Diversin regulates heart formation and gastrulation movements in development

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Canonical and noncanonical Wnt signaling regulate crucial events in the development of vertebrates and invertebrates. In this work we show that vertebrate Diversin, a potential orthologue of *Drosophila* Diego, controls fusion of heart precursors and gastrulation movements in zebrafish embryogenesis. These events are regulated by noncanonical Wnt signaling, which is independent of β-catenin. We found that Diversin directly interacts with Dishevelled and that this interaction is necessary and sufficient to mediate signals of the noncanonical Wnt pathway to downstream effectors like Rho family GTPases and Jun N-terminal kinase. The ankyrin repeats of Diversin are required for the interaction with Dishevelled, for the activation of noncanonical Wnt signaling, and for the biological responses. The mutation K446M in the DEP domain of vertebrate Dishevelled, which mimics a classical *Drosophila* loss of function mutation, prevents functional interaction with Diversin's ankyrin repeats. Diversin also affects planar cell polarity in *Drosophila*, which is controlled by the noncanonical Wnt signaling pathway. Our data thus demonstrate that Diversin and Dishevelled function together in a mutually dependent fashion in zebrafish gastrulation and organ formation.

convergence and extension | Dishevelled | embryogenesis | noncanonical Wnt signaling | Rho family GTPases

Signaling by ligands of the Wnt family controls a variety of crucial cell changes and morphogenetic events in development. Two branches of the Wnt pathway exist: a β-catenin-dependent so-called canonical pathway and several β-catenin-independent noncanonical pathways (1–3). Noncanonical Wnt pathways regulate planar cell polarity (PCP) in *Drosophila* and gastrulation movements and cardiogenesis in vertebrates (4–7). In zebrafish, Wnt genes that activate the noncanonical Wnt pathway are pipetail/Wnt5 and silberblick/Wnt11 (8–10). Further upstream genes in noncanonical Wnt signaling in zebrafish are knypek/glypican and trilobite/Strabismus/VanGogh (11, 12). An essential downstream effector of noncanonical Wnt signaling is Dishevelled: loss of function mutations of the DEP domain of Dishevelled perturb PCP in *Drosophila*, and a dominant-negative Dishevelled mutation lacking the DEP domain prevents convergence and extension (CE) in gastrulation and precursor fusion in heart formation in zebrafish embryos (6, 13–15). Downstream effectors of Dishevelled that control CE are Rho, Rac, and JNK, and RhoA controls cardiogenesis (6, 13, 16).

The ankyrin repeat protein Diversin of vertebrates is related to Diego of *Drosophila*, which controls PCP during fly development (17, 18). Diego has been identified as one of the core PCP genes in *Drosophila* that are involved in noncanonical Wnt signaling (19). Like Frizzled and Dishevelled, Diego stimulates PCP signaling and prevents the antagonist Prickle from binding to Dishevelled (20, 21). Diversin also acts in canonical Wnt signaling (18), a function that has not been assigned to Diego of *Drosophila*. Diversin is a modular protein containing N-terminal ankyrin repeats, a central casein kinase-binding domain, and a C-terminal domain that binds axin/conductin (Fig. 1*A*) (18). It has been shown that the central casein kinase-binding domain and the axin/conductin-binding domain control canonical Wnt signaling in zebrafish embryos. It has not been determined which domain(s) are involved in Diversin's action in noncanonical Wnt signaling.

