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Review

Calcium signaling in vertebrate embryonic patterning and morphogenesis

Diane C. Slusarski [a,1], Francisco Pelegri [b,*]

[a] Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, USA
[b] Laboratory of Genetics, University of Wisconsin–Madison, Madison, WI 53706, USA

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Abstract

Signaling pathways that rely on the controlled release and/or accumulation of calcium ions are important in a variety of developmental events in the vertebrate embryo, affecting cell fate specification and morphogenesis. One such major developmentally important pathway is the Wnt/calcium signaling pathway, which, through its antagonism of Wnt/β-catenin signaling, appears to regulate the formation of the early embryonic organizer. In addition, the Wnt/calcium pathway shares components with another non-canonical Wnt pathway involved in planar cell polarity, suggesting that these two pathways form a loose network involved in polarized cell migratory movements that fashion the vertebrate body plan. Furthermore, left–right axis determination, neural induction and somite formation also display dynamic calcium release, which may be critical in these patterning events. Finally, we summarize recent evidence that propose a role for calcium signaling in stem cell biology and human developmental disorders.

A great variety of developmental processes, from fertilization to organ formation and function, are dependent on the dynamic release of calcium (Ca^{2+}) ions. This review will focus on the role of Ca^{2+}-mediated signals in patterning events in animal embryos, such as cell fate specification and morphogenesis. The reader is referred to reviews that address the role of Ca^{2+} signaling in other biological processes, such as egg activation and fertilization (Santella et al., 2004), cellular cleavage (Webb and Miller, 2003; Baluska et al., 2006), neuronal development (Archer et al., 1998) and cell death (Berridge et al., 1998; Chinopoulos and Adam-Vizi, 2006). We will first describe current models of Ca^{2+}-mediated cellular signaling, such as the organelles and proteins important for Ca^{2+} dynamics and their interpretation by Ca^{2+}-sensitive factors. Later, we summarize current knowledge on the role of Ca^{2+} signaling in cell fate decisions in the vertebrate embryo, from the cellular blastoderm through organogenesis and the stem cell niche. Finally, we present current known associations between Ca^{2+} signaling pathways and human developmental disorders.

An overview of calcium signaling pathways

Ca^{2+} ions are not metabolized by the cell. Instead, Ca^{2+} acts as a second messenger in the cell by forming ionic gradients within or outside the cell. Such gradients originate through Ca^{2+} mobilization across membranes, either the plasma membrane or the membrane of intracellular Ca^{2+}-storing organelles (Fig. 1). The resulting Ca^{2+} increases are regulated by the location, extent and duration of the ion channel opening and when interpreted by Ca^{2+}-sensitive mediators result in local or global signaling events that implement cellular responses.

In non-excitable (non-neuronal) cells, the majority of intracellular Ca^{2+} release occurs through inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} channels present in the endoplasmic reticulum (ER) membrane. Other channels, present in other cellular organelles, can also contribute to intracellular Ca^{2+} release, such as the ryanodine receptors (RyR) in the ER, NAADP-triggered receptors in lysosome-like organelles and ion exchange channels in mitochondria (reviewed in Berridge et al., 2003). There is extensive feedback between Ca^{2+} release circuits. For example, Ca^{2+} released from the ER can bind back to receptors (IP_3 receptors (IP_3Rs) and RyRs) and stimulate Ca^{2+}-induced Ca^{2+} release influencing neighboring receptors and potentially triggering a regenerative Ca^{2+} wave (Berridge, 1997; Berridge et al., 2003; Roderick et al., 2003a). In addition,

* Corresponding author. Fax: +1 608 262 2976.
E-mail addresses: diane-slusarski@uiowa.edu (D.C. Slusarski), fjpelegri@wisc.edu (F. Pelegri).
[1] Fax: +1 319 335 1069.

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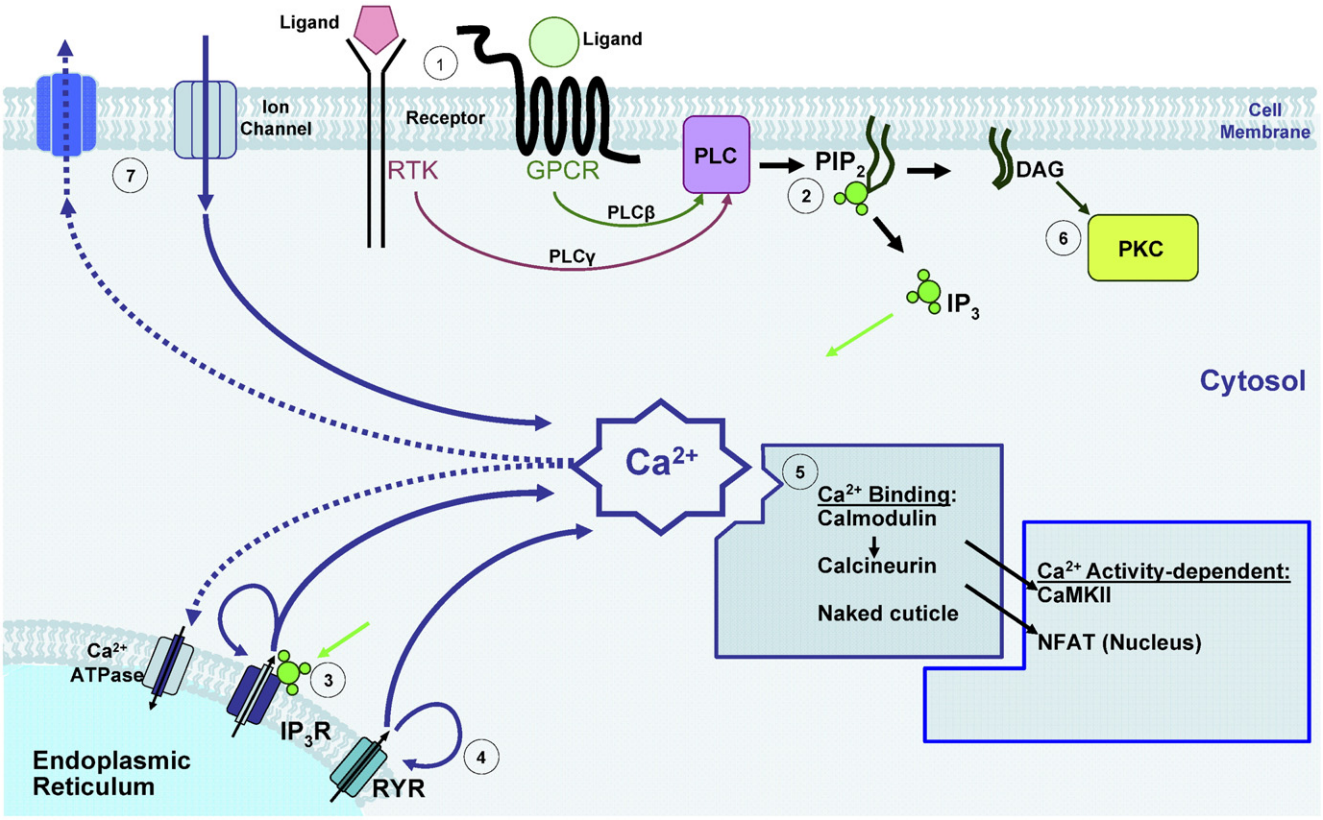

