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Spatial and temporal control of NODAL signaling

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Embryonic development is orchestrated by the activity of signal transduction pathways, amongst which are those downstream of the transforming growth factor β (TGF-β) family. Here I focus on signalling by one of these ligands, NODAL, which is essential for early embryonic axis patterning. I review recent advances in our understanding of how NODAL signalling is transduced from the plasma membrane to the nucleus to regulate the transcription of target genes, and how domains of NODAL activity are established and refined during embryonic development. The duration of signalling is emerging as a key determinant of the specificity of downstream responses in terms of cell fate decisions and I will discuss what is currently known about the underlying mechanisms.

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Introduction

Cell communication, mediated by signal transduction pathways, is fundamental to the exquisite patterning of embryos that is reproducibly achieved, with tissues of the appropriate size developing in the right place at the correct time. The concept of morphogens, ligands that diffuse from a localised source into the surrounding tissue, providing positional information in a dose-dependent manner, has established a framework for understanding these issues. A handful of signalling pathways activated by these ligands are involved in embryonic patterning, amongst which are those downstream of the transforming growth factor β (TGF-β) family of growth and differentiation factors [1]. Here, I will focus on signalling by one of these family members, NODAL. The last few years has seen some important new insights into the mechanism of NODAL signalling, and a new understanding of how domains of NODAL signalling evolve in space and time during embryonic development. In turn, novel concepts in NODAL signalling are beginning to explain how one signalling pathway can have qualitatively different outputs depending on duration and strength of signalling, which is shedding new light on the physiological responses downstream of NODAL.

A brief overview of NODAL signalling and function

NODAL is found in all deuterostomes, where it is essential for early axis patterning [2,3]. In fact in *Xenopus*, zebrafish and sea urchins, a gradient of NODAL signalling, counteracted by a gradient of bone morphogenetic protein (BMP) signalling (another TGF-β family member), is sufficient to organise a complete embryonic axis [3–5]. NODAL was originally thought to be restricted to deuterosomes, but recently was shown to be expressed in hydra, where it is essential for establishing axial asymmetry along the main body axis [6] and in snails where it is involved in specifying left–right asymmetry [7]. In vertebrates, the major functional roles of NODAL are in mesoderm and endoderm specification and in controlling left–right asymmetry [8,9]. In addition, in pre-implantation mouse embryos, NODAL is also required for maintaining expression of genes encoding the determinants of pluripotency, such as *Oct4* (also known as *Pou5f1*) and *Nanog* [10]. NODAL is not normally expressed in adult tissues, with the exception of organs that undergo widespread remodelling, such as the placenta, endometrium and lactating mammary gland [11]. However, it has been reported to be re-expressed in cancer in aggressive tumours, where it may be required to drive cancer stem cell self-renewal and to promote a less differentiated and more plastic phenotype [11].

As with all TGF-β family members, NODAL is expressed as a precursor, with a long N-terminal prodomain. The mature domain is cleaved from the prodomain by proteases of the proprotein convertase family, in particular, FURIN and PCSK6 (also known as Pace4) [12,13]. For most TGF-β family members, this is thought to occur intracellularly, but for NODAL there is some evidence that it might occur extracellularly [13]. Evidence is also emerging that NODAL may form heterodimers with other TGF-β family ligands, in particular, with GDF1, which potentiates NODAL's activity [14,15]. In common with all other TGF-β family members, NODAL binds a complex of serine/threonone kinase receptors comprising a type II and a type I, which localise to the lateral surface in polarised cells [16]. The NODAL type II receptors are thought to be ACVR2A and ACVR2B (also called ACTRII and ACTRIIB) [17], although a recent report showed that NODAL can

bind BMPR2 with high affinity [18]. The type I receptors recognised by NODAL are ACVR1B and ACVR1C (also called ALK4 and ALK7) [17]. In addition, a GPI-linked co-receptor of the EGF-CFC family, TDGF1 or CFC1 (previously known as Cripto and Cryptic respectively), is essential for NODAL signalling [17] (Figure 1).

