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Calcium dynamics integrated into signalling pathways that influence vertebrate axial patterning

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Many aspects of animal development including fertilization as well as organ formation and function are dependent upon the dynamic release of calcium (Ca^{2+}) ions. Although the controlled release and/or accumulation of Ca^{2+} ions has been extensively studied, how the release dynamics produce a specific biological output in embryonic development is less clear. We will briefly summarize Ca^{2+} sources, highlight data on endogenous Ca^{2+} release in vertebrate embryos relevant to body plan formation and cell movement, and integrate pharmacological and molecular-genetic studies to lend insight into the signalling pathways involved. Finally, based on *in vivo* imaging in zebrafish genetic mutants, we will put forward the model that distinct Ca^{2+} release dynamics lead to antagonism of the developmentally important Wnt/β-catenin signalling pathway, while sustained Ca^{2+} release modulates cell polarization or directed migration.

Keywords: calcium; Wnt; β-catenin; zebrafish; dorsal–ventral axis; left–right patterning

1. CALCIUM SOURCES

Although critical for many processes, calcium ions (Ca^{2+}) are not metabolized by the cell. Instead, Ca^{2+} enters the cell across either the plasma membrane or the membrane of intracellular organelles. Depending on the location of the ion channels and the extent and duration of the channel opening, local or global changes in Ca^{2+} levels in the cytosol of the cell can result. Intricate crosstalk and feedback between release circuits can stimulate Ca^{2+}-induced Ca^{2+} release, influencing neighbouring receptors and potentially triggering a regenerative wave (Berridge 1997; Berridge *et al.* 2003; Roderick *et al.* 2003). In addition, continued stimulation and/or depletion of endoplasmic reticulum (ER) stores activate a store-operated Ca^{2+} entry influx pathway located at the plasma membrane (Parekh & Putney 2005).

In non-excitable (non-neuronal) cells, a majority of intracellular Ca^{2+} release occurs through inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} channels present in the ER membrane (reviewed in Berridge *et al.* 2003). The phosphatidylinositol (PI) cycle is activated in response to many hormones and growth factors that bind to cell surface receptors. Two predominant receptor classes are the G-protein-coupled receptor class and the receptor tyrosine kinase class. Extracellular ligand stimulation of these receptors activates a PI-specific phospholipase C (PLC). Activated PLC converts membrane-bound phosphatidylinositol (4,5)-bisphosphate (PIP_2) into IP_3 and lipophilic diacylglycerol (DAG). IP_3 subsequently binds to receptors (IP_3R) located principally on the ER triggering the rapid release of Ca^{2+} into the cytosol of the cell. At the same time, DAG produced by PIP_2 hydrolysis can act as an additional second messenger to further activate downstream targets such as protein kinase C (PKC).

Relevant to this discussion is the fact that Ca^{2+} release is heterogeneous. Specific cellular responses can be triggered by differences in the amplitude, frequency and duration of intracellular Ca^{2+} oscillations. Such oscillations can be derived from changes in upstream steps within the PI cycle, such as G-protein activity, PLC activity and IP_3 levels (Hirose *et al.* 1999; Luo *et al.* 2001; McCarron *et al.* 2004; Thore *et al.* 2004; Nomikos *et al.* 2005; Rey *et al.* 2005). Oscillatory small molecules such as IP_3 may be transmitted to other cells via gap junctions (Lin *et al.* 2004), a phenomenon that may be of significance in the regulation of axis induction in the zebrafish blastula (see below). Feedback from activated Ca^{2+}-binding proteins adds another layer of complexity to the dynamics of Ca^{2+} release and removal. For example, IP_3R activity integrates signals from small molecules and proteins, including PKC and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Nadif Kasri *et al.* 2002; Assefa *et al.* 2004; Patterson *et al.* 2004).