Fig. 1. Schematic diagram of cellular Ca^{2+} sources in non-excitable cells. (1) Stimulation of the cells with agonists and growth factors leads to the activation of GPCR and RTK. (2) This leads to activation of PLC isoforms, which catalyze the hydrolysis of PIP_2 giving rise to IP_3 and DAG. (3) IP_3 binds to its receptor (IP_3R) on the ER and triggers Ca^{2+} release from the store. (4) One aspect of CICR involves Ca^{2+} binding to the high affinity Ca^{2+} activation sites on IP_3R and RyR inducing the channels to open. (5) Intracellular Ca^{2+} is rapidly bound by Ca^{2+} binding proteins, which leads to their activation. (6) DAG is another second messenger which activates PKC among other targets. (7) Clearance of cytoplasmic Ca^{2+}, shown by dashed lines, occurs by Ca^{2+} extrusion via plasmalemmal pumps and Na^{+}/ Ca^{2+} exchange as well as by uptake into intracellular stores, such as the endoplasmic reticulum. GPCR, G-protein-coupled receptor; RTK, receptor protein tyrosine kinase; PLC, phosphoinositide-specific phospholipase C; PIP_2, membrane phosphatidylinositol-4,5-biphosphate; PLC, protein kinase C; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; RYR, ryanodine receptor; CaMK II, Ca^{2+}calmodulin-dependent kinase II; CICR, Ca^{2+}-induced Ca^{2+} release; NFAT, nuclear factor of activated T cells.

continued stimulation and/or depletion of ER stores activates a store operated Ca^{2+} entry (SOC) influx pathway located at the plasma membrane (Parekh and Putney, 2005).

A number of studies have implicated a signal transduction pathway dependent on the phosphatidylinositol (PI) cycle leading to Ca^{2+} release from intracellular organelles in early developmental cell decisions. This is corroborated by studies that demonstrate broad expression of IP_3R subtypes beginning at early developmental stages (Kume et al., 1993; Kume et al., 1997b; Rosemblit et al., 1999). In comparison, the RyR is thought to have a major role in striated muscle function and its expression only occurs as organogenesis proceeds, particularly in skeletal and cardiac muscle. The 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 (GPCR) and the receptor tyrosine kinase (RTK) class. Extracellular ligand stimulation of these receptors activates a PI-specific phospholipase C (PLC) (Fig. 1). GPCRs generally activate PLC-β while RTKs generally stimulate PLC-γ. Activated PLC converts membrane bound phosphatidylinositol (4,5) bisphosphate (PIP_2) into IP_3 and lipophilic diacylglycerol (DAG). IP_3 subsequently binds to receptors located principally on the endoplasmic reticulum (ER) and activates the IP_3R, 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 pathway downstream targets such as Protein Kinase C (PKC; see below).

Effectors and interpretation of calcium signals

Relative to cytosolic Ca^{2+} levels, cellular stimulation has been shown to induce a transient increase or oscillations of Ca^{2+} (Bootman et al., 2001), and in some systems these two responses may occur simultaneously (Gerbino et al., 2005). Much of the newly released cytosolic Ca^{2+} is quickly bound by Ca^{2+} binding proteins (Falcke, 2003). Some of these proteins act as Ca^{2+} buffers while other proteins become activated components of signal transduction pathways. For example, calmodulin, a member of the EF-hand protein family that represents the most abundant family of eukaryotic Ca^{2+} binding proteins (Haiech et al., 2004), is activated by cooperative binding of Ca^{2+} ions and subsequently activates protein kinases, phosphatases, ion transporters and cytoskeletal proteins. One

particularly notable class is the Ca^{2+}/calmodulin-dependent kinase (CaMK) family (Hoeflich and Ikura, 2002; see Table 1 for a summary of Ca^{2+} signaling regulators described in this review).

Another major target of activated calmodulin is the protein phosphatase calcineurin, which activates the nuclear factor of activated T cells (NFAT). Calcineurin phosphorylates NFAT proteins, promoting their nuclear localization and assembly with partner proteins to form transcription complexes. Rephosphorylation by an unknown priming kinase and glycogen synthase kinase-3 (GSK-3) leads to NFAT export from the nucleus (Beals et al., 1997; Graef et al., 1999), ending their cycle of activation (reviewed in Schulz and Yutzey, 2004). Another set of molecular targets of PI cycle activation is constituted by the protein kinase C (PKC) isozymes, which are activated by both DAG (produced by PIP_2 hydrolysis) and free intracellular Ca^{2+} (Sakai et al., 1997; Oancea and Meyer, 1998; Shirai et al., 1998; Violin et al., 2003). In addition to triggering specific cellular inductive responses, intracellular Ca^{2+} concentrations can affect the general state of the cell, for example the levels of protein synthesis and folding (Roderick et al., 2003b) and the decision to undergo apoptosis (Berridge et al., 1998). A review of other known Ca^{2+}-sensitive factors can be found in Ikura et al., (2002).

A particularly important emerging concept is the idea that ubiquitous Ca^{2+} can trigger various specific cellular responses by virtue of 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 (Luo et al., 2001; Rey et al., 2005), PLC activity (Thore et al., 2004; Nomikos et al., 2005) and IP_3 levels (Hirose et al., 1999; McCarron et al., 2004). 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., 2004; Patterson et al., 2004).

Many Ca^{2+}-binding proteins sense the frequency of intracellular Ca^{2+} increases. In the case of CaMKII, such ability has been shown to depend on the synergism between Ca^{2+}/calmodulin bound to each of the multimeric CaMKII subunits and the activity of the kinase domain (De Koninck and Schulman, 1998; Dupont and Goldbeter, 1998). Of interest, the frequency-dependent response to Ca^{2+} oscillations can be modulated by the use of alternative CaMKII splice variants (Bayer et al., 2002), suggesting that gene regulation may further modify the cellular response to variations in intracellular Ca^{2+}. The transcriptional regulatory activity of NFAT has also been shown to be exquisitely sensitive to the frequency of IP_3 and Ca^{2+} oscillations, presumably via changes in calcineurin activity (Dolmetsch et al., 1997; Dolmetsch et al., 1998; Li et al., 1998). Other studies have shown that Ca^{2+} oscillation frequencies mediate Ca^{2+}-dependent activation of Ras family effector G-proteins and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade (Walker et al., 2004; Kupzig et al., 2005).

