Zebrafish Dapper1 and Dapper2 play distinct roles in Wnt-mediated developmental processes

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Summary

Wnt signaling pathways in vertebrates use the phosphoprotein Dishevelled (Dvl). The cellular responses to Wnt signaling may in part be modulated by Dvl-associated proteins, including Dapper (Dpr). We have cloned and characterized the zebrafish Dpr paralogs Dpr1 and Dpr2. Loss-of-function studies reveal that endogenous Dpr1 but not Dpr2 is required to enhance Wnt/β-catenin activity in zebrafish embryos that are hypomorphic for Wnt8. Conversely, Dpr2 but not Dpr1 is required for normal convergence extension movements in embryos that are hypomorphic for Stbm or Wnt11, supporting a functional interaction of Dpr2 with Wnt/Ca^{2+}-PCP signaling. In gain-of-function experiments, Dpr1 but not Dpr2 induces Wnt/β-catenin target genes. Dpr1 synergizes with zebrafish Dvl2, and with the Dvl-interacting kinases CK1ε, Par1 and CK2, in activating target genes. We conclude that two Dvl-associated paralogs, Dpr1 and Dpr2, participate in distinct Wnt-dependent developmental processes.

Key words: Dishevelled, Dapper, Zebrafish, Wnt signaling

Introduction

Wnt signaling through Frizzled (Fz) serpentine receptors controls a plethora of developmental and adult processes in metazoa (Lustig and Behrens, 2003; Myers et al., 2002; Veeman et al., 2003a; Wodarz and Nusse, 1998). However, the number of distinct Wnt/Fz pathways in vertebrates is unresolved. The best characterized is the Wnt/β-catenin pathway, which regulates cell proliferation, survival and fate in a context-dependent manner. For example, this pathway controls developmental events in frog and fish embryos including early dorsoventral and anteroposterior patterning (Erter et al., 2001; Kelly et al., 2000; Larabell et al., 1997; Lekven et al., 2001). Another vertebrate Wnt pathway is the Wnt/Ca^{2+}pathway, which may functionally and mechanistically overlap with a vertebrate counterpart of the *Drosophila* planar cell polarity (PCP) pathway. In *Drosophila* the PCP pathway controls wing hair, ommatidia and bristle polarity. In *Xenopus* and zebrafish, the Wnt/Ca^{2+}pathway and orthologs of the *Drosophila* PCP pathway control proper cell movements during gastrulation that are required for convergence and extension (CE) (reviewed by Veeman et al., 2003a). To reflect shared functions in regulating CE, we refer to these pathways as the Wnt/Ca^{2+}-PCP pathway, leaving open the issue of whether they are mechanistically unique or overlapping pathways.

In addition to a Wnt ligand, Wnt/β-catenin signaling involves a seven transmembrane receptor, Fz, and a transmembrane co-receptor, LRP receptor-related protein-5 or -6 (LRP5/6) (Mao et al., 2001; Tamai et al., 2000). In the absence of Wnt signaling, β-catenin is phosphorylated in a complex of proteins that includes the scaffolding proteins Axin and APC, and the kinases GSK-3β and CK1α. Phosphorylation of β-catenin leads to its ubiquitination and degradation (Liu et al., 1999; Marikawa and Elinson, 1998). In response to a Wnt signal, Dishevelled (Dvl) is activated, promoting the stabilization of β-catenin. As β-catenin accumulates in the cytoplasm and nucleus, it associates with the LEF/TCF transcription factors to activate transcription of targets genes (Huelsken and Behrens, 2002). In vertebrates, the Wnt/Ca^{2+}-PCP pathway also appears to involve a Wnt ligand and Fz, but does not involve LRP-5/6 co-receptors. Instead, stimulation of this pathway leads to the association of Dvl with proteins that control cytoskeletal remodeling, including Rac and the Rho-interacting protein Daam (Habas et al., 2003; Habas et al., 2001). Vertebrate Dvl also interacts with orthologs of *Drosophila* proteins that regulate PCP signaling, including Strabismus/Trilobite (Stbm/Tri) and Prickle (Pk) (reviewed by Wharton, 2003). However, the general mechanisms of Wnt/Ca^{2+}-PCP signaling remain unclear (reviewed by Veeman et al., 2003a).

The phosphoprotein Dvl is therefore involved in the Wnt/β-catenin and Wnt/Ca^{2+}-PCP pathways. Both genetic and biochemical screens have identified a host of Dvl-interacting proteins (reviewed by Wharton, 2003). In addition to those proteins previously mentioned, this list includes Naked cuticle (Nkd) (Rousset et al., 2001), Casein kinase 1ε (CK1ε) (Peters et al., 1999), Protein kinase CK2 (CK2) (Willert et al., 1997),

Par1 (Sun et al., 2001), GBP (Li et al., 1999), FRODO (Gloy et al., 2002) and Dapper (Dpr; Dact – Zebrafish Information Network) (Cheyette et al., 2002). CK1ε, CK2, Par1, GBP and FRODO have been described as positive regulators of Wnt/β-catenin signaling, while Nkd and Dpr have been characterized as inhibitors of Wnt signaling. A number of these Dvl-interacting proteins have been proposed to act as switches that regulate Dvl functions in different Wnt/Fz signaling pathways. For example, it has been proposed that Par1 promotes Wnt/β-catenin signaling at the expense of Wnt/Ca^{2+}-PCP signaling, while vertebrate Nkd has been proposed to do the opposite (Sun et al., 2001; Yan et al., 2001). Clearly an understanding of Dvl-associated proteins is central to understanding how Wnt pathways are regulated.

We have previously reported the characterization of a Dvl-interacting protein, *Xenopus* Dpr1 (Cheyette et al., 2002), and presented evidence that it is required for notochord formation. More recently, Hikasa and Sokol (Hikasa and Sokol, 2004) have presented evidence that a Dpr homolog, FRODO, is involved in aspects of organizer formation and neural patterning. To understand better the functions of the Dpr family in vertebrate embryos, in the present study we have characterized two zebrafish Dpr paralogs, Dpr1 and Dpr2, which we analyze through their loss and gain of function. Our data suggest that the Dpr1 and Dpr2 paralogs have different roles in early vertebrate development.

Materials and methods

Cloning, expression analysis and phylogenetic analysis

A fragment of zebrafish Dpr1 was cloned from a degenerate PCR screen of cDNA. cDNAs encoding the complete open reading frames for zebrafish Dpr1, Dpr2 and Dvl2 were cloned using the Clontech SMART RACE Kit (BD Biosciences, Palo Alto, CA). In situ procedures were performed as previously described (Oxtoby and Jowett, 1993). RT-PCR was performed using cDNA made from RNA of 25 embryos of each stage using the Thermoscript kit (Invitrogen, Carlsbad, CA). All sequences of primers are available upon request. Alignment of sequences was performed using ClustalW via MacVector. Neighbor joining phylogenetic analysis was performed using PAUP* 4.0.

Expression constructs

All genes used except for β-galactosidase, CK1ε, CK1D→N, *Drosophila* Par1 and Par1-KN were cloned into pCS2P+. The *Xenopus* CK1ε and CK1D→N in pCS2 were gifts from J. Graff (Peters et al., 1999). CK2α and β subunits, gifts from D. Seldin, were subcloned into pCS2P+. *Drosophila* Par1 and Par1-KN were in pCS2+, as reported previously (Sun et al., 2001). GBP RNA was a gift of D. Kimelman and H. Gist Farr. The zebrafish Dvl2ΔPGB construct deletes amino acids 231-252. Capped mRNAs for injection were prepared using the Message Machine kit (Ambion, Austin, TX) and dissolved in water.

RT-PCR analysis of Wnt/β-catenin target genes

For analysis of the Wnt/β-catenin target genes *siamois* (*sia*) and *Xenopus nodal-related 3* (*Xnr3*), *Xenopus* embryos were injected, animal caps isolated, and RT-PCR conducted essentially as described (Cheyette et al., 2002). Controls omitting RT were conducted for all experiments (not shown).

Morpholino injections of zebrafish

Stock morpholinos (MO; Genetools, Philomath, OR) were dissolved in 1×Danieu's buffer to a concentration of 10 ng/nl (Nasevicius and Ekker, 2000). The diluted morpholino stocks (2 nl) were injected into one-cell zebrafish embryos. *strabismus/trilobite* MO (Park and Moon, 2002), *wnt11/silberblick* MO (Lele et al., 2001), *wnt8-orf1* and *wnt8-orf2* MOs (Lekven et al., 2001) have been reported previously. Morpholino sequences for *dpr1* and *dpr2* are as follows:

dpr1 MO, 5′-AACAAATACTAACCAGAGCTTGGCC;
dpr1 MO(2), 5′-TATAAATCCAATGAAAGCAATAGAC;
dpr2 MO, 5′-TCAGACGTGCCGTTTAGACATATCC;
dpr2 MO(2), 5′-GGCATGTGTAGTCACCTGAACTGG.

Immunocytochemistry

For immunocytochemistry, *Xenopus* embryos were injected, and animal caps were isolated, fixed in phosphate-buffered saline and processed by standard methods (Cheyette et al., 2002). The anti-Myc antibody was the monoclonal 9E10 and the secondary antibody was the goat-anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR).

Xenopus embryo lysates and immunoprecipitation

Embryos injected with ~3 ng of each RNA were lysed with 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.5% CHAPS, 1 mM EDTA, 1 mM EGTA and Complete protease cocktail (Roche, Indianapolis, IN). For immunoprecipitation (IP), the lysis buffer did not include EDTA and EGTA. Anti-HA antibody was a commercial monoclonal antibody.