2. CALCIUM AND THE VERTEBRATE BODY PLAN

After fertilization, the next major developmental programme involves the establishment of the primary axes, in which regions of the embryo receive signals to determine the cells that will contribute to the dorsal (back) or ventral (belly) tissue as well as anterior (head/top) and posterior (tail/bottom) regions. A number of studies have linked PI-cycle activity with

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body plan formation. Classical work using lithium, an inhibitor of inositol turnover (Berridge *et al.* 1989), induced expansion of dorsal structures in *Xenopus* (Kao *et al.* 1986; Kao & Elinson 1989, 1998), and similar effects were obtained in the zebrafish (*Danio rerio*) embryo (Stachel *et al.* 1993; Aanstad & Whitaker 1999). Lithium-induced embryonic defects are rescued by supplying an intermediate of the PI cycle, *myo*-inositol (Busa & Gimlich 1989). In further support, zebrafish and *Xenopus* embryos injected with antibodies that disrupt IP_3R function displayed expanded dorsal structures with the loss of ventral structures (Kume *et al.* 1997; Westfall *et al.* 2003*b*). Treatment of zebrafish embryos with inhibitors that target different steps in the PI cycle generated dorsalized or axis-duplication phenotypes (figure 1*a*, inset). The dorsalization defects generated by the use of pharmacological inhibitors that target inositol turnover in the zebrafish were likewise rescued by *myo*-inositol (Westfall *et al.* 2003*b*). The findings of an involvement for PI-cycle activity in axis induction are consistent with the observed spontaneous increase in IP_3 levels in the *Xenopus* embryo at the blastula stage (Busa & Gimlich 1989; Maslanski *et al.* 1992). Moreover, *in vivo* imaging of calcium release dynamics in the zebrafish embryo identified rapid aperiodic Ca^{2+} release that persists until the midblastula transition stage (Reinhard *et al.* 1995; Slusarski *et al.* 1997*b*; Slusarski & Corces 2000). The idea that increased IP_3 levels may trigger Ca^{2+} release during these stages has been corroborated by drug inhibition studies (Slusarski *et al.* 1997*a*). The effects of lithium were most pronounced when exposure occurred on the ventral side of the embryo, suggesting that in the embryo PI-cycle activity is normally high on the ventral side and low on the dorsal side. Consistent with this is the described role of activated CaMKII for ventral fates in *Xenopus* embryos (Kühl *et al.* 2000*a*).

3. THE WNT SIGNALLING NETWORK

The Wnt family of growth factors and components of their signalling pathways have diverse roles in development and disease. Wnt signalling influences many aspects of embryonic patterning, cell proliferation as well as the maintenance and differentiation of stem cells, and is critical in axis formation (figure 1*b*; reviewed in Moon *et al.* 2004; Kohn & Moon 2005; Clevers 2006). In the absence of the so-called canonical Wnt signalling (Wnt/β-catenin), β-catenin is rapidly sequestered in a cytoplasmic degradation complex containing axin, the adenomatous polyposis tumour suppressor protein (APC) and the serine threonine kinase GSK-3β. GSK-3 phosphorylation of β-catenin targets the latter for proteasomal degradation (figure 1*b*). Wnt binding to its co-receptors Frizzled and LRP5/6 (low-density lipoprotein receptor-related protein) activates a cytoplasmic phosphoprotein (Dishevelled, Dsh) which downregulates GSK-3 and inhibits the degradation of β-catenin. Stabilized β-catenin protein interacts with the members of the LEF/TCF transcription factor family in the nucleus to promote the activation of downstream target genes involved in axis specification. Another endogenous target of lithium is the β-catenin degradation complex component GSK-3, which when inhibited promotes dorsal axis induction (Klein & Melton 1996; Stambolic *et al.* 1996). The fact that exogenous *myo*-inositol can suppress the effects of GSK-3 inhibition (Hedgepeth *et al.* 1997) further supports the notion of communication between PI-cycle activity and Wnt/β-catenin signalling to regulate axis induction.