Calcium transients and axis induction

Axis induction in vertebrates has been shown to be dependent on the activity of the Wnt signaling network (Fig. 2) (reviewed in Pelegri, 2003; Weaver and Kimelman, 2004; see also Tao et al., 2005). Activation of the so-called canonical Wnt pathway results in the inhibition of a complex; composed of GSK-3, Axin/Conductin, the adenomatous polyposis tumor suppressor protein (APC) and Diversin, which normally targets the β-catenin pro-

Table 1
Regulators of calcium signaling with an inferred developmental role

Factor	Type	Role	Process affected	References
Wnt-5/Ppt	Extracellular ligand	Activates Ca^{2+} transients	Axis induction/convergence extension	Slusarski, et al., 1997a,b; Westfall et al., 2003a,b
hecate	Unknown	Regulates Ca^{2+} transient frequency	Axis induction	Lyman-Gingerich et al., 2005
CaMKII	EF-hand Ca^{2+}-binding kinase	Regulates target protein factors	Axis induction/convergence extension	Kühl et al., 2000a,b
Calcineurin	Ca^{2+}-dependent phosphatase	Promotes NFAT nuclear translocation	Axis induction/organ formation	Saneyoshi et al., 2002; Yoshida et al., 2004
NFAT	Transcription factor	Regulates target gene expression	Axis induction/stem cell maintenance/organ formation	Saneyoshi et al., 2002; Kawano et al., 2006; Shulz and Yutzey, 2004; Wilkins and Molkentin, 2004
Pkd-2	Ca^{2+}-permeable ion channel	Required for Ca^{2+}-asymmetry in the node	Left–right asymmetry	McGrath et al., 2003
CaR	Seven-transmembrane Ca^{2+}-sensing receptor	Required for import of extracellular Ca^{2+}	Stem cell homing	Adams et al., 2005
DYRK1A	Nuclear serine/threonine kinase	Prevents nuclear translocation of NFAT	Defects associated with Down's syndrome	Arron et al., 2006
SHP-2/PTPN11	Src homology tyrosine phosphatase	Activates Ca^{2+} transients	Defects associated with Noonan syndrome	Uhlén et al., 2006

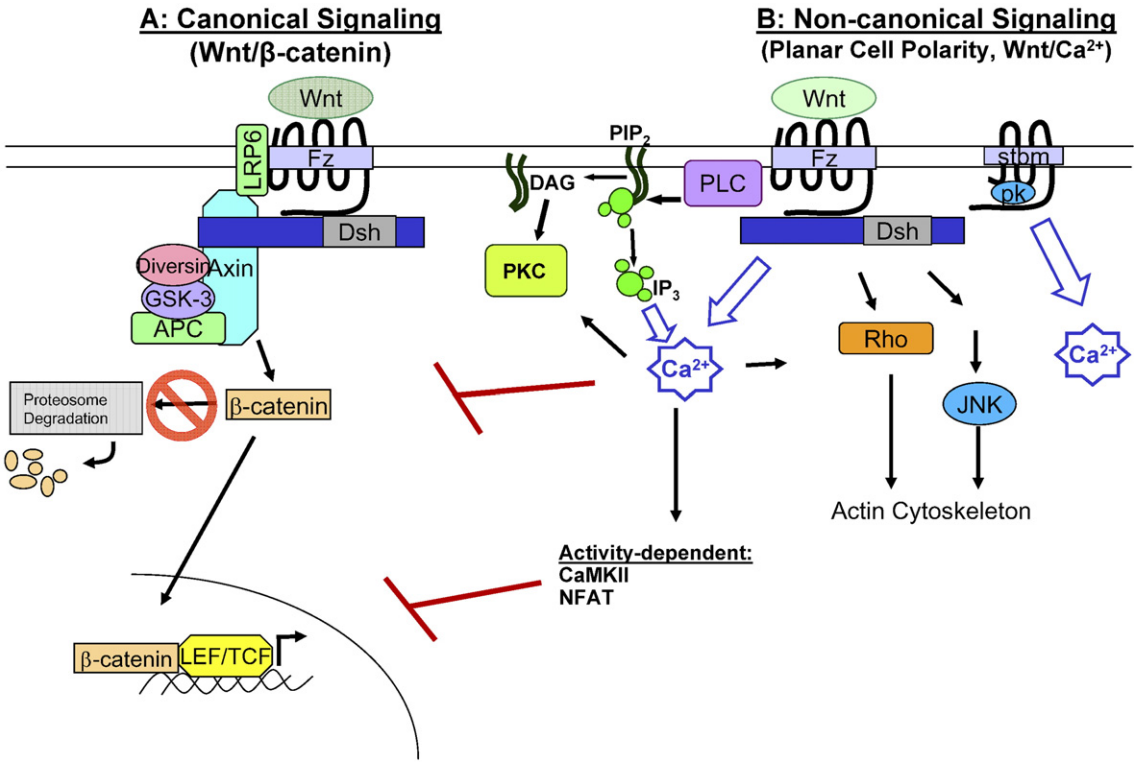

Fig. 2. Schematic diagram of the Wnt signaling network. Highlighted are key components identified in the (A) Wnt/β-catenin and the (B) Wnt/Ca^{2+} signaling pathways. When the so-called "canonical" or Wnt/β-catenin path is inactive, a degradation complex, including Axin, GSK-3 and APC, phosphorylates β-catenin inducing its rapid destruction by the proteasome. Once the Frizzled(Fz)/LRP co-receptor complex is bound by Wnt, Fz interacts with Dsh, which modifies the destruction complex and leads to β-catenin stabilization. Nuclear β-catenin interacts with LEF/TCF to promote the transcription of Wnt target genes. The so-called "non-canonical" Wnts are thus named as they appear to act independently of β-catenin. Wnt binding to Fz leads to activation of Dsh, an increase in intracellular Ca^{2+} and activation of PKC. Increased intracellular Ca^{2+} can then lead to a secondary activation of PKC as well as to activation of CaMKII and NFAT. Increased intracellular Ca^{2+} and activated calcium sensors have been shown to antagonize β-catenin, noted as red bars. The PCP pathway also signals through Fz and Dsh which then signals through small GTPases (Rho) and C-Jun N-terminal kinase (JNK) to modulate cytoskeletal elements. The PCP pathway utilizes core components, shown are stbm and pk. Fz, Dsh and pk are all capable of activating Ca^{2+} release. Fz, Frizzled; LRP, low density lipoprotein receptor; APC, adenomatous polyposis coli; GSK-3, glycogen synthase kinase 3; Dsh, Dishevelled; TCF, T cell factor; LEF, lymphoid enhancer factor; PKC, protein kinase C; PLC, phospholipase C; JNK, c-jun NH_2-terminal kinase; stbm, Strabismus; pk, Prickle.

