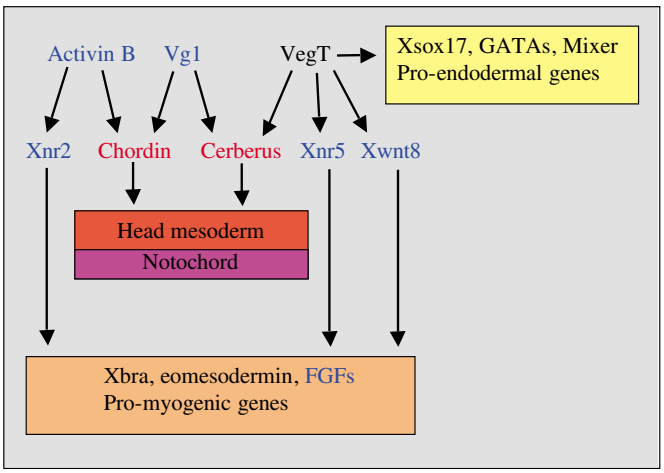

Fig. 3. The endodermal regulatory network. VegT activates the transcription of pro-endodermal transcription factors, including Xsox17, GATA5 and Mixer, and of signaling molecules, including Xnr5, Wnt8 and the signaling antagonist cerberus. Vg1 and activin B also activate signaling pathways that lead to the transcription of the signaling antagonists chordin and cerberus, and the signaling molecule Xnr2. Xnr2, Xnr5 and Wnt8 are important mesoderm-inducing molecules, responsible for the transcriptional activation of pro-myogenic genes *Xbra* and *eomesodermin*, and FGF genes, which can regulate specific myogenic genes such as *MyoD*. Cerberus suppresses Xnr, Wnt and BMP signals extracellularly, and is required for head (prechordal) mesoderm formation, while BMP suppression by chordin is required for notochord formation. Signaling molecules are shown in blue and signaling antagonists in red.

of pro-endodermal transcription factors, including the HMG-box gene *Xsox17*, and GATA factors 4, 5 and 6. It also activates the transcription of genes encoding mesoderm-inducing molecules (such as *Xnr5* and *Wnt8*) and of *cerberus* (the BMP and Wnt antagonist), raising the issue of how the domains of mesodermal and endodermal gene expression downstream of VegT are dictated. One likely regulator is the homeodomain protein Mixer, a target of VegT that induces endodermal (*Xsox17*) gene expression while repressing mesodermal genes (such as those encoding the T-box transcription factor eomesodermin and Fgf8) (Kofron et al., 2004b).

Most endodermal and mesodermal gene expression can be rescued in VegT-depleted embryos by the reintroduction of *Xnr* mRNAs, but not by the reintroduction of FGF or *activin* mRNAs (Kofron et al., 1999; Xanthos et al., 2001). This, as well as many other studies, suggests a pivotal role for Xnr proteins downstream of VegT in mesoderm and endoderm formation (for a review, see Agius et al., 2000).

As well as VegT-target TGFβ proteins, two other TGFβ family members, Vg1 and activin, play essential roles in patterning the gastrula (Fig. 3). For many years, Vg1 function was not clear because the original gene product was poorly translated and processed (Tannahill and Melton, 1989), and did not rescue the Vg1-depleted phenotype (Birsoy et al., 2006). By contrast, a second *Vg1* allele has recently been characterized, called Vg1-ser, which is more efficiently processed than the first allele (Vg1-pro) and does partially rescue the Vg1-depletion phenotype (Birsoy et al., 2006). Consistent with the dorsal enrichment of *Vg1* mRNA, dorsally localized BMP antagonist mRNAs (*chordin*, *cerberus*, *noggin*) are severely depleted in Vg1-depleted embryos, while general endoderm markers are less affected. Smad2-phosphorylation and gastrulation are delayed in Vg1-depleted embryos and they develop microcephaly.

The fact that Vg1 activates the same pathway as the nodal proteins raises the question of why it does not alleviate the phenotype of embryos lacking VegT function. One likely explanation is that, as discussed above, *VegT* mRNA also has a role in the oocyte, maintaining the localization of other maternal mRNAs. Its depletion reduces *Vg1* mRNA and protein, as well as *VegT* (Heasman et al., 2001; Kloc et al., 2005). Thus the original 'VegT phenotype' is likely to be due to the loss of both Vg1 and VegT. New studies are required to determine the specific role of VegT alone, using morpholino oligos, which block VegT protein synthesis but do not degrade *VegT* mRNA, and do not disrupt *Vg1* mRNA localization (Heasman et al., 2001).

The function of activin B also took a long time to clarify. Loss of function studies show it is essential for normal development, and regulates the dorsal zygotic genes, particularly *goosecoid*, *chordin* and the anterior endodermal marker *Xhex*. Unlike Vg1, it regulates the transcription of other TGFβ proteins. In particular, *Xnr2* mRNA expression is increased and the Vg1-related *derriere* mRNA is decreased by the loss of activin function (Piepenburg et al., 2004). Derriere, in turn, regulates the expression of the promesodermal gene *Fgf4* (Sun et al., 1999).