The Wnt network has layers of complexity including the fact that different Wnt ligands can activate distinct cellular outputs. In vertebrate embryos, overexpression of a subset of Wnts induces hyperdorsalization and ectopic axes by virtue of increased Wnt/β-catenin signalling activity (Moon *et al.* 1993*b*; Moon & Kimelman 1998). Additional Wnts (including *Wnt*-5, -4 and -11) appear to act independently of β-catenin function (Dale 1998; Kühl *et al.* 2000*b*). Emerging evidence suggests that the ability of Wnt ligands to activate different signalling pathways, β-catenin dependent (canonical) and β-catenin independent (non-canonical), appears to be controlled by the timing of expression and receptor context, not to mention the correct combination of intracellular effectors. In the zebrafish embryo, *Wnt-5* overexpression results in an increase in the frequency of intracellular Ca^{2+} release in a manner that is dependent on G-protein activity and the PI cycle (Slusarski *et al.* 1997*a*,*b*), thus linking Wnt activity to IP_3-dependent Ca^{2+} release and defining the Wnt/Ca^{2+} signalling pathway.

The concept that Wnt/Ca^{2+}, either in parallel or as part of a complex signalling network, appears to interact with the Wnt/β-catenin pathway in early axis specification was initially suggested by the apparent antagonism of certain pairs of Wnt ligands when expressed in *Xenopus* and zebrafish embryos (Moon *et al.* 1993*b*; Slusarski *et al.* 1997*b*). Expression of ligands that activate Wnt/β-catenin signalling in these embryos, such as Wnt-8, results in ectopic axis induction. However, when Wnt-5 is co-expressed with Wnt-8, the Wnt-8 axis induction phenotype is suppressed. Stimulating Ca^{2+} release, via activated serotonin receptor, also antagonizes Wnt-8-induced expansion of dorsal domains (Slusarski *et al.* 1997*b*), supporting that Wnt-5 antagonism of Wnt/β-catenin is mediated by Ca^{2+} release. On the other hand, pharmacological or genetic reduction of the Wnt/Ca^{2+} pathway in zebrafish embryos or mouse limb buds generates ectopic accumulation of nuclear β-catenin and activation of β-catenin transcriptional targets (Topol *et al.* 2003; Westfall *et al.* 2003*a*,*b*). Additionally, inhibition of G-protein function dorsalizes *Xenopus* embryos (Kume *et al.* 2000). These observations are consistent with a model in which IP_3-dependent Ca^{2+} release, promoted by Wnt/Ca^{2+} signalling activity, negatively regulate the Wnt/β-catenin signalling pathway and therefore axis induction (figure 1*b*).

Various studies have shown that there are common components between the Wnt/Ca^{2+} and the planar cell polarity pathways (Wnt/PCP), another non-canonical Wnt pathway involved in the polarization of cells in *Drosophila* and vertebrate species (Kohn & Moon 2005; Solnica-Krezel 2005). During gastrulation, vertebrate