tein for degradation via ubiquitination and the proteasome complex (Fig. 2A) (reviewed in Polakis, 2000). Inactivation of the β-catenin degradation complex by Wnt signaling results in the stabilization and nuclear accumulation of β-catenin protein; thus, this pathway has been termed the Wnt/β-catenin pathway. Nuclear β-catenin in turn interacts with members of the LEF/TCF transcription factor family to promote the activation of downstream target genes involved in axis specification.

However, other Wnt pathways, either in parallel or as part of a complex signaling network, appear to interact with the Wnt/β-catenin pathway in the early specification of the embryonic axis. In *Xenopus* and zebrafish, one of these pathways involves the PI cycle and Ca^{2+} release (Fig. 2B). Classical studies linking PI cycle activity to body plan determination reported the ability of lithium, an inhibitor of inositol turnover (Berridge et al., 1989), to induce dorsal cell fates in *Xenopus* (Kao et al., 1986; Kao and Elinson, 1989; Kao and Elinson, 1998), and similar effects were obtained in the zebrafish embryo (Stachel et al., 1993; Aanstad and Whitaker, 1999). Lithium-induced expansion of dorsal structures in the embryo can be rescued by supplying an intermediate of the PI cycle (*myo*-inositol; Busa and Gimlich, 1989), indicating that indeed the PI cycle is a primary target with regards to the effects of this agent on dorsal cell fate specification. Moreover, 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 in the dorsal side. Subsequent findings indicated that another endogenous target of lithium is the β-catenin degradation complex component GSK-3, which when inhibited promotes dorsal axis induction (Klein and Melton, 1996; Stambolic et al., 1996). Exogenous *myo*-inositol can also suppress the effects of GSK-3 inhibition (Hedgepeth et al., 1997), further supporting the notion that PI cycle activity and Wnt/β-catenin signaling act in parallel to regulate axis induction. It remains to be determined whether lithium affects additional targets involved in axis induction.

Several pieces of evidence in zebrafish and *Xenopus* further support a requirement for PI cycle activity in dorsoventral patterning. *Xenopus* embryos injected with antibodies that disrupt IP_3R function displayed expanded dorsal structures with the loss of ventral structures (Kume et al., 1997a). A similar dorsalization effect can be observed in the zebrafish after injection of IP_3R blocking antibodies as well as treatments with

other PI-cycle inhibitors (Westfall et al., 2003b). Together, these studies suggested that high levels of PI cycle activity promote ventral cell fates, possibly by counteracting the axis-inducing Wnt/β-catenin signaling pathway.

The findings of an involvement for PI cycle activity in axis induction agree well with the observed spontaneous increase in IP_3 levels in the *Xenopus* embryo at the blastula stage (Busa and Gimlich, 1989; Maslanski et al., 1992). Moreover, beginning at the 32-cell stage, the zebrafish embryo exhibits rapid aperiodic Ca^{2+} release that persists until the midblastula transition stage (Reinhard et al., 1995; Slusarski et al., 1997a; Slusarski et al., 1997b), consistent with the idea that the increased IP_3 levels may trigger Ca^{2+} release during these stages. This idea has been corroborated by drug inhibition studies that indicate that these Ca^{2+} transients depend on PLC activity and IP_3-dependent Ca^{2+} release from the ER (Slusarski et al., 1997a; Slusarski et al., 1997b).

Inhibition of G-protein signaling suppresses Ca^{2+} release in zebrafish (Slusarski et al., 1997a; Ahumada et al., 2002), indicating that the Ca^{2+} release pathway occurs downstream of a G-protein-coupled receptor (as opposed to a G-protein-independent pathway of PLC activation such as that triggered by fibroblast growth factor; see below). Of interest are Ca^{2+} transients in the zebrafish blastula that originate in external cellular layers, the enveloping layer (EVL) and yolk syncytial layer (YSL) (Reinhard et al., 1995; Slusarski et al., 1997b). Although the EVL and YSL are extraembryonic (Kimmel et al., 1995), it has been proposed that signaling from these layers becomes transmitted into the blastula cells that will form the embryo proper. There is accumulating evidence that this does occur between the YSL and the overlying deep cells (Mizuno et al., 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999) and has been proposed to occur between the EVL and the cells below (Westfall et al., 2003a; Westfall et al., 2003b; Lyman-Gingerich et al., 2005). The mechanism of the intercellular transmission of this Ca^{2+} remains unknown, although it is possibly mediated by gap junctions present in zebrafish blastula cells (Bozhkova and Voronov, 1997), which have been shown to be involved in the transmission of Ca^{2+}-releasing small molecules such as IP_3 (Clair et al., 2001).

In vertebrate embryos, while overexpression of a subset of Wnts induces hyperdorsalization and ectopic axes by virtue of Wnt/β-catenin signaling activity (Moon et al., 1993b; Du et al., 1995; Kelly et al., 1995; Dale, 1998; Moon and Kimelman, 1998), a second Wnt class (including *Wnt-5A*, *-4* and *-11*) appears to act independently of β-catenin function (Kühl et al., 2000b). Emerging evidence suggests that the ability of Wnt ligands to activate different signaling pathways, β-catenin-dependent (or canonical) and β-catenin-independent (or non-canonical) appears to be dependent on timing of expression and receptor context. 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., 1997a; Slusarski et al., 1997b), thus linking this Wnt family activity to IP_3-dependent Ca^{2+} release and defining the Wnt/Ca^{2+} signaling pathway. Various studies have shown that there are common components, between the Wnt/Ca^{2+} and another non-canonical Wnt pathway, the planar cell polarity (Wnt/PCP) pathway, involved in the polarization of cells in *Drosophila* and vertebrate species (reviewed in Wallingford et al., 2002; Strutt, 2003). These common components suggest that non-canonical Wnt signaling activity can be viewed as a complex network with cellular outputs identified by Ca^{2+} modulation and polarized cell movement (Mlodzik, 2002).

