Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/β-catenin activity

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We provide genetic evidence defining a role for noncanonical Wnt function in vertebrate axis formation. In zebrafish, misexpression of Wnt-4, -5, and -11 stimulates calcium (Ca\(^{2+}\)) release, defining the Wnt/Ca\(^{2+}\) class. We describe genetic interaction between two Wnt/Ca\(^{2+}\) members, Wnt-5 (pipetail and Wnt-11 (silberblick), and a reduction of Ca\(^{2+}\) release in Wnt-5/pipetail. Embryos genetically depleted of both maternal and zygotic Wnt-5 product exhibit cell movement defects as well as hyperdorsalization and axis-duplication phenotypes. The dorsalized phenotypes result from increased β-catenin accumulation and activation of downstream genes. The Wnt-5 loss-of-function defect is consistent with Ca\(^{2+}\) modulation having an antagonistic interaction with Wnt/β-catenin signaling.

Introduction

The Wnt family of growth factors and components of their signaling pathways have diverse roles in development and disease. Wnt signaling influences cell fate, migration, polarity, and neural patterning while inappropriate activation has been implicated in several human cancers (Huelsken and Birchmeier, 2001). The Wnt gene family can be grouped into mechanistically distinct classes based on overexpression assays. The canonical Wnt class (also referred to as Wnt/β-catenin) consists of the Drosophila wingless (wg) and vertebrate Wnt-1 and -8 (Dale, 1998). In zebrafish and Xenopus embryos, overexpression of canonical Wnts induce hyperdorsalization and ectopic axes (Moon et al., 1995; Kelly et al., 1995; Moon and Kimelman, 1998). Stimulation of the canonical Wnt path activates a cytoplasmic phosphoprotein (dishevelled [dsh]), which then inhibits the function of a degradation complex including glycogen synthase kinase 3 and Axin. Down-regulation of glycogen synthase kinase 3 activity leads to accumulation of β-catenin, a multifunctional protein that interacts with cadherins as well as transcription factors (lymphoid enhancer factor/α multifunctional protein that interacts with cadherins as well as transcription factors (lymphoid enhancer factor/lymphoid enhancer factor/large T-cell factor, DNA binding proteins) influencing gene expression (Bienz and Clevers, 2003). In addition, canonical Wnt signaling may be regulated by events Gsk-independent but Axin-dependent to modulate β-catenin nuclear entry (Tolwinski et al., 2003). The end result is tight regulation of Wnt/β-catenin activity and downstream target genes.

The common element of noncanonical Wnt signaling is that this class (including Wnt-5A, -4, and -11) appears to be β-catenin-independent (Kuhl et al., 2000b). Noncanonical Wnt activity can be viewed as a complex network with several cellular outputs identified by calcium (Ca\(^{2+}\)) modulation and polarized cell movement (Wnt/Ca\(^{2+}\) and planar cell polarity [PCP]; Modzik, 2002). Stimulation of the Wnt/Ca\(^{2+}\) pathway triggers the release of Ca\(^{2+}\) (Slusarski et al., 1997a,b), activating Ca\(^{2+}\) sensitive proteins including PKC (Sheldahl et al., 1999), Ca\(^{2+}\)/calmodulin-dependent kinases (CaMKII; Kuhl et al., 2000a) and calcineurin-dependent nuclear factor of activated T cells (Saneyoshi et al., 2002). More recently, a PCP-specific component, Prickle, has been shown to modulate cell movement and stimulate Ca\(^{2+}\) release in zebrafish, and a PCP-specific form of dsh has been shown to activate the Wnt/Ca\(^{2+}\) cascade in Xenopus and zebrafish (Sheldahl et al., 2003; Veeman et al., 2003). This work raises the intriguing possibility that Wnt/Ca\(^{2+}\) and PCP either substantially overlap or are part of the same signaling network.