Cell culture and reporter assays

Human embryonic kidney 293T cells were transfected using Lipofectamine Plus (Invitrogen) with the reporter construct, Super(8X)TOPFLASH (Veeman et al., 2003b), an internal control construct encoding Renilla luciferase and the indicated plasmids and empty vector to maintain constant amounts of DNA. Luciferase activity was measured 24 hours after transfection, using the Dual-Luciferase Reporter Assay System (Promega).

GenBank Accession numbers

Zebrafish Dpr1, AY545443; zebrafish Dpr2, AY545444; zebrafish Dvl2, AY552332.

Results

Vertebrate genomes have multiple Dpr paralogs

We initially conducted phylogenetic analysis of the two Dpr homologs. Alignment of the sequences using ClustalW, followed by neighbor-joining (shown) or maximum parsimony (not shown), which showed identical relationships, revealed two ortholog groups (Fig. 1A and see Fig. S1 in the supplementary material). The Dpr homologs that formed a monophyletic group and clustered with the previously published Dpr members, XDpr1 and FRODO, were assigned as the Dpr1 orthologs. The other monophyletic group was assigned Dpr2. Based on the available genomic sequences, each vertebrate species, except for *Xenopus*, has only two full-length Dpr paralogs. The phylogenetic analysis revealed that FRODO and XDpr are a duplication event (Graf and Kobel, 1991) that is specific to *Xenopus*, hence we refer to XDpr1 as XDpr1a, and FRODO as FRODO/XDpr1b. The Dpr family has seven conserved domains (Fig. 1B and see Fig. S1 in the supplementary material). Only two conserved domains have defined motifs: the leucine zipper and the PDZ binding motif, which is required to interact with the Dvl PDZ domain.

Expression patterns of zebrafish Dpr1 and Dpr2

By RT-PCR, *dpr1* is expressed only zygotically, while *dpr2* is expressed both maternally and zygotically (Fig. 2). With regard

to expression patterns, both genes are expressed on the future dorsal side of the embryos at sphere stage (Fig. 2C, part a; Fig. 2D, part a). At shield stage, both *dpr1* and *dpr2* are expressed in the shield as well as the margin (Fig. 2C, part b; Fig. 2D, part b). *dpr1* is more highly expressed in the involuted hypoblast, but is expressed at low levels in the marginal hypoblast and epiblast (compare Fig. 2C, part b with 2D, part b). *dpr2*, however, is highly expressed throughout the shield and immediately lateral areas (Fig. 2D, part b). At 80% epiboly, *dpr1* and *dpr2* are expressed in the axial and lateral mesoderm and the ectoderm (Fig. 2C, part c; 2D, part c).

At approximately tailbud stage, *dpr1* is expressed in specific ectodermal and mesodermal domains. *dpr1* is expressed in a stripe in the brain anterior to *pax2.1* (arrows; Fig. 2C, part d). *dpr1* staining is also seen in the posterior hindbrain and anterior spinal cord (arrowheads; Fig. 2C, part d). To determine the localization of *dpr1* expression in the brain, in situ hybridization was performed with probes for *dpr1* (blue), *pax6.1* (red), a diencephalic marker, and *krox20* (red), a marker for rhombomeres 3 and 5 (arrowheads; Fig. 2C, part e). At tailbud stage, *dpr1* expression completely overlaps that of *pax 6.1* (not shown). By the five-somite stage, *dpr1* (black arrow; Fig. 2C, part e) and *pax6.1* (red arrow; Fig. 2C, part e) resolve into separate domains, with *dpr1* anterior and *pax6.1* posterior (Fig. 2C, part e). After tailbud stage, *dpr1* is no longer expressed in the axial mesoderm, but is expressed in specific areas of the somites during somitogenesis, as is evident at the 12 somite stage. The anterior, older 3 or 4 somites have *dpr1* staining throughout the somites (black arrow; Fig. 2C, part f). The more posterior, younger somites have *dpr1* staining in the posterior of the somites (black arrowhead; Fig. 2C, part f). In the posterior, *dpr1* stains the paraxial mesoderm (asterisk), but is excluded from the tailbud (yellow arrow; Fig. 2C, part f). Later expression patterns for *dpr1* are presented in Fig. S2 in the supplementary material.

At tailbud stage, *dpr2* is maintained in the axial mesoderm and in the anterior brain. This brain expression is transient and

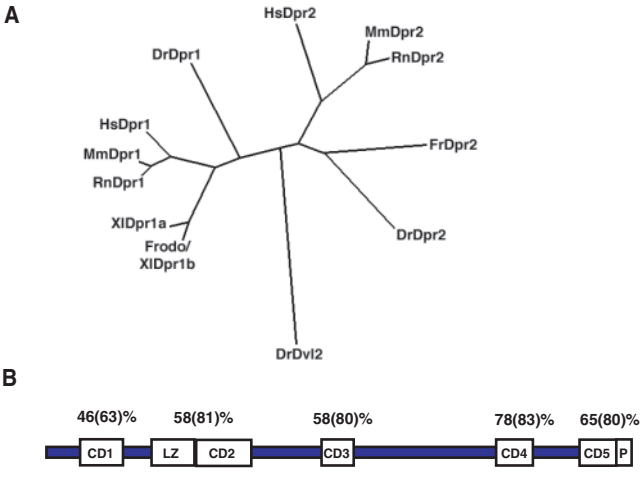

Fig. 1. Relationship of Dpr family members. Four Dpr sequences had been published previously, while five others were determined from a combination of the GenBank sequence databases. (A) Neighbor joining phylogenetic analysis of 11 Dpr members reveal two monophyletic groups. Each vertebrate except *Xenopus* has two paralogous Dpr genes. XDpr1 (XDpr1a) and FRODO (XDpr1b) were found to be a *Xenopus* specific duplication. Zebrafish Dvl2 was defined as the outgroup. Species are: Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Xl, *Xenopus laevis*; Fr, *Fugu rubripes*; Dr, *Danio rerio*. (B) The conserved domains of Dpr family members. The number above the conserved domains is the percent identity and overall similarity (brackets-taking into account conservative changes) from comparison of zebrafish Dpr1 and Dpr2. CD, conserved domain; L2, leucine zipper; P, PDZ binding domain.

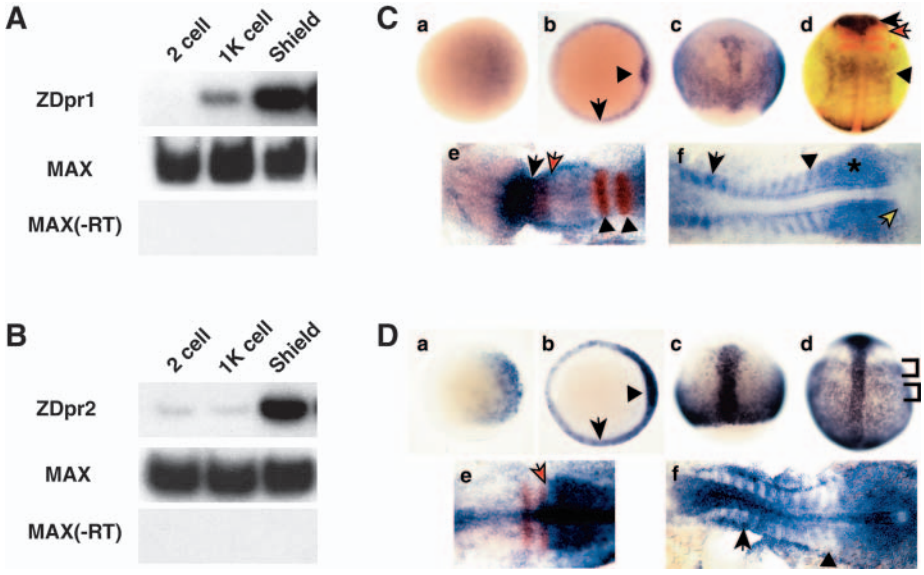

Fig. 2. RT-PCR and in situ hybridization analysis of zebrafish *dpr1* and *dpr2*. (A,B) RT-PCR analysis (25 embryos per stage) of *dpr1* (A) and *dpr2* (B). Zebrafish *max* is used as a loading/positive control. –RT is a control for genomic DNA contamination. (C,D) In situ hybridization for expression of *dpr1* (C, parts a-f) and *dpr2* (D, parts a-f), with embryos shown that are representative of at least 25 analyzed per stage. Stages: (a) sphere stage; (b) shield stage; (c) 80% epiboly; (d) ~tailbud; (e) five somites (in C), three somites (in D); (f) ~12 somites. Orientation: (a,b) animal views, dorsal is rightwards; (c,d) dorsal views, anterior is upwards; (e,f) anterior is leftwards. (C, part b) Arrow indicates margin and arrowhead indicates shield. (C, part d) *dpr1* (blue), *pax2.1* (red, midbrain/hindbrain boundary) and *krox20* (red, –r3 and 5). Black arrow indicates *dpr1* expression in brain. Black arrowhead indicates *dpr1* expression in anterior spinal cord. Red arrow indicates *pax2.1*. Red asterisk indicates *krox20*. (C, part e) Black arrow is posterior limit of *dpr1*(blue) expression. Red arrow is posterior limit of *pax6.1*(red) expression. Arrowheads indicate r3 and r5. (C, part f) Arrow indicates anterior older somite. Arrowhead indicates staining of posterior, younger somite. Asterisk indicates presomitic mesoderm. Yellow arrow indicates tailbud. (D, part b) Arrow indicates margin and arrowhead indicates shield. (D, part d) Brackets indicate mediolateral banding pattern of staining in lateral mesoderm. (D, part e) Arrow indicates boundary of *krox20* (red) and *dpr2* (blue) staining. (D, part f) Arrow indicates staining in the anterior of the older somites. Arrowhead indicates presomitic mesoderm.

is predominantly lost by three somites (not shown). *dpr2* expression is maintained in the lateral mesoderm, and occurs in a banding pattern that is reminiscent of *wnt11* (arrowhead; Fig. 2D, part d) (Kilian et al., 2003). At the three somite stage, *dpr2* is expressed posterior to a sharp boundary in the posterior hindbrain and anterior spinal cord. Double staining with *dpr2* (blue) and *krox20* (red) reveals this sharp boundary is adjacent to r5 at three somites (red arrow; Fig. 2D, part e). Similar to *dpr1*, *dpr2* expression is quite specific during somitogenesis (Fig. 2D, part f). By the 12 somite stage, *dpr2* is upregulated in the anterior of the older somites (Fig. 2D, part f, arrow), but there is little *dpr2* expression in the nascent somites (arrowhead; Fig. 2D, part f). *dpr2* is also expressed in the posterior presomitic mesoderm and tailbud. Later expression patterns for *dpr2* are presented in Fig. S3 in the supplementary material. The data of Gillhouse et al. (Gillhouse et al., 2004), published during revision of this manuscript, support these patterns of expression of *dpr1* and *dpr2*.

