Dishevelled activates Ca\textsuperscript{2+} flux, PKC, and CamKII in vertebrate embryos

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Wnts and Frizzled (Fz) receptors have been shown to activate multiple intracellular signaling pathways. Activation of the Wnt–\β-catenin pathway has been described in greatest detail, but it has been reported that Wnts and Fzs also activate vertebrate planar cell polarity (PCP) and Wnt–Ca\textsuperscript{2+} pathways. Although the intracellular protein Dishevelled (Dsh) plays a dual role in both the Wnt–\β-catenin and the PCP pathways, its potential involvement in the Wnt–Ca\textsuperscript{2+} pathway has not been investigated. Here we show that a Dsh deletion construct, XDsh\text{ΔDIX}, which is sufficient for activation of the PCP pathway, is also sufficient for activation of three effectors of the Wnt–Ca\textsuperscript{2+} pathway: Ca\textsuperscript{2+} flux, PKC, and calcium/calmodulin-dependent protein kinase II (CamKII). Furthermore, we find that interfering with endogenous Dsh function reduces the activation of PKC by Xfz7 and interferes with normal heart development. These data suggest that the Wnt–Ca\textsuperscript{2+} pathway utilizes Dsh, thereby implicating Dsh as a component of all reported Fz signaling pathways.

Introduction

Wnts are a large family of secreted signaling molecules that play a number of important roles during animal development (for review see Miller et al., 1999). These genes are often expressed in spatially and temporally overlapping patterns. It is not surprising, therefore, that Wnts are able to signal via several intracellular pathways. The Wnt–\β-catenin pathway has been studied in greatest detail, and its signaling is initiated by Wnts interacting with serpentine Frizzled (Fz)* receptors and LRP5/6 coreceptors. Signaling by Fz is thought to activate the function of the intracellular protein Dishevelled (Dsh; for review see Wharton, 2003), leading to the stabilization of \β-catenin, which in turn translocates into the nucleus and binds Lef/Tcf family transcription factors to activate the transcription of target genes. Two other Wnt–Fz pathways have recently been described: the Wnt–Ca\textsuperscript{2+} pathway and the planar cell polarity (PCP) pathway.

The vertebrate Wnt–Ca\textsuperscript{2+} pathway is stimulated by certain Wnt ligands and Fz receptors and involves the mobilization of intracellular Ca\textsuperscript{2+} and the activation of the calcium-responsive enzymes PKC and calcium/calmodulin-dependent protein kinase II (CamKII) (for review see Kühl et al., 2000a,b; Huelsken and Behrens, 2002). Wnt–Ca\textsuperscript{2+} activity functions in promoting ventral cell fate and antagonizing dorsal cell fate during early Xenopus development, in regulating gastrulation movements, and in regulating heart development (Kühl et al., 2000a,b; Pandur et al., 2002). The PCP pathway has been studied primarily in Drosophila and involves an array of proteins, two of which are also required for Wnt–\β-catenin signaling: Fz receptors and the cytoplasmic protein Dsh (for reviews see Shulman et al., 1998; Peifer and McEwen, 2002; Wharton, 2003).

The term “noncanonical Wnt signaling” has been used to describe both the Wnt–Ca\textsuperscript{2+} pathway and the PCP pathway, primarily to differentiate either pathway from the Wnt–\β-catenin pathway. In vertebrates, noncanonical Wnt signaling has been shown to be very important for the control of convergent extension movements during gastrulation (for reviews see Smith et al., 2000; Darken et al., 2002; Kühl, 2002;
Wallingford et al., 2002; Yamanaka et al., 2002). Regulation of convergent extension by Wnts has been attributed to the activation of the PCP pathway (Smith et al., 2000). However, activation of the Wnt–Ca\(^{2+}\) pathway has also been implicated in the regulation of convergent extension movements (Torres et al., 1996; Kühl et al., 2001), and its effectors (Ca\(^{2+}\), PKC, and CamKII) act downstream of Wnts and Fzs that reportedly also activate the PCP pathway (Kühl et al., 2000a; Medina et al., 2000; Winklbauer et al., 2001). Thus, these two vertebrate pathways may overlap in terms of function and, conceivably, mechanisms of signaling.