In zebrafish, overexpression of Xwnt-5A is antagonistic to the Wnt/β-catenin class in that coinjection of RNA encoding...
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Results

Demonstrate an increase in (CaMKII). Through loss-of-function analyses, our data Wnt/Ca

vation of downstream genes supporting a functional role for ability to stimulate Ca

transformation (Olson and Gibo, 1998). Furthermore, Drosophila Wnt 4 (Dwnt-4) is antagonistic to wg as injection of antisense Dwnt-4 RNA resembles wg gain-of-function mutations and Dwnt-4 antagonizes wg in Xenopus axis-inducing

assays (Gieseler et al., 1999; Buratovich et al., 2000). Dwnt-4 also functions in cell movement (Cohen et al., 2002). Misexpression of zebrafish Wnt-5 and Wnt-4 alters morphogenetic movements (Ungar and Moon, 1995; Slusarski et al., 1997b) and genetic mutations in zebrafish Wnt-5/pipetall (ppt) and Wnt-11/silberblick (sib) have been shown to influence convergence extension movements during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003). Thus, in both vertebrates and invertebrates, noncanonical Wnts have dual functions; they are antagonistic to canonical Wnt signaling and modulate cell movement/polarity.

Although there is genetic and biochemical evidence for how the Wnt-β-catenin pathway works in both vertebrates and invertebrates, there is little genetic evidence for how noncanonical Wnt signaling pathways work in axis formation in vertebrates. Our paper provides a genetic demonstration of a maternal requirement for a Wnt in vertebrate dorsal-ventral (D-V) patterning. We identify zebrafish Wnt/Ca

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members

lection of zebrafish Wnt/Ca

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sufficient to increase the frequency of Ca

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release at least twofold over endogenous levels (zWnt-11; Fig. 1 A). Conversely, the canonical Wnt-8 does not change the levels of Ca

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release is consistent with our earlier studies with Wnts and Frizzles from other species (Slusarski et al., 1997a,b; Ahumada et al., 2002). Given that subtle differences in the amplitude and/or frequency of Ca

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release can have profound effects on cell behavior (Berridge et al., 1998), we next characterized the impact Wnt-5-stimulated Ca

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release on embryonic patterning.

Ventralization phenotypes with Wnt-5 gain of function

For gain-of-function analyses, we manipulated Wnt-5 activity by DNA or RNA injections into wild-type embryos and subsequently monitored Ca

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release frequency. In this manner, we can correlate the immediate physiological output of Ca

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release with morphological or molecular changes evaluated hours later. Wnt-5 RNA injected embryos typically demonstrated robust early Ca

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release activity (from 64- to 1,000-cell) resulting in cell movement defects as described in Slusarski et al. (1997b) and is consistent with morphogenetic defects described for zebrafish Wnt 4 (Ungar and Moon, 1995) and Xenopus Wnt-5A (Moon et al., 1993a). At the highest RNA concentrations, we induce hyperdorsalization defects (unpublished data) consistent with phenotypes described for high dose injections (>100 pg/embryo) in zebrafish (Kilian et al., 2003). Because we have

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Zebrafish embryos overexpressing Wnt-5A produce developmental defects similar to those induced by agents that stimulate phosphatidylinositol cycle activity (Slusarski et al., 1997b). Stimulation of the phosphatidylinositol cycle leads to intracellular Ca

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-sensitive dye along with the respective RNA are subjected to image analysis. Overexpression of zebrafish noncanonical Wnts (Wnt-4, -5, and -11) is

Figure 1. Ca

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release dynamics in zebrafish embryos expressing Wnts and in Wnt-5/ppt mutants. Non-canonical Wnt members stimulate Ca

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release in zebrafish and these changes were monitored with Fura-2 in live embryos. The representative embryo shown is a two-dimensional topographic image of the location of all the Ca

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fluxes that occurred during the time course (50 min). Surface plots of Ca

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release activity in embryos mis-expressing (A) Wnt-11 and (B) Wnt-8 RNA. Ca

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release profile of endogenous activity in (C) heterozygous (ppt-/) and (D) mutant (ppt-/-) embryos from the same clutch. Height and color of peaks indicate the number of Ca

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fluxes observed over the course of the experiment with the embryos oriented in a lateral position. The color bar indicates the pseudo-color representation of the number of transients from low (purple, 1) to high (red, 40).

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comitant range of developmental defects. Although Wnt-5 misexpression implicates a role in D-V patterning, we wanted to confirm with loss-of-function analyses.