The link between non-canonical Wnt pathway activation and axis induction was initially suggested by the apparent antagonism of certain pairs of Wnt ligands when expressed in *Xenopus* and zebrafish embryos (Moon et al., 1993b; Slusarski et al., 1997b). Expression of ligands that activate Wnt/β-catenin signaling in these embryos, such as Wnt-8, results in ectopic axis induction. However, coexpression of these Wnt ligands with others that when expressed on their own do not promote Wnt/β-catenin activation, such as Wnt-5A, suppresses this axis-induction effect. Stimulating Ca^{2+} release, for example via activation of the Serotonin receptor, also antagonizes *Xwnt-8* induced expansion of the dorsal domains (Slusarski et al., 1997b), suggesting 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 generates ectopic accumulation of nuclear β-catenin and activation of β-catenin transcriptional targets (Westfall et al., 2003a; Westfall et al., 2003b), and G-protein inhibition is able to dorsalize *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+} signaling activity, negatively regulates the Wnt/β-catenin signaling pathway and therefore axis induction (Fig. 2).

Further support of this idea comes from the analysis of a mutation in the zebrafish maternal gene *hecate*, where an increase in Ca^{2+} release frequency is associated with a strong inhibition of dorsal axis induction (Lyman-Gingerich et al., 2005). Pharmacological inhibition studies indicated that the ectopic Ca^{2+} release observed in *hecate* embryos depends on Wnt/Ca^{2+} pathway components, and interference with Ca^{2+} dynamics was shown to rescue the defects in dorsal cell fate specification observed in these mutants. Importantly, the level of Wnt/β-catenin activity does not affect the frequency of endogenous Ca^{2+} transients (Westfall et al., 2003a; Lyman-Gingerich et al., 2005), in agreement with a causal relationship between Ca^{2+} release and the inhibition of dorsal axis induction.

In the zebrafish, Wnt-5 has been shown to correspond to the genetically defined gene *pipetail* (*ppt*; Rauch et al., 1997), a gene which when mutated results in zygotic defects in the extension of the axis during somitogenesis (Hammerschmidt et al., 1996; Kilian et al., 2003). The possibility that Wnt-5/Ppt itself is the endogenous activator of Wnt/ Ca^{2+} signaling in the zebrafish embryo was determined by testing for maternal effects caused by germ line homozygosity for *Wnt-5*/*ppt*. Zebrafish embryos lacking maternal *Wnt-5*/*ppt* function exhibit a reduction in the frequency of Ca^{2+} transients and a stabilization of nuclear β-catenin, as well as dorsalized phenotypes, which become more prevalent if they are additionally mutant for zygotic *Wnt-5*/*ppt* (Westfall et al., 2003a). Thus, the gain- and

loss-of-function effects of Wnt-5 suggest that this factor is an early endogenous signal involved in Wnt/Ca^{2+} activation and the regulation of dorsal axis induction.

Several Ca^{2+}-sensitive factors have been implicated as potential downstream mediators of Wnt/Ca^{2+} antagonism of Wnt/β-catenin signaling. In *Xenopus*, CaMKII is activated by Wnt and Frizzled (Fz) receptors to promote ventral cell fates (Kühl et al., 2000a). In the zebrafish embryo, expression of constitutively active CaMKII can similarly lead to axis induction defects (Westfall and Slusarski, unpublished observations). Moreover, CaMKII activation rescues the zygotic *Wnt-5*/*ppt* phenotype, showing that CaMKII activity occurs downstream of Wnt/Ca^{2+} pathway activation, at least during the gastrulation stages. Other studies in *Xenopus* have shown that Wnt-5A induces nuclear translocation of the calcineurin target transcription factor NFAT (Saneyoshi et al., 2002). The same studies also show that the expression of activated NFAT ventralizes *Xenopus* embryos and antagonizes Wnt/β-catenin activity, while conversely expression of dominant-negative NFAT induces ectopic axis formation and expression of dorsal target genes. Additionally, the *Drosophila* segment polarity gene *naked cuticle* (*nkd*) has been shown to antagonize Wnt/β-catenin activity in a manner dependent on its EF-hand Ca^{2+}-binding motif (Zeng et al., 2000; Rousset et al., 2001; Wharton et al., 2001; Li et al., 2005). Thus, multiple Ca^{2+}-sensitive factors may be likely candidates to regulate Wnt/Ca^{2+} signaling and axis induction, although some of these studies have the caveat that the observed effects on axis induction depend on the expression of activated or dominant-negative proteins, or are context-dependent. For example, maternally provided Wnt-11 has been shown to be the endogenous signal involved in Wnt/β-catenin activation and axis induction in *Xenopus* (Tao et al., 2005), and Wnt-5A, when coexpressed with the appropriate Frizzled receptor, can also induce Wnt/β-catenin signaling (Mikels and Nusse, 2006). Yet genetic loss of Wnt-11 function in the zebrafish supports a clear role in cell movement and no indication of a role in axis formation (Heisenberg et al., 2000). Loss of function studies using genetic mutations or functional knockdown approaches should be helpful in discerning the identities of the endogenous factors involved in this process.

The precise nature of the regulation of the Wnt/β-catenin pathway by Ca^{2+}-sensitive mediators is also not fully understood. In the zebrafish blastula embryo, this regulation may occur upstream or at the level of β-catenin accumulation, as suggested by the reduction of nuclear β-catenin in *hecate* mutant embryos (Lyman-Gingerich et al., 2005), and the ectopic accumulation of nuclear β-catenin in embryos where Ca^{2+} release is inhibited (Westfall et al., 2003b). In *Xenopus*, calcineurin/NFAT activity appears to regulate Wnt/β-catenin signaling by modulating the activity of the GSK-3-dependent β-catenin degradation complex (Saneyoshi et al., 2002), suggesting a possible mechanism for this regulation. However, there is also precedent for other modes of GSK-3-independent regulation of β-catenin stability, as in the vertebrate limb, where Wnt-5A promotes the degradation of β-catenin in a manner dependent instead on the Siah-APC-Ebi E3 ubiquitin ligase complex (Topol et al., 2003). The protease calpain has also been shown to mediate the Ca^{2+}-dependent degradation of β-catenin independently of the GSK-3-containing β-catenin degradation complex (Li and Iyengar, 2002). Similarly, activated PKC can promote β-catenin degradation through a GSK-3-independent mechanism (Gwak et al., 2006). Furthermore, it remains a possibility that Wnt/ Ca^{2+} may also regulate dorsal induction in a manner independent of β-catenin itself, as has been proposed in various cellular systems where CaMKII acts through a mitogen-activated protein kinase (MAPK) pathway to directly regulate the activity of Tcf family transcription factors (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Ishitani et al., 2003a,b).