Dsh plays a dual role in the Wnt–β-catenin and PCP pathways, and has been termed a molecular switch between the two (for review see Wharton, 2003). Dsh is comprised of different domains, including the DEP, PDZ, and DIX domains (Fig. 1 h), and analysis of Dsh deletion constructs and Drosophila genetics have been used to separate the role of Dsh in the Wnt–β-catenin pathway versus the PCP pathway (Axelrod et al., 1998; Boutros et al., 1998; Penton et al., 2002). That the PCP pathway is downstream of Wnt signaling in vertebrates was first suggested when Dsh was found to affect Wnt and Fz function during gastrulation in zebrafish (Heisenberg et al., 2000) and Xenopus (Tada and Smith, 2000; Wallingford et al., 2000). One construct, DshΔDIX, which is not competent to activate the Wnt–β-catenin pathway but can signal via the PCP pathway, has been found to rescue loss of Wnt-11 function during gastrulation in Xenopus (Tada and Smith, 2000) and zebrafish (Heisenberg et al., 2000). Finally, data from PCP signaling in flies suggest that activation of the small GTPase Rho and jun-N-terminal kinase (JNK) lies downstream of Dsh (for review see Adler and Lee, 2001). In vertebrates, activation of Rho by Wnts and Fz also involves Dsh, and the protein Daam1, which links Dsh to Rho (Habas et al., 2001).

It is notable that although Dsh functions in both the PCP and Wnt–β-catenin pathways, there have been no indications of whether it also functions in the Wnt–Ca\(^{2+}\) pathway. Resolving whether Dsh functions in all Fz-mediated signaling pathways, or only a subset, is important to understanding how the specificity of signaling pathways is generated in response to Wnts and activation of Fzs. We therefore asked whether Dsh, an activator of both β-catenin and PCP signaling, is able to activate the Wnt–Ca\(^{2+}\) pathway in vertebrate embryos.

Results

**XDshΔDIX activates PKC and is pertussis toxin insensitive**

We have previously monitored the activation of the Wnt–Ca\(^{2+}\) pathway by three different means: visualizing intracellular Ca\(^{2+}\) release (Slusarski et al., 1997a,b), and measuring the activation of two different Ca\(^{2+}\)-sensitive enzymes, PKC (Sheldahl et al., 1999) and CamKII (Kühl et al., 2000a). To test whether Dsh is capable of activating the Wnt–Ca\(^{2+}\) pathway, we initially tested wild-type (wt) Dsh and three deletion constructs (XDshΔDIX, XDshΔPDZ, and XDshΔDEP; Axelrod et al., 1998) for their ability to induce the relocalization of Xenopus PKC (XPKC) in Xenopus animal cap cells. In this assay, membrane localization of epitope-tagged XPKC is used as a measure of kinase activation, as common and novel PKC isoforms are known to translocate from the cytoplasm to the plasma membrane upon activation by the second mes-

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**Figure 1.** **XDshΔDIX activates PKC in a PTX-insensitive manner.** Two-cell-stage Xenopus embryos were injected with RNAs encoding XPKCo–myc (0.5–1 ng) plus XDsh–GFP (0.5–1 ng), XDshΔDEP–GFP (0.5–1 ng), XDshΔDIX–GFP (0.5–1 ng), Rfz2 (0.5–1 ng), PTX (1–2 ng), and/or LacZ (as a control for normalizing levels of injected RNA, 0–4 ng) and cultured to stage 8, and animal caps were explanted. (a) XPKC (red) is localized primarily in the cytoplasm under control conditions. (b) Ectopic XDsh–GFP (green), which is localized to punctate structures, is relatively weak at activating translocation of XPKC (red) to the membrane. (c) XDshΔDEP–GFP (green) as well as XDshΔPDZ–GFP (not depicted) do not activate XPKC (red) membrane translocation. (c, inset) XDsh–GFP (lane 1) and XDshΔDIX–GFP (lane 2) are expressed equally, as detected by an anti-GFP Western blot. Although the protein in lane 2 migrates more rapidly on gels compared with lane 1 (not depicted), it is aligned with lane 1 to facilitate the comparison of signal intensities. (d) Expression of XDshΔDIX–GFP (green) activates translocation to the membrane of PKC (red). (e) Rfz2 (untagged) activates membrane translocation of XPKC (red). (f) Rfz2–mediated membrane translocation of XPKC (red) is partially blocked by PTX. (g) The ability of XDshΔDIX–GFP (green) to induce membrane translocation of XPKC (red) is not blocked by PTX. (h) A schematic representing the three domains of Dsh discussed in the text.
sengers Ca\(^{2+}\) and/or diacylglycerol (Mellor and Parker, 1998; Sheldahl et al., 1999). Myc-tagged XPKC and GFP-tagged Xenopus Dsh (XDsh) constructs were injected into the animal pole of two-cell Xenopus embryos, animal caps were dissected at stage 8, and PKC localization was visualized using confocal microscopy (Sheldahl et al., 1999). We found that expression of XDshDIX leads to the translocation of XPKC to the plasma membrane (red signal in Fig. 1 d, compared with control, a), whereas wt XDsh was less active in promoting membrane translocation of XPKC (Fig. 1 b). Similarly, expression of XDshDEP (Fig. 1 c) and XDshPDZ (not depicted) does not activate XPKC translocation to the membrane. The greater activity of XDshDIX–GFP was not due to differences in levels of protein expression, as XDsh–GFP and XDshDIX–GFP were expressed equally, as measured by Western blot analysis (Fig. 1 c, inset).