Results and Discussion

We have previously reported that the ankyrin repeat protein Diversin of vertebrates is related to Diego of *Drosophila*, which controls noncanonical Wnt signaling and PCP during fly development (17, 18). In the present study we first elucidated the biological implications of the action of Diversin in noncanonical Wnt signaling. Early zebrafish embryos were injected with Diversin mRNA that encodes a protein lacking the ankyrin repeat domain (Div-ΔANK) (Fig. 1*A*), and these embryos were found to develop cardia bifida, i.e., two separately beating hearts (Fig. 1*B*; see also Movie 1, which is published as supporting information on the PNAS web site, to monitor two separately beating hearts at 48 h after fertilization). Cardia bifida in fish is produced when, late in gastrulation, the bilateral heart anlagen fail to fuse because of defective migration of myocardial precursors to the dorsal midline, which is regulated by noncanonical Wnt signaling (6, 22, 23). That the two heart primordia in Div-ΔANK-injected embryos failed to fuse could already be observed at 20 h after fertilization by *in situ* hybridization using heart-specific markers (Fig. 1 *C* and *D*; see quantification in Fig. 1*F*). Apparently, Diversin that lacks the ankyrin repeat domain inhibits normal heart development, possibly by acting in a dominant-negative fashion. We were not able to produce cardia bifida phenotypes by injection of Diversin antisense morpholinos (MO) (data not shown). We could not use appropriate concentrations of MO, because these interfered with canonical Wnt signaling (see ref. 18). Div-ΔANK mRNA injection also disturbed CE in zebrafish embryos (data not shown; see also below). CE represents the migration of mesendodermal cells toward the dorsal midline and the lengthening of the anterior/posterior axis during gastrulation (24, 25). CE is also regulated by noncanonical Wnt signaling (9, 10, 26).

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Abbreviations: PCP, planar cell polarity; CE, convergence and extension; MO, morpholino.

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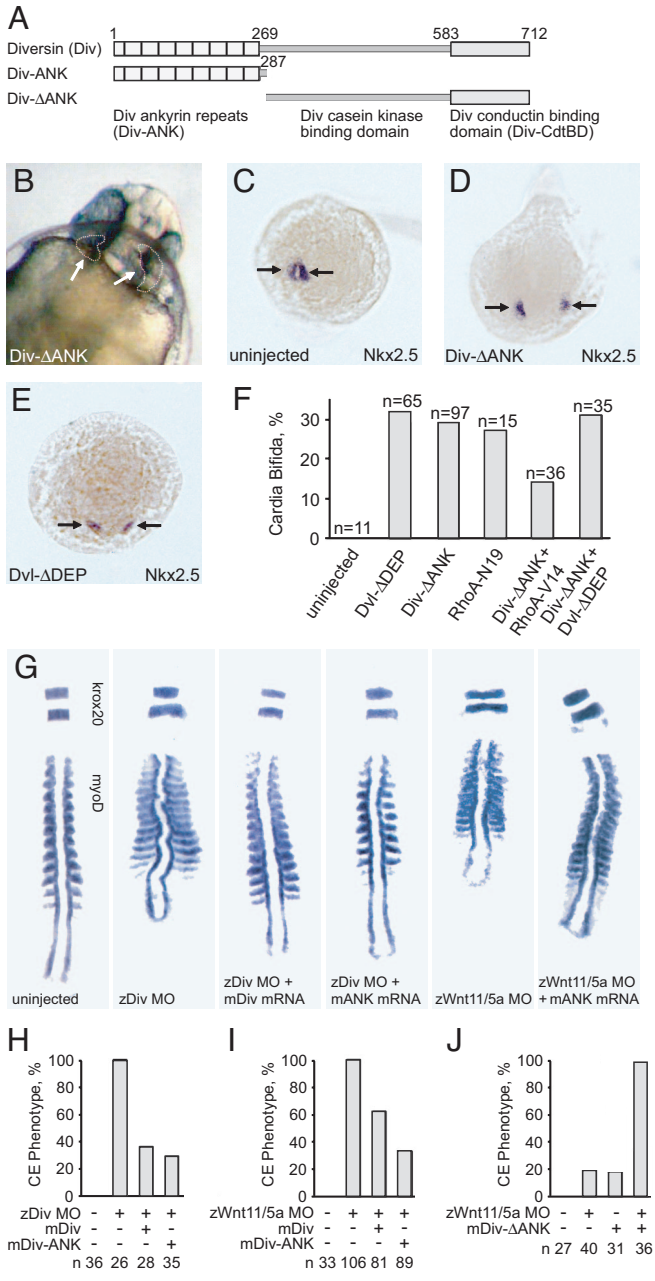