Although the precise roles of all the TGFβ proteins remain to be resolved, what is clear is that, individually or together, the Xnr proteins, derriere, Vg1 and activin activate several signal transduction cascades during the mid-late blastula stages, leading to the transcription of many zygotic genes. First, they cause the phosphorylation of Smad2 in receiving cells. Phospho-Smad2 acts as a co-activator of many transcription factors, including the maternal cell cycle regulator transcription factor, p53 (Cordenonsi et al., 2003), the transcriptional activator and repressor FoxH1 (Kofron et al., 2004a), and the VegT target homeodomain transcription factor Mixer (Kofron et al., 2004b), all of which are essential for early embryonic patterning. Second, they activate TGFβ-activated kinase 1 (TAK1), which in turn activates [through nemo-like kinase (NLK)] another essential transcription factor, signal transducer and activator of transcription (STAT3) (Ohkawara et al., 2004). Third, Xnr proteins induce the expression of FGF3, FGF4 and FGF8, which bind FGF receptors and activate several transcription factors, including activator protein 1 (AP1).

The FGF signaling pathway

Although some FGF mRNAs are expressed maternally, there are no known maternal transcripts that localize to the equatorial zone of the oocyte. The earliest equatorial-specific factors appear at the late blastula stage and include zygotic T-box genes, *brachyury* (*Xbra*), *eomesodermin* and *antipodean* (Ryan et al., 1996; Smith et al., 1991; Stennard et al., 1996). Their expression is dependent on both Xnr signaling (Xanthos et al., 2002; Xanthos et al., 2001), and the maternal Wnt pathway (Vonica and Gumbiner, 2002). *eomesodermin* is expressed first and is enriched on the dorsal side, and engrailed-repressor experiments suggest it regulates FGF and *Xbra* expression, which then act in cross-regulatory loops (Ryan et al., 1996). The boundaries of the expression domains of the T-box genes are constrained by several animally localized regulators (see below), and by Mixer vegetally. In agreement with this, MAP kinase immunostaining shows that high FGF signaling is restricted to the equatorial region at this time (Schohl and Fagotto, 2002), and FGF loss of function causes reduced somites and notochord and defects in convergence extension movements (see below) (Amaya et al., 1991; Conlon et al., 1996; Fisher et al., 2002).

The Wnt signaling pathway

The third major influence in the mid-blastula is the maternal Wnt signaling pathway. Without its activity, the embryo develops with three layers, but lacks dorsal, anterior or posterior structures. What information does this signal provide? Because of the enrichment of Wnt11 and its dorsal secretion, the expression of *siamois*, *goosecoid*, *Xhex*, *Xnr3*, and of the signaling antagonists *noggin*, *chordin* and *cerberus* is specifically localized. These proteins regulate head, notochord and somite formation (see below). In addition, *chordin*, *noggin* and *siamois* are expressed in the embryonic dorsal animal cap, as well as the marginal zone, and this expression is essential for anterior neural induction (Kuroda et al., 2004).

Although the Wnt signal is required for their expression, each zygotic gene is regulated differently, by multiple factors. For example, *cerberus*, is directly regulated by at least four transcription factors: Xlim1, a target of VegT activation; the orthodenticle-related protein Otx1; and homeodomain proteins Siamois and Mix1 (Yamamoto et al., 2003). Such combinatorial regulation may explain why the Wnt target genes are not expressed in identical locations. For example, *Xnr5* is expressed in dorsal vegetal cells and *Xnr3* is expressed above the dorsal lip of the blastopore. Alternatively, more than one maternal Wnt signal may regulate their expression.

The BMP signaling pathway

The BMP signaling pathway is initially activated at MBT throughout the mid-blastula, downstream of maternal BMP2 and BMP7 activity, except in the embryonic dorsal animal quadrant (Faure et al., 2000; Schohl and Fagotto, 2002). The restriction of BMP signaling from this quadrant may be the result of the early expression of the BMP antagonists *noggin* or *chordin* (Kuroda et al., 2004). Animal cap regions explanted from mid-blastulae follow

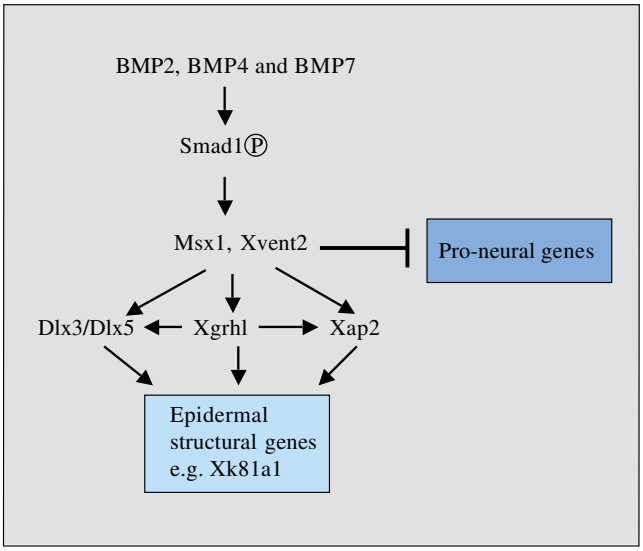

Fig. 4. The epidermal regulatory network. BMP signaling activates target genes directly, including *Xvent2* and *Msx1* (which activate the epidermal pathway and suppress neural fates when ectopically overexpressed). Xvent2 and Msx1 regulate the more-restricted pro-epidermal genes *Xap2*, *Dlx3* and *Xgrhl1*, which can directly regulate epidermal structural genes such as the cytokeratin gene *Xk81.1a*, but which do not have neural-inhibiting functions.