Heterotrimeric G proteins have been shown to be downstream of Wnt–β-catenin and Wnt–Ca\(^{2+}\) pathways (Slusarski et al., 1997a,b; Sheldahl et al., 1999; Kühl et al., 2000a; Liu et al., 2001), but the hierarchy between G proteins and Dsh remains unclear. The A protomer subunit of pertussis toxin (PTX) catalyzes the ADP ribosylation of specific G protein α subunits of the G, family and prevents the interaction of the receptor and G protein (Gilman, 1987). Co-expression of the A protomer of PTX partially inhibits the rat Fz-2 (Rfz2)–mediated membrane translocation of XPKC (red signal in Fig. 1 f, compared with Rfz2 alone, e; Sheldahl et al., 1999), yet does not block activation of XPKC by XDshDIX (red signal in Fig. 1 g, compared with d).

**XDshDIX activates intracellular calcium flux in a PTX-insensitive manner**

We have previously demonstrated that misexpression of both XWnt-5A and Rfz2 stimulates an increase in intracellular Ca\(^{2+}\) release in zebrafish embryos (Slusarski et al., 1997a,b). We therefore asked whether XDsh and XDshDIX would also stimulate Ca\(^{2+}\) flux in this in vivo assay. Injection of RNA encoding wt XDsh is sufficient to stimulate a mild increase in Ca\(^{2+}\) release (Fig. 2 c), above levels observed in uninjected embryos (Fig. 2 a), in the region of the embryo injected with RNA and Texas red lineage tracer (Fig. 2 d, n = 6). By comparison, misexpression of XDshDIX (Fig. 2 e) stimulates Ca\(^{2+}\) release well above endogenous levels, as further demonstrated by the localized distribution of the RNA and Texas red lineage tracer (Fig. 2 f, n = 11) spatially overlapping the localized elevated Ca\(^{2+}\) flux (Fig. 2 e).

As Dsh functions downstream of G proteins to activate PKC, we then asked if the ability of Dsh to activate Ca\(^{2+}\) release is also independent of G protein signaling. Previously, we showed that ectopic expression of Rfz2 increases the frequency of Ca\(^{2+}\) release at least twofold over endogenous levels and that this activity is inhibited by PTX (Slusarski et al., 1997a). To extend these studies to Dsh, embryos were injected to achieve a uniform distribution of RNA (encoding Rfz2, XWnt-5A, or XDshDIX) and then were injected at a later stage with A protomer RNA mixed with lineage tracer. A broad distribution of A protomer activity in the embryo was sufficient to inhibit Rfz2-induced Ca\(^{2+}\) release (Fig. 2 g, compared with Rfz2 alone, b), whereas the A protomer–free regions (Fig. 2, compare g with h) still demonstrated a partial increase in Ca\(^{2+}\) release. Localized injection of A protomer and lineage tracer to the right side of the embryo (Fig. 2 j) was sufficient to reduce XWnt-5A–induced Ca\(^{2+}\) fluxes, whereas the left side maintained robust Ca\(^{2+}\) release (Fig. 2 i). Similar analyses with XDshDIX demonstrated that its ability to stimulate Ca\(^{2+}\) release is PTX insensitive. Uniform injection of XDshDIX with unilateral expression of A protomer and lineage tracer on the right side of the embryo (Fig. 2 l) did not significantly reduce XDshDIX-induced Ca\(^{2+}\) release (Fig. 2 k, n = 5). We conclude that Dsh activates Ca\(^{2+}\) flux independently of G proteins.