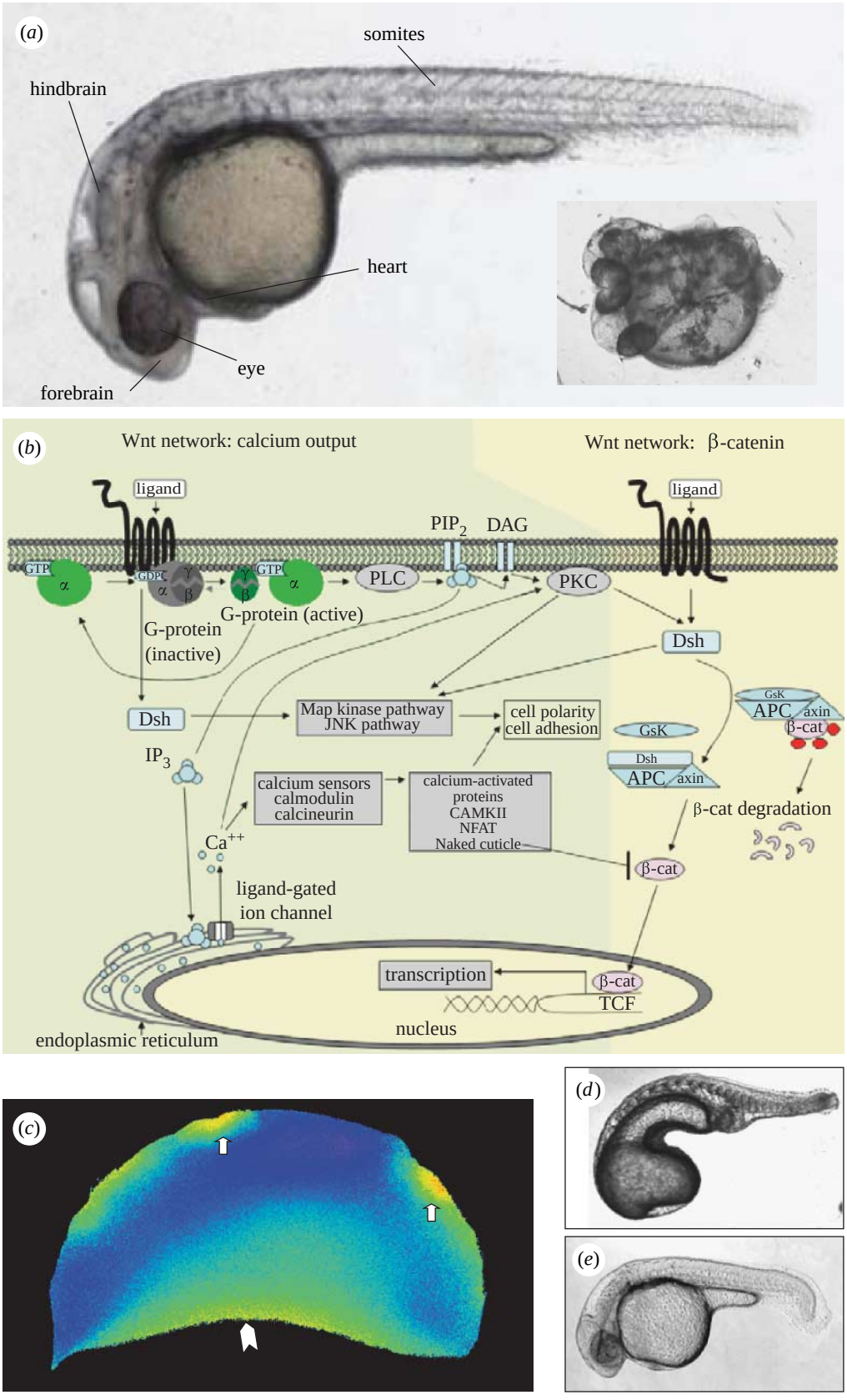

Figure 1. Signal transduction pathways in vertebrate axis formation. During embryogenesis, the basic body plan generates a dorsal–ventral and anterior–posterior orientation of tissues and organs. (*a*) Lateral view of a wild-type zebrafish 48 hour larvae with normal axial patterning with anterior to the right and dorsal to the top. Key tissues are noted. Inset: a two-headed embryo as a result of PI-cycle inhibition. (*b*) A simplified schematic of the Wnt signalling network. β-catenin-independent pathways are encased in green region and β-catenin-dependent components are in the yellow region. β-cat, β-catenin. (*c*) A single ratiometric image taken from a time course during the cellular blastoderm stage of a fura-2-injected zebrafish embryo. The image is pseudo-coloured to represent high calcium levels as warm colouring (yellow) and low calcium levels as blue. Arrows denote transient fluxes observed in the enveloping layer and the arrowhead designates the yolk syncytial layer region. (*d*) *hecate* mutant embryo lacking dorsal–anterior structures such as the eyes and brain reflective of a ventralized phenotype. (*e*) *ppt* mutant embryo with a shortened anterior–posterior axis and kinked tail.