The best characterised signalling pathway downstream of the NODAL receptors is the SMAD pathway [19] (Figure 1). In the receptor complex, the type II receptor phosphorylates and activates the type I receptor, which then phosphorylates two receptor-regulated SMADs (R-SMADs), SMAD2 and SMAD3 on two serines at their extreme C-terminus. Once phosphorylated, SMAD2 and SMAD3 form complexes with SMAD4 that accumulate in the nucleus and regulate the transcription of target genes. Although SMAD3–SMAD4 complexes are capable of directly binding DNA, SMAD2–SMAD4 complexes are unable to bind DNA alone, and hence require other transcription factors (TFs) to recruit them to DNA [20]. NODAL signalling is regulated extracellularly by ligand antagonists, LEFTY1 and LEFTY2, which are themselves members of the TGF-β family, and also two members of the DAN family, CER1 (also called Cerberus) and DAND5 (also called Coco) [17]. The DAN family antagonists are thought to function by sequestering ligand [18,21]. LEFTY1 and LEFTY2 have also been shown to bind NODAL, but in addition bind TDGF1/CFC1, suggesting that they may function predominantly by interferring with the activity of these co-receptors [12] (Figure 1).

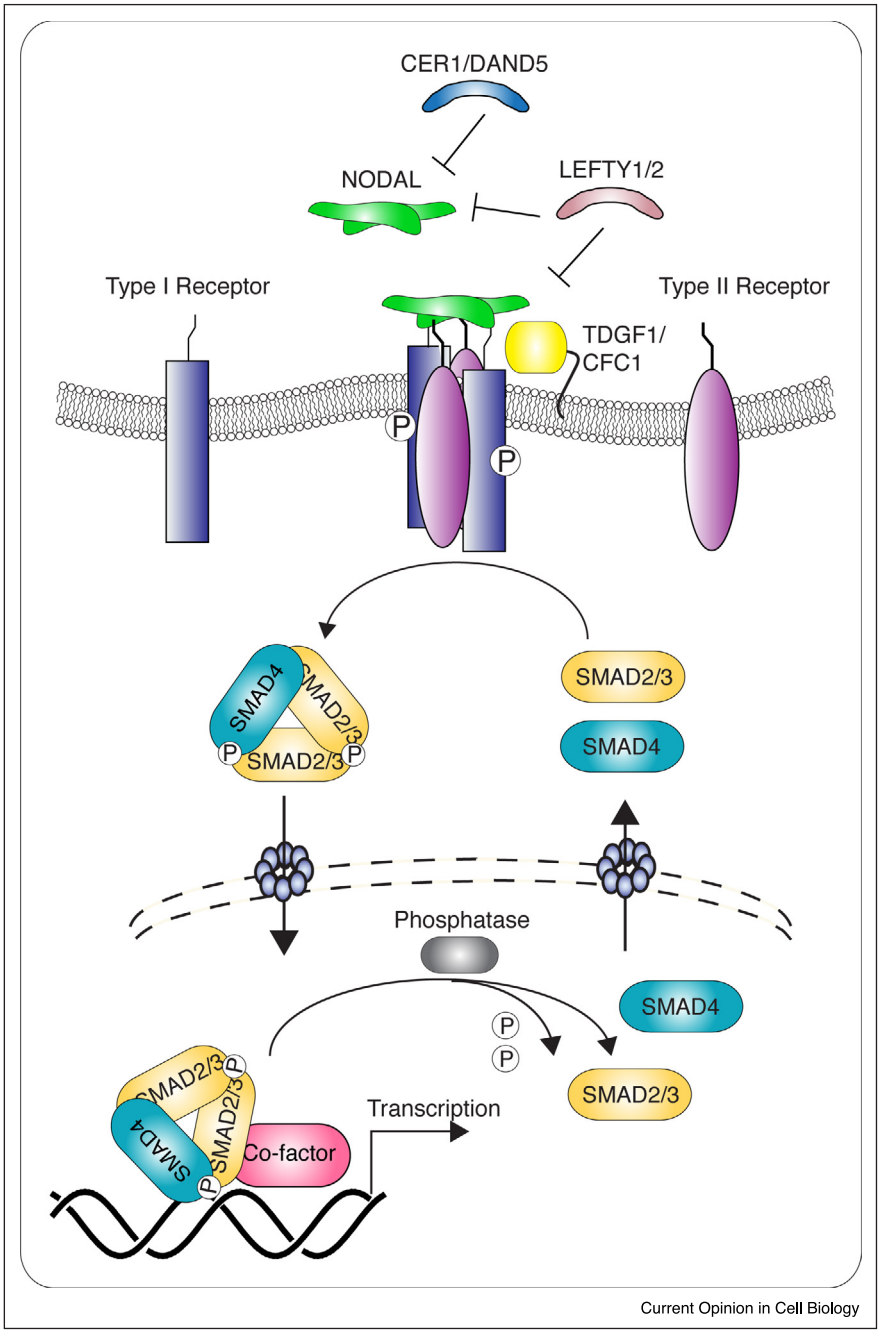
New mechanistic insights into NODAL signalling