Dpr1 and Dpr2 have unique functions in development

In order to study the requirements for endogenous Dpr1 and Dpr2 in zebrafish development, we used morpholino antisense oligonucleotides (MOs) (Draper et al., 2001; Nasevicius and Ekker, 2000). The *dpr1* MO was designed to block the proper splicing of the second intron/exon boundary. The *dpr1* MO was able to significantly abrogate proper splicing by the appearance of an ~1700 bp band as analyzed by RT-PCR (Fig. 3A). The morpholino to *dpr2* was designed to the 5′-UTR. To test if this specifically blocked translation, the 5′-UTR was cloned upstream of luciferase (5′Dpr2-luc). The *dpr2* MO specifically blocked the in vitro translation of the 5′-UTR Dpr2-luciferase construct, relative to an internal control β-galactosidase (Fig. 3B).

During gastrulation *dpr1* morphants (morpholino-injected embryos) have apparently normal domains of expression of expression of *bozozok*, a dorsal marker and β-catenin target gene (data not shown). At approximately the 10 somite stage, *dpr1* morphants are slightly smaller (*n*=162/178 – 91%), but still do not have significant problems with specification of cell fate, as assayed by a cocktail of probes for in situ hybridization containing *catL* (hatching gland), *opl* (telencephalon), *en2* (midbrain-hindbrain boundary), *krox20* (rhombomeres 3 & 5), and *myod* (adaxial cells and somites) (compare Fig. 3C, part b

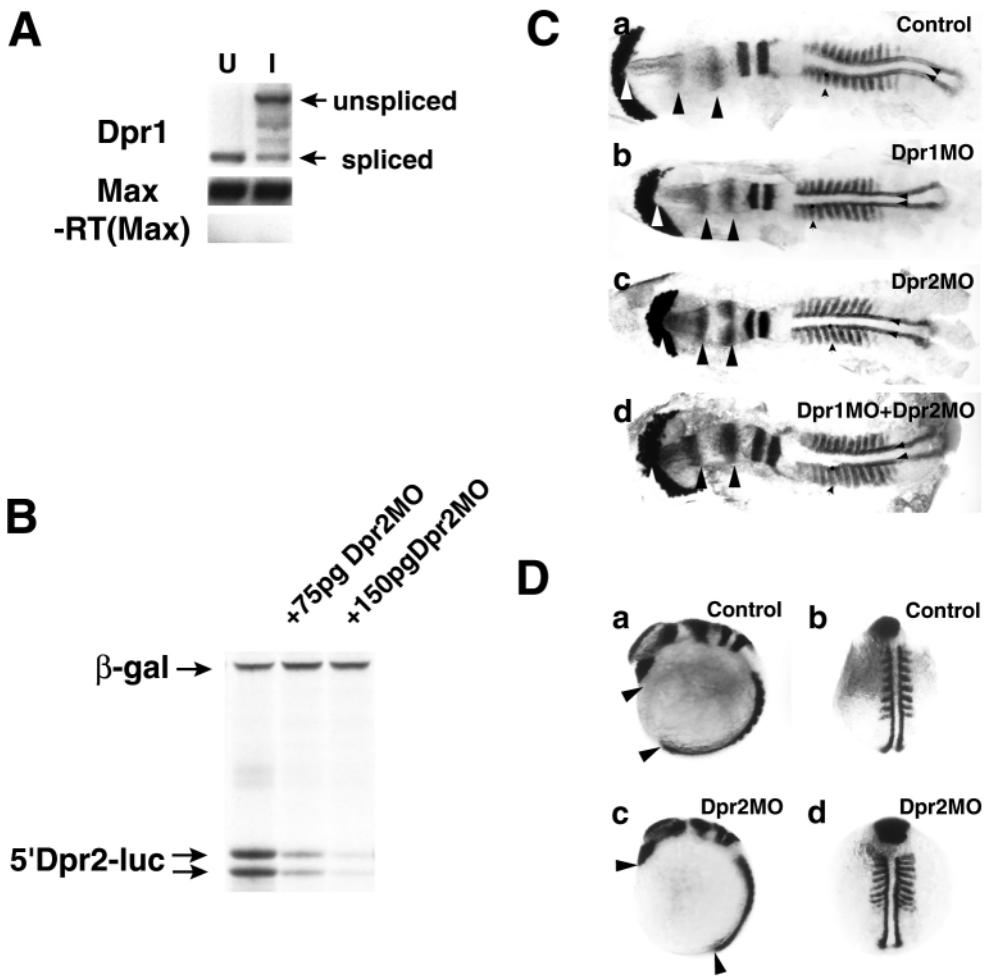

Fig. 3. Zebrafish *dpr1* and *dpr2* morphant phenotypes. (A) RT-PCR analysis of efficacy of *dpr1* MO. *dpr1* splice blocking MO induces the predicted shift to an ~1700 band, indicating it abrogates proper splicing of the transcript. Spliced band is at ~500 bp. U, uninjected; I, injected. (B) 5′-Dpr2-luciferase construct is blocked in an in vitro transcription/translation reaction. β-Galactosidase was used as an internal control. (C) *dpr1* morphants (b, injected with ~12 ng MO) are discernibly smaller but have no major change of cell fates compared with control (a). *dpr2* morphants (c, injected with ~8 ng MO) have convergent extension defects, which are more apparent in whole embryos (D, parts c,d). However, the notochord is noticeably wider – compare distances between the small posterior arrowheads in c with a and b. *dpr1*+*dpr2* morphants (d) do not have novel phenotypes, indicating they are not redundant. White arrow indicates anterior limit of head. Black arrows indicate distance between *opl* and *en2*. Small arrowhead indicates somite. Posterior arrowheads indicate width of notochord. Embryos were flatmounted, with anterior towards the left. (D) Whole-mount in situ hybridizations of *dpr2* morphants indicate they are shorter (compare arrowheads in a and c) and the somites and notochord are wider (compare *myod* staining in b and d), but major specification events are not affected. For C and D, the experiments were repeated four times with comparable results, see text for penetrance of phenotypes. In situ probes used from anterior to posterior were*: cathepsinL* (*catL*-hatching gland), *opl* (telencephalon), *en2* (midbrain/hindbrain boundary), *krox20* (rhombomeres 3 and 5) and *myod* (adaxial cells and somites).

with 3C, part a). The most noticeable defect is that the brain anterior to the midbrain-hindbrain boundary is smaller. This can been seen by comparing the distance between the *catL* and *opl*, and *opl* and *en2* (compare distances between arrowheads in Fig. 3C, part b and 3D, part a).

dpr2 morphants (*n*=258/289 – 89%) have a different phenotype that is indicative of aberrant convergent extension movements during gastrulation (Fig. 3C, part c; 3D, parts c,d). The body axes are short and wide, and there are no major changes in cell fates. Consistent with this observation, the anterior of the embryos does not extend as far around the yolk (compare arrowheads in Fig. 3D, part c with part a), the notochord is wider and somites are elongated (compare distance between small, posterior arrowheads in Fig. 3C, part c with those in 3C, parts a,b; note elongated somites in Fig. 3D, part d compared with those in 3D, part b). Similar results were obtained during somitogenesis with a different MO, *dpr2* MO(2), that abrogates proper splicing of this gene (data not shown).

To see if Dpr1 and Dpr2 are functionally redundant we injected both *dpr* morpholinos. The phenotype (*n*=121/137 – 88%) was strictly additive (Fig. 3C, compare part d with parts a-c). We conclude that *dpr1* morphants have a subtle phenotype leading to reduction of the midbrain and diencephalon, while *dpr2* appears to regulate convergent extension movements. However, repeating the morpholino injections in sensitized, hypomorphic, backgrounds proved to be informative, as discussed below.

