Stony Brook University

Differential mediation of the Wnt canonical pathway by mammalian Dishevelleds-1, -2, and -3

A Dissertation Presented

by

Yi-Nan Lee

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Physiology and Biophysics

Stony Brook University

December 2007

Stony Brook University

The Graduate School

Yi-Nan Lee

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Hsien-yu Wang, Ph.D., Research Associate Professor, Advisor
Department of Physiology and Biophysics

Craig C. Malbon, Ph.D., Professor, Committee Chair
Department of Pharmacological Sciences

Roger A. Johnson, Ph.D., Professor
Department of Physiology and Biophysics

W. Todd Miller, Ph.D., Professor
Department of Physiology and Biophysics

Nicolas Nassar, Ph.D., Research Assistant Professor
Department of Physiology and Biophysics

Ken-Ichi Takemaru, Ph.D., Assistant Professor, Outside Committee Member
Department of Pharmacological Sciences

This Dissertation is accepted by the Graduate School.

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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In *Drosophila*, a single copy of the gene encoding the phosphoprotein Dishevelled (Dsh) is found. In the genomes of higher organism (including mammals), three genes encoding isoforms of Dishevelled (Dvl1, Dvl2, and Dvl3) are present. In the fly, Dsh functions in the Wnt-sensitive stabilization of intracellular β-catenin and activation of the Lef/Tcf-sensitive transcriptional response known as the Wnt "canonical" pathway. In this study we explore the expression of Dvls in mammalian cells and provide an estimate of the relative cellular abundance of each Dvl. In mouse F9 cells, all three Dvls are expressed. Dvl2 constitutes more than 95% of the cellular pool, the sum of Dvl1 and Dvl3 constituting the remainder. Similarly, Dvl2 constitutes more than 80% of the Dvl1-3 pool in mouse P19 and human HEK 293 cells. Suppression of cellular protein expression can be catalyzed by use of small interfering RNA (siRNA) that target specific protein mRNA. siRNA-induced knock-down of individual Dvls was performed and the Wnt3a-sensitive canonical pathway in F9 cells employed as the read-out. Activation of the canonical signaling pathway by Wnt3a was dependent upon the presence of Dvl1, Dvl2, and Dvl3, but each to a variable extent. Wnt3a-sensitive canonical transcription was suppressible, by knock-down of Dvl1, Dvl2, or Dvl3. Conversely, the overexpression of any one of three Dvls individually was found to be capable of promoting Lef/Tcf-sensitive transcriptional activation, in the absence of Wnt3a, *i.e.*, overexpression of Dvl1,

Dvl2, or Dvl3 is Wnt3a-*mimetic*. Graded suppression of individual Dvl isoforms by siRNA was employed to test if three Dvls could be distinguished from one another with regard to mediation of the canonical pathway. Canonical signaling was most sensitive to changes in the abundance of either Dvl3 or Dvl1. Changes in expression of Dvl2, the most abundant one of the three isoforms, resulted in the least effect on canonical signaling. Dvl-based complexes were isolated by pull-downs from whole-cell extracts with isoform-specific antibodies and were found to include all three Dvl isoforms. Rescue experiments were conducted in which depletion of any one of three Dvl isoforms suppresses Wnt3a activation of the canonical pathway and the ability of a Dvl isoform to rescue the response was evaluated. Rescue of Wnt3a-stimulated transcriptional activation in these siRNA-treated cells occurred only by the expression of the very same Dvl isoform depleted by the siRNA. Thus, Dvls appear to function cooperatively as well as uniquely with respect to mediation of Wnt3a-stimulated canonical signaling. Dvl3 plays the most obvious role, whereas the most abundant Dvl (*i.e.*, Dvl2) plays the least obvious role. These observations suggest that individual Dvl isoforms in mammals operate as a network with some features in common and others rather unique.