The mechanism whereby the EGF-CFC co-receptors, TDGF1 and CFC1 function has been a matter of debate. The original view was that these co-receptors promoted NODAL binding to the receptor complex, and consistent with this, TDGF1 was shown to bind NODAL and ACVR1B via two different motifs [22]. An alternative model was then put forward which suggested a dual role for EGF-CFCs, where they were required for NODAL processing and for internalisation of NODAL into endosomes, where NODAL signalling is thought to predominantly occur [13]. In this view, TDGF1 at the plasma membrane recruits the proprotein convertases to cleave the promature form of NODAL and also concentrates NODAL in endosomes, where it binds the receptors and initiates signalling. A very recent paper investigating *in vitro* how TDGF1 binds ligand, shed more light onto the mechanism of TDGF1 function and drew interesting parallels with other TGF-β family co-receptors [23]. These authors showed that TDGF1 binds ligand on surfaces that are recognized by type I or type II receptors, and they demonstrated that soluble TDGF1 inhibited NODAL activity *in vitro*, whilst membrane-bound TDGF1 promoted it. They highlighted provocative similarities with another GPI-linked co-receptor family, the RGMs, which function in BMP signalling pathways [24]. RGMs have been shown to bind to BMP2 in such a way as to block BMPR1A binding, but *in vivo* they promote BMP signalling [25]. The model for RGM function proposed suggests that upon BMP2 stimulation, BMP2–RGM complexes, possibly also containing BMPR2, are targeted to endosomes, which are enriched with BMP type I receptors. The acidic environment of the endosomes promotes dissociation of RGM from the complex and its replacement by the BMP type I receptor leading to activation of SMAD signalling [25]. Intriguingly, a similar scenario has very recently been demonstrated for another BMP co-receptor, ENG (endoglin). In this case, a crystal structure has revealed that ENG-bound BMP9 can interact with ACVRL1 (ALK1), but not with the type II receptor, ACVR2B [26]. It is tempting to speculate that these TGF-β family co-receptors, the EGF-CFCs, the RGMs and ENG may all function via a common mechanism, where they bring together in a ternary complex the ligand with one receptor, and are then replaced in this complex by the second receptor, possibly in the endosomal compartment.