Fig. 1. Diversin is essential for heart formation and gastrulation movements in zebrafish embryogenesis. (*A*) Diversin domain structure and deletion mutants used for mRNA injections. Numbers indicate amino acid positions of mouse Diversin. (*B*) Zebrafish embryo (at 48 h after fertilization) that was injected with 75 pg of Div-ΔANK mRNA at the one- to two-cell stage shows two separate hearts (marked by arrows and dashed lines). Live beating hearts in this embryo are shown in Movie 1. (*C–E*) *In situ* hybridization of *Nkx2.5* of zebrafish embryos at 20 h after fertilization indicates single or double heart primordia in the injected embryos (arrows, dorsal view). (*C*) Heart primordia of uninjected wild-type embryos are paired at the dorsal midline. (*D* and *E*) Embryos injected with 75 pg of Div-ΔANK or Dvl- ΔDEP mRNA show two heart primordia located laterally of the dorsal midline. (*F*) Diversin controls heart formation via the RhoA signaling pathway. Shown is quantification of cardia bifida phenotypes of zebrafish embryos that were injected with the indicated mRNAs. Amounts of injected mRNAs: Div-ΔANK and Dvl-ΔDEP, 75 pg in single injections and 37.5 pg in double injections; RhoA(N19), 30 pg; RhoA(V14), 2 pg. (*G*) The ankyrin repeat domain of Diversin is crucial for regulation of gastrulation movements in zebrafish embryos. *In situ* hybridizations of *krox20* and *myoD* of flat-mounted zebrafish embryos are shown (12- to 15-somite stage, dorsal view). One- to two-cell-stage embryos were injected with antisense MO against zebrafish Diversin (Div MO, 1.5 ng) or against zebrafish Wnt11 and Wnt5a (Wnt11/5a MO, 1 + 2 ng), or were coinjected with MO and 50 pg of the indicated mouse Diversin mRNAs. (*H* and *I*) Quantification of experiments in *G*. Embryos were classified as with CE phenotype (not rescued) when anterior/posterior body axis length, shape of the notochord, and compression of somites were similar to MO-injected embryos (in *G* compare embryos 2 and 5 with embryos 1, 3, 4, and 6). (*J*) Diversin lacking the ankyrin repeat domain, Diversin-ΔANK, synergistically enhances the induction of CE defects induced by Wnt11 and Wnt5a MO. Zebrafish embryos were injected with Wnt11 and Wnt5a MO (0.5 + 1 ng) or Diversin-ΔANK mRNA (50 pg) or coinjected with both (experiments were quantified as described for *G* and *H*). *n*, number of embryos.

It had been previously reported that injection of dominant-negative Dishevelled lacking the DEP domain (Dvl-ΔDEP) into zebrafish embryos also induced cardia bifida phenotypes and CE defects (Fig. 1 *E* and *F*) (6). We could show that cardia bifida induced by dominant-negative Diversin could be rescued by coinjection of an activated form of RhoA (RhoA-V14) (Fig. 1*F*), suggesting that Diversin controls heart formation through RhoA signaling. It had previously been shown that Dishevelled also regulates heart formation via the activation of RhoA (Fig. 1*F*) (6). Coinjection of both dominant-negative molecules, Div-ΔANK and Dvl-ΔDEP, did not synergize; i.e., the frequency of cardia bifida was not increased (Fig. 1*F*). These results suggest that both Diversin and Dishevelled control heart formation and noncanonical Wnt signaling in zebrafish embryogenesis by similar mechanisms.

We then analyzed the importance of the domains of Diversin in CE movements, because we could rescue Diversin MO knockdowns by coinjections of heterologous mRNAs. We have previously shown that injection of antisense MO directed against zebrafish Diversin disturbed CE, which was reflected in broad-shaped somites, undulated notochords, and shortened anterior/posterior axes (Fig. 1*G*) (18). Here we found that the Diversin MO-induced defects in CE were rescued by coinjection of mouse Diversin mRNA (Fig. 1*G*; see quantification in Fig. 1*H*). Remarkably, mRNA encoding a truncated molecule that contains only the ankyrin repeat domain of mouse Diversin also rescued CE (Fig. 1 *G* and *H*). These results show that the ankyrin repeat domain of Diversin is sufficient to control CE in zebrafish embryogenesis and that Diversin lacking the ankyrin repeat domain induces CE defects and cardia bifida.