Dpr1 is an enhancer of Wnt/β-catenin signaling

Although *dpr1* morphants do not have a severe phenotype, the pattern of *dpr1* expression could be consistent with it playing a role in early Wnt-8/β-catenin signaling that is required for ventralization and posteriorization of embryos (Lekven et al., 2001). To determine if *dpr1* functionally interacts with the Wnt8 signal during early zebrafish development, we injected the *dpr1* MO with a low amount of two *wnt8* morpholinos

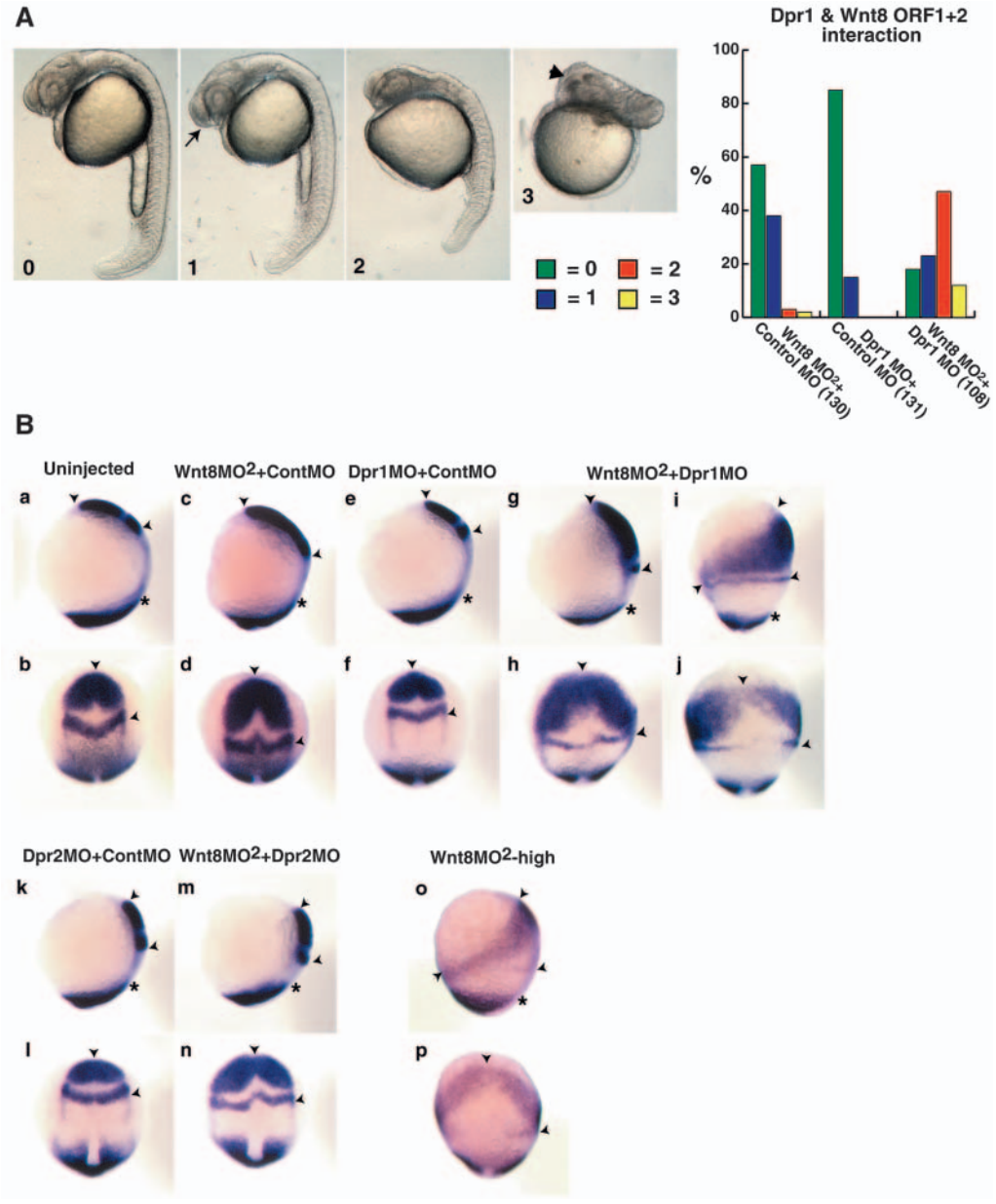

Fig. 4. Dpr1 is an enhancer of Wnt/β-catenin signaling required for ventral and posterior cell fates. (A) Representative embryos for scoring of ventroposteriorization at 24 hours. 0, wild type; 1, slight enlargement of the telencephalon (arrow); 2, strong anteriorization phenotype, enlargement of the head and reduction of the tail; and 3, very strong anteriorization phenotype, super enlargement of the telencephalon (arrowhead) and major loss of trunk and tail. Graph shows percentage of embryos with the respective phenotypes (numbers in brackets indicate the number of embryos used). (B) *dpr1*, *dpr2* and *wnt8*MO^2 morphant phenotypes at the one- to two-somite stage. In situ markers used were *opl* (telencephalon), *pax2.1* (midbrain/hindbrain boundary) and *tbx6* (ventrolateral mesoderm). Arrowheads indicate distance between anterior limit of *opl* and posterior limit of *pax2.1*. Asterisks indicate anterior limit of *tbx6*. (a,c,e,g,i,k,m,o) Dorsal is towards the right. (b,d,f,h,j,l,n,p) Dorsal is out of the plane of the page. In all figures, anterior is upwards. Suboptimal dose of wnt8 MO^2 is 0.45 ng each MO (0.9 ng total). *dpr1* MO dose is 12 ng. *dpr2* MO dose is 8 ng. The experiments were repeated five times with comparable results; see text for penetrance of phenotypes.

(MO^2, ORF1 MO+ORF2 MO), a dose that produces no phenotype or a weak hypomorphic phenotype (Lekven et al., 2001). Embryos were first examined at 24 hours, and scored on a 4 point scale with 0 being wild type, 1 having modest enlargement of the telencephalon, 2 having enlargement of the anterior and reduction of the posterior, and 3 having severe enlargement of the anterior and reduction of the posterior (Fig. 4A). Injection of *wnt8* MO^2 plus control MO yielded embryos with a slightly larger telencephalon than controls (Fig. 4A, arrow in panel 1 and graph). *dpr1* MO plus control morpholino injected embryos are equivalent to *dpr1* MO alone (Fig. 4A, graph). However, when the *dpr1* MO was injected with the *wnt8* MO^2, the morphants phenocopied strong loss of Wnt8 function (Fig. 4A, graph). These embryos had enlarged telencephalons (arrowhead in panel 3), eyes, and reduced posterior (trunk and tail). We confirmed these results using a second *dpr1* morpholino (*dpr1* MO2, not shown).

To ensure that this phenotype was due to problems in specification of ventral and posterior cell fates attributable to *wnt8* ORF1+2 function, embryos were examined at the one- to two-somite stage by in situ hybridization using probes for *opl*, *pax2.1* and *tbx6* (ventrolateral mesoderm). In agreement with the 24 hour phenotype and a role for Dpr1 in early specification events, co-injection of suboptimal amounts of *wnt8* MO^2 plus *dpr1* MO yielded embryos that had large expansion of *opl* coupled with reduction of *tbx6*, phenocopying strong Wnt8 loss of function (compare Fig. 4B, parts g-j with 4B, parts o,p). The embryos depicted in Fig. 4B, parts g,h are representative of a score of 2, while 4B, parts i,j are representative of a score of 3 at 24 hours. *wnt8* MO^2 plus a control morpholino yielded embryos with a moderate expansion of anterior tissues as indicated by the posterior expansion of *opl* and *pax2.1* (compare Fig. 4B, parts c,d with parts a,b). Injection of *dpr1* MO plus a control morpholino yielded embryos with no major changes in cell fate (compare Fig. 4B parts e,f with 4B, parts a,b). Although the *dpr1* morphant phenotype is subtle, when *dpr1* perturbation is coupled with moderate *wnt8* attenuation, the resulting *dpr1* + *wnt8* morphants phenocopy strong loss of *wnt8* function. We conclude that Dpr1 is required as an enhancer of Wnt8 signaling.

Given that *dpr2* morphants have convergence extension problems, we would not expect to see the same interaction with Wnt8. To test this hypothesis, we injected the hypomorphic dose of *wnt8* MO^2 plus a maximal dose of the *dpr2* MO. In *dpr2* MO plus control morpholino, the embryos did not extend as far around the yolk and their bodies are wider (Fig. 4B, parts k,l). Injection of *dpr2* MO plus *wnt8* MO^2 is strictly additive (compare Fig. 4B, parts m,n with parts k,l and parts c,d). The embryos are shorter and wider, coupled with modest expansion of *opl*, like *wnt8* MO^2 alone. We conclude that Dpr2 does not play a role in Wnt8 signaling in early zebrafish development.