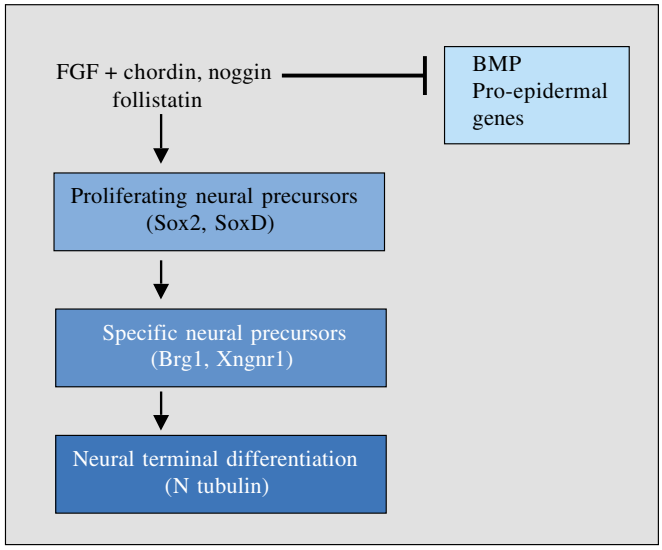

Fig. 5. The neural regulatory network. Animal cells are directed towards a neural fate beginning at the late blastula stage and continuing through gastrulation. Neural induction requires both suppression of the BMP-Smad1-Msx pathway by the BMP antagonists chordin, noggin and follistatin, and the activation of FGF signaling. Cells begin to express proneural genes (*Sox2*, *SoxD*) at the gastrula stage. By the end of gastrulation, the deeper layer of the pro-neural ectoderm activates specific neuronal precursor markers, including Xngnr1, which in turn regulates primary neuronal differentiation via XneuroD and N-tubulin. Primary neurogenesis requires the function of the maternal chromatin remodeling protein Brg1.

an epidermal differentiation pathway that is dictated by BMP signaling (Fig. 4). When all BMPs and the dorsally expressed BMP-like molecule anti-dorsalizing morphogenetic protein (ADMP) are depleted, the entire outer layer of the embryo expresses neural markers (Reversade and DeRobertis, 2005). What then causes neural specification?

An old idea was that no specific signal activates neural fates; that it is the 'default state', as disaggregated animal cells express neural markers (Godsave and Durston, 1997). However, it has recently been shown that cell dissociation actually activates FGF signaling and inhibits Smad1 by MAP kinase phosphorylation of its linker region (Kuroda et al., 2005). This raises the question, 'is FGF the activator of neural specification, or does it act solely as a BMP signaling antagonist?'. Definitive evidence that FGF has proneural roles would be obtained by identifying specific FGF transcriptional targets required for neurogenesis. Recent in vivo studies suggest that this is the case; the proneural genes *Sox2* and neural cell-adhesion molecule (*Ncam*) expression depend on low levels of FGF signaling at the blastula stage, independently of BMP antagonism (Delaune et al., 2005).

The epidermal regulatory network downstream of BMP signaling includes the transcriptional activators *Xvent2* (Onichtchouk et al., 1996) and *Msx1* (Suzuki et al., 1997), which activate the epidermal pathway and also suppress pro-neural genes when ectopically overexpressed. These genes in turn activate more restricted pro-epidermal genes, which can directly regulate epidermal structural genes, but do not have neural repressive roles (Tao et al., 2005a), (Fig. 4). Thus, epidermal fate is determined by BMP signaling, while neural specification may require FGF signaling and BMP antagonism (Fig. 5).

The classical animal cap assay illustrates how easily mid-blastula animal cells can be diverted to mesodermal or endodermal fates by added growth factors. Moreover, several Xnr proteins have been shown to have long signaling ranges (White et al., 2002; Williams et al., 2004). But even after suppression of BMP signaling, or after suppressing both BMP signals and their antagonists, animal cells express neural, rather than mesodermal, markers (Reversade and De Robertis, 2005). So what prevents animal cells from undergoing mesoderm induction?

Recently, several intrinsic mesoderm antagonism mechanisms have been identified. One essential maternal regulator is the RING-type ubiquitin ligase, ectodermin, which regulates Smad4 degradation (Dupont et al., 2005). As Smad4 heterodimerizes with both Smad1 and Smad2, its degradation reduces both BMP and nodal-type TGFβ signaling. The field of influence of ectodermin is dictated by its localized pattern of expression in the animal half of the oocyte and blastula, and its depletion causes the ectopic expression of the mesodermal gene *eomesodermin* and the expanded expression of the endodermal gene *Mixer* into the animal hemisphere. The neural marker *Xsox2* is also downregulated by ectodermin depletion. Thus, the animal localization of ectodermin dictates the lower margin of the ectoderm precursor region and favors neural specification. *ectodermin* expression becomes asymmetrically enriched in the embryonic dorsal animal quadrant at the gastrula stage, where the abrogation of Xnr and BMP signaling is required for neural specification (Dupont et al., 2005).