embryos undergo a variety of morphogenetic movements to thicken (dorsal convergence) and elongate (axis extension) the embryo, also called convergence extension (CE; Keller 2002). Ca^{2+} mobilization associated with waves of tissue contraction can be observed in *Xenopus* explants (Wallingford *et al.* 2001*a*). In intact zebrafish embryos, intercellular Ca^{2+} waves have been observed at the margin during gastrulation (Gilland *et al.* 1999). The relationship between Ca^{2+} waves and cell movement is supported by the finding that, in *Xenopus* embryos, pharmacological inhibition of such waves results in CE defects without altering cell fate (Wallingford *et al.* 2001*a*,*b*). Wnt genes that result in the activation of Ca^{2+} release in the blastula embryo, such as *Wnt-5* (Slusarski *et al.* 1997*b*; Westfall *et al.* 2003*a*), can also alter morphogenetic movements later during gastrulation when misexpressed (Moon *et al.* 1993*a*; Ungar & Moon 1995). Core components involved in the polarization of epithelial cells in the *Drosophila* cuticle are also necessary for the polarization of migrating cells during vertebrate gastrulation (Solnica-Krezel 2005). Indeed, several core components of Wnt/PCP have been shown to activate Ca^{2+} release in zebrafish including Frizzled-2, Dsh and the intracellular protein Prickle (Slusarski *et al.* 1997*a*; Sheldahl *et al.* 2003; Veeman *et al.* 2003). The fact that interference with either Ca^{2+} release or Wnt/PCP signalling results in CE defects suggests non-canonical Wnt signalling activity can be characterized as a complex network with cellular outputs defined by Ca^{2+} modulation and polarized cell movement (figure 1*b*).

4. CALCIUM DYNAMICS AND BIOLOGICAL OUTPUTS

The Ca^{2+} activity in the zebrafish blastula is observed in the enveloping layer (EVL) and yolk syncytial layer (YSL; figure 1*c*; Reinhard *et al.* 1995; Slusarski *et al.* 1997*b*; Slusarski & Corces 2000). Although present in overlapping stages, the EVL-specific Ca^{2+} fluxes are present in a cell or small cluster of cells lasting for short intervals. The YSL-specific Ca^{2+}, on the other hand, displays sustained elevation in a population of cells. The distinct dynamics of Ca^{2+} increases and the bimodal role of Wnt/Ca^{2+}, in β-catenin antagonism and polarized cell movement, led us to hypothesize that the rapid aperiodic Ca^{2+} release is coupled to Wnt/β-catenin antagonism and the sustained Ca^{2+} levels integrates into polarizing cells or their directed migration.

Support for this theory comes from the analysis of Ca^{2+} release dynamics in zebrafish mutants. A mutation in the zebrafish maternal gene *hecate* produces ventralized embryos (figure 1*d*) that lack nuclear β-catenin (Lyman Gingerich *et al.* 2005). Consistent with our theory, these mutant embryos display increased Ca^{2+} release frequency in the EVL (Lyman Gingerich *et al.* 2005). Suppression of the Ca^{2+} dynamics with pharmacological reagents was sufficient to rescue the defects in dorsal cell fate specification observed in these mutants (Lyman Gingerich *et al.* 2005), highlighting the relationship between Ca^{2+} release and β-catenin antagonism in a genetic context.

In the zebrafish, Wnt-5 has been shown to correspond to the genetic mutation *pipetail* (*ppt*; Rauch *et al.* 1997). The *ppt* zygotic mutants have axis extension defects, reflected in a shorter anterior–posterior length and kinks in the tail, resembling a pipe (figure 1*e*; Hammerschmidt *et al.* 1996; Kilian *et al.* 2003). Analysis of zygotic *ppt* mutant embryos revealed reduced Ca^{2+} levels in the YSL region (Westfall *et al.* 2003*a*). Suggestive that reduced Ca^{2+} levels are central to the *ppt* defects is the ability to rescue the mutant phenotype with the expression of activated CaMKII (Westfall *et al.* 2003*a*). These data raise the possibility that the YSL-specific Ca^{2+} dynamics contributes to polarized cell movements during vertebrate gastrulation.

5. CALCIUM AND LEFT–RIGHT ASYMMETRY

There are many cases in development where a signalling cassette is used in multiple processes. Thus, it would be predicted that transient Ca^{2+} release will correlate with β-catenin antagonism or sustained Ca^{2+} signalling with cell polarization at additional stages or in different tissues. Section 6 describes the orientation of organs relative to the body axis and the implications of both transient and sustained Ca^{2+} modulations in this process. Although vertebrates appear bilaterally symmetrical from the outside, the heart, lungs, liver and gut are carefully positioned across the left–right (LR) axis. The development of this asymmetry is highly conserved across species and most likely will use conserved signalling molecules.