In early mouse embryos, the main R-SMAD downstream of NODAL appears to be SMAD2, as it is expressed earlier than SMAD3 and as a result, *Smad2* knockout mice have a strong embryonic phenotype, whilst *Smad3*-null mice develop normally [2]. The early importance of SMAD2 versus SMAD3 is also evident in *Xenopus* where Smad3 is much less abundant at early stages than Smad2 [27]. This has now also been confirmed in zebrafish, since a recent paper has shown that a maternal–zygotic *smad2* mutant phenocopies loss of Nodal signalling, suggesting a minimal or no role for Smad3 at early stages [28].

As mentioned above, NODAL-activated SMAD2–SMAD4 complexes rely on other TFs to recruit them to DNA. The first such TF to be discovered was FOXH1 (originally called FAST-1) [29]. New insights into how Foxh1 functions in *Xenopus tropicalis* have come from genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) experiments [30••]. This work has revealed that in early blastula *Xenopus* embryos Foxh1 is bound to enhancers in the absence of signalling, complexed with the transcriptional repressor Tle, which is a member of the Groucho family. The data suggest that after zygotic transcription is initiated, Nodal signalling results in phosphorylated Smad2/3 replacing Tle at a subset of these enhancers to drive Nodal-induced transcription. At gastrulation stages, another member of the Fox family, one of the Foxa proteins, then replaces Foxh1, whose expression drops dramatically at the beginning of gastrulation. Thus in this system, Foxh1 marks enhancers before RNA polymerase II enrichment and the transcription of the target gene. Interestingly, in another recent paper investigating NODAL signalling in mouse

Figure 1



Schematic of the NODAL signalling pathway. NODAL requires type I and type II receptors and a co-receptor, TDGF1/CFC1 to signal. The activated type I receptor phosphorylates SMAD2 and/or SMAD3, which form complexes with SMAD4 that accumulate in the nucleus, where they cooperate with other transcription factors (denoted co-factors) to regulate transcription. In the nucleus a phosphatase dephosphorylates SMAD2/3 allowing them to recycle to the cytoplasm. NODAL activity is regulated extracellularly by DAN family members and by LEFTY1/2.
Source: Adapted from Ref. [17].

P19 cells, FOXH1 and SMAD2 were shown to bind together inducibly upon ligand stimulation [31]. A clue as to why FOXH1 might behave differently in different species comes from an analysis of the sequences of FOXH1 proteins. Only the *Xenopus tropicalis* and *Xenopus laevis* Foxh1 proteins contain the Tle binding motif, FMIDSLL ([30••] and see alignments in [32]); it is absent in the human, mouse and zebrafish versions. This suggests a specific requirement during *Xenopus* development to actively keep Nodal target genes silent during cleavage

stages, which is not necessary during the early stages of human, mouse and zebrafish development.

A similar theme, whereby a SMAD-recruiting TF interacts with a repressor prior to the SMAD complexes has recently emerged with the bHLH protein, HEB [33•]. HEB associates with mesendoderm enhancers in pluripotent mouse ES cells (ESCs) together with the polycomb complex, PRC2. Upon NODAL signalling these enhancers become associated with HEB–SMAD2/3 complexes. HEB therefore cooperates with PRC2 to effectively mark developmental enhancers for eventual SMAD2/3 occupancy.