Animally localized maternal and zygotic transcription factors also regulate the boundary between pro-ectodermal and mesodermal areas. The depletion of the maternal, animally localized, *Zic2*

transcript results in increased Xnr expression (Houston and Wylie, 2005), while the *Xenopus* X-box binding protein 1 (*Xbp1*) regulates BMP4 expression and suppresses mesodermal and neural gene expression (Cao et al., 2006). In addition, the Forkhead-family member Foxi1E (also known as Xema) activates epidermal differentiation and represses endodermal and mesodermal gene expression in animal cap cells (Suri et al., 2005).

BMP signaling occurs in the ventral equatorial zone, as well as in the animal cap, at the blastula stage. What then are the states of determination of equatorial cells when BMP function is blocked? A key analysis here has been to manipulate BMP signaling in a temperature-sensitive manner (Marom et al., 2005). BMP suppression at the blastula stage causes upregulation of organizer genes (*goosecoid*), causing secondary axis (neural and somite tissue) formation. This indicates that a major, organizer-suppressing role of the BMP pathway occurs early at the blastula stage.

Thus, at the late blastula stage, the animal cap is already divided into areas of BMP signaling and BMP antagonism, the equatorial zone is a site of dynamic interactions of all four pathways, and the vegetal mass has high Smad1 and Smad2 activity and low Wnt and FGF signaling. During gastrulation, the results of these interactions begin to be realized.

Gastrulation

The timing of gastrulation

During gastrulation, the cell cycle expands from 55 minutes to 4 hours, a lengthening that is essential for further development (Fig. 1). The control of cytostasis is not understood, although TGFβ signaling is likely to be involved as it is known to limit re-entry into the cell cycle (Siegel and Massague, 2003) and is necessary downstream of VegT for gastrulation to occur (Zhang et al., 1998). WEE1, an antagonist of M-phase re-entry, is clearly required because its depletion causes an increased mitotic index from 10% to 25% during gastrulation and results in abnormalities in gastrulation movements. Zygotic gene expression continues, although the positioning of *Xbra* and *chordin* expression is disrupted, which may be crucial for correct cell movements (see below) (Murakami et al., 2004). WEE1 may regulate the mitotic activity of bottle cells, the shape changes of which in response to Xnr/Vg1/activin are responsible for the first invagination movements of gastrulation. These cells are the earliest non-mitotic population at gastrulation, and promoting mitosis arrests bottle cell formation (Kurth, 2005). WEE1 is a maternal protein, which may explain a long-standing observation that the timing of MBT and gastrulation onset are not linked (Smith and Howard, 1992). The timing of gastrulation is also dependent on several other maternal inputs. Abrogating either the maternal Wnt or Vg1 pathway delays formation of the dorsal lip (Birsoy et al., 2006; Heasman et al., 1994), and maternal CREB depletion slows ventral lip formation (Sundaram et al., 2003).

Events in the embryonic dorsal mid-line

Blastopore invagination resulting from bottle cell formation is the first external sign that gastrulation is under way. Internally, gastrulation movements are also beginning. Initial movement occurs not in the marginal zone but in the vegetal mass, which undergoes an active inward surging in the animal direction, causing an increase in the blastocoel floor area (Winklbauer and Schurfeld, 1999), and driving firstly the involution of the prechordal (head) mesoderm, followed by chordamesoderm (presumptive notochord) (Fig. 6). The fact that these two domains can be physically separated suggests that AP patterning in the dorsal mesoderm is established by this time. After involution, prechordal mesoderm cells become actively migratory and move animally (Shook et al., 2004). This behavior is regulated by multiple factors but the definitive endogenous combination is not yet known.

One secreted protein known to be involved in chordamesoderm cell behavior is platelet-derived growth factor, PDGFA, which is secreted by blastocoel roof cells. PDGFA depletion causes random protrusive activity of prechordal mesoderm and loss of head structures (Nagel et al., 2004). A second regulator of prechordal mesoderm is the Wnt antagonist dickkopf (Kazanskaya et al., 2000). Inhibition of its activity results in microcephaly, while overexpression of dickkopf expands the size of the prechordal plate