**XDshDIX activates CamKII in a PTX-insensitive manner**

As a third independent assay for the activation of the Wnt–Ca\(^{2+}\) pathway, we monitored activation of CamKII (Kühl et al., 2000a). Injection of XDshDIX or Rfz2 RNA at the two-cell stage resulted in a twofold activation of CamKII activity in Xenopus embryos at stage 7 before the onset of zygotic transcription (Fig. 3). Consistent with the observed increase in Ca\(^{2+}\) fluxes, injection of RNA encoding wt XDsh
slightly increases CamKII activity. Whereas activation of CamKII by Rfz2 was sensitive to the A protomer of PTX, the effect of XDsh/H9004DIX was insensitive to this treatment (Fig. 3). In summary, our data reveal that XDsh/H9004DIX is able to activate three effectors of the Wnt–Ca²⁺ pathway, Ca²⁺, PKC, and CamKII, in a PTX-insensitive manner.

Dsh function is required for Xfz7-mediated activation of PKC

Having shown that ectopic Dsh lacking the DIX domain, a form of Dsh previously shown to activate the PCP pathway, is also sufficient to activate the Wnt–Ca²⁺ pathway, we next used three independent assays to ask whether Dsh is required for the activation of Wnt–Ca²⁺ signaling. The first method involved the expression of putative dominant-interfering Dsh deletion constructs. We therefore injected two-cell Xenopus embryos with RNAs encoding one of the three domain deletions of XDsh (XDshΔDIX, XDshΔDEP, or XDshΔPDZ) along with RNAs encoding Xenopus Fz-7 (Xfz7) and XPKC. Although the mechanism of action of these constructs to modulate PCP or Wnt–β-catenin signals is likely complex, XDshΔDEP and XDshΔPDZ have nevertheless been found to disrupt PCP signaling (Axelrod et al., 1998; Boutros and Mlodzik, 1999; Wallingford et al., 2000). None of these constructs block Xfz7-mediated activation of ectopic XPKC, measured by its translocation to the plasma membrane (red XPKC signals in Fig. 4, b and c, compared with effects of Xfz-7 alone in a; XDshΔDIX not depicted). When interpreting these data, it is important to note that interference with PCP can be achieved both by inhibition or overstimulation of the PCP pathway (Adler and Lee, 2001), whereas we would expect inhibition of XPKC activity to occur only with the inhibition of Ca²⁺ flux.

As the DEP domain has been shown to be necessary and sufficient for membrane relocalization of Dsh (Axelrod et al., 1998), we injected RNA encoding this domain (XDEP–GFP) along with RNAs encoding Xfz7 and XPKC to ask whether it would block translocation to the membrane of ectopic XPKC. Unlike the other deletion constructs of XDsh, we found that activation of XPKC was partially inhibited by XDEP–GFP (Fig. 4 e shows higher levels of cytoplasmic XPKC, red signal, compared with effects of Xfz7 control, a). Injection of this minimal construct has also been shown to
interfere with PCP signaling (Tada and Smith, 2000), presumably by interfering with the relocalization of endogenous Dsh to the plasma membrane.

As an independent method to test whether Dhs function is required for Wnt–Ca²⁺ signaling to activate PKC, we then asked whether decreasing levels of endogenous XDsh would decrease translocation to the membrane of ectopic XPKC. To accomplish this, we injected an antisense morpholino (MO) oligonucleotide, designed to hybridize with the 5′ untranslated region of XDsh mRNA and inhibit its translation (for review see Heasman, 2002). We found that this MO (XDshMO), when injected into fertilized Xenopus eggs, was capable of reducing levels of endogenous XDsh protein in animal caps explanted from stage-8 embryos, without affecting the levels of a control protein (Fig. 5 e). Furthermore, translocation of ectopic XPKC to the membrane in response to Xfz7 was largely inhibited by this MO (Fig. 5 b, compared with control, a, shows reduced membrane localization of XPKC and elevated cytoplasmic XPKC). When a control MO was injected, membrane translocation of XPKC induced by Xfz7 was still observed (Fig. 5 c, compared with control, a). Coinjection of XDshMO with XDsh–HA RNA, which does not contain the 5′ untranslated region targeted by XDshMO, rescues the ability of Xfz7 to activate XPKC translocation to the membrane (Fig. 5 d, compared with b and a). Injection of this RNA also restores levels of Dsh to those found in control MO-injected animal caps (Fig. 5 e). Injection of XDshMO did not block membrane translocation of XPKC by the pharmacological PKC activator PMA (unpublished data), suggesting that the MO does not act upon XPKC directly.