The emerging picture is made additionally complex by the possibility that Ca^{2+}-sensitive targets may not only affect Wnt/β-catenin activity but may also feed back to modify the activity of Wnt/Ca^{2+} signaling. For example, increased DAG and Ca^{2+} levels caused by Wnt/Ca^{2+} pathway activation trigger the recruitment of PKC to the plasma membrane in early vertebrate embryos (Berridge, 1993; Sheldahl et al., 1999; Sheldahl et al., 2003) and this activated kinase both regulates common Wnt pathway components such as Dishevelled (Dsh; Kinoshita et al., 2003) and provides negative feedback on Ca^{2+} oscillations (Codazzi et al., 2001; Halet et al., 2004).

While the role of Wnt/Ca^{2+} in axis induction is becoming increasingly substantiated in the vertebrate embryo, less certain is the significance of Ca^{2+} release mediated by other signaling pathways such as fibroblast growth factor (FGF). As with other members of the RTK family, ligand stimulation of FGF receptors activates PLC-γ (Mohammadi et al., 1991), hydrolyzes PIP_2, into IP_3 and DAG and leads to the subsequent release of Ca^{2+} from IP_3-sensitive intracellular stores (Fig. 1). In *Xenopus*, activation of FGF signaling induces mesoderm in the blastula embryo (Kimelman and Kirschner, 1987; Slack et al., 1987; Kimelman et al., 1988) as well as Ca^{2+} efflux in oocytes (Muslin et al., 1994). However, although phosphorylation of PLC-γ by the FGF receptor has been shown to be developmentally associated with mesoderm induction in *Xenopus* (Ryan and Gillespie, 1994; Ryan et al., 1998), a mutation in the FGF receptor that renders it unable to either activate PLC-γ or trigger Ca^{2+} release does not interfere with its mesoderm-inducing ability (Muslin et al., 1994). Thus, PLC-γ activation, and presumably FGF-induced Ca^{2+} release, does not appear to be necessary for mesoderm induction. Studies in the zebrafish system have shown an additional role for FGF, which is dorsally expressed during gastrulation, in the establishment of dorsoventral patterning (reviewed in Thisse and Thisse, 2005). This later role appears to occur independently of the early Wnt/β-catenin pathway involved in axis induction and instead occurs by the repression of the ventral inducing BMP factors in dorsal regions. Of interest, Palma et al. (2001) have shown a role for Ca^{2+} signaling in determining *dorsal* cell fates during gastrulation and not ventral cell fates as suggested by the Ca^{2+}-dependent inhibition of axis induction normally observed in the blastula embryo (Westfall et al., 2003a,b; Lyman-Gingerich et al., 2005). Further studies are needed to determine whether FGF-mediated Ca^{2+} signaling has a role in the promotion of dorsal fates in the gastrulating embryo.

Global waves and morphogenesis during vertebrate gastrulation

During gastrulation, vertebrate embryos undergo a variety of morphogenetic movements instrumental for the development of the body plan (reviewed in Keller, 2002; Wallingford et al., 2002), including the dorsally directed migration that results in axis thickening (dorsal convergence) and the lateral intercalation of axial cells that results in its elongation (axis extension). Recent studies suggest that Ca^{2+} release may be involved in the orchestration of such morphogenetic movements involving cell polarization. Waves of Ca^{2+} mobilization, associated with waves of tissue contraction, can be observed in dorsal explants of gastrulating *Xenopus* embryos (Wallingford et al., 2001). Similarly, intercellular Ca^{2+} waves have been observed at the margin of gastrulating zebrafish embryos (Gilland et al., 1999). A causal relationship between Ca^{2+} waves and morphogenesis is supported by the finding that, in *Xenopus* embryos, pharmacological inhibition of such waves results in convergent extension defects without affecting cell fate (Wallingford et al., 2001). Similarly, zebrafish embryos zygotically mutant for *Wnt-5*/*ppt*, which exhibit a reduction in Ca^{2+} transient frequency (Westfall et al., 2003a), exhibit defects in axis extension (Hammerschmidt et al., 1996; Kilian et al., 2003). As mentioned above, expression of activated CaMKII can rescue the convergence extension defect characteristic of *Wnt-5*/*ppt* mutants (Westfall et al., 2003a), indicating that CaMKII may mediate the effects of Ca^{2+} in this process. These data suggest the possibility that these Ca^{2+} waves coordinate convergent extension (C-E) during vertebrate gastrulation.

Convergent extension in the vertebrate embryo, a result of the polarization of migrating cells, is considered analogous to the PCP pathway involved in the polarization of epithelial cells in the *Drosophila* cuticle (Solnica-Krezel, 2005). Wnt genes that result in the activation of Ca^{2+} release in the blastula embryo, such as Wnt-5 (Slusarski et al., 1997b; Westfall et al., 2003b), can also alter morphogenetic movements later during gastrulation (Moon et al., 1993a; Ungar et al., 1995). Recent studies indicate that that Wnt/Ca^{2+} and Wnt/PCP pathways share common components and may even be part of a loosely connected network (Sheldahl et al., 2003). The observations that interference with either Ca^{2+} release or Wnt/PCP signaling results in convergence extension defects suggests that this non-canonical Wnt signaling network is involved in convergence extension (Fig. 2B).

Indeed, in addition to *Wnt-5*/*ppt*, mutations in other genes involved in non-canonical Wnt signaling result in cell movement defects in zebrafish. This is the case for Wnt-11/silberblick (Heisenberg et al., 2000), the Wnt receptor Frizzled-2 (Oishi et al., 2006), the putative transmembrane protein Strabismus/trilobite (Jessen et al., 2002; Park and Moon, 2002) and the intracellular protein Prickle (Veeman et al., 2003). In addition, expression of Prickle (Veeman et al., 2003), Frizzled-2 (Slusarski et al., 1997a), Strabismus (DCS unpublished) and Wnt-4, -5 and -11 (Westfall et al., 2003a) all stimulate Ca^{2+} release in zebrafish. Likewise, the mutant form of Dsh that retains the ability to signal through the PCP pathway but not the Wnt/β-catenin pathway is also able to activate the Wnt/Ca^{2+} cascade in *Xenopus* and zebrafish (Sheldahl et al., 2003). On the other hand, pharmacological reagents that suppress Fz2-induced Ca^{2+} release in zebrafish lead to altered gastrulation movements (Slusarski et al., 1997a; Ahumada et al., 2002). Similarly, a requirement for G-protein signaling in gastrulation was recently demonstrated by antisense morpholino oligonucleotide knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ and the use of dominant-negative constructs (Lin et al., 2005). These observations are consistent with the possibility that Wnt/Ca^{2+} signaling, possibly dependent upon G-protein activity, is important for cell polarization involved in vertebrate morphogenesis.