**Genetic interaction between zebrafish Wnt-5 (ppt) and Wnt-11 (slb)**

Genetic mutations have been isolated for Wnt-5 and -11 (Rauch et al., 1997; Heisenberg et al., 2000). Given that both are expressed in the early embryo and capable of stimulating Ca²⁺ release, we decided to test for genetic interaction. The slb mutation maps to Wnt-11 and molecular analysis revealed that an extreme allele, slb\(^{pp265}\), was a loss-of-function allele (Heisenberg et al., 2000). ppt maps to the Wnt-5 gene and the most severe allele (ppt\(^{pp265}\)) phenotypically has a premature stop codon eliminating three conserved cysteines in the COOH terminus (Rauch et al., 1997). Due to the fact that the ppt\(^{pp265}\) mutation is a truncation, we wanted to verify that the ppt\(^{pp265}\) allele was not antimorphic. Expression of ppt\(^{pp265}\) mRNA in wild-type embryos did not generate any ppt\(^{−−}\)− mutant phenotypes (unpublished data) leading us to conclude that ppt\(^{pp265}\) is a loss-of-function mutation.

The slb mutation affects forebrain patterning and homozygous slb\(^{pp265}\) mutant embryos often have incomplete separation of the eyes (Fig. 3 B, arrow pointing to a fused lens) compared with wild-type (Fig. 3 A). Homozygous ppt\(^{pp265}\) mutant embryos display shortened body length and undulating notochords but most commonly tail defects (deformed tip of tail resembling a pipe; Fig. 3 C, arrow). To test for genetic interaction, we generated adult fish doubly heterozygous for ppt\(^{pp265}\) and slb\(^{pp265}\) and crossed to obtain doubly homozygous mutant embryos, confirmed by PCR of genomic DNA. The double homozygous mutant embryo phenotype is markedly more severe than the additive of the single mutant combinations. The eye anlagen fuse more frequently in the double mutant embryos than in slb\(^{−−}\)− embryos (Fig. 3 D). Double homozygous mutant embryos also have more severe tail and trunk defects than observed in ppt\(^{−−}\)− (Fig. 3 D).

**Reduced Ca²⁺ release in Wnt-5 (ppt) mutant embryos**

Given that Wnt-5 misexpression is sufficient to modulate Ca²⁺ release and induce ventralization, we next ask if Wnt-5 function is necessary for endogenous Ca²⁺ release activity in embryos. In vivo image analysis of Ca²⁺ release identifies a reduced frequency in ppt\(^{−−}\)− embryos (Fig. 1 D, average of 0.76 new transients/min) compared with ppt\(^{+−}\)− siblings (Fig. 1 C, average of 1.2 new transients/min). Interestingly, the embryonic region displaying the greatest reduction of Ca²⁺ activity in ppt\(^{−−}\)− embryos includes the yolk syncytial layer (YSL). The embryos are imaged in a lateral orientation and the YSL region would be included in the bottom portion of the half-moon–like topographical plot and note the lack of transients in that region (Fig. 1 D). The YSL has been shown to play important roles in epiboly, early induction and patterning in zebrafish (for review see Sakaguchi et al., 2002).

Alterations in cell movement have been described in slb mutant embryos (Heisenberg and Nusslein-Volhard, 1997; Heisenberg et al., 2000) and in embryos homozygous for an

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**Figure 2. Hyperventralization phenotypes of Wnt-5-injected embryos.** Embryos mis-expressing Wnt-5 were evaluated for changes in D-V patterning by morphology and whole mount in situ. (A) Wild-type morphology with the arrow designating the anterior-most region. Loss of dorsal-anterior tissue and expansion of ventral-posterior tissue is evident by lack of head tissue arrow in B and an onion-like mass of ventralized tissue in C. Dual-hybridization whole mount in situ at shield stage with the dorsal-specific chordin domain (red) denoted by arrowheads flanked by the ventral-specific eve (blue) expression domain. Reduction of dorsal domains and expansion of ventral domains compared with (D) wild-type is seen in (E) Wnt-5-injected embryos. (A–C) Lateral orientation with anterior to the left and (D–E) animal pole orientation with dorsal to the right.

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| A | B | C |
|---|---|---|
| D | E | F |
embryos. Late treatments with phosphoinositide cycle inhib-
treated) embryos.

triphosphate receptor (IP3R) channels with Xestospongin C

mutant (Wnt-11) treated embryos. In Wnt-11 mutant (slb−/−)
embryo has a fused eye, the arrow indicates the lens. (C) In Wnt-5
mutant (ppt−/−), the dashed arrow demarcates the shortened-curled
tail defect and in the (D) double mutant (slb−/−; ppt−/−), the block
arrow indicates the fused eye, whereas the dashed arrow marks the
shortened-twisted tail. The notochord and one set of somites are
highlighted by a dashed line in (E) IP3R-inhibited (XeC), and (F) ppt−/−
embryos. The presence of a protruding yolk is an indication of
incomplete epiboly cell movements. Fused eyes and shortened
twisted trunks can also be observed in (G) Ca2+-inhibited (L-690,330-
treated) embryos.