We then tested the effects of XDshMO on endogenous XPKC activity by using an in vitro kinase activity assay (Sheldahl et al., 1999). Animal caps from injected Xenopus embryos were lysed, and the lysates were measured for their ability to incorporate radiolabeled phosphate into a peptide substrate. Phosphatidylinositol–serine (in mixed Triton micelles) was added as a required PKC cofactor, and basal kinase ac-
tivity in the absence of phosphatidyl-1-serine and calcium was subtracted from all measurements to generate PKC-specific kinase activity. Confirming our results with ectopic XPKC in the membrane translocation assay, we found that XDshMO, as compared with a control MO, reduced by several-fold the ability of Xfz7 to activate endogenous XPKC (P < 0.05 in a t test; Fig. 5 f, n = 7). Furthermore, coinjection of XDsh RNA rescued the PKC activity that had been reduced by XDshMO (P < 0.01; Fig. 5 f, n = 6). We therefore conclude that endogenous XDsh is necessary for maximal activation of endogenous XPKC by Xfz7.

As Wnt-11–mediated activation of noncanonical Wnt signaling through PKC and JNK is required for heart development in Xenopus (Pandur et al., 2002), we investigated whether injection of XDshMO into eight-cell embryos leads to heart defects, as monitored by reduced or missing contracting tissue. Indeed, after injecting XDshMO into early Xenopus embryos, nearly 86% of embryos had a reduced or missing heart, compared with 20% of embryos injected with a control MO (Fig. 6, a and b). Interestingly, nearly 30% of XDshMO-injected embryos completely lacked contracting tissue, whereas this phenotype was not observed in embryos injected with the control MO. We confirmed the reduced heart phenotype independently by analyzing the expression of the cardiac marker gene cardiac troponin I (TnIc) by whole mount in situ hybridization. Indeed, XDshMO-injected embryos showed a strong reduction in TnIc staining (Fig. 6 c; 64% of XDshMO-injected embryos, n = 53, vs. 16% of control MO-injected embryos, n = 50). These data indicate that disrupting the function of XDsh interferes with normal heart formation, though it is unknown at present whether XDsh is required within the developing heart or in noncardiac cells. In addition, embryos injected with XDshMO display a penetrant moderate to weak shortening of the axis, suggestive of an effect on convergent extension movements of gastrulation (unpublished data).

Discussion

Dsh is a multifunctional protein known to play a pivotal role in both the Wnt–β-catenin pathway and the PCP pathway. Here we use several independent assays to establish that Dsh also functions in the Wnt–Ca²⁺ pathway. First, we show that XDshΔDIX, which is incapable of activating the Wnt–β-catenin pathway but is sufficient for activation of the PCP pathway, is also capable of stimulating three components of the Wnt–Ca²⁺ pathway: Ca²⁺ flux, PKC, and CamKII. The ability of XDshΔDIX to activate Wnt–Ca²⁺ signaling is likely due to its inability to bind Wnt–β-catenin signaling molecules, such as axin (Kishida et al., 1999; Zhang et al., 2000), while retaining other functions of Dsh. Second, we have shown that Dsh-induced activation of Ca²⁺ flux, PKC, and CamKII is insensitive to PTX, which suggests that Dsh is either downstream of heterotrimeric G protein function or acting in parallel. Third, we show that XDsh function is required for full activation of XPKC by Xfz7. Finally, we have shown that endogenous XDsh function is required for proper development of the heart in Xenopus.