Oishi et al., (2006) report that the knockdown of the putative phosphorylation-dependent cytoskeletal regulatory molecule, *duboraya* (*dub*), synergizes with a *Frizzled-2* knockdown to produce embryos with shorter anteroposterior axes and undulating notochords, a phenotype consistent with convergence extension defects. These studies also show that phosphorylation of *dub*, known to be essential for its function, is influenced by the expression of proteins that stimulate Ca^{2+} release in zebrafish embryos (Liu et al., 1999; Ahumada et al., 2002; Sheldahl et al., 2003). Thus, it is possible that Wnt/ Ca^{2+} signaling results in the activation of *dub* via phosphorylation, although further study is required to confirm this hypothesis.

Ca^{2+} as a second messenger regulating cellular movements has been demonstrated in many cell types and most likely has a multifold role in coordinating epiboly and gastrulation movements in the embryo. Drawing a parallel between neural outgrowth and gastrulation, transient Ca^{2+} release has been proposed to influence neuronal outgrowth by regulating cellular secretion and organization of the cytoskeleton (reviewed in Spitzer, 2006). Thus, secretion of diffusible molecules, such as the Wnts, and the generation of new cell contacts could enable inductive interactions among cells. In addition, cellular microdomains (including receptors, their associated proteins and Ca^{2+} pumps) have been described in polarized epithelial cells (reviewed in Kiselyov et al., 2006). The polarized distribution of Fz and other core PCP components could lead to differential Ca^{2+} dynamics across a cell, or sheet of cells, and influence cell adhesion and motility. Further insight into downstream targets could also be drawn from the growing tips of plants, which integrate small GTPases, PI cycle, Ca^{2+} and protein kinases to mediate actin cytoskeletal reorganization and membrane trafficking (reviewed in Cole and Fowler, 2006). Investigation of Ca^{2+} release dynamics in zebrafish epiboly and convergence extension mutants may further correlate intracellular Ca^{2+} with coordinated or polarized cell movements.

Calcium, cilia and left–right patterning

Evidence from several vertebrate model systems suggests that the positioning of the internal organs across the left–right (L–R) axis, presaged by the asymmetric expression of a group of genes (Levin, 2005), is modulated by Ca^{2+} signaling. In mice, the symmetry-breaking event in left–right polarity is thought to arise from a directional flow generated by the rotation of monocilia in the embryonic node (Nonaka et al.,

1998; Okada et al., 1999). Similar monocilia are observed in the chick node and the zebrafish Kupffer's vesicle (KV), where they are proposed to serve a similar function as in the mouse node. In these analogous structures, cilia beat in the same direction, creating a leftward nodal flow. In the mouse, this flow has been proposed to stimulate mechanosensory cilia to trigger an elevation in intracellular Ca^{2+} levels in cells along the left edge of the node (McGrath et al., 2003). Intracellular Ca^{2+} increases with a left-sided bias near the zebrafish KV have also been detected (Sarmah et al., 2005). Elevated intracellular Ca^{2+} is thought to act as a second messenger, via an unknown mechanism, to ultimately induce left-sided gene expression. This model is further supported by the observation that the asymmetry in node Ca^{2+} levels is lost in mouse embryos homozygous for mutations in the polycystic kidney disease gene (*Pkd-2*), a Ca^{2+}-permeable ion channel, and that these mutants exhibit laterality defects (McGrath et al., 2003).

In chick embryos, it is not known if there is a similar asymmetry of intracellular Ca^{2+} as observed in the mouse node and zebrafish KV. However in chick, it appears that *extracellular* Ca^{2+} levels may be higher transiently on the left side. This asymmetry was abolished after treatment with ompremazole, an inhibitor of H^+/K^+ ATPase, which also caused L–R defects, specifically the reversal of heart looping. These results led the authors to propose that differential H^+/K^+ ATPase activity sets up a spatial gradient of extracellular Ca^{2+}, which is subsequently transduced to activate asymmetric gene expression on the left side (Raya et al., 2004). Thus, evidence of a role for Ca^{2+} in L–R patterning is very tantalizing, but many questions and issues remain to be addressed; such as the Ca^{2+} sources, the Ca^{2+}-dependent responders and the precise role of extracellular versus intracellular Ca^{2+} in the induction and maintenance of laterality signals.

Recently, PCP components have been linked with cilia function and laterality. It has long been known that PCP-mediated cell polarization is required for the proper placement of cilia in *Drosophila* wing cells. However, only very recent studies suggest a similar function for PCP signaling in vertebrate cells. Indeed, *Frizzled-2* knockdown, in addition to C-E defects, results in a reduction in cilia length and number within the zebrafish KV (Oishi et al., 2006). The same authors report a similar defect caused by functional knockdown of the cytoskeletal regulator *duboraya*. Although the precise role of the Ca^{2+} releasing factor Fz2 and its proposed target *duboraya* in PCP signaling (see above) and ciliogenesis needs to be better substantiated, these findings suggest an association of Wnt/PCP and Ca^{2+}-releasing genes with cilia generation, maintenance and/or function.

Calcium signaling and organogenesis

Other aspects of organogenesis impacted by Ca^{2+} release involve the induction of the neural precursor cells, which will give rise to the Peripheral and Central Nervous Systems. The role of Ca^{2+} in neural induction has been extensively described in a recent review (Webb et al., 2005) and we describe here only some basic findings. Periodic Ca^{2+} fluxes are observed in anterior dorsal ectoderm during stages of presumptive neural patterning in *Xenopus*, where they increase in amplitude at a time coincident with neural induction (Leclerc et al., 2000). Similarly, zebrafish embryos also exhibit intercellular Ca^{2+} waves in the prospective dorsal region (Créton et al., 1998; Gilland et al., 1999). Ca^{2+} release from L-type Ca^{2+} channels present in the plasma membrane is required to induce neural specific genes in *Xenopus* (Leclerc et al., 1999, 2000, 2003) and the newt *Pleurodeles waltl* (Moreau et al., 1994). However, manipulations that inhibit Ca^{2+} release and neural induction also alter gastrulation movements (Leclerc et al., 2000; Palma et al., 2001; Wallingford and Harland, 2001), making it difficult to use pharmacological agents to separate the effects of Ca^{2+} signaling on gastrulation and neural patterning.