It has previously been reported that Wnt5a/pipetail and Wnt11/silberblick are the ligands of the noncanonical Wnt signaling pathway that control CE in zebrafish embryogenesis (8–10, 27). We found that mRNAs encoding full-size mouse Diversin or the ankyrin repeats rescued CE phenotypes induced by Wnt11/5a MO (Fig. 1 *G* and *I*), indicating that Diversin acts downstream of the two Wnt ligands that signal via the noncanonical Wnt signaling pathway. Moreover, combinations of low concentrations of Wnt11/5a MO and Div-ΔANK, which alone were virtually ineffective, acted synergistically in inducing strong CE phenotypes (Fig. 1*J*).

We also investigated the epistatic relationship between Diversin and Dishevelled in controlling CE in zebrafish embryogenesis. For this purpose, we attempted to rescue the CE defects caused by the loss of function of one of these proteins by injecting mRNA encoding the other. Diversin mRNA was unable to rescue the defects caused by Dishevelled lacking the DEP domain. Similarly, Dishevelled did not rescue the defects caused by Diversin MO (Table 1). It has previously been shown that the small GTPases RhoA and Rac1 act downstream of Dishevelled in *Xenopus* gastrulation (16). We found that the defects caused by dominant-negative Dishevelled mRNA and those caused by Diversin MO were rescued by coinjection of mRNAs that encode constitutively active variants of RhoA

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Table 1. Epistasis of Diversin and Dishevelled in zebrafish embryogensis

Type of injection	No. of embryos	CE embryos (not rescued), %
Uninjected	208	0
Diversin MO (1.5 ng)	86	100
Diversin MO (1.5 ng) + Dvl-2 (50 pg)*	50	100
Diversin MO (1.5 ng) + Rac1-V12 (2 pg)	60	100
Diversin MO (1.5 ng) + RhoA-V14 (2 pg)	86	98
Diversin MO (1.5 ng) + RhoA-V14 (1 pg) + Rac1-V12 (1 pg)	50	64
Dvl-2ΔDEP (100 pg)	127	99
Dvl-2ΔDEP (100 pg) + Diversin (50 pg)	58	98
Dvl-2ΔDEP (100 pg) + Rac1-V12 (2 pg)	56	93
Dvl-2ΔDEP (100 pg) + RhoA-V14 (2 pg)	60	95
Dvl-2ΔDEP (100 pg) + RhoA-V14 (1 pg) + Rac1-V12 (1 pg)	108	63

Experiments were performed and data were quantified as described for Fig. 1 *G* and *H*.

*The Dvl-2ΔDIX mutant was used in order not to interfere with canonical Wnt/β-catenin signaling (38).

(V14) and Rac1 (V12) (Table 1). Together, these data indicate that both Diversin and Dishevelled act downstream of Wnt11 and Wnt5a and upstream of the small GTPases Rac and Rho. The epistatic relationship between Diversin and Dishevelled could not be resolved, suggesting that the two molecules act as partners on the same level, rather than in a linear pathway.

Diego of *Drosophila* is related to vertebrate Diversin and controls the establishment of PCP during development of the fly eye (5, 17). To assess a potential functional conservation, we compared the functions of Diversin and Diego in the *Drosophila* eye. Strikingly, overexpression of Diego and Diversin interfered with the establishment of normal PCP in a very similar manner (Fig. 2 *B* and *C*). Overexpression in the R3 and R4 photoreceptors in the eye leads to chirality reversals and rotation defects and thus causes typical changes associated with deficits in establishment of PCP. However, the phenotype caused by the loss-of-function allele dgo^{380} is rescued by the expression of *Drosophila* Diego but could not be rescued by the expression of Diversin (Fig. 2 *D–F*). These data indicate that vertebrate Diversin can interact with components of the signaling pathway that establishes PCP in *Drosophila*. However, differences between Diversin and Diego exist, and thus not all functions of Diego can be replaced by Diversin. In line with this finding, the ankyrin repeats of Diego could not rescue the dgo^{380} loss of function mutation (Fig. 4, which is published as supporting information on the PNAS web site). Diego lacking the ankyrin repeat domain did not rescue dgo^{380} but was weakly dominant-negative, as is Diversin-ΔANK in zebrafish embryos (see above). Further functional differences are apparent because Diversin, unlike Diego, participates also in the control of the canonical Wnt signaling pathway (18).