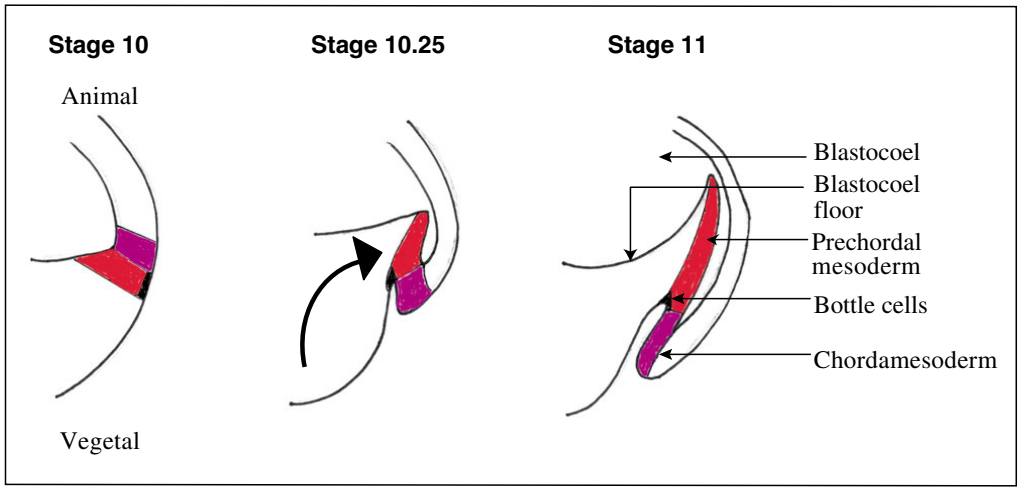

Fig. 6. Cell movements at the dorsal lip of the blastopore during early gastrulation. During the first 2 hours of gastrulation, the vegetal mass undergoes an active inward surging (black arrow) in the animal direction, such that the animal part of the vegetal mass expands, increasing the area of the blastocoel floor, while the vegetal part contracts. This movement drives the first phase of involution of the prechordal mesoderm (red). After involution, prechordal mesoderm cells become actively migratory and migrate animally. Their movement is stopped by mid-gastrula stage by their firm adhesion to the substrate, the proneural animal cap cells. The chordamesoderm (purple) then undergoes convergence extension movements to cover the vegetal mass and close the blastopore. The bottle cells (black) indicate the site of dorsal lip formation.

at the neurula stage, without affecting chordamesoderm formation. An important issue here is which Wnt signal is being antagonized by dickkopf. Third, the migrating prechordal mesoderm zone is an area of active repression of *Xbra* expression. Repression is achieved by several inputs, including the binding of the transcriptional repressor goosecoid to the *Xbra* promoter (Yao and Kessler, 2001). *Xbra* overexpression in the prechordal mesoderm prevents cell adhesion to the extracellular matrix protein fibronectin (Kwan and Kirschner, 2003). As with PDGFA depletion, abrogation of goosecoid activity or of its transcriptional activator *siamois* blocks prechordal migration, presumably by increasing *Xbra* expression. Last, the size, shape and correct placement of the entire involuted region is regulated by nodal, BMP and Wnt antagonists, particularly by the TGFβ family member antivin/lefty (Branford and Yost, 2002). Antivin-depleted embryos have increased and expanded *Gsc*/*Xnr3* and *Xbra* expression, exogastrulate at the mid-gastrula stage and fail to form heads (Branford and Yost, 2002).

The period of prechordal mesoderm migration is brief and is limited by the direct adhesion of the prechordal mesoderm to the head neuroectoderm (Koide et al., 2002) (Fig. 2), so the major force in extending the AP axis in gastrulation is the involution and convergence extension of the chordamesoderm (Figs 2 and 6). Many factors regulate this convergence extension, as described in Box 2. In summary, many essential early zygotic patterning genes are not tissue differentiation genes, but are involved in regulating cell behavior. For example, loss of *dickkopf*, *siamois* or *goosecoid* activity abrogates prechordal mesoderm migration and prevents the formation of head structures, while *Xbra* is required for convergence extension of the chordamesoderm.

Events in the non-organizer marginal zone

Meanwhile, away from the organizer region, convergence extension movements are delayed. Several pathways are involved in establishing muscle precursor fates. First, FGF signaling and *Xbra* expression are required and maintain each others expression, as described above (Fig. 7). The expression pattern of *Xbra* mRNA, which forms a ring around the blastopore of the gastrula, is evidence that its role is not confined to dorsal convergence extension activity. Promoter studies show that dorsal and ventrolateral *Xbra* expression are differently regulated (Latinkic and Smith, 1999; Lerchner et al., 2000; Papin et al., 2002). By mid-gastrulation the myogenic markers *Mespo*, *Myf5* and *MyoD* mRNA are all expressed in an equatorial ring similar to *Xbra* and FGF, and depletion of FGF4, FGFR1 or Xbra causes severe reduction in their expression (Conlon et al., 1996; Fisher et al., 2002; Yokota et al., 2003).

How does *Xbra* function in both myogenic and convergence extension regulation? The expression of one target gene, the cytoplasmic regulator *sprouty*, may be particularly important in this regard. Sprouty is a cytoplasmic antagonist of FGF signaling that blocks convergence extension movements by interfering with protein kinase C (PKC) function, without blocking activation of myogenic gene expression. It can thus separate the convergence extension and muscle specification functions of *Xbra* (Sivak et al., 2005). A second receptor tyrosine kinase regulator, Spred, has the opposite effect; it is required for somite specification but not for convergence extension movements (Sivak et al., 2005). Muscle specification during gastrulation also depends on the repression of BMP signaling, as triple depletions of noggin, chordin and follistatin eliminate muscle precursor gene expression (Khokha et al., 2005).

Box 2. Factors regulating chordamesoderm formation

Convergence extension (CE) movements of the chordamesoderm close the blastopore during gastrulation (see Fig. 2A and Fig. 6). Several factors have essential roles in this process:

(1) The establishment of two domains in the chordamesoderm, marked anteriorly by *chordin* and posteriorly by *Xbra* expression. Mixing *chordin*-and *Xbra*-expressing cells prevents convergence extension until the two populations are sorted (Ninomiya et al., 2004).