Dpr2 functionally interacts with the Wnt/Ca^{2+}-PCP pathway(s)

The *dpr2* morphant phenocopies loss of PCP components like *strabismus/trilobite* (*stbm*), *kynpek* (*kny*) and *prickle* (*pk*) (Fig. 5A) (Jessen et al., 2002; Park and Moon, 2002; Topczewski et al., 2001; Veeman et al., 2003b). At 24 hours *dpr2* morphants are noticeable shorter, with block-like somites (Fig. 5A, arrow) and they have eyes that have not properly separated owing to improper migration of underlying mesodermal tissues (Fig. 5A, arrowhead). To test if *dpr2* functionally interacts with *stbm*, we injected a low hypomorphic dose of the *stbm* MO with a low hypomorphic dose of the *dpr2* MO. Embryos were then scored on a scale of 0 to 2, with 0 being wild-type, 1 having mild convergence extension (CE) problems and 2 having strong CE problems (Fig. 5B). Injection of either morpholino alone (plus control morpholino) yielded 10% and 15% of the embryos with mild CE problems for *stbm* MO and *dpr2* MO, respectively (Fig. 5C). None of the embryos had strong CE problems (Fig. 5C). However, co-injection of the *stbm* MO and *dpr2* MO yielded 30% of the embryos having mild CE problems and 55% of the embryos with strong CE defects. Thus, we conclude there is a functional interaction between Dpr2 and Stbm.

dpr2 morphants have anteriorly improperly placed eyes, but they rarely have cyclopia (Fig. 5D, parts g,h). Although perturbations in genes affecting CE may not individually have a high percentage of cyclopia, in combination they can genetically interact to yield a high percentage of cyclopic embryos (Marlow et al., 1998). To test if Dpr2 functionally interacts with Wnt11 by increasing the percentage of cyclopic embryos, we used a *wnt11* MO that phenocopies *wnt11/slb* mutants (Lele et al., 2001). A suboptimal dose of *wnt11* MO was injected with a suboptimal dose of *dpr2* MO. Embryos were scored on a cyclopia index of 0 to 2, with 0 being wild type, 1 having forwardly positioned narrow eyes, and 2 being cyclopic and having fusion of the lenses (Fig. 5D, parts a-f). Injection of *wnt11* MO plus control morpholino yielded a low percentage of cyclopic embryos (17%; Fig. 5E). However, 65% of embryos co-injected with *wnt11* MO and *dpr2* MO were cyclopic (Fig. 5E). The percent of cyclopic embryos was increased without affecting the posterior of the embryos (not shown). Therefore, we conclude that *dpr2* functionally interacts with *wnt11/slb* as well as with *stbm/tri*.

To test if these functional interactions with *stbm/tri* and *wnt11/slb* are specific to Dpr2 and not Dpr1, we co-injected embryos with a higher dose of *dpr1* MO(2) and either the *stbm* MO or *wnt11* MO (Fig. 5C,E). The scoring of these embryos was almost identical to the *stbm* MO or *wnt11* MO plus control morpholino. Therefore, there is not a functional interaction between *stbm* or *wnt11* and *dpr1*.

Association with zebrafish Dvl2 and subcellular localization of Dpr orthologs

After injection of *dpr1-myc* or *dpr2-myc* RNA with zebrafish *dvl-HA* RNA into *Xenopus* embryos, followed by immunoprecipitation with anti-HA antibodies, both Dpr1-myc and Dpr2-myc proteins were detected by western blot (Fig. 6A,B). Thus, both zebrafish Dpr family members can associate either directly or indirectly with Dvl2, as reported for *Xenopus* Dpr family members (Cheyette et al., 2002; Gloy et al., 2002).

To determine the subcellular localization of ectopic Dpr1 and Dpr2, immunochemistry was performed using *Xenopus* animal caps injected with the *dpr1-myc* or *dpr2-myc* RNA. In this heterologous system, chosen in order to take advantage of the large size of the cells, both ectopic proteins are cytoplasmic (compare Fig. 6C with 6D). However, Dpr2 is also visible in the nuclei of some cells (small arrows) and has more intense staining at the cell membrane (large arrow).

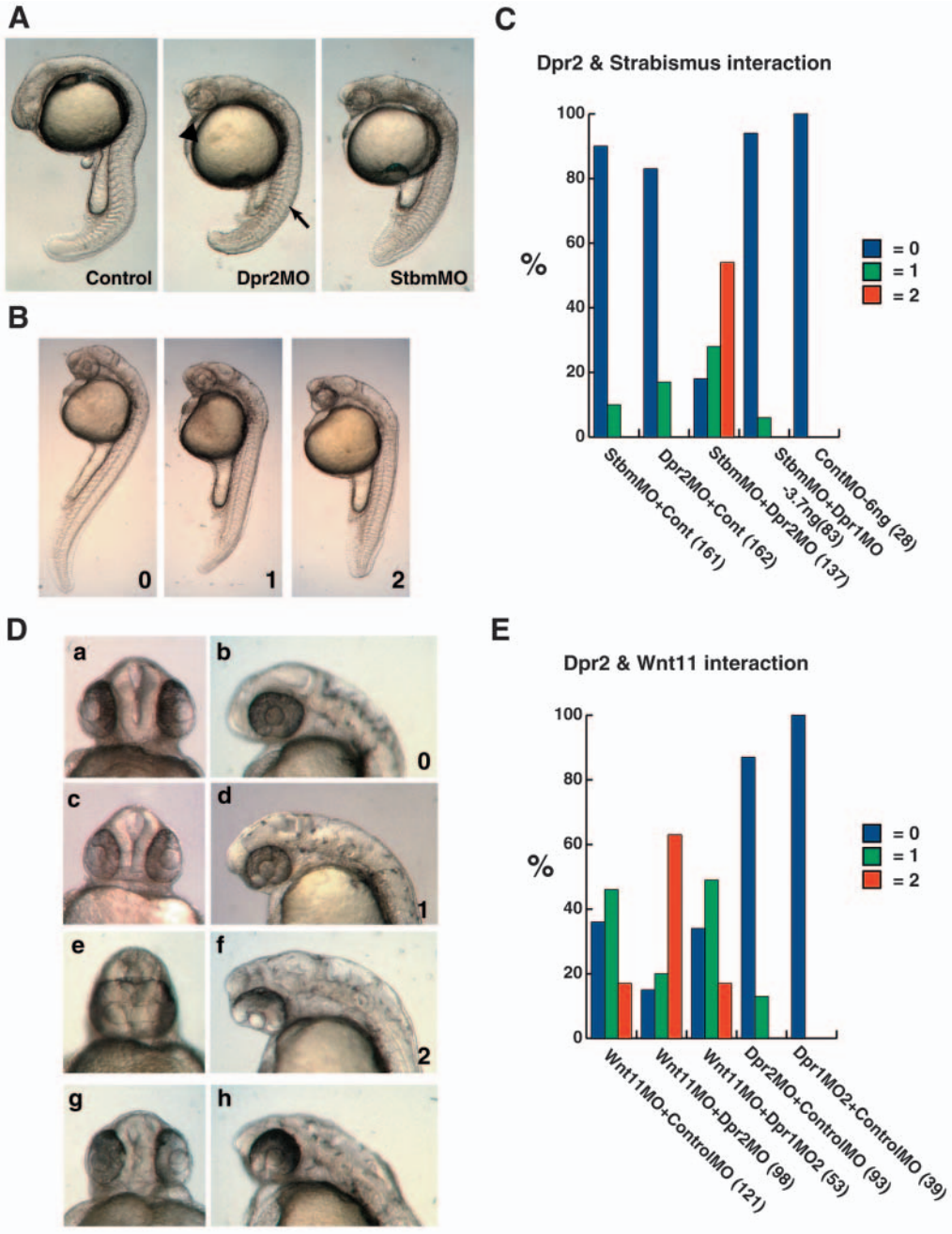

Fig. 5. *dpr2* morphants phenocopy *strabismus/trilobite (stbm)* morphants, and functionally interact with *stbm* and *wnt11/silberblick*. (A) *dpr2* morphants (middle-injected with 7.5 ng *dpr2* MO) phenocopy *stbm* morphants (right-injected with 3 ng). Arrow indicates flattened, non-chevron-shaped somite. Arrowhead indicates narrowed eyes. (B) Scoring of embryos that were co-injected with suboptimal doses of *stbm* and *dpr2* MOs. 0, wild type; 1, moderate CE defects; 2, severe CE defects. (C) Percentage of embryos injected with MOs exhibiting phenotype. (D) Scoring of cyclopia index of embryos co-injected with *dpr2* MO and *wnt11* MO (a-f). *dpr2* MO (normal dose, g,h). 0, wild type; 1, narrowing of the eyes; 2, fusion of the lens. (E) Percentage of embryos injected displaying respective phenotypes. Suboptimal (hypomorphic) dose of *dpr2* MO is 3 ng, *stbm* MO is 0.4 ng and *wnt11* MO is 1.5 ng. All injections were balanced with control MO. The numbers in brackets indicate the numbers of embryos used.

Gain of function of Dpr1 and Dpr2 in *Xenopus* and HEK293T cell assays

Overexpression of signaling proteins is useful in establishing the activity of a protein in a specific context, and thus learning its potential involvement in a given pathway. We asked whether gain-of-function of Dpr1 and Dpr2 might affect Wnt/β-catenin signaling. Unfortunately the overexpression studies could not be conducted in zebrafish, as injection of Dpr RNAs into embryos leads to a high percentage of early embryonic lethality and non-specific toxicity. Therefore, the gain-of-function assays were conducted in *Xenopus* embryos and in HEK293T cells, both of which are responsive to Wnt signals.

We first tested whether Dpr1 and Dpr2 induce Wnt/β-catenin target genes in *Xenopus* animal caps and found that *dpr1* but not *dpr2* RNA induces *sia* and *Xnr3* (Fig. 6E, compare lane 1 with lanes 2 and 3). Consistent with this result, ventral injection of *dpr1* RNA induced partial secondary axes in 36% of *Xenopus* embryos (n=78), while *dpr2* RNA induced partial secondary axes in only 5% of *Xenopus* embryos (n=52) (not shown). Injection of less than 4 ng of *dpr1* or *dpr2* RNA did not induce Wnt/β-catenin target genes or secondary axes (not shown).

While injecting embryos for these animal cap experiments we observed that injection of *dpr2* RNA into the animal pole resulted in aggregation of pigment around the site of injection, whereas injection of *dpr1* RNA and β-gal control RNA did not (see Fig. S4 in the supplementary material). This result, which we do not understand in terms of mechanisms, further

suggests functional differences in the activities of the two Dpr proteins.