In the second part of our work we wanted to provide a molecular mechanism for the action of Diversin in noncanonical Wnt signaling and to explain why Diversin and Dishevelled act in a similar fashion during gastrulation and organogenesis. The important domain of Diversin involved in noncanonical Wnt signaling is the ankyrin repeat domain (see above). We therefore performed a yeast two-hybrid screen using the Diversin ankyrin repeats as bait to identify essential interaction partners. Screening a human brain cDNA library (HY4004AH; Clontech, Palo Alto, CA), we identified Dishevelled-2 (Dvl-2) as a protein that binds to the ankyrin repeats of Diversin (data not shown). Coimmunoprecipitation in

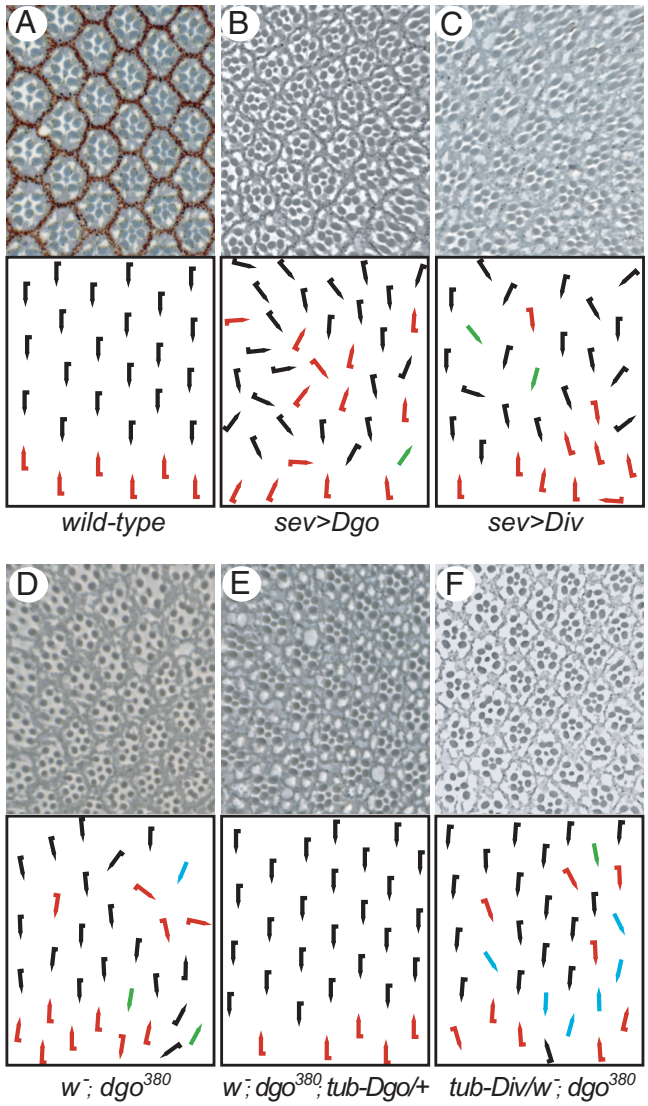

Fig. 2. Diversin affects PCP signaling in *Drosophila*. All panels show tangential sections of adult eyes of indicated genotypes, with corresponding schemes below. Arrows indicate dorsal chiral forms in black, ventral chiral forms in red, and symmetric ommatidia in green (R3/R3 type) and blue (R4/R4 type). Compared with a wild-type eye (*A*), overexpression of Dgo (*sev→Dgo* in *B* and *sev→Diversin* in *C*) at the time of PCP signaling leads to typical PCP phenotypes, including rotation and chirality defects. (*D*) Section of a dgo^{380} mutant eye. In contrast to Dgo (*E*), Diversin (*F*) cannot rescue the dgo-null mutation. Both transgenes were expressed under the control of the ubiquitous tubulin promoter.