Neural induction involves interaction between bone morphogenetic proteins (BMPs) and their antagonists, such as chordin and noggin (De Robertis and Kuroda, 2004). In *Pleurodeles* explants, noggin application triggers an increase in Ca^{2+} release (Leclerc et al., 1999). Whether this Ca^{2+} transient occurs by the direct activation of Ca^{2+} release by noggin or via other noggin-modulated pathways, such as BMP signaling, has yet to be determined, as well as whether these events occur in the context of the whole animal.

In addition to neural induction, Ca^{2+} signaling has been implicated in the formation of the somites, which will give rise to muscle, cartilage and bones. Somites are derived from paraxial mesoderm, where Ca^{2+} release activity has been reported during the segmentation period (Créton et al., 1998; Webb and Miller, 2000). Ca^{2+} release activity has also been reported in isolated *Xenopus* myocytes (Ferrari and Spitzer, 1999) and in mature somites in whole zebrafish embryos (Ashworth, 2004). Ca^{2+} release inhibition alters myotome patterning (Ferrari and Spitzer, 1999). In addition, elimination of calcineurin activity in *Xenopus* embryos abolished somite formation and led to additional later organogenesis defects in the heart, kidney and gut looping (Yoshida et al., 2004). Recent work has linked bilateral somite formation to L–R asymmetry signals (Kawakami et al., 2005; Vermot et al., 2005; Vermot and Pourquie, 2005). It has yet to be determined whether this coupling of L–R and somite formation processes is directly linked to Ca^{2+} fluxes.

There is significant evidence suggesting a role for the calcineurin/NFAT pathway in the development of the cardiovascular and skeletal muscle systems, which has been presented in extensive recent reviews (Hogan et al., 2003; Wilkins and Molkentin, 2004). Future studies should aim at clarifying the regulatory pathways involved in Ca^{2+} release and modulation involved in these processes.

Calcium and the stem cell niche

Several studies are beginning to show a role for Ca^{2+} signaling in stem cell development. Human bone marrow-derived mesenchymal stem cells (hMSDs) show Ca^{2+} oscillations that are dependent on both Ca^{2+} release from IP_3Rs in the ER as well as Ca^{2+} entry and extrusion via plasma membrane ion pumps and Na^+–Ca^{2+} exchangers (Kawano et al., 2002,

2003). Further studies found that the Ca^{2+} oscillations depend on an autocrine/paracrine signaling pathway, where secreted ATP stimulates P2Y1 receptors to activate PLC-β to produce IP_3 (Kawano et al., 2006). These same studies showed that the translocation of the downstream transcription factor NFAT is dependent on the ATP-induced Ca^{2+} oscillations, and that these oscillations and NFAT nuclear translocation disappeared as hMSCs differentiated into adipocytes. Conversely, increases in intracellular Ca^{2+} result in the inhibition of differentiation of human adipocytes (Ntambi and Takova, 1996). These studies suggest a link between intracellular Ca^{2+} oscillations and the maintenance of undifferentiated hMSCs.

Another interesting report has shown a role for extracellular Ca^{2+}, present in the endosteal surface of the bone marrow and sensed by the seven transmembrane-spanning Ca^{2+}-sensing receptor (CaR), in the migration and homing of mammalian hematopoietic stem cells (HSCs; Adams et al., 2005). In this case, however, Ca^{2+} signaling does not appear to influence the ability of HSCs to proliferate or differentiate. As stem cells corresponding to other cell types are studied, it will be interesting to determine how common the involvement of Ca^{2+} signaling is in stem cell specification, homing and maintenance.

Human developmental disorders involving calcium-sensitive factors

Defects in the regulation of Ca^{2+}-sensitive factors may underlie a variety of developmental human syndromes. Two genes within the critical region responsible for Down's syndrome, DSCR1 and the nuclear serine/threonine kinase DYRK1A, act synergistically to prevent the nuclear translocation of the calcineurin target NFAT (Arron et al., 2006). This and previous studies have shown that calcineurin- and NFAT-deficient mice, as well as Dscr1- and Dyrk1a-overexpressing mice, show phenotypes similar to those of human Down's syndrome, including neurological, skeletal, cardiovascular and immunological defects (Arron et al., 2006). The authors propose that a 1.5-fold increase in dosage of the DSCR1 and DYRK1A genes destabilizes a regulatory circuit leading to reduced NFAT activity and Down syndrome features. A potential for disrupted Ca^{2+} regulation of the calcineurin/NFAT pathway resulting in Down's syndrome is further supported by the conservation across species of pathways regulating NFAT nuclear localization, namely activation by intracellular Ca^{2+} increase and calcineurin and inhibition by DYRK kinases (Gwack et al., 2006a,b). However, further analysis will be required to determine the precise role of Ca^{2+} signaling in Down's syndrome.

Recent studies have implicated a role of Ca^{2+} signaling misregulation in another human developmental disorder, Noonan syndrome, which is associated with facial dysmorphia, disproportionate short stature, increased risk of leukemia and congenital heart defects (Noonan, 1968; Allanson, 1987). This syndrome is thought to be caused by mutations in a src homology 2-containing protein tyrosine phosphatase (SHP-2/PTPN11), which cause its constitutive activation (Tartaglia et al., 2001, 2003; Araki et al., 2004). Gain-of-function mutants of SHP-2/PTPN11 enhanced FGF-2-mediated Ca^{2+} oscillations in fibroblasts, as well as spontaneous Ca^{2+} oscillations in cardiomyocytes (Uhlén et al., 2006). Together with the known role of the calcineurin/NFAT pathway in cardiac morphogenesis (Hogan et al., 2003; Schulz and Yutzey, 2004; Wilkins and Molkentin, 2004), these data suggest that at least some aspects of Noonan syndrome may be caused by increased frequency of Ca^{2+} oscillations and overactivation of calcineurin/NFAT signaling.

Conclusion

One of the most intriguing questions in biology is how ubiquitous signals can be used to convey specific information. Ca^{2+} signaling constitutes an excellent example of this challenge since it is important for basic cellular processes, from cell division to cell death, and also appears to regulate a variety of specific events involved in patterning and morphogenesis. An important part of the solution to this problem appears to be that information can be encoded through variations in amplitude, length and frequency of Ca^{2+} oscillations. Our understanding of the mechanisms that regulate these oscillations, and the processes involved in translating their effects into cellular responses, is still in its infancy. Other important avenues of research will address how such basic information branches into coordinated pathways involving both cell fate specification and morphogenesis. The exciting recent findings that suggest that misregulation of Ca^{2+} signaling pathways is involved in a number of human developmental disorders impart significant urgency to the quest toward their understanding, as it may result in therapies to treat these genetic disorders.

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