allele of ppt that replaces one conserved cysteine (pptΔCys)
leaving the rest of the Wnt-5 sequence intact (Heisenberg et
al., 2000; Kilian et al., 2003). In addition to the prototypical
ppt tail defect (Fig. 3 C), we also observe some epiboly de-
fects in the pptΔCys allele genetic background. As the pptΔCys
is the most severe allele identified thus far, we believe it uncovers
additional roles for Wnt-5 in cell movement. Consistent with altered Ca2+
modulation in ppt−/− embryos contributing to the mutant phenotypes is the fact that we reproduce,
in part, the phenotypic defects in Ca2+ release suppressed
embryos. Late treatments with phosphoinositide cycle inhib-
itors (after 125–256 cell stage) result in anterior brain de-
fects and eye fusions similar to those described for slb (West-
fall et al., 2003). We target phosphoinositide cycle turnover
with L-690,330 and block Ca2+ release from inositol 1,4,5-
triphosphate receptor (IP3R) channels with Xestospongin C
(XeC; Atack et al., 1993; Gafni et al., 1997). Inhibitor-
treated embryos were analyzed for in vivo Ca2+ release dy-
namics and we selected a dose that approximately mimics
the reduced Ca2+ release frequency observed in ppt−/− em-
byos. In both Ca2+ release–inhibited (Fig. 3 E) and in ppt−/−
(Fig. 3 F) embryos, we observe cell movement defects re-
sulting in split somites encircling the yolk typically fusing
back together at the most posterior tip of the tail. We also
observe twisted posterior notochord defects, thus, contort-
ing the tail similar to ppt−/− phenotypes and also generated
embryos with trunk and tail defects similar to ppt−/−; slb−/−
phenotypes (Fig. 3 G).

Morphological phenotypes in Wnt-5/ppt and Ca2+-
inhibited embryos. Lateral view, anterior to the right of 24–36 hpf
embryos. (A) In wild-type, the block arrow indicates one eye, the
other is out of the focal plane and the dashed lined demarcates the
tail extending posteriorly off the yolk. (B) The Wnt-11 mutant (slb−/−)
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Maternal depletion of Wnt-5 (ppt) reveals a necessary
role in ventral patterning.

Strong inhibition of IP3R function with blocking antibodies
leads to expanded dorsal structures in Xenopus (Kume et al.,
1997). Consistent with the Xenopus studies, inhibition of ei-
ther IP3R or phosphoinositide cycle turnover results in hyper-
dorsalization in zebrafish (Westfall et al., 2003). In compar-
ison, we use relatively mild doses of inhibitor (XeC and
L-690,330) to match the reduced Ca2+ release frequency in the
zygotic ppt−/− embryos. This raises the possibility that there
may be residual Wnt-5 in these zygotic ppt−/− embryos. In
support of this notion, Wnt-5 transcript is maternally depos-
ited and ubiquitously expressed in zebrafish (Blader et al.,
1996) but protein distribution is unknown. Antisense mor-
pholino knockdown of Wnt-5 generates phenotypes similar
to the zygotic mutant (Lele et al., 2001). It should be noted
that the antisense approach does not eliminate maternal pro-
tein. Therefore, in order to determine if maternally supplied
Wnt-5 is contributing to early patterning, we set out to gen-
erate ppt−/− females by gene product rescue.

Assuming that maternal product would be present in em-
byos from a heterozygous ppt cross, we injected Wnt-5 DNA
(∼200 pl of 10–12 ng/ul) at a concentration that activates
mild yet sustained Ca2+ release. We score rescue by suppres-
sion of the ppt morphological phenotypes. Injection sets with
less than 10% ppt-like phenotypes, compared with 25% pheno-
types in clutch controls, were allowed to develop. A small
number of the rescued embryos developed swim bladders, a
necessary organ for zebrafish viability and were raised to
adulthood. Mature ppt−/− females were PCR genotyped.

Consistent with maternal effect mutations, embryos col-
lected from ppt−/− females exhibit phenotypes regardless of
the paternal genotype. Embryos from ppt−/− females crossed
to heterozygous ppt−/− males fell into two general phenotypic
groups. One class, accounting for >50% of the defects, is
similar to the tail defects observed in mutant embryos from
heterozygous females (Fig. 4 B), however, embryos from ppt−/−
females have pointedly more severe defects with extreme
shortened axis, undulating notochord and tail defects (Fig. 4
C). This class of phenotypes was scored as ppt−/zygotic–like.