(2) Xbra and FGFR1 activity. Their abrogation causes CE defects and reduces notochord and somite formation, illustrating the importance of the FGF/Xbra loop in gastrulation movements (Amaya et al., 1991; Conlon et al., 1996).

(3) Zygotic *Wnt11*mRNA expression, which is regulated by Xbra (Tada and Smith, 2000). Wnt11 causes Xdsh localization at the cell cortex in a PAR1/PKC dependent fashion (Habas et al., 2003; Kusakabe and Nishida, 2004; Tada and Smith, 2000) and also mobilizes the cytoskeleton by activating Rac and RhoA (Habas et al., 2003; Habas et al., 2001).

(4) The Wnt target gene *Xnr3*, which activates the dorsal *Xbra* expression domain (Yokota et al., 2003). *Xnr3* underexpression causes CE defects and head reduction, indicating that it has an additional role in prechordal mesoderm migration/specification.

(5) Xlim1, a VegT-target LIM-domain transcription factor specifically expressed in the organizer. Its depletion causes head reduction and CE defects (Hukriede et al., 2003). Xlim1 regulates the dorsal paraxial protocadherin expression, which is required for dorsal RhoA and Jun kinase activation and for CE movements. Xlim1-depleted embryos still make posterior notochord, indicating that the anterior chordamesoderm is Xlim1 dependent, while posterior chordamesoderm may be regulated by Xbra/Wnt11/Dsh.

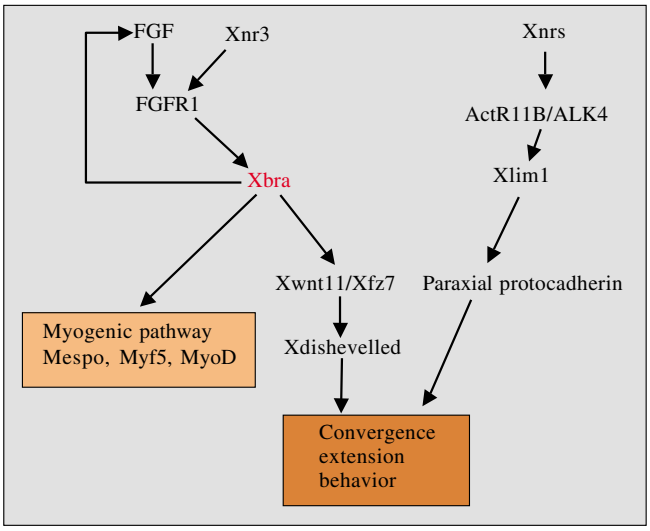

Fig. 7. The central role of Xbra in convergence extension and somite formation. FGF activates pathways through FGFR1 and Xbra, leading to convergence extension movements and myogenic specification. Xnr3 also activates FGFR-dependent *Xbra* expression, but only in the embryonic dorsal area. Xbra causes convergence extension movements by activating the expression of zygotic *Wnt11*. Wnt11 signaling causes convergence extension in a dishevelled-dependent manner that does not involve β-catenin, the so-called 'non-canonical Wnt pathway'. A separate pathway regulating convergence extension involves the activation of paraxial protocadherin downstream of Xlim1. The cytoplasmic kinase regulator genes *sprouty* and *spred* regulate whether cells undergo convergence extension or somite formation in response to FGFR stimulation (see text for details).

An essential aspect of mesoderm patterning is the interaction of several pathways in the initiation of Hox gene expression during gastrulation and neurulation. The AP patterning in the definitive trunk region results from the expression of nine co-linear Hox genes, which are expressed in a specific temporal order in the non-organizer mesoderm, beginning at the early gastrula stage with *HoxD1* expression (Wacker et al., 2004a). The early Hox expression determines the length of the trunk region, and depletion of HoxA1, HoxB1 and HoxD1 results in reduced *MyoD* expression (Wacker et al., 2004a; Wacker et al., 2004b). Hox expression depends on the FGF/Xbra pathway (Fig. 7), and is limited by BMP signaling (Wacker et al., 2004b). Another important mesodermal regulator is retinoic acid (RA). Depletion of retinoic acid receptor Xrarα2 causes both microcephaly and tailless embryos, and reduces expression of both FGF receptors FGFR1 and FGFR4, and posterior Hox gene expression (HoxB9) (Shiotsugu et al., 2004). The *Xenopus* caudal family member *Xcad3* is essential for tail somite formation, and its transcriptional regulation is complex and involves maternal CREB, FGF signaling and RA activity (Isaacs et al., 1998; Shiotsugu et al., 2004; Sundaram et al., 2003).

Unexpectedly, a key BMP antagonist, the frizzled-related protein sizzled, is expressed not dorsally, but in the ventral lip of the blastopore. Sizzled acts as a competitive inhibitor of the chordin metalloproteinases, Xlr1 and BMP1 (Lee et al., 2006). Its expression is activated by BMP signaling and is repressed by VegT/Vg1 via the BMP antagonists (Birsoy et al., 2006; Lee et al., 2006; Salic et al., 1997). Depletion of sizzled causes an expansion of ventral blood islands and does not affect the expression of *MyoD* or of organizer genes. It has an essential role in regulating epidermal versus neural cell fates (Lee et al., 2006).