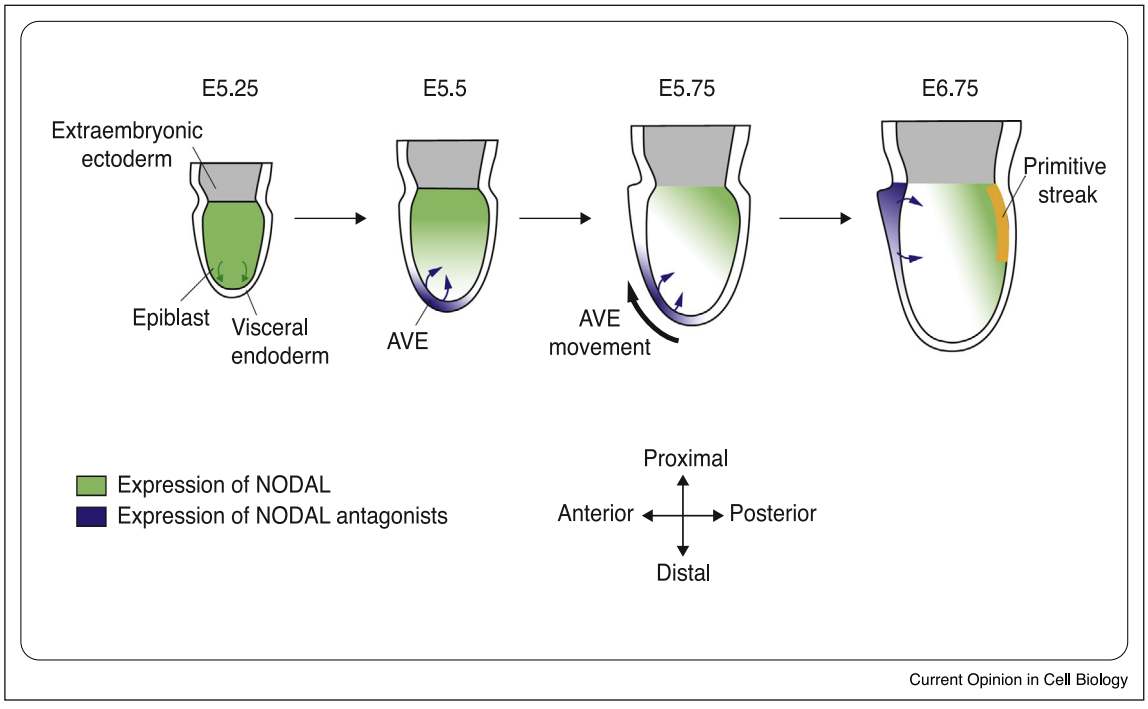
Another bHLH protein that is crucial for recruiting activated SMAD complexes to enhancers is E2a [34]. In *Xenopus* it was known to be required for mesoderm induction, and has now been shown to function through two separate mechanisms. Firstly, it is important for positioning the Smad2/3–Smad4 complexes correctly at the *lefty* genomic locus to give appropriate levels of *lefty* transcription, and it is essential for activating Smad2/3 target genes such as *eomes*, *xbra* and *epha4*.

The TFs that recruit activated SMAD complexes to chromatin can also act to integrate NODAL signalling with other signalling pathways and recent work in mouse ESCs has provided a nice demonstration of this [35•]. These authors found that WNT3-activated TCF3 and NODAL-activated SMAD2/3 depend on each other for binding to enhancers of mesendoderm genes, for example to the *Eomes* enhancer (see also [36]). Importantly, induction of *Wnt3* and the WNT receptor *Fzd1* in these cells absolutely requires expression of p53 family members. This work has thereby established p53 family members as key players for integrating WNT and NODAL signalling to drive mesendoderm differentiation of pluripotent ESCs.

Graded NODAL signalling — establishing and refining domains of NODAL activity

In mice NODAL signalling is dynamic. By embryonic day 5.5 (E5.5), it is expressed throughout the epiblast and overlying visceral endoderm (VE), and then becomes restricted to the posterior epiblast. By early gastrulation (E6.5) Nodal is expressed only in the epiblast cells that will ingress through the primitive streak (Figure 2) [2,12]. *Nodal* transcription in the early epiblast is regulated by two enhancers, one of which is autoregulatory and contains a FOXH1 site, and the other is responsive to Wnt signalling [2]. Later on in development, NODAL expression in the node that is required for left–right asymmetry

Figure 2



Spatial regulation of NODAL signalling in the early mouse embryo. At early stages *Nodal* expression is ubiquitous in the epiblast, and then becomes restricted to the posterior by the action of secreted antagonists, predominantly, LEFTY1 and CER1. Green arrows denote autoregulation. Blue arrows denote signalling inhibition by the antagonists. *Abbreviation*: AVE, anterior visceral endoderm.
Source: Adapted from Ref. [12].

is driven by Notch signalling [2,12]. The domains of NODAL signalling in the early mouse embryo are predominantly established by the activity of soluble ligand antagonists. CERl and LEFTY1 produced initially by the distal VE (DVE) inhibit NODAL signalling in the epiblast. Additional VE cells then form the AVE which together with the DVE migrate anteriorly, restricting NODAL signalling to the posterior (Figure 2) [2,12].