Despite differences in the ability of injected *dpr1* and *dpr2* RNAs to induce Wnt/β-catenin target genes, we found that both Dpr orthologs synergize with zebrafish Dvl2 to induce Wnt/β-catenin target genes in *Xenopus* animal caps (Fig. 6F, compare lanes 2-7 with control lane 8; see Fig. S5 in the supplementary material for similar results with *Xenopus* Dpr orthologs). However, *dpr1* synergizes more strongly (n=6 experiments). This is probably not due to preferential expression of Dpr1, as Dpr1 and Dpr2 translate at similar levels in vitro (Fig. 6G) and tagged proteins Dpr1-myc and Dpr2-myc, which are functional equivalent to untagged Dpr1 and Dpr2, are expressed similarly in frog embryos (not shown and Fig. 6G). This synergistic activation of Wnt/β-catenin target genes by Dpr1 and Dvl2 was blocked by co-expression with Axin (Fig. 6H), consistent with its being a β-catenin-dependent synergy.

As Dpr1 and Dpr2 synergize with Dvl2, a component of Wnt signaling pathways, we next asked if either would synergize with Wnt itself to activate Wnt/β-catenin target genes. Neither zebrafish ortholog synergized with Wnt8, nor did either inhibit activation of target genes (Fig. 6I). We then turned to an independent assay system to investigate effects of co-expression of Dpr orthologs with Dvl2 or Wnt. HEK293T cells are responsive to Wnt/β-catenin signaling, which can be monitored by the luciferase activity of Super(8X)TOPFlash, a β-catenin-responsive reporter construct (Veeman et al., 2003b). Consistent with what we observed in *Xenopus* animal cap experiments, both zebrafish Dpr1 and Dpr2 synergize with zebrafish Dvl2 after transient transfection in HEK293T cells (Fig. 6J). However, both Dpr orthologs were inhibitory to the Wnt-mediated activation of the luciferase reporter in this cell line (see Fig. S6 in the supplementary material).

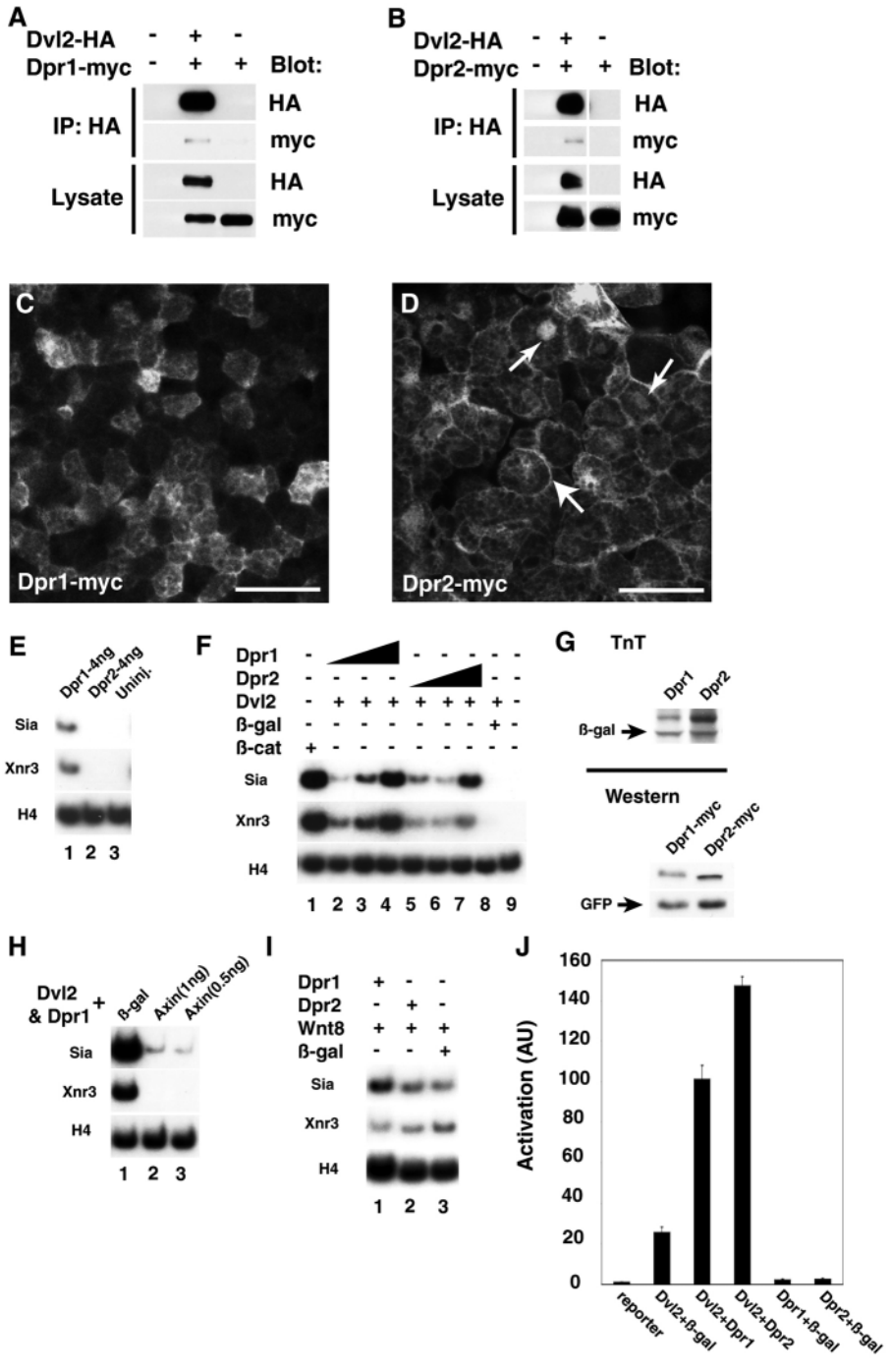

Fig. 6. Dpr immunoprecipitation, localization and activation of Wnt/β-catenin target genes. (A) Dpr1-myc and (B) Dpr2-myc immunoprecipitate with Dvl2-HA from *Xenopus* lysates. (C) Dpr1-myc and (D) Dpr2-myc localization in *Xenopus* animal caps. Small arrow in D indicates nuclear staining. Large arrow indicates membrane staining. Scale bar: 20 μm in C,D. (E) Injection of 4 ng *dpr1* RNA induces β-catenin target genes, but 4 ng *dpr2* RNA does not. (F) Dpr1 and Dpr2 synergize with suboptimal dose of zebrafish Dvl2. *dpr1* and *dpr2* low dose is 0.25 ng, medium dose is 0.5 ng and high dose is 1.0 ng. β-*galactosidase* RNA dose is 1.0 ng. (G) Dpr1 and Dpr2 with internal control β-gal in TnT reaction (upper). Dpr1-myc and Dpr2-myc western from *Xenopus* lysates with internal control GFP (lower). (H) Dpr1-Dvl2 synergy is inhibited by Axin. (I) Dpr1 and Dpr2 do not inhibit Wnt8 activation of β-catenin target genes. (J) Dpr1 and Dpr2 synergize with Dvl2 in activating a β-catenin-dependent luciferase reporter in HEK 293T cells. The experiment is representative of three independent transfections, with each data point being conducted in triplicate. Unless noted otherwise, 1 ng of *dpr1-myc*, *dpr2-myc* and β-*galactosidase* RNA, and 0.4 ng *dvl2* RNA were used. For I, 1 pg of *wnt8* RNA, and 4 ng of *dpr1* and *dpr2* RNA were used. For J, 400 ng of Dpr1, Dpr2 or β-galactosidase, and 20 ng of zebrafish Dvl2 were transfected. AU, arbitrary light units.

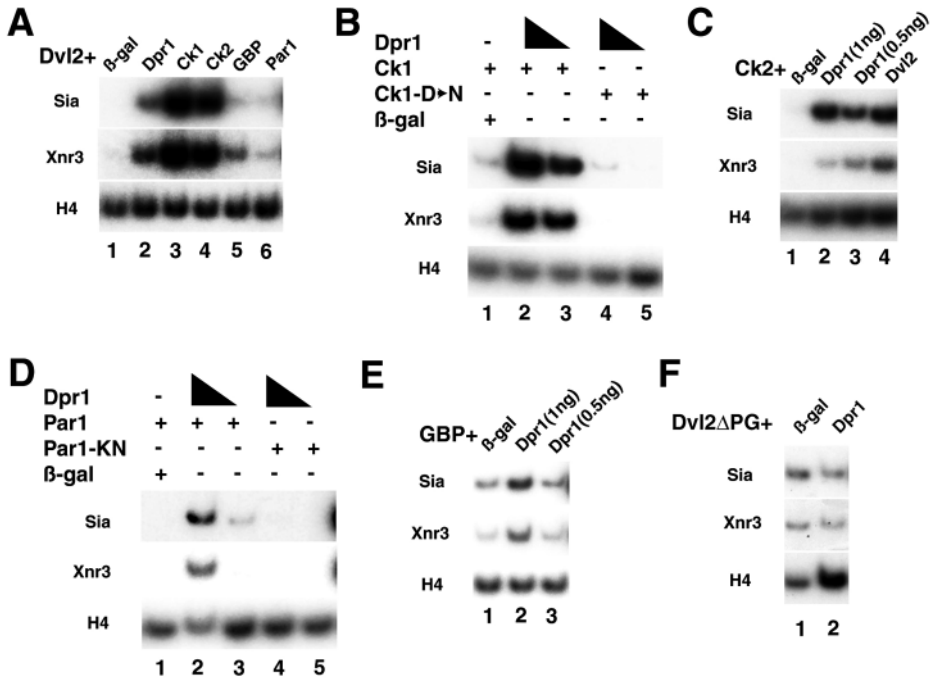

Fig. 7. Dpr synergizes with Dvl-interacting kinases in *Xenopus* animal caps. For all injections, unless noted, 1 ng of *dpr1*, 0.1 ng of *ck1ε,* 0.1 ng of *ck1-DN*, 1 ng of *par1*, 2 ng of *par1-KN*, 0.4 ng of *dvl2*, 1 ng of *ck2α,* 1 ng of *ck2*β, 1 ng of *gbp*, 1 ng of β-*galactosidase*, 0.5 ng of *dvlΔpgb* RNA were used. Low dose of *dpr1* RNA is 0.5 ng for B and E. (A) Dvl2 synergizes with Dpr1, CK1ε, CK2, GBP and Par1. (B) Zebrafish Dpr1 synergizes with CK1ε, but not CK1-D→N. (C) CK2 can synergize with Dpr1 (Dvl2 is shown as a positive control). (D) Dpr1 synergizes with Par1, but not Par1-KN. Low dose of *dpr1* RNA is 0.5 ng. (E) Dpr1 does not synergize well with GBP. (F) Dpr1 does not synergize with the Dvl2ΔPGB.