Events in the animal cap

While these complex events are occurring in mesodermal precursors, cells in the animal cap remain set to become epidermis, as dictated by continuing BMP signaling, providing that they are not underlain by involuted prechordal mesoderm or chordamesoderm. Switching from epidermal to proneural fate absolutely requires BMP suppression, as described previously (Khokha et al., 2005); the entire ectoderm becomes neural when all BMP signaling is depleted (Reversade et al., 2005; Reversade and De Robertis, 2005). The epidermis is a bilayer at the gastrula stage, and both layers overlying the chordameosderm express proneural genes, although only the inner layer undergoes primary neuronal differentiation at the neurula stage (Chalmers et al., 2002) (Fig. 5).

It is clear that neural specification, like that of muscle, is an active process, involving a complex network of transcription factors (Fig. 5). Transcription factors of the Sox class are expressed in ectoderm from the late blastula stage, and dominant-negative Sox2 inhibits neural induction when it is expressed specifically during the gastrula stage (Kishi et al., 2000). SoxD and Smad interacting protein 1, SIP1, a member of the EF1/ZFH family, maintain expression of each other after the gastrula stage. Loss-of-function experiments show that they are necessary for neural induction, with SIP1 preventing the activation of pro-epidermal genes (Nitta et al., 2004). The three paralogous Hox group 1 genes, *HoxA1*, *HoxB1* and *HoxD1*, are essential for neural patterning and begin to be expressed at the gastrula stage; their simultaneous depletion has severe effects on hindbrain and neural crest patterning (McNulty et al., 2005). FGF8 and RA are the most likely candidate activators of Hox gene expression (Christen and Slack, 1997; Shiotsugu et al., 2004). Cell cycle withdrawal is also important for further neural differentiation. Depletion of the maternally expressed SWI/SNF remodeling protein BRG1 causes the proliferation and expansion of proneural gene expression (*Sox2*), but reduces neural differentiation. BRG1 also directly binds to and co-activates several bHLH transcription factors in the differentiation pathway (Seo et al., 2005).

As gastrulation proceeds, anterior- and posterior-specific neural genes begin to be expressed in the neural anlagen, a process that depends on the anterior repression of Wnt signaling via both intrinsic and extrinsic pathways. An important regulator of neural AP patterning is the zinc-finger protein Xsalf, which is activated in the anterior neural ectoderm in the late gastrula. Xsalf both activates the expression of anterior neural genes (*Otx1*) and represses posteriorizing Wnt signals by activating the expression of Wnt repressors *Xtcf3* and *GSK3β*, so that Xsalf-depleted embryos have reduced heads and lack forebrain gene expression (Onai et al., 2004). The picture that emerges is different from the original activation/transformation model of neural induction, which suggested that the activation step turns the entire neural anlagen into a pro-anterior neural state, which is later transformed by a posteriorizing gradient. The activation/transformation model predicts that Xsalf would be expressed throughout the neural ectoderm, which it is not. This lends support to an alternative, regional activation model, as described further elsewhere (Onai et al., 2004).

Events in the vegetal mass

The vegetal mass, the cells of which are determined towards endodermal fates by the early gastrula stage (Heasman et al., 1984), is the least studied region of the gastrula. Immunostaining of the vegetal mass identifies the major signaling activities as those continuing from the blastula stages: TGFβ proteins that activate Smad2 and Wnt signaling (Faure et al., 2000; Schohl and Fagotto, 2002). It remains to be shown whether the nuclear β-catenin present at this time is the result of maternal Wnt11 signaling or of new zygotic Wnt pathways. FGF signaling is very low in this region, consistent with the fact that overexpressed FGF causes reduced endoderm formation, and blocking FGF signaling expands the expression of endodermal genes into the equatorial zone (Cha et al., 2004). BMP signaling activity is almost completely excluded from the dorsal vegetal area, suggesting its antagonism is necessary for endoderm specification. In agreement with this, chordin and noggin treatment of animal caps causes ectopic endodermal gene expression (Sasai et al., 1996).

Gene expression in the gastrula stage is dictated by the four pathways that are activated in the blastula vegetal mass. These pathways can be placed into three groups; those involved in boundary formation between mesoderm and endoderm [such as the Bix and Mix homebox transcription factors (Casey et al., 1999; Kofron et al., 2004b), which are expressed during gastrulation only]; a dorsally localized group whose expression domain includes the dorsal prechordal mesoderm (often described as anterior mesendodermal genes), including *cerberus*, *Xlim1*, *dickkopf* and *Xhex,* the main function of which may be in establishing correct gastrulation movements; and those that are distributed throughout the vegetal mass (*Xsox17*, *Gata4*, *Gata5* and *Gata6*). This last group of transcription factors continues to establish regulatory networks required for endoderm specification and maintenance after gastrulation (Afouda et al., 2005; Xanthos et al., 2002; Xanthos et al., 2001). A new player on the signaling scene in the presumptive endoderm is the short-range signal receptor Notch. Notch suppression leads to the expansion of mesodermal molecular markers and to the loss of endodermal markers, endodermin and the HMG box transcription factor Xsox17 (Contakos et al., 2005).