In zebrafish the situation is different. Whereas in early mouse embryos NODAL and the antagonists are expressed in adjacent cell populations [37], in fish they are co-expressed in the same cells in the marginal domain. This has given rise to the idea that the marginal domain of Nodal signalling, which is required for mesendoderm induction, is generated through a Turing Reaction–Diffusion model [8,38]. Recent work, however, exploiting a Nodal signalling reporter, immunostaining for phosphorylated Smad2 and consideration of other signalling pathways operating at the zebrafish margin has led to a different model for establishing this domain of graded Nodal signalling [39••]. The two Nodal ligands, Ndr1 and Ndr2 are first expressed zygotically in yolk syncytial layer (YSL), which underlies the blastoderm and are under the control of the TF, Mxtx2, itself controlled by Wnt signalling [39••,40]. These Nodal ligands induce more *ndr1/2* expression in the blastoderm and the signalling domain spreads by a relay mechanism through autoregulation [39••]. The genes encoding Lefty1 and Lefty2 (*Lft1/2*) are expressed with *ndr1/2* in the blastoderm, as they are Nodal target genes, but are not translated due to activity of a microRNA, miR-430. During a window of ~1.3 hours from sphere stage to 50% epiboly, the domain grows to about five cell tiers from the YSL. By 50% epiboly, levels of *Lft1/2* mRNA overcome the suppression by miR-430, *Lft1/2* are translated and the domain grows no further. The result is a gradient of Nodal signalling within the Nodal ligand expressing domain (Figure 3a,b). One of the key results that led to the idea that the Nodal signalling domain was generated by a Reaction–Diffusion mechanism was that a number of Nodal target genes were expressed beyond the domain of Nodal ligand expression and thus thought to be regulated by extremely low levels of Nodal signalling arising from diffusion. However, expression of these target genes has now been shown to be due to FGF signalling, which results from the induction of FGF ligand expression by Nodal in the first 4–5 cell tiers of the margin, and gives rise to a broad domain of FGF signalling as read out by phosphorylated ERK MAP kinase (Figure 3b) [39••].

Another layer of regulation of the Nodal signalling domain at the zebrafish margin has been recently reported to be mediated by the LIM domain binding protein, Lbd2a [41]. *lbd2a* is a Nodal-inducible gene which encodes a transcriptional regulator that represses the expression of Ndr1, whilst simultaneously activating the expression of the inhibitory Smad, Smad7 that acts to inhibit Nodal signalling, thus fine tuning Nodal signalling at the margin.