As the Dvl-associated protein Dpr1 synergizes with Dvl2, and does so to a greater extent than Dpr2, we next investigated whether Dpr1 could synergize with other proteins that interact with Dvl. We focused on CK1ε, protein kinase CK2 (here referred to as CK2), Par1 and GBP/FRAT-1. We expected that these Dvl-interacting proteins should synergize with Dvl2 in animal cap assays to activate Wnt/β-catenin target genes, which is what we observed (Fig. 7A, compare lanes 2-6 with lane 1).

We next asked whether Dpr1 synergizes with any of three different Dvl-associated kinases beginning with CK1ε, which is a potent activator of Wnt/β-catenin signaling (Hino et al., 2003; McKay et al., 2001; Peters et al., 1999). We found that CK1ε and Dpr1 synergize in inducing Wnt/β-catenin target genes in *Xenopus* animal caps (Fig. 7B, compare lanes 2 and 3 with lane 1). To determine whether this Dpr1-CK1ε synergy requires the kinase activity of CK1ε, we co-injected *dpr1* RNA with *ck1-D→N* (a less active form of CK1ε) RNA. The *ck1-D→N* was substantially less active in synergizing with Dpr1 (Fig. 7B, compare lanes 4 and 5 with lanes 2 and 3). Like its antagonization of the synergy between Dpr1-Dvl2, Axin was also able to inhibit Dpr1-CK1ε synergy (not shown).

We then tested whether Dpr1 could synergize with a second Dvl-associated kinase, CK2, to induce Wnt/β-catenin target genes. As CK2 functions as a hetero-tetramer, 'CK2' indicates that we injected RNA encoding both α and β subunits (Pinna, 2002) unless otherwise noted. Although *ck2* alone did not induce the target genes, co-injection of *ck2* RNA with *dpr1* RNA induced β-catenin target genes (Fig. 7C, compare lanes 2 and 3 with lane 1). As CK2 functions as a hetero-tetramer, with the β subunit being required to stabilize the α subunit (Pinna, 2002), we tested the specificity of the Dpr1-CK2 synergy by co-injecting just the *ck2α* subunit RNA with *dpr1* RNA. No synergy was observed (data not shown).

We next tested whether there is a functional interaction between Dpr1 and the Dvl-associated kinase Par1. At any dose tested, *par1* alone did not activate Wnt/β-catenin target genes in animal caps. However, when *par1* RNA was co-injected with *dpr1* RNA, there was a synergistic activation of Wnt/β-catenin target genes (Fig. 7D, compare lanes 2 and 3 with lane 1). To determine whether this functional interaction was dependent upon Par1 kinase activity, we co-injected *dpr1* RNA with a higher dose of *par1-KN* (a kinase dead form of Par1) RNA. Dpr1 did not synergize with Par1-KN (Fig. 7D, compare lanes 4 and 5 with lanes 2 and 3).

Finally, we tested whether GBP/FRAT-1 and Dpr1 synergize to activate Wnt/β-catenin target genes. We only observed a mild enhancement between Dpr1 and GBP/FRAT-1 when co-injected (Fig. 7E). As Par1 synergizes with Dpr1, while the GBP/FRAT-1-mediated gene activation was weakly enhanced by Dpr1, we deleted the Par1-GBP/Frat-1-binding domain (Hino et al., 2003) in zebrafish Dvl2, resulting in the construct Dvl2ΔPGB (ΔPar1 and GBP Binding domain). In principle, this should result in a Dvl2 protein that would be unable to interact with endogenous GBP or Par1. Like Hino et al, (Hino et al., 2003), we found this construct could activate Wnt/β-catenin target genes alone (not shown). However, we found that Dvl2ΔPGB did not synergize with Dpr1 in the *Xenopus* animal cap assay (Fig. 7F, compare lane 2 with lane 1).

In *Xenopus* explant assays, the above data show that Dpr1 can functionally interact with Dvl2 and the Dvl-interacting proteins CK1ε, Par1 and CK2, but it does not functionally interact as well with GBP. We next turned to prospective loss-of-function studies using the CK1ε inhibitor CKI-7 (Peters et al., 1999). CKI-7 had no apparent effect on gene induction by stabilized β-catenin, a positive control for inducing *sia* and *Xnr3* (Fig. 8A, compare lane 5 with lane 6). Whether gene activation by Dvl requires CK1ε has not been explored. In addressing this, we found that Dvl-mediated induction of target genes was not affected by CKI-7 injection (Fig. 8A, compare lane 3 with lane 4), though it has been reported that Dvl-mediated axis duplication in *Xenopus* can be inhibited by CKI-

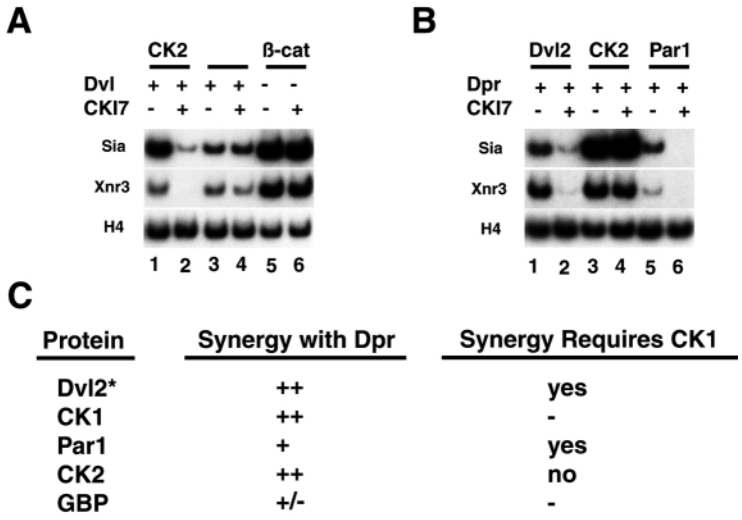

Fig. 8. Dpr synergy requires CK1ε or CK2. (A) Dvl2 or β-catenin activation of canonical target genes does not require CK1ε, but Dvl2-CK2 synergy does require CK1ε. Lanes 1 and 2, 0.4 ng of *dvl2*, 1 ng of *ck2α* and 1 ng of *ck2*β RNA; lanes 3 and 4, 0.8 ng *dvl2* RNA; lanes 5 and 6, 0.1 ng stabilized β-catenin RNA. (B) Dpr1-Dvl2 and Dpr1-Par1 synergy requires CK1ε, but Dpr1-CK2 synergy does not. The amounts of RNA injected were 1 ng of *dpr1*, 1 ng of *par1*, 0.4 ng of *dvl2*, 1 ng of *ck2α* and1 ng of *ck2*β. CKI-7 (5 nl of 2.5 mM) was co-injected in experiments in A and B. (C) Table of Dpr1 synergistic interactions and requirement on CK1ε. Asterisk indicates higher doses of Dvl2 RNA that could activate alone did not require CK1ε, but Dvl2-CK2 synergy did require CK1ε.

7 (Peters et al., 1999). We then tested if Dpr1-Dvl2 synergy requires CK1ε. Dpr1 and Dvl2 did not synergize in gene induction when CKI-7 was co-injected (Fig. 8B, compare lane 1 with lane 2). Thus, Dpr1 requires endogenous CK1ε to enhance Dvl activity. We also found that the synergy of Dpr1-Par1 requires endogenous CK1ε activity (Fig. 8B, compare lane 5 with 6). Thus, Dpr1 is dependent on CK1ε for synergistic interactions with both Dvl2 and Par1.

Last, we determined if Dpr1-CK2 synergy in inducing Wnt/β-catenin target genes in *Xenopus* explants requires CK1ε, and found that it does not (Fig. 8B, compare lane 3 with lane 4). However, CK2-Dvl2 synergy is dependent upon CK1ε (Fig. 8A, compare lane 1 with lane 2). Therefore, both Dpr1 and CK2 synergistically interact with Dvl2 in a CK1ε-dependent manner, but excess Dpr1 and CK2 overcomes a requirement for CK1ε to enhance activation of Wnt/β-catenin target genes (summarized in Fig. 8C).