The timing and order of patterning events in the endoderm, from the gastrula stage onwards, is not clear. Although early explant studies showed that AP patterning is autonomous to the endoderm (Gamer and Wright, 1995), this has not been supported by more recent work, which suggests that mesodermal signals as late as the tailbud stage are necessary to specify foregut versus hindgut fates (Horb and Slack, 2001). It is nevertheless the case that blocking the establishment of the embryonic dorsal axis by UV treatment of the fertilized egg causes a loss of expression of the late anterior gut marker *Pdx1*, as well as of early gastrula dorsal endoderm markers, indicating that the early regionalization of the endoderm foreshadows later events (Henry et al., 1996). Future studies will determine the extent to which endoderm patterning and shape changes are regulated by the same signaling networks and their antagonists that operate in the mesoderm and ectoderm.

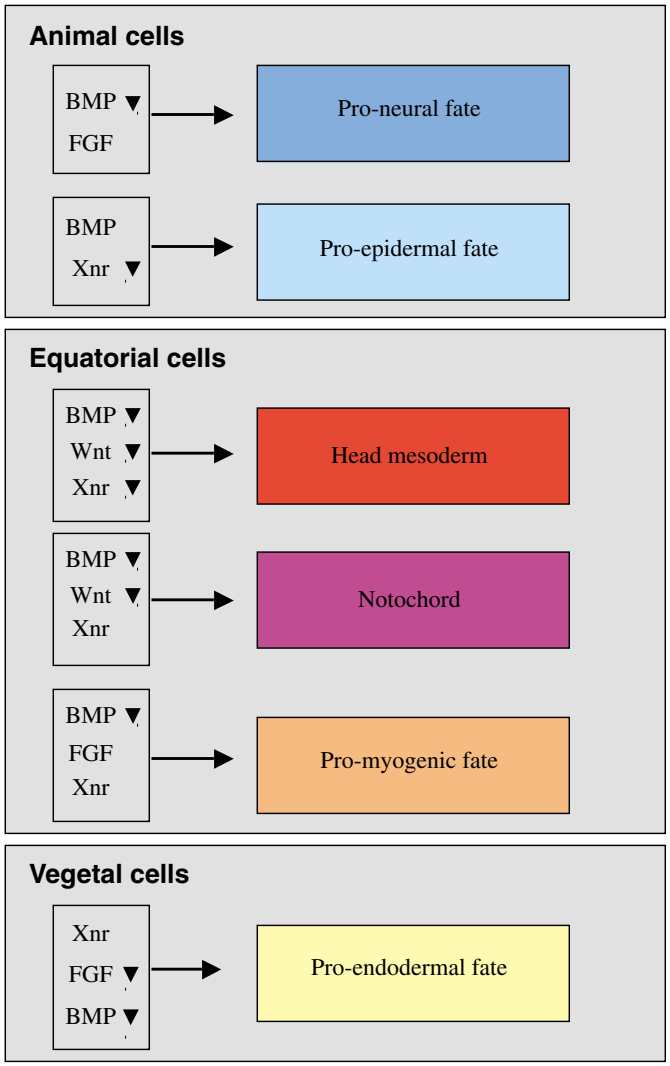

Fig. 8. Signaling combinations that influence cell fate in the early *Xenopus* embryo. Mid-blastula cells are pluripotent, and many factors determine the fate of their progeny. Of major importance in regulating the expression of proneural, myogenic, endodermal and epidermal transcription factors, are the BMP, Xnr (+activin+Vg1), FGF and Wnt signaling pathways. As described in the text, loss-of-function experiments support the combinations of signals shown here as being crucial for ectodermal (neural and epidermis), mesodermal (head mesoderm, notochord, somite) and endodermal fates, at the late blastula and gastrula stages. The specification of blood islands, heart, intermediate and lateral plate mesoderm are not considered in this review. Arrowhead indicates required repression of the activity of the ligand.

Summary

Important advances have been made in our understanding of the events that pattern the early *Xenopus* embryo. We appreciate the central importance of maternal regulators in this process and recognize the crucial roles of antagonists, such as transcriptional and cell cycle repressors, and a surprising number of signaling inhibitors. We are perceptibly nearer to understanding the essential roles of the four signaling pathways in ectoderm, mesoderm and endoderm specification (Fig. 8), and the mechanism of gastrulation. Nevertheless, early development continues to surprise. Outstanding mysteries include: the mechanism by which *VegT* mRNA carries out an architectural role in mRNA localization during oogenesis; the control of vegetal rotation movements in the endoderm; and how Wnt11 can have both canonical and non-canonical roles. And what would be the state of determination of gastrula cells in which BMP, Xnr, Wnt and FGF signaling was prevented?

The next phase of investigation involves an embarrassment of riches. The *Xenopus* Genome Initiative and array technology mean that we will all have long lists of target genes to study. Mutagenesis studies in *Xenopus tropicalis* (Grammer et al., 2005) and easy transgenesis methods (Pan et al., 2006) will provide us with a new wave of phenotypes to analyze. Some reassurance that it will be possible to place the new knowledge of gene function into correct regulatory networks comes from the fact that we can validate our findings by rescue experiments. Furthermore, basic principles are emerging that show a similarity of process among all the germ layers. Examples include the regulation of AP patterning by Hox genes, repression of cell division preceding cell movement and differentiation, and the recurring theme of global transcriptional repression and localized activation. And, if we finally master gastrulation, the next challenge will be organogenesis.

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