HEK293 cells confirmed that Diversin and Dishevelled-2 interact and that the ankyrin repeat domain but not the conductin-binding domain of Diversin is sufficient for binding of Dishevelled-2 (Fig. 3 *A* and *B*). We also found that Dishevelled-1 can be coimmunoprecipitated with Diversin (Fig. 5, which is published as supporting information on the PNAS web site). JNK activation can be used to monitor noncanonical Wnt signaling in cell culture (13, 28). We found that the expression of Diversin and Dishevelled-2 act synergistically to activate JNK in HEK293 cells (Fig. 3*C*). The ankyrin repeat domain of Diversin was essential and sufficient to cooperate with Dishevelled-2 in the activation of JNK (Fig. 3 *A* and *C*). We also used siRNA-mediated silencing of Diversin and Dishevelled to explore their relationship in the JNK reporter assay. We used SW480 cells, which express

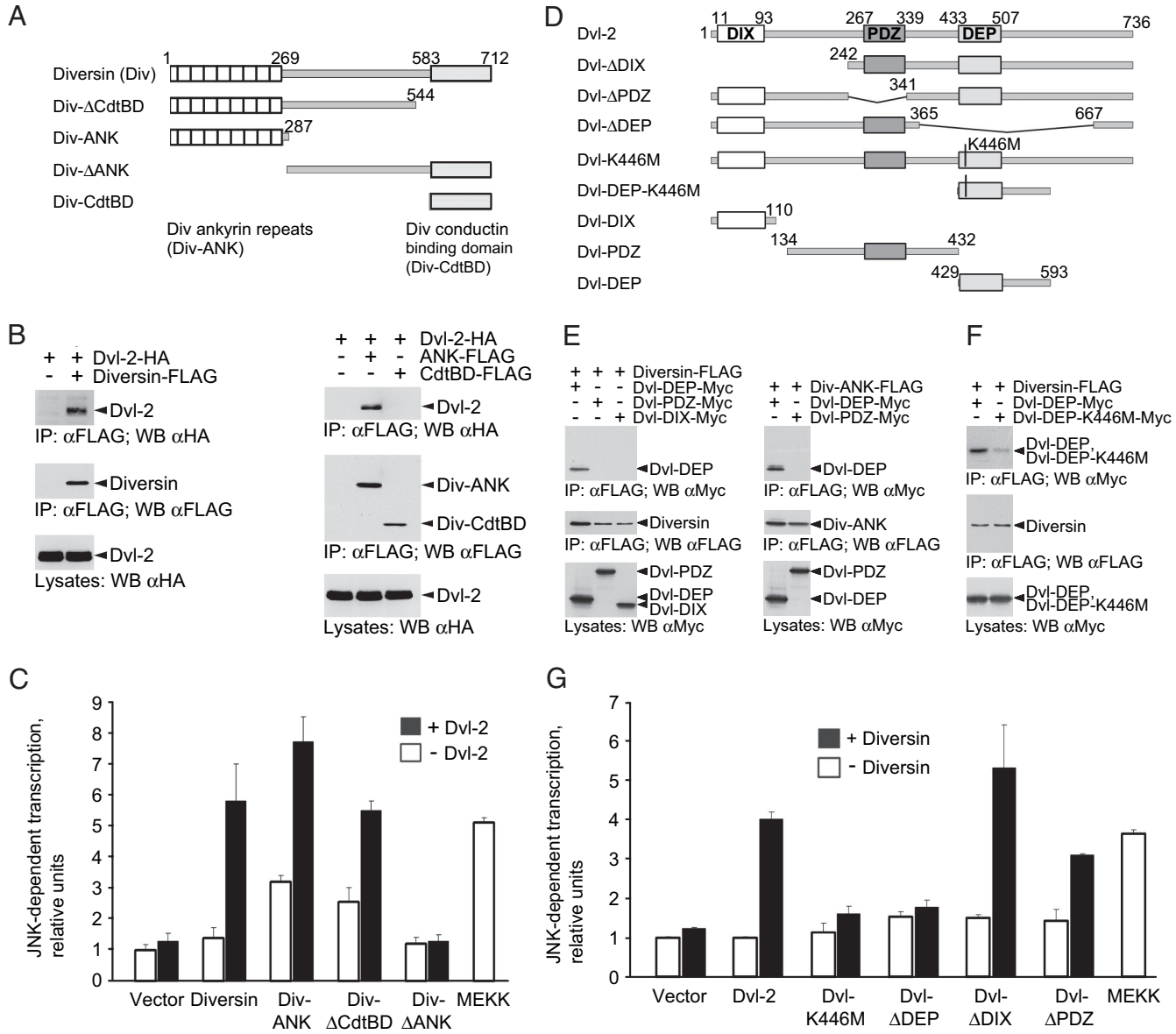

Fig. 3. The ankyrin repeat domain of Diversin binds to the DEP domain of Dishevelled, and both domains are required and sufficient to activate noncanonical Wnt/JNK signaling in a mutually dependent fashion. (*A*) Domain structure of Diversin construct and mutants used. (*B*) Interaction of Diversin with Dishevelled in mammalian cells (HEK293). Shown are Western blots of anti-Dishevelled or anti-Diversin immunoprecipitates with the indicated antibodies. (*C*) The ankyrin repeats of Diversin are functionally important for noncanonical Wnt/JNK signaling. HEK293 cells were cotransfected with the indicated Diversin constructs (0.25 μg) and either Dishevelled (0.25 μg, black bars) or empty vector (white bars). JNK-dependent transcription was measured by luciferase reporter activity. Error bars indicate standard deviations. A MAPK kinase kinase (0.25 μg) construct that activates JNK activity served as control. (*D*) Overview of domain structure of mouse Dishevelled-2 and of deletion constructs and mutants of Dishevelled-2. (*E* and *F*) The DEP domain of Dishevelled binds to Diversin. Western blot analysis of immunoprecipitated Diversin and Dishevelled constructs