Potential Dpr mechanisms

We further attempted to determine any mechanisms by which Dpr1 and Dpr2 might affect Wnt pathways. With respect to the Wnt/Ca^{2+}-PCP pathway, Rho activation has been proposed to be a potential indicator of this poorly understood pathway(s) (Habas et al., 2001). However, neither Dpr1 nor Dpr2 affected Rho activation in HEK 293T cells or in *Xenopus* embryo lysates (not shown). With respect to Wnt/β-catenin signaling, CK1ε has been shown to increase GBP association with Dvl-1 (Hino et al., 2003). However, we did not find that CK1ε enhanced Dpr1 or CK2 association with Dvl2, nor did we find that Dpr increased the association of other Dvl-interacting molecules with Dvl2 (not shown). Increased CK2 activity is correlated with increased Dvl-2 stability (Song et al., 2000). However, we did not find that Dpr or any other Dvl-interacting protein (including CK2) could enhance the stability of Dvl (not shown). Finally, Dvl has been proposed to undergo an electrophoretic mobility shift in correlation with Wnt or Fz activation (Lee et al., 1999; Rothbacher et al., 2000; Sun et al., 2001). We also did not find that Par1 or CK2, in the presence or absence of ectopic Dpr, increased the phosphorylation state of Dvl, as monitored by electrophoretic mobility (not shown). With the caveats associated with any negative data, we suspect that these potential mechanisms of action of Dpr orthologs are not the most likely to explain how Dpr family members participate in Wnt signaling pathways.

Discussion

Zebrafish Dpr paralogs can regulate different Wnt-dependent pathways

Paralogous vertebrate Wnt and Fz genes have been found to function in Wnt/β-catenin and/or in Wnt/Ca^{2+}-PCP pathways. For example, zebrafish Wnt8 is thought to activate β-catenin signaling and to be required for regulation of gene expression and cell fates (Erter et al., 2001; Lekven et al., 2001). Wnt/Ca^{2+}-PCP signals, which are elicited by Wnt-11/Silberblick and Wnt-5/Pipetail, are not involved in specification of cell fates, but control the cell movements during gastrulation that allow the embryo to elongate (Heisenberg et al., 2000; Rauch et al., 1997). In this report, we have provided data consistent with zebrafish Dpr1 functioning as an enhancer of Wnt8/β-catenin signaling that specifies ventral and posterior cell fates. We have also provided evidence that Dpr2 functions as an enhancer of the Wnt/Ca^{2+}-PCP components Stbm/Tri and Wnt-11/Slb, thereby controlling cell movements during gastrulation. The primary conclusion of this study is that the two zebrafish Dpr paralogs appear to be required for processes regulated by β-catenin or Wnt/Ca^{2+}-PCP pathways. This parallels the requirement for Wnt and Fz proteins in each of these pathways.

Gain- and loss-of-function of Dvl, as well as structure-function analysis are consistent with the idea that specific Wnt and Fzs somehow activate Dvl in both vertebrate Wnt pathways. How Dvl regulates multiple Wnt pathways is not known. One hypothesis, based primarily on gain-of-function assays in cell culture, predicts that Dvl-interacting proteins direct Dvl function into a specific Wnt/Fz pathway (Sun et al., 2001; Yan et al., 2001). Although this is an attractive hypothesis, there is yet little evidence to support it. Most loss-of-function perturbations of the Wnt/Ca^{2+}-PCP pathway do not produce gain-of-function phenotypes of the Wnt/β-catenin pathway and vice versa. Examples include *dsh* mutants in *Drosophila*, *wnt11/slb* and *stbm/tri* mutants in zebrafish, and

daam1 Xenopus morphants (Axelrod et al., 1998; Habas et al., 2001; Heisenberg et al., 2000; Jessen et al., 2002). In addition, *Drosophila nkd* is not required for the PCP pathway (Rousset et al., 2001). Nevertheless, a few recent reports provide some evidence that in specific contexts there may be opposition of these pathways, though they do not point to the opposition occurring at Dvl (Topol et al., 2003; Westfall et al., 2003). Thus, in some contexts there might be mechanisms by which Dvl is free to regulate either β-catenin or Wnt/Ca^{2+}-PCP pathway without affecting the other.

In the current study, we provide evidence that the zebrafish Dpr paralogs represent examples of Dvl-interacting paralogs that appear to be functionally associated with separate Wnt pathways. Importantly, they do not appear to have redundant functions, yet they have largely overlapping expression patterns in the early zebrafish development and both can interact with zebrafish Dvl2. Therefore, the Dpr morphant phenotypes are consistent with a hypothesis whereby the Wnt/β-catenin and Wnt/Ca^{2+}-PCP pathways can co-exist independently of each other.

Zebrafish Dprs are enhancers of Wnt signaling

The loss-of-function data in zebrafish support the general conclusion that Dpr orthologs are enhancers of multiple Wnt pathways. The Dpr1 morphants have subtle phenotypes that do not lead to any discernable loss of cell fates, yet they have strong Wnt loss-of-function phenotype in a mildly hypomorphic Wnt8 background. These results are reminiscent of those obtained in CK1 RNAi experiments, which also acts as a signaling enhancer in nematodes (Peters et al., 1999). Alone, RNAi of CK1 gives a low percentage of worms with the more-mesoderm (MOM) phenotype. However, when combined with loss of another component of the Wnt pathway, there is a strong MOM phenotype. Likewise, while loss of Dpr2 has a phenotype similar to Wnt/Ca^{2+}-PCP mutants, the strongest affected *dpr2* morphants are not as severe as the strongest affected *stbm/tri* alleles or morphants (Jessen et al., 2002; Park and Moon, 2002). Nevertheless, there is a strong functional interaction between *dpr2* and *stbm/tri* or *wnt11/slb*, indicating that *dpr2* may act to promote Wnt/Ca^{2+}-PCP pathway activity.

How is it possible that the zebrafish *dpr2* morphant indicates a loss of Wnt/Ca^{2+}-PCP activity, but when overexpressed it can synergize with Dvl2 to activate Wnt/β-catenin targets? We do not interpret these results as being mutually exclusive. Wnt components have been demonstrated to be context-dependent activators of Wnt/β-catenin or Wnt/Ca^{2+}-PCP pathways. *Wnt5a/pipetail* is probably the best example. Wnt5a overexpression in *Xenopus* leads to improper convergent and extension movements (Du et al., 1995). Similarly, a loss-of-function mutation of *wnt5/ppt* in zebrafish leads to improper convergence extension movements in the tail (Rauch et al., 1997; Westfall et al., 2003). Thus, the overt phenotype of loss or gain of Wnt/Ca^{2+}-PCP components is similar. However, overexpression of Wnt5a along with Fz5 or LRP6 activates Wnt/β-catenin target genes in animal caps and duplicates axes in *Xenopus* embryos (He et al., 1997; Tamai et al., 2000). Furthermore, expression of high levels of Wnt11 and some Fzs can weakly duplicate axes and induce β-catenin target genes, emphasizing a caveat for overexpression of paralogous proteins (Du et al., 1995; Ku and Melton, 1993; Sheldahl et al., 1999).

Comparison with the *Xenopus* Dpr family

We have previously reported that *Xenopus* Dpr1a could function as an inhibitor of Wnt/β-catenin signaling (Cheyette et al., 2002). Gloy et al. (Gloy et al., 2002) initially reported that the related FRODO/XDpr1b acted like an activator of Wnt signaling. In an effort to resolve the apparent discrepancies between the first two studies, the Sokol laboratory has compared the signaling activities of the two Dpr family members side by side in gain- and loss-of-function studies. They conclude that both XDpr1a and FRODO/XDpr1b can act as either activators or inhibitors, depending on the level at which the pathway is activated, and the doses of the proteins (Hikasa and Sokol, 2004). To understand better the normal functions of Dpr family members we undertook the present study in zebrafish in part to exploit the ability to conduct loss-of-function of Dpr orthologs in sensitized backgrounds. Although our new experiments support the hypothesis that endogenous Dpr orthologs are positive modulators of Wnt signaling in zebrafish under the conditions assayed, we do not dispute the conclusions of Cheyette et al. (Cheyette et al., 2002) and Hikasa and Sokol (Hikasa and Sokol, 2004) that *Xenopus* Dpr family members can act as inhibitors in some contexts. We propose that further analysis of Dpr family members in other contexts besides frog and fish embryos may help determine the mechanisms of action of these interesting proteins.

Conclusions

In conclusion, we provide loss-of-function evidence that Dpr1 but not Dpr2 is required for proper dorsoventral and anteroposterior patterning in zebrafish that are mildly hypomorphic for Wnt8, indicating that Dpr1 probably acts as an enhancer of Wnt8/β-catenin signaling in early development. Dpr2 but not Dpr1 acts as an enhancer of *stbm/tri* and *wnt11/slb* in the Wnt/Ca^{2+}-PCP pathway(s). Thus, in early zebrafish development, loss-of-function evidence indicates Dpr1 and Dpr2 are not redundant, and they function to regulate Wnt/β-catenin and Wnt/Ca^{2+}-PCP pathways, respectively. Regarding mechanisms of action, how Dpr2 functions is unclear, largely because of the lack of understanding of the Wnt/Ca^{2+}-PCP pathway and the lack of robust assays. With regard to Dpr1 orthologs, in the majority of contexts they function as positive regulators of Wnt/β-catenin signaling, though they can inhibit this pathway in various overexpression assays. We extend our analysis of the Dvl-associated Dpr1 orthologs by demonstrating that zebrafish Dpr1 synergizes in gene induction with three Dvl-associated kinases, CK1ε, Par1 and CK2. Moreover, we demonstrate that gene regulation by Dpr1 in some contexts is dependent upon CK1ε. We look forward to genetic and proteomic approaches being employed to further test these findings on the functions and possible mechanisms of action of Dapper family members.

Note added in proof

During the final preparation of this manuscript, Zhang et al. (Zhang et al., 2004) reported *dpr2* morphants have a similar phenotype as reported here.

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Supplementary material

Supplementary material for this article is available at
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