Embryonic organ laterality is preceded by molecular and physiological asymmetries. Shown in figure 2*a* is a schematic of developmental structures and gene products implicated in LR patterning with a focus on zebrafish. At the morphological level, non-involuting dorsal mesoderm cells, the dorsal forerunner cells (DFCs), migrate ahead of the dorsal blastoderm during gastrulation (figure 2*b*, arrow; Cooper & D'Amico 1996; Melby *et al.* 1996). At the start of gastrulation, these cells express key signalling molecules such as nodal-related *squint*, a Brachyury homologue *no tail* and *left–right dynein* (*lrdr1*; Schulte-Merker *et al.* 1994; Feldman *et al.* 1998; Essner *et al.* 2002). The DFCs are highly endocytotic and readily take up vital dyes (Cooper & D'Amico 1996) allowing for the visualization of their migration to the tailbud region where they undergo morphogenesis and form a ciliated structure, the Kupffer's vesicle (KV), during early somite stages (figure 2*c*; Cooper & D'Amico 1996; Melby *et al.* 1996). Ablation of the DFCs or mechanical disruption of the KV disrupts LR patterning (Amack & Yost 2004; Essner *et al.* 2005). Endogenous Ca^{2+} release activity in and around the DFC region during epiboly suggests striking similarities to the transient activity both in a cell or a few cells (figure 2*d*, arrow) and in a region of sustained high Ca^{2+} (figure 2*d*, arrowhead) previously described during the cellular blastoderm stages (figure 1*c*; Schneider *et al.* 2008). Whether this activity correlates with β-catenin antagonism and polarized cell movement bears further study.

The LR signals have been proposed to be modulated by gap junctional communication and/or asymmetrical H^+/K^+-ATPase expression during *Xenopus* early cleavage (reviewed in Levin 2005).