is shown. Equal expression of transfected cDNAs (3–12 μg) in HEK293 cells was verified by immunoblotting with the indicated antibodies. (*G*) The DEP domain of Dishevelled is essential for Diversin-dependent activation of JNK. Experiments were performed as described in *C*. Indicated cDNAs (0.25 μg) were cotransfected with empty vector (white bars) or Diversin (0.25 μg, black bars).

Diversin and, in particular, Dishevelled-3. Both Diversin siRNA and Dishevelled-3 siRNA inhibited Wnt5a/11-induced JNK-dependent transcription (Fig. 6, which is published as supporting information on the PNAS web site).

Various deletion constructs and point mutations were used to determine the essential domain(s) of Dishevelled-2 that are necessary for Diversin binding and for the functional cooperation with Diversin in noncanonical Wnt signaling and JNK activation (Fig. 3*D*). The DEP domain of Dishevelled was found to be essential to bind Diversin or the Diversin ankyrin repeats (Fig. 3*E*). All variants that contained the DEP domain of Dishevelled-2 coprecipitated with Diversin, whereas the presence or absence of the DIX or PDZ domain did not affect coprecipitation. Moreover, a DEP domain point mutation of Dishevelled-2, K446M, displayed decreased affinity and coprecipitated less efficiently with Diversin (Fig. 3*F*). An analogous mutation in *Drosophila* Dishevelled, dsh[1], was previously found to be defective in PCP signaling in *Drosophila* (13–15). It has also been shown that the DEP domain of Dishevelled is sufficient to activate JNK (29, 30). Furthermore, we observed that Dishevelled-2 variants containing a wild-type DEP domain cooperated with Diversin to activate JNK, whereas the K446M mutant was virtually inactive (Fig. 3 *D* and *G*). In contrast, Dishevelled-2 variants that lack the DIX or

PDZ domains were able to cooperate with Diversin to activate JNK. Taken together, these results demonstrate that the ankyrin repeat domain of Diversin binds the DEP domain of Dishevelled and that these two domains are necessary and sufficient to mediate JNK activation in cultured cells.