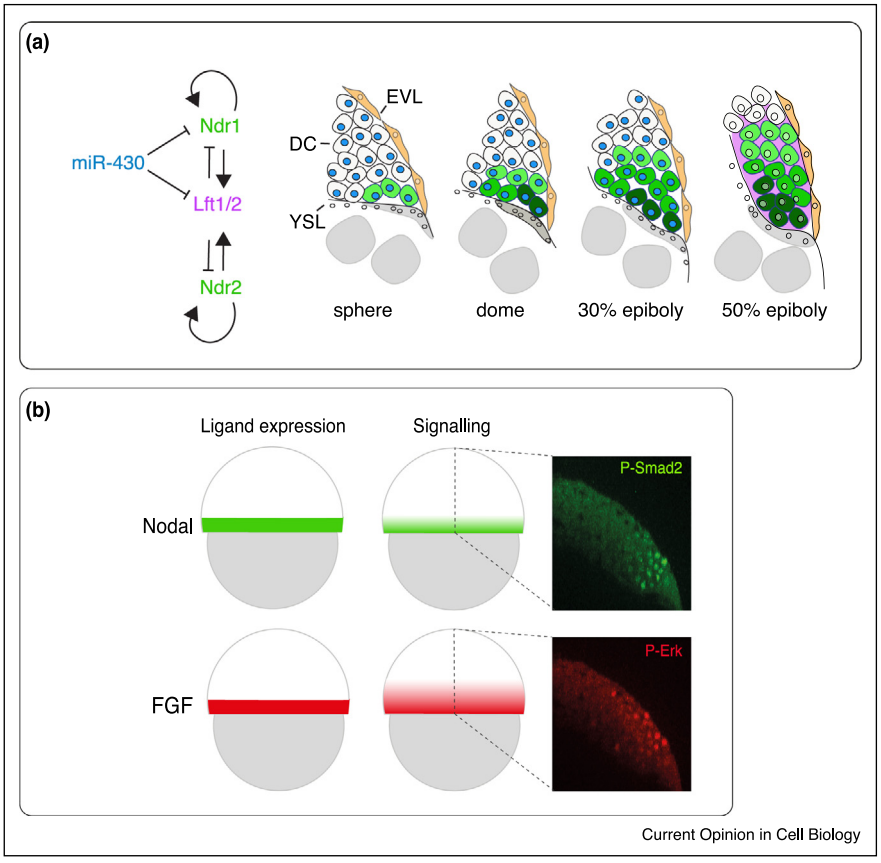
In sea urchins, as in vertebrates, Nodal is required for axis specification. Whereas in zebrafish and *Xenopus*, Nodal signalling is higher on the dorsal side, and BMP signalling higher on the ventral side, in sea urchins it is the opposite. Nodal specifies ventral ectoderm and then induces expression of BMP2/4 dorsally, which acts in a relay to specifiy dorsal ectoderm [42]. How the initial ventral domain of Nodal expression is established was not known, but a recent paper has now shed light on this issue [43••]. The authors show that a molecule called Panda, which is a maternal GDF15-like TGF-β family ligand, directs formation of the dorsal–ventral axis by restricting Nodal expression, acting through BMP type I receptors. Their model suggests that a dorsal to ventral gradient of Panda activity restricts Nodal expression to the ventral blastomeres. This is then maintained by Lefty expression, which is thought to prevent the Nodal domain from expanding dorsally. Subsequently, BMP2/4 expression downstream of Nodal acts to restrict Nodal activity to the ventral side, as well as inducing expression of dorsal genes [43••]. It is interesting in sea urchins that Nodal and Lefty alone are not sufficient to create asymmetry. Panda is required to restrict the Nodal expression first, and then Lefty expression can maintain it.

Determinants of specificity in downstream physiological responses to NODAL signalling

In vertebrates, NODAL is essential for mesoderm and endoderm specification and for left–right asymmetry. In the interests of space I will focus here on new insights into mesendoderm induction, but point the reader to an excellent recent review that discusses NODAL's role in controlling left–right asymmetry and the downstream effects that drive asymmetries in organ formation [9].

In zebrafish, a subset of the cells closest to the YSL will be specified as endoderm, whilst cells further away will become mesoderm [44]. It was traditionally thought that the concentration of Nodal ligand was the key determinant of these cell fate choices [45]. A direct test of this idea, however, demonstrated that ligand concentration was insufficient to predict the response of target genes [28]. Instead, these authors concluded that the kinetics of target gene induction and the timing of the onset of induction predicted the spatial range of gene expression. From the analysis of how the graded domain of Nodal evolves over time, it has become clear that, as well as generating a spatial domain of Nodal signalling at 50% epiboly, this mechanism generates a temporal Nodal gradient, with the cells closest to YSL signalling the longest, and those more distant, signalling for a shorter length of time [39••]. This is evident in the phosphorylated Smad2 staining, since the levels of phosphorylated