The other phenotypic group (38%, 193/506) demonstrated
dorsalized mutant phenotypes similar to those described in
Mullins et al. (1996), as well as axis duplication phenotypes.
These include expansion of somites, shortened and twisted
tail (piggy-tail–like; Fig. 4 D) and severe curling of the tail
over the trunk (snail-house–like; Fig. 4 F) instead of straight
extension off of the yolk as in wild-type (Fig. 4, A and E). The
ppt−/− embryo in Fig. 4 G has a partial secondary axis with an
ectopic pair of otic vesicles associated with a duplicated beat-
ing heart. Organ duplication was verified in several maternal-

Figure 3. Morphological phenotypes in Wnt-5/ppt and Ca2+-
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zygotic \( \text{ppt}^{-/-} \) (mzppt) embryos by whole mount in situ with a cardiac specific probe (Fig. 4 I; Chen and Fishman, 1996). Based on morphology, the hyperdorsalized and duplicated axis defect frequency of 38% is consistent, yet lower than the expected 50% frequency for embryos depleted of both maternal and zygotic product. The lower frequency could be the result of a maternal effect that is not fully penetrant or that we were conservative in scoring a phenotype as dorsalized and instead classified it as a severe ppt-zygotic–like defect. To distinguish between these two possibilities, we employed a more sensitive molecular analysis of dorsal patterning.

**Wnt-5 (ppt) maternal phenotypes are the result of ectopic dorsal signaling centers**

The hyperdorsalization and partial secondary axis phenotypes observed in maternally depleted ppt embryos suggest an expanded and/or ectopic dorsal signaling center. Confirmation that loss of Wnt-5 function results in expansion of dorsal tissue was obtained by whole mount in situ hybridization analysis with the dorsal-specific probe chordin (Miller-Bertoglio et al., 1997). In wild-type embryos, chordin is typically expressed in the shield region at 6 h post fertilization (hpf; Fig. 4 J, white arrowheads). 64% of the embryos \( (n = 73) \) from ppt\(^{-/-}\) females crossed to ppt\(^{+/-}\) males display expanded (Fig. 4 K) and/or ectopic (Fig. 4 L) domains of chordin expression.

**Maternal loss of Wnt-5 (ppt) results in \( \beta \)-catenin stabilization**

One mechanism leading to the dorsalization phenotypes described in the previous paragraph is activation of the canonical Wnt path leading to accumulation of \( \beta \)-catenin protein. Although there is no evidence for a maternal canonical Wnt ligand in zebrafish, there is nuclear accumulation of \( \beta \)-catenin protein on the future dorsal side
To determine if there is a change at the level of β-catenin, embryos are analyzed for β-catenin protein distribution. In wild-type embryos, we typically observe nuclear β-catenin spanning ~20% the circumference of the embryo when observed from the animal pole. Fig. 5 A represents one frame of a confocal series at lower magnification with the regions of nuclear β-catenin localization (dots) confirmed at higher magnification. A representative frame from images collected around the circumference of a wild-type embryo at higher magnification is shown in Fig. 5 C with an arrowhead marking a cell with nuclear β-catenin. In wild type, we observe an average of 11 β-catenin-positive nuclei/embryo, (n = 10 embryos). In contrast, β-catenin-positive nuclei in embryos from ppr<sup>−/−</sup> females span >50% the embryo circumference, in some embryos, the domains are opposite the putative endogenous dorsal domain (Fig. 5 B, dots). Embryos from ppr<sup>−/−</sup> females demonstrate a dramatically higher number of cells with nuclear β-catenin (Average of 48 nuclei/embryo, n = 10 embryos) with a noticeable increase in the overall level of protein (Fig. 5 D, arrows denoting a few of the cells with nuclear β-catenin). The individual panels of wild type at higher magnification typically have 1–2 nuclei/frame in the dorsal domain, whereas ppr panels can have upwards of 6–10 nuclei/frame.