We have here shown that both Diversin and Dishevelled control cardiogenesis and gastrulation movements in zebrafish embryogenesis through small GTPases. Both Diversin and Dishevelled act downstream of Wnt11/silberblick and Wnt5a/pipetail in embryogenesis, and they cooperate to activate JNK in cell culture. Knockdown of both Diversin and Dishevelled by siRNA in cell culture inhibited activation of JNK by Wnt5a/11. Moreover, Diversin and Dishevelled can bind directly. The responsible interaction domain in Diversin contains ankyrin repeats, which are often found in protein–protein interaction domains (31). In Dishevelled, the DEP domain is required for binding to Diversin, and mutational analysis of this domain has been shown to be crucial for noncanonical Wnt signaling (13, 16, 30, 32, 33). These data indicate that Diversin and Dishevelled function together in the control of noncanonical Wnt signaling. It has recently been shown that the Diversin orthologue of *Drosophila*, Diego, genetically interacts with and physically binds Dishevelled (21). An involvement of the PDZ domain of *Drosophila* Dishevelled in binding Diego has been demonstrated. We show here that Diversin can interfere with Diego function in *Drosophila* but cannot rescue the Diego loss-of-function phenotype. This finding indicates that not all domains of Diversin and Diego are functionally interchangeable.

In conclusion, we have here provided a molecular mechanism for the action of Diversin. Diversin and Dishevelled are mutually dependent players of noncanonical Wnt signaling, both in cell culture and in embryogenesis. Diversin and Dishevelled directly bind, and we have identified the crucial domains in this interaction. We further demonstrated that the functional interaction of Diversin and Dishevelled is essential in two phases of embryonic development: gastrulation and cardiogenesis.

Materials and Methods

Cell Culture and Biochemical Experiments. Coimmunoprecipitation experiments were performed as previously described (18) with the following alterations: 1.5×10^6 HEK293 cells were transfected with 10–15 μg of the indicated cDNAs. Anti-FLAG beads (Sigma, St. Louis, MO) were used for precipitation of FLAG-tagged proteins according to the manufacturer's instructions. Western blot analysis was performed by using peroxidase-conjugated anti-HA (1:5,000) and anti-FLAG (1:1,000) antibodies (Sigma) and goat-anti-c-Myc (1:2,000) antibody (A14G; Santa Cruz Biotechnology, Santa Cruz, CA). A JNK luciferase assay was performed as previously described (18) with the following alterations: 0.75×10^5 of HEK293 cells per well of a 12-well plate were transfected by using Lipofectamine reagent (Invitrogen, Karlsruhe, Germany). Samples were normalized by measuring β-galactosidase activity. Experiments were carried out in duplicates and repeated at least three times. Dishevelled mutants Dvl-ΔDEP, Dvl-DIX, Dvl-PDZ, and Dvl-DEP have been previously described (34), Dvl-ΔDIX and Dvl-ΔPDZ mutants were generated by restriction digests, and the Dvl-K446M mutant (13–15) was generated by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA) using the following primers: forward, 5′-GCATGTGGCTCATGATCACCATCCCAAACGC-3′; reverse, 5′-GCGTTTGGGATGGTGATCATGAGCCACATGC-3′.

Zebrafish and *Drosophila* Embryogenesis. *In situ* hybridizations, mRNA synthesis, and microinjections of zebrafish embryos were performed as described previously (8, 18, 27). MO sequences used for zebrafish experiments were as described (18, 27). For experiments in *Drosophila*, pCaspTubPA Diversin and pUAST Diversin were cloned by inserting a KpnI/XbaI fragment of pcDNA-Fg-Diversin (18) into pCaspTubPA and pUAST (35), respectively. A DraI fragment of EP Dgo(2619) (17) was inserted into pCAspTubPA to give pCaspTubPA Dgo. UAS Dgo was as described (36). Transgenic flies were generated by standard P-element-mediated transformation. Overexpression studies in *Drosophila* were performed by using a sev-Gal4 driver (37) for the Gal4/UAS system (35) with flies grown at 29°C. w^{1118} (w^-) was used as control. Rescue crosses were grown at 25°C. Three independent transgene insertions were analyzed. For more details see *Supporting Methods*, which is published as supporting information on the PNAS web site.

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