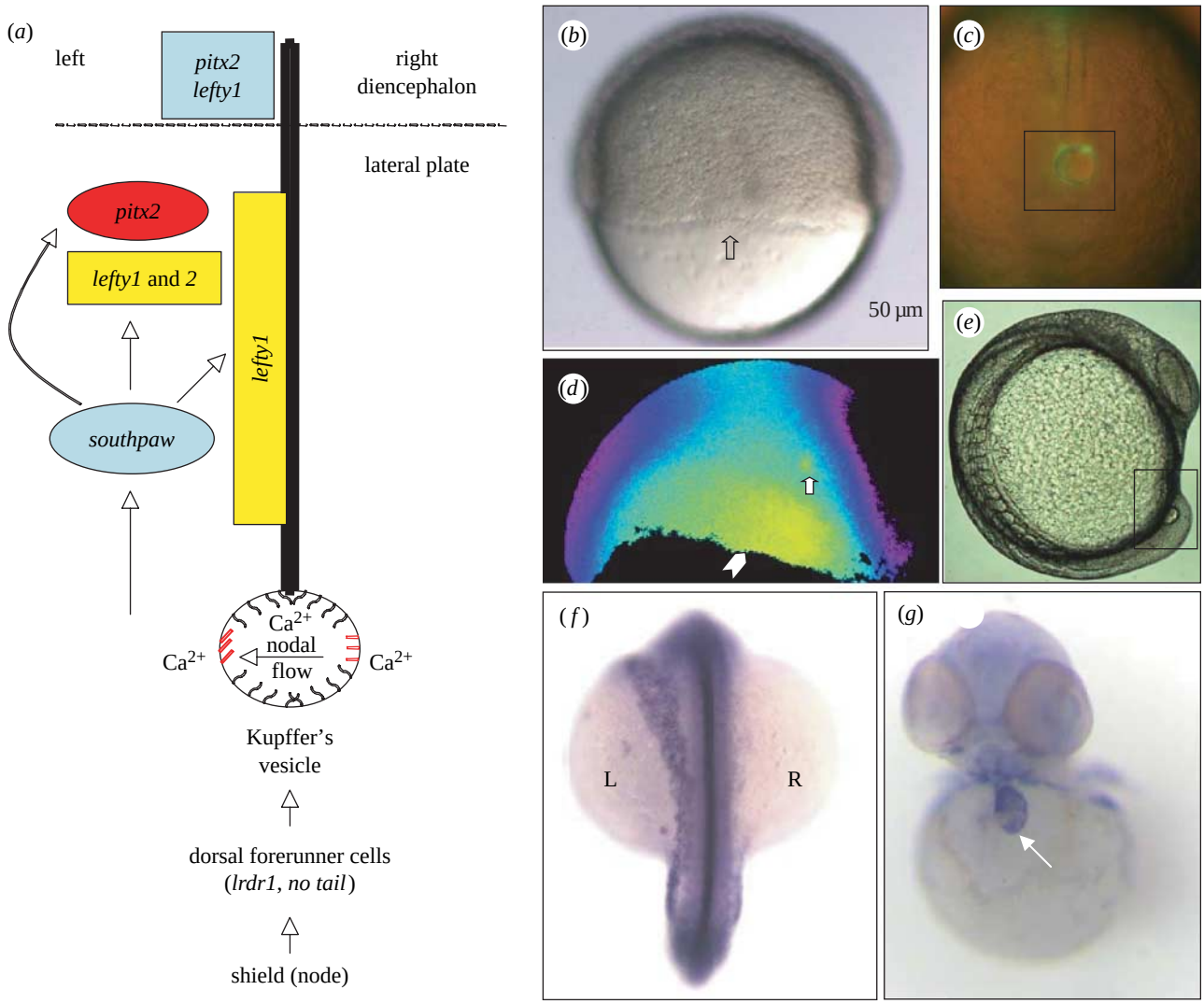

Figure 2. Left–right pattern formation in the zebrafish. (*a*) Schematic noting key structures and gene products implicated in LR patterning. (*b*) Epiboly-staged embryo oriented with the animal pole towards the top, yolk region at the bottom and the dorsal shield region in the centre. The arrow notes the DFCs migrating ahead of the shield region. (*c*) Vital-dye-labelled DFCs (with Syto-11) at the early somite stages. The combined bright field and fluorescent image focused on the tailbud showing the DFCs forming the KV in the black box. The midline of the embryo can be distinguished by the compact notochord cells above the KV. (*d*) A ratiometric image taken from a time course of an epiboly-staged fura-2-injected embryo. Arrow notes a transient flux while the arrowhead notes a region of high sustained Ca^{2+}. (*e*) Lateral view of a somite-stage embryo at a time molecular asymmetric markers begin to be expressed. The KV is morphologically visible in the tail (black box). (*f*) Whole-mount *in situ* hybridization at approximately 22 somite stage with the *southpaw* and *no tail* probes. A dorsal view, anterior to the top, shows left-sided expression of *southpaw* and midline expression of *no tail*. (*g*) Whole-mount *in situ* hybridization of a 48 hours post-fertilization larva with cardiac myosin light chain to illustrate heart morphogenesis. The arrow points to the heart tube.

However, conserved molecular asymmetries in vertebrates become apparent only during later somite stages after the KV/node has formed (figure 2*e*). Of note is the conserved left-sided expression of the secreted transforming growth factor-β (TGF-β) related factor *nodal* (reviewed in Ahmad *et al.* 2004). In zebrafish, the *nodal*-related gene *southpaw* (*spaw*; Long *et al.* 2003) is the earliest asymmetric marker expressed in the left lateral plate mesoderm (figure 2*f*). Downstream targets of *southpaw*, including *lefty1* and *pitx2*, also have conserved asymmetric expression in vertebrates (reviewed in Hamada *et al.* 2002; Wright & Halpern 2002). *lefty1* contributes to a barrier at the midline of the embryo, preventing the left-sided signal from propagating to the right, and *pitx2* contributes to the subsequent morphogenetic changes in the organs, such as looping to form the chambers of the heart (figure 2*g*).