To analyze increased Wnt/β-catenin activity at the molecular level, we monitored expression of a downstream target, the homeodomain protein bozozok (Fekany et al., 1999). boz zygotic expression initiates at the dorsal blastoderm and dorsal YSL indicating the zebrafish organizer at 4 hpf (Fig. 6 A). 59% of embryos from ppr<sup>−/−</sup> females have expanded and/or ectopic boz expression (n = 147; Fig. 6, B–D). The ppr<sup>−/−</sup> embryos in Fig. 6 (B and C) display lateral expansion (arrowheads) of the boz expression domain as well as an increased number of boz-positive cells. Additionally ectopic domain of boz expression can be observed as a group of cells (Fig. 6 B) or as individual boz-positive cells (Fig. 6 D). Because a milder ppr allele (ppr<sup>409</sup>) has redundant functions with slb in anterior cell movements (Kilian et al., 2003), we wanted to confirm genetic interaction between ppr<sup>1293</sup> and slb<sup>2216</sup> or potential functional redundancy between the Wnt-5 and -11 products in earlier embryonic stages. We evaluated boz distribution in embryos collected from a cross between doubly heterozygous ppr<sup>1293</sup> and slb<sup>2216</sup> parents. In the doubly homozygous ppr<sup>1293</sup>; slb<sup>2216</sup> mutant embryos, we observed an increase in the boz expression domain with a larger number of strongly expressing boz-positive cells forming a dark trapezoid shape (14%, n = 96; Fig. 6 F) compared with the linear pattern of weakly expressing boz-positive cells in wild-type (Fig. 6 E) and single mutant ppr<sup>1293</sup> or slb<sup>2216</sup> embryos (0%, n = 146). The mild increase in the boz expression domain suggests that there may be a zygotic contribution from this class of Wnts to maintain or refine, in part, the maternally established D-V boundaries.
CaMKII activity can partially rescue Wnt-5 (ppt) mutants

If the ppt\(^{-/-}\) phenotype is the result of reduced Ca\(^{2+}\) release, it may be possible to suppress the mutant defect by artificially activating a Ca\(^{2+}\) cascade. CaM is a predominant Ca\(^{2+}\) binding protein in the cell, which, when bound to Ca\(^{2+}\), stimulates CaM-dependent enzymes including Ca\(^{2+}\)/calmodulin-dependent protein kinases. Due to its proposed role in Xenopus ventral patterning (Kuhl et al., 2000a), we tested whether CaMKII was sufficient to rescue the Wnt-5 loss-of-function phenotype. Injection of full-length CaMKII expression did not completely rescue the phenotype. Expression of an activated form of CaMKII, truncated to remove a regulatory domain (CamKIItr; Hansen et al., 2003), suppressed the ppt\(^{-/-}\) phenotype (Fig. 7 B) compared with uninjected sibling ppt\(^{-/-}\) embryos (Fig. 7 C). However, CaMKIItr expression did not completely rescue the phenotype relative to wild-type (Fig. 7 A). As in the Wnt-5 experiments, embryos from a standard ppt heterozygous cross were injected with CamKIItr and rescue was scored in injection sets with suppressed ppt-like tail and trunk phenotypes (ppt phenotypes in clutch controls are typically at 25%, whereas rescue sets had fewer than 10%). Embryos from injection sets with increased frequency of wild-type–like morphology were individually photographed and PCR genotyped. PCR genotyping of wt-looking embryos revealed 19% homozygous ppt in CamKIItr rescue sets, whereas no homozygous ppt genotypes were identified among phenotypic wild-type clutch embryos. Thus, constitutively active CaMKII can result in straightened tails and increased trunk length (within 90% of wt length). Additional rescued embryos \((n > 180)\) were raised, whereas CaMKII-injected ppt\(^{-/-}\) heterozygotes survived, no CaMKII-injected ppt\(^{-/-}\) reached maturity.

Discussion

Recent work has highlighted the importance of Wnt/Ca\(^{2+}\) in ventral specification through misexpression of dominant negative Wnt-11, DN X nuclear factor of activated T cells and a truncated frizzled 8 in Xenopus (Itoh and Sokol, 1999; Kuhl et al., 2000a; Saneyoshi et al., 2002). A genetic loss of Wnt-5 function facilitates greater understanding of subcellular interactions, in the context of the whole embryo under endogenous regulatory dynamics. Our findings demonstrate that homozygous ppt/Wnt-5 mutant embryos have reduced frequency of Ca\(^{2+}\) release and mzppt/Wnt-5 mutant embryos display hyperdorsalization phenotypes. The ectopic axes observed in mzppt\(^{-/-}\) embryos are partial, not complete axis duplications, possibly due to contributions from Wnt-11 or other maternally supplied Wnt/Ca\(^{2+}\) members. The synergy in the ppt\(^{-/-}\)-silb\(^{-/-}\) double mutant suggests redundant/overlapping function with Wnt-11. Regardless, this is a genetic demonstration for a role of a Wnt in D-V patterning in vertebrate embryos.