Figure 3



Establishment of the Nodal signalling domain in the zebrafish embryo. **(a)** Interplay between the two zebrafish Nodal ligands, Ndr1 and Ndr2, the two Lefty antagonists, Lft1 and 2 and miR-430 explain the evolution of the Nodal signalling domain at the margin. Adapted from Ref. [39••]. YSL-expressing Ndr1/2 is shown in grey; cells responding to Nodal are in green, with darker shade indicating those experiencing the longest signalling duration. Cells expressing miR-430 are denoted with blue nuclei, and pink shading denotes cells in which Lft1/2 is translated and thus inhibitory to Nodal signalling. **(b)** A schematic showing that the Nodal signalling gradient is within the Nodal expression domain. For FGF ligands, which are induced in the first 4–5 cell tiers from the YSL, FGF signalling induces a gradient of phosphorylated Erk that reaches up to about 10 cell tiers from the YSL. To the right is shown immunostaining for phosphorylated Smad2 and for phosphorylated ERK in the zebrafish margin at 50% epiboly. Z reconstructions of a confocal stack are shown.

Smad2 are proportional to the duration of signalling for the NODAL pathway (Figure 3b) [39••]. This new understanding of how the Nodal gradient is established suggests that duration of signalling might determine cell fate specification, rather than ligand concentration *per se*. Indeed, timed Nodal inhibition experiments performed in zebrafish concluded that Nodal signalling specifies sequentially, the somites, notochord, blood, Kupffer's vesicle, hatching gland, heart and endoderm, suggesting a linkage between cell fate and length of exposure to Nodal signals [46]. A recent paper has now used optogenetics to test the importance of signal duration for dictating the specificity of downstream responses [47••]. These authors observed that expression of the transcriptional repressor, *gsc*, was high in the precordal plate where levels of the endoderm progenitor marker, *sox17* were lower. Noting that Nodal signalling is most sustained in the precordal plate progenitors, they tested the effect of extending the duration of Nodal signalling artificially with light using photoactivatable receptors. The result was that precordal plate was specified at the expense of endoderm, confirming the importance of signal duration in cell fate specification.

How is the duration of signalling read out at the level of gene expression? The answer lies in the TF network downstream of Nodal signalling. Target genes are dependent on sustained signalling for their induction if they require a TF for their induction that is itself induced (directly or indirectly) by the pathway — a phenomenon that has been called a self-enabling response [48]. It has been known for some time that the earliest marker of endoderm progenitors, *sox32*, requires the TFs Gata5 and Mixl1 for its induction, both of which are Nodal target genes [49]. The TF network downstream of Nodal has recently been further fleshed out using whole genome

approaches [50•]. The results reveal a temporal hierarchy to gene expression downstream of Nodal and show that *sox32*, which is induced in the cells experiencing the longest duration of Nodal signalling, requires a combination of maternal TFs, Nanog and Eomesa, their direct targets, Mxtx2 and Pou5f3, the Nodal-induced TF, Mixl1, as well as activated Smad2 [50•]. *mixl1* transcription was also shown to require the T-box TFs, Ta and Tbx16, which themselves are Nodal-dependent genes, further explaining the necessity of sustained Nodal signalling for endoderm progenitor specification.

Conclusions

The last few years have seen a dramatic increase in our knowledge of how Nodal signalling is regulated, both spatially and temporally, at the level of its intracellular signal transduction pathway and more broadly in terms of how domains of Nodal signalling are established and modulated during embryonic development, and we are starting to understand how signal duration may be interpreted by downstream gene regulation networks. The next major challenge will be to determine how Nodal signalling interacts with other signalling pathways to reproducibly achieve the exquisite patterning of embryos in all species from hydra to humans.

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