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List of Abbreviations

APC	Product of the *adenomatous polyposis coli* gene
CK1	casein kinase-1
CK2	casein kinase-2
CRD	cysteine-rich domain
DAG	diacylglyceride
Daam1	Dishevelled-associated activator of morphogenesis 1
Dsh	Drosophila Dishevelled
Dvl	vertebrate Dishevelled
EV	empty vector
Fz	Frizzled
GBP	GSK-3β binding protein
GFP	green fluorescence protein
GPCR	G protein-coupled receptor
GSK-3β	glycogen synthase kinase-3β
GST	glutathione S-transferase
HA	hemagglutinin antigen
IP_3	inositol trisphosphate
IP_5	inositol pentakisphosphate
IP3K	inositol 1,4,5-trisphosphate 3-kinase
IPMK	inositol polyphosphate multikinase
JNK	c-Jun N-terminal kinase
KD	knock-down
Lef	lymphoid enhancer-binding factor
LRP	low density lipoprotein receptor-related protein
MARCKS	myristoylated alanine-rich C-kinase substrate
MED	membrane effector domain
M_r	molecular weight
MuSK	muscle-specific kinase
NF-AT	nuclear factor of activated T cells
PIP_2	phosphatidylinosital 4,5-bisphosphate
PLCβ1	phospholipase Cβ-1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SRFP	solubile Frizzled-related proteins
Tcf	T-cell factor

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Introduction

Wnts are secreted, glycosylated and palmitoylated ligands that bind to members of the Frizzled class of heptahelical G protein-coupled receptors (GPCR) to regulate fundamental aspects of embryonic development, growth, stem cell renewal, and adult tissue homeostasis [1-4]. In development, Wnt signals govern body axis formation, germ layer patterning, neural tube closure, axonal processes protrusion, and retinal angiogenesis [5-7]. Many cancers and human genetic diseases, such as colon cancer, leukemia, and osteoporosis pseudoglioma syndrome are consequences of mis-regulation of Wnt signaling pathways [8].

In mammals, 19 Wnt genes have been found by genome sequencing. The solubility of the Wnt protein is determined by palmitoylation of a conserved cysteine residue (*e.g.,* Cys77 in Wnt3a) [9, 10]. For Wnt-secreting cells, the *Porcupine* gene encoding an O-acyltranferase is required for Wnt palmitoylation [11]. The palmitoylation restricts diffusion of Wnt proteins and enhances their interaction with cell membranes. Wnts thus act as morphogens, which function over long distances yet in a concentration-dependent manner. Wnt secretion also requires *Wntless* and *Evenness interrupted* cargo proteins for proper trafficking in the default secretory pathway [12, 13]. The activation of Wnt signaling is initiated by the binding of Wnt ligand to a G protein-coupled receptor Frizzled (Fz), and coreceptor LRP5/6. An extracellular N-terminal CRD domain present in each Fz constitutes the site of high-affinity binding of Wnt [14]. In addition to Wnt, two polypeptides, Norrin and R-spondin, functioning as agonists have been reported. Norrin can bind to Fz4 and initiate the canonical Wnt pathway. Mutation of Norrin, which loses the ability to bind Fz4 found in Norrin disease, results in abnormal vasculogenesis in the eye and in blindness [18]. R-spondin, binding to Wnt coreceptor LRP5/6, can cooperate with Wnt to promote β-catenin accumulation and Wnt-dependent gene induction [19, 20]. Several Wnt antagonists have been reported. Dickkopf and Wise are secreted proteins which inhibit Wnt signaling via direct binding to LRP5/6 [15, 16]. Soluble Frizzled-Related Proteins (SFRPs) containing ligand-binding CRD domains similar to those of Frizzleds compete with Fz for binding to Wnts, act as Wnt inhibitors. Thus, secreted proteins have been reported that function as either agonists or antagonists of Wnt in Wnt signaling pathways.

Currently, three Wnt signaling pathways have been identified: the canonical pathway (*i.e.,* Wnt/β-catenin/Lef-Tcf-sensitive transcription pathway), the planar cell polarity pathway (*i.e.,* Wnt/small GTPase/JNK pathway), and the non-canonical pathway (*i.e.,* Wnt5a/cGMP/Ca^{2+} pathway) (fig. 1) [21-23]. The activation of canonical Wnt pathway leads to accumulation of cellular β-catenin and the activation of Lef/Tcf-sensitive gene transcription [24, 25]. The planar cell polarity pathway is involved in the organization of cytoskeleton to establish cell polarity [6, 26, 27]. The non-canonical Wnt signaling pathway leads to elevation of intracellular Ca^{2+} levels. The activation of Wnt/Ca^{2+} pathway results in induction of NF-AT dependent transcription [28, 29]. All three pathways operate by heterotrimeric G proteins.