Wnt-5 loss-of-function mimics activation of Wnt/\(\beta\)-catenin signaling most likely by relieving negative regulation of some component(s) of the Wnt–\(\beta\)-catenin pathway (Fig. 8). We observe an accumulation of \(\beta\)-catenin protein and ectopic activation of target genes. Although further studies in the mz-ppt embryos are needed to confirm if the impact on \(\beta\)-catenin and target genes is a direct effect of Wnt-5/Ca\(^{2+}\) activity, antagonism of \(\beta\)-catenin levels is consistent with Wnt-5 misexpression resulting in reduced chordin expres-
sion domains. As there has not been a clear demonstration of a maternally provided canonical Wnt, the level of antagonism may lie within the cell. Recent demonstration of dsh activating Wnt/Ca\textsuperscript{2+} provides one candidate for maternal influence (Sheldahl et al., 2003). The cellular response to Ca\textsuperscript{2+} release most likely involves a network of proteins activating multiple components. Data suggests an antagonistic role for CaMKII, interfering with Xenopus gastrulation movements (Kuhl et al., 2001) and by CaMKII-dependent activation of a β-catenin/Tcf inhibiting nemolink kinase (Ishitani et al., 2003). The partial rescue of the ppp\textsuperscript{-/}/p phenotype suggests that although CaMKII is a downstream responder to Wnt/Ca\textsuperscript{2+} activation, other Ca\textsuperscript{2+}-sensitive components may also be required. In particular, PKC (Kuhl et al., 2001) and naked cuticle (Zeng et al., 2000; Yan et al., 2001) proteins, which could lead to β-catenin accumulation or those that could influence β-catenin nuclear import (Tolwinski et al., 2003), are perhaps required.

Misexpression of high levels of XWnt-5A with human Fz5 in frog embryos is sufficient to induce axis duplication (He et al., 1997). It is possible that, upon overexpression, Wnt5 and Fz5 can stimulate both canonical and noncanonical Wnt pathways. However, misexpression of Wnt5 in melanoma cells activates PKC but not β-catenin and, furthermore, treatment of these melanoma cells with antibodies that block Fz5 function show decreased Wnt5A-induced PKC activation and metastasis (Weeraratna et al., 2002), supporting the hypothesis that the Fz5 couples into the Wnt–Ca\textsuperscript{2+} path. Whether axis duplication is the result of hyperactivation of the Wnt–Ca\textsuperscript{2+} path resulting in depletion of Ca\textsuperscript{2+} stores and subsequent Wnt/β-catenin activation or due to direct activation of the Wnt–β-catenin path has not been determined. Critical to the depletion hypothesis is that reduction of GSK-3 activity suppresses β-catenin accumulation or those that could influence β-catenin nuclear import (Ishitani et al., 2003). In summary, Wnt/Ca\textsuperscript{2+} functions in D-V axis specification. Our in vivo studies reveal that changes in intracellular Ca\textsuperscript{2+} concentrations as a result of Wnt-5 modulation can lead to rapid and sustained events by establishing feedback loops to sharpen boundaries or to coordinate cell movement. Wnt-5 is maternally required and its biological effects are in part due to negative regulation of Wnt/β-catenin signaling. Because activation of β-catenin signaling has been implicated in cancer, our work provides genetic evidence that the Wnt–Ca\textsuperscript{2+} pathway may be a tumor suppressor pathway. This is further supported by the recent demonstration that Ca\textsuperscript{2+} activity suppresses β-catenin in human colon carcinomas (Chakrabarty et al., 2003). Knowledge of the developmental processes of Wnt/Ca\textsuperscript{2+} signaling will lay the foundation for understanding complex developmental events, as well as the oncogenic role of the Wnt signaling family.

### Materials and methods

#### Embryo manipulation

Fertilized eggs were collected from natural spawning of adults and microinjected with ~200 pl volumes at the 1-cell stage and 50~100 pl at the 8–32-cell stage. Capped RNA was prepared with the Ambion mRNAMessage Machine kit after template linearization. Wnt-4, -5, and -11 RNA was injected as ~200 pl drops of a 5–40-ng/μl stock solution. CaMKII and CaMKIItr (provided by S.H. Green, University of Iowa, Iowa City, IA) were injected at a 30–60-ng/μl stock solution. Wnt-5 DNA was injected at 8–12 ng/μl in a 100–200-pl drop. Live embryos were photographed with Nomarski optics after orienting in 3% methylcellulose and staged according to Kimmel et al. (1995).