Asymmetric Ca^{2+} levels across the mouse and chick node have been implicated in LR axis determination (McGrath *et al.* 2003; Raya *et al.* 2004). Elevated Ca^{2+} is thought to act via an unknown mechanism to induce left-sided gene expression in concert with or independent of nodal activity (Brennan *et al.* 2002; Hashimoto *et al.* 2004; Marques *et al.* 2004). Rotation of monocilia generates a leftward fluid flow in the node (Nonaka *et al.* 1998; Okada *et al.* 1999) and has been proposed to stimulate mechanosensory cilia and trigger elevated intracellular Ca^{2+} levels at the left edge of the mouse node (McGrath *et al.* 2003). The zebrafish KV contains monociliated cells similar to those found in the mouse node (Supp *et al.* 1997, 1999;

Brueckner 2001; Hashimoto *et al.* 2004; Essner *et al.* 2005) and these cilia have been shown to beat in the same direction, possibly establishing Ca^{2+} asymmetries (Kramer-Zucker *et al.* 2005). Indeed, an intracellular Ca^{2+} flux with a left-sided bias near the zebrafish KV has been detected and is proposed to be required for normal LR patterning (Sarmah *et al.* 2005). In an alternate model, leftward flow of vesicular particles containing *sonic hedgehog* (*shh*) acts in a distinct atypical signalling pathway to activate Ca^{2+} on the left side of the node (Tanaka *et al.* 2005).

In contrast to intracellular Ca^{2+} release, a role for left-sided elevation of extracellular Ca^{2+} has also been proposed. In the chick node, extracellular Ca^{2+} levels appear to be higher transiently on the left side, although it is unclear whether intracellular Ca^{2+} is also increased. This asymmetry was abolished after a treatment with ompremazole, an inhibitor of H^+/K^+ ATPase, which also caused LR defects. This led to the proposal that differential H^+/K^+ ATPase activity during gastrulation sets up a spatial gradient of extracellular Ca^{2+}, which is subsequently transduced through Notch to activate asymmetric gene expression (Raya *et al.* 2004). In zebrafish, pharmacological disruption of H^+/K^+ ATPase activity leads to LR asymmetry defects (Kawakami *et al.* 2005) and early H^+/K^+ ATPase inhibition disrupts cilia number and length in the KV (Adams *et al.* 2006). However, immunodetection of H^+/K^+ ATPase did not display obvious asymmetries in zebrafish (Kawakami *et al.* 2005). Moreover, the H^+/K^+ ATPase inhibitor used, ompremazole, also induces ectopic *shh* expression in chick (Raya *et al.* 2004). Although it is unclear whether extracellular Ca^{2+} regulation is conserved in other organisms, mouse embryos that are homozygous for mutations in the *Polycystin-2* (*Pkd2*) gene, a Ca^{2+}-permeable cation-selective channel, exhibit loss of asymmetric Ca^{2+} levels across the node and LR defects (McGrath *et al.* 2003). Thus, several vertebrate models support a role for Ca^{2+} signalling in the establishment of LR asymmetry, but many questions and issues remain to be addressed, such as the Ca^{2+} sources, the Ca^{2+}-dependent responders and the mechanism linking the Ca^{2+} flux in and around the node to asymmetry in the lateral plate mesoderm. Also, it has not been investigated whether there are multiple stages of Ca^{2+}-dependent events involving the polarized migration and coalescence of the DFCs into a KV, initiation of asymmetric expression and the subsequent maintenance of laterality signals.

6. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, progression from egg to embryo requires a dynamic interaction of signal transduction networks. The continuous exchange of information between cells, through both direct contact and diffusible molecules, influences gene expression and cell behaviour. *In vivo* imaging studies are a critical step in the comprehensive analysis of Ca^{2+} signalling in development. Coupling *in vivo* imaging with molecular, genetic or pharmacological tools will determine the mechanism by which Ca^{2+} signalling is modulated and interpreted in the embryo. Future studies will reconstruct the spatial and temporal dynamics of Ca^{2+} release and incorporate this activity into known signalling pathways, thus providing the capacity to discern the true nature of the cellular basis of pattern formation. Knowledge about how intricate Ca^{2+} signals are integrated into developmental pathways, in particular the Wnt growth factors, raises the possibility to address the pathophysiology of diseases that result from misregulation of this network.

Animal care and experiments were performed in accordance with institutional guidelines for animal experiment ethics.

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