What can one conclude from the observation that a known activator of PCP signaling, XDshΔDIX, also activates three effectors of Wnt–Ca²⁺ signaling? Moreover, what is the relevance of finding that interfering with the function of Dsh reduces the activity of at least one of these effectors, PKC? Answers to these questions, based on the available literature, suggest that either Dsh functions in three vertebrate Wnt–Fz signaling pathways, i.e., the Wnt–β-catenin, PCP,
and Wnt–Ca^{2+} pathways, or that the PCP and Wnt–Ca^{2+} pathways are overlapping. We favor this latter possibility because in Xenopus and zebrafish embryos, both Wnt–Ca^{2+} signaling and vertebrate orthologues of the Drosophila PCP pathway modulate convergent extension movements during gastrulation. Moreover, a mutant form of XDsh lacking the DIX domain activates both the PCP and Wnt–Ca^{2+} pathways. Further supporting the overlap of these pathways, zebrafish Prickle, a component of the PCP pathway, stimulates Ca^{2+} signaling in embryos, albeit not as rapidly as some Wnts or Fzs (Veeman et al., 2003). That the PCP and Wnt–Ca^{2+} pathways were discovered independently, but may overlap mechanistically, is likely attributable to their being initially described by genetic versus nongenetic approaches, and in different organisms.

Besides asking whether an activator of PCP signaling can activate the Wnt–Ca^{2+} pathway, it is worth considering the reciprocal question of whether effectors of the Wnt–Ca^{2+} pathway are known to be involved in PCP signaling. XDsh has been reported to transduce Wnt-11/Fz7 signals to affect convergent extension movements during vertebrate gastrulation, perhaps via activation of the PCP pathway (Djiane et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000). As Wnt-11 and Fz7 have both been shown to activate the Wnt–Ca^{2+} pathway (Kühl et al., 2000a; Medina et al., 2000) and we have shown here that XDsh is both necessary and sufficient for activation of PKC and other effectors of the Wnt–Ca^{2+} pathway, this raises the question as to whether at least PKC is a part of the PCP pathway. Supporting, in the present study, we found that a mutant form of Dsh that should inhibit the PCP pathway, XDEP–GFP, at least partially inhibits Fz-mediated activation of ectopic PKC. Interestingly, both Dsh and PKC are required downstream of Drosophila Wnt-4 and Fz2 to regulate focal adhesion contacts during ovarian morphogenesis (Cohen et al., 2002), though this may not involve the PCP pathway. PKC activity is also required for the ability of Dsh to regulate amyloid precursor protein processing (Mudher et al., 2001), which at least further links Dsh with PKC. During Xenopus tissue separation, however, activation of PKC has been reported to be independent of XDsh (Winklbauer et al., 2001), and we have found that the Dsh1 mutation, which prevents activation of PCP signaling by Dsh in Drosophila, does not prevent XDshΔDIX from relocalizing XPKC in Xenopus (unpublished data).

The GTPase Cdc42 has a potential, though unresolved, role in PCP signaling and a more apparent role in cell polarity. In regulation of cell polarity, it is interesting that Cdc42 does not prevent XDsh from relocalizing XPKC in Xenopus (unpublished data). Cdc42 may be regulated by PKC and the Wnt–Ca^{2+} pathway (Choi and Han, 2002). In both flies and vertebrates, activation of the PCP pathway by Dsh leads to activation of JNK (for review see Adler and Lee, 2001), and Cdc42 is a known activator of JNK (Levi et al., 1998). It is important to note, however, that activation of JNK by Dsh can occur independently of Cdc42 (Li et al., 1999).

Our data show that the Wnt–Ca^{2+} pathway, like other Fz-mediated pathways, involves Dsh. Whether the Wnt–Ca^{2+} and PCP pathways substantially overlap will still require further study, as will elucidating the mechanisms by which Dsh promotes Ca^{2+} release and activation of PKC and CamKII.

Materials and methods

**RNA injections into Xenopus embryos**

Xenopus embryos were injected with RNAs or MO, as described, and cultured to stage 8. 20 animal caps from injected embryos were cut and lysed in 20 mM extraction buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 50 μM NaF, 10 μg/ml PMSF). Lysates were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in TBS with 0.1% Tween 20 and 5% dried milk, probed with anti-myc antibody (1:250 dilution) or anti-Dvl-1 (1:1,000 dilution), followed by an HRP-conjugated secondary antibody (1:5,000 dilution), and exposed using the ECL Western blotting detection system (Amersham Biosciences).