#### Mutant identification

Stock families of heterozygous adults for ppp\textsuperscript{217} (Hammerschmidt et al., 1996), sb\textsuperscript{276} (Heisenberg et al., 1996), and ppp\textsuperscript{276}, sb\textsuperscript{276} are maintained under standard conditions. Mutants are identified by test crosses back to ppp\textsuperscript{217}/sb\textsuperscript{276}, as well as by PCR analysis. Genomic DNA isolated from single embryos or tail-snipped adults were proteinase K-treated (2 ng/ml in PCR buffer + Tween + NP-40). The PCR primers used for ppp\textsuperscript{217} in exon 4 (5’-CTACACCATCATGATATTTCCAC-3’ and 5’-CTATACACGCGACACCG-TACAC-3’) identify an A to T transition at position 1045 and the primers specific for sb\textsuperscript{276} identify the truncation at glycine 153 (5’-ACGGTTTTGTTG TTTCTCTGG-3’ and 5’-TCTCATGTGTGCTAGTTCAG-3’). The PCR products were isolated and sequenced by standard techniques.

#### Pharmacological reagents

L-690,330 (Tocris), an inositol monophosphatase inhibitor (Atack et al., 1993), was injected to ~1.5–2 ng/embryo after the 8-cell stage. XeC (Calbiochem), a membrane-permeable blocker of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release (Galin et al., 1997), was used at 1–2 μM doses. For the cell movement defects, treatment was typically after the 128-cell stage.

#### Whole mount in situ hybridization

For all the manipulations, embryos at the appropriate developmental stage, sphere/dome or 50% epiboly, were placed in 4% PFA/PBS fixative. In situ hybridization was done as described in Thisse et al. (1993) and double label in situ as described in Long and Rebagliati (2002). After probe detection, embryos were mounted and photographed.

#### β-Catenin immunolocalization

Embryos were fixed in 4% PFA/XB PBS at the sphere/dome stage. Overnight incubation with anti-β-catenin (P14L, provided by Dr. T. Kurth, MPI für Entwicklungsbiologie, Tübingen, Germany); Schneider et al., 1996), followed by secondary antibody conjugated with a fluorescent label (Texas-red or Alexa633; Molecular Probes). Nuclei were identified by counter stain with the 5 μM Sytox Green (Molecular Probes). Whole mounts or embryos in an animal pole orientation were optically sectioned using two-channel imaging on a scanning laser confocal microscope system (20×/0.7 Plan Apo and 63×/1.2 water objectives; model TCS-NT; Leica). The image stacks collected at 4-μm intervals were evaluated and nuclear β-catenin in nonoverlapping cells was counted as positive.

#### Calcium image analysis

The ratiometric Ca\textsuperscript{2+}-sensing dye Fura-2 (dextran conjugated; Molecular Probes) was injected into 1-cell zebrafish embryos. Indicated RNAs or pharmacological reagents were either coinjected with the Fura-2 at the 8–32-cell stage. Capped RNA was prepared with the Ambion mRNAMessage Machine kit after template linearization. Wnt-4, -5, and -11 RNA was injected as ~200 pl drops of a 5–40-ng/μl stock solution. CaMKII and CaMKIItr (provided by S.H. Green, University of Iowa, Iowa City, IA) were injected at a 30–60-ng/μl stock solution. Wnt-5 DNA was injected at 8–12 ng/μl in a 100–200-pl drop. Live embryos were photographed with Nomarski optics after orienting in 3% methylcellulose and staged according to Kimmel et al. (1995).

We wish to thank B. Hjertos for earlier contributions to this work and Dr. M.E. Dailey for assistance with the confocal analysis. We also thank Dr. Y. Yang for sharing information before publication; and Dr. M. Rebagliati and Dr. R. Cornell for critical reading of the manuscript.

This work was supported by March of Dimes (MOD) (grant 5-FY99-806) and the American Cancer Society (grant IN-122V) administered through...
the Holden Comprehensive Cancer Center at the University of Iowa. D.C. Slusarski is a MOD Basil O’Conner Research Scholar.

Submitted: 17 March 2003
Accepted: 2 July 2003

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