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**MO injections into Xenopus embryos**

XDshMO oligonucleotide was obtained from Gene Tools, LLC and resuspended in sterile water. The sequences are as follows: XDshMO, 5′-TCATTTATGCTCCGGATCTTCCCCG-3′ (sequence corresponding to the start codon of XDsh is underlined); control MO, 5′-CTAAACTTGTGGT-GTCTGGCGGATA-3′. For biochemistry and cell biology experiments, 4–8 ng MO oligonucleotide was injected into one-cell Xenopus embryos plus 0.5–1 ng Xfz7, 0.5–1 ng PKCa–myc, and either 1–2 ng XDsh–GFP or 1–2 ng GFP RNA. For phenotypic analysis of XDshMO, a total of 50 ng was injected into all four of the vegetal blastomeres of eight-cell Xenopus embryos.

**PKC activity assays**

Two- to four-cell embryos were injected as described in MO injections. 20 stage animal caps from stage-8 embryos were homogenized in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 50 μM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and assayed for activity using the following reagents: [32P]-ATP (NEN Life Science Products) and a biotinylated substrate peptide (Promega), phospho-(S)erine (Avanti Polar Lipids, Inc.) as a cofactor, and GF109203X (BIOMOL Research Laboratories, Inc.) as a specific inhibitor. Standard assay conditions were as follows: 20 mM Tris, pH 7.5, 1.0 μM ATP, 20 mM MgCl2, 200 mM free Ca^{2+} (calculated using the CHELATE computer program), 100 μM biotinylated peptide substrate, 0.3% mixed micelles with 3% Triton X-100 and 50 μg/ml phosphatidyl-c-serine, 1 μM autoinhibitory peptide, and 10 μM KN93 (both CamKII inhibitors) for a final volume of 25 μl incubated for 10 min at 30°C and then spotted onto SAM4 Biotin Capture Membranes (Promega). [32P]-ATP not incorporated into substrate peptide was washed away before counting membranes in a scintillation counter. Samples were run in duplicate for each experiment.

**In situ hybridization**

Whole mount in situ hybridization with Tnlc cDNA (provided by P. Krieg, University of Arizona, Tucson, AZ) was performed using a standard protocol (Drysdale et al., 1994).
the one-cell stage. RNA solutions included ~10–40 pg wt Dsh or DshΔDIX, and 5–10 pg of A protomer, as previously described (Slusarski et al., 1997a). The Dsh constructs were cleaved with ClaI to remove the GFP domain before in vitro transcription to prevent interference with the Fura channels. For experiments with unilateral expression, Fura-injected embryos were injected again at the 8–16-cell stage with RNA mixed with lineage marker Texas red-dextran.

Injected embryos were transferred to a coverslip-bottomed heated chamber on an inverted epifluorescence microscope. Embryos with equal fluorescence intensity were selected for analysis and oriented in a lateral position. Image pairs were collected at 340- and 380-nm excitation wavelength (510-nm emission) at 15-s intervals. The ratio image, a pixel by pixel match of both excitation wavelengths, was calculated by computer software (RatioTool; Invision), and the sequence of ratio images was processed. Ca\(^{2+}\) fluxes (transients) were determined by a subtractive analogue, as previously described (Slusarski and Corces, 2000), compiled for the duration of the time course (50–75 min), and represented as a two-dimensional topographical image. The spatial distribution of the Ca\(^{2+}\) transients was mapped along the surface of the embryo. The peak height and color represent the total number of transients that occurred in that location, where a low peak, purple color, represents a small number (1) and a high peak, red color, represents a high number (25) of Ca\(^{2+}\) transients. When needed, Texas red distribution was collected.

L.C. Sheldahl and this research were supported by a grant from the National Institutes of Health (RO1 HD27525) to R.T. Moon, who is an Investigator of the Howard Hughes Medical Institute. D.C. Slusarski was supported by the March of Dimes and the University of Iowa Biosciences Initiative. P. Pandur and M. Kühl were supported by Deutsche Forschungsgemeinschaft (SFB 497). J.R. Miller was supported by the March of Dimes (5-FY01-427).

Submitted: 20 November 2002
Revised: 8 April 2003
Accepted: 8 April 2003

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Submitted: 20 November 2002
Revised: 8 April 2003
Accepted: 8 April 2003

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Submitted: 20 November 2002
Revised: 8 April 2003
Accepted: 8 April 2003

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