In the absence of Wnts (*e.g.,* Wnt3a), β-catenin is phosphorylated by GSK-3β and CK-1α in an Axin/APC degradation complex. Axin and APC scaffold proteins are predicted to chaperone the optimal conformation for β-catenin to enable its phosphorylation [30]. The function of APC is to efficiently remove phosphorylated β-catenin from the "active" site of the complex. CK1α acts as primer kinase to facilitate the subsequent phosphorylation of β-catenin by GSK-3β [31]. Phosphorylated β-catenin is recognized by β-Trcp, a component of an E3 ubiquitin ligase complex, and is earmarked with ubiquitin for degradation by the proteosome [32]. Thus, cytoplasmic levels of β-catenin are stringently kept low to prevent the uncontrolled stimulation of Wnt canonical signaling.

The canonical pathway is initiated by the engagement of Wnt3a binding to Fz1 and LRP5/6, although the ternary complex has not been identified biochemically. Activation of Wnt canonical pathway results in the accumulation of β-catenin in the cytoplasm. The lack of a nuclear localization signaling sequence in β-catenin suggests that the cytosolic β-catenin directly interacts with nuclear pore components and then translocates into the nucleus to interact with Tcf [33]. In F9 teratocarcinoma embryonal stem cells, β-catenin accumulation and Lef/Tcf-dependent transcription are required for primitive endoderm formation [24, 34]. Pioneering studies uncovered heterotrimeric G proteins as necessary for canonical pathway using a Fz-β2-adrenergic receptor chimera [24, 35]. Recently, other studies also showed the requirement of G proteins in Wnt

signaling [28, 40, 41]. Dishevelled (Dvl) is an element downstream of G protein in Wnt canonical pathway, although the interaction and hierarchy among Fz/LRP, G protein and Dvl are still elusive. By using genetic and biochemical approaches, Dvls are found to be necessary for Wnt pathways. Three murine Dvl genes were cloned and predicted to encode scaffold proteins without known catalytic motifs. For the past 10 years, although more than 20 Dvl-associated proteins have been identified by using native purification, yeast-two-hybrid screen, or GST-Dvl pull down assays, we still do not have a definitive understanding about how Dvl regulates Wnt signaling [22]. Dvls have been hypothesized to recruit GBP/Frat, displacing GSK-3β from Axin/APC degradation complex [36]. However, mice lacking all of three GBP/Frat isoforms are viable with normal phenotype, suggesting GBP/Frat is not essential for Dvl to regulate canonical pathway [37]. It has been proposed that initiation of Wnt signaling may induce Dvl to recruit Axin from Axin/APC/GSK-3β degradation complex [30]. Activation by Wnt eliminates the optimal conformation accommodated for β-catenin phosphorylation in the Axin/APC degradation complex and promotes β-catenin accumulation [38]. However, there is no evidence to prove an interaction between Dvl and Axin/APC/GSK-3β degradation complex. It also is not clear whether or not the interaction of Dvl with the degradation complex is regulated by Wnt signaling. Wnt3a stimulation of canonical pathway recently has shown inositol pentakisphosphate (IP_5) as a second messenger, catalyzing a novel mechanism for control of β-catenin accumulation. The binding of Wnt3a to Fz1 activates PLCβ1/3 via the action of Gαq. PLCβ1/3 hydrolyzes membrane lipid PIP_2 to generate DAG and IP_3. Some IP_3 is converted to IP_5 by the action of IP3K and IPMK. IP_5 exerts differential effects on GSK-3β and CK2, the two canonical signaling target kinases. In pull-down assay, IP_5 inhibits GSK-3β activity. Conversely, IP_5 stimulates CK2 to phosphorylate the Thr393 residue of β-catenin and stabilize it. Thus, IP_5 coordinately regulates GSK-3β and CK2 to promote β-catenin accumulation [39]. Interestingly, IPMK was found to be associated with Dvl2 in our preliminary study. Determination of the interaction between Dvl and Gαq, as well as that of Dvl with associated proteins will provide valuable information for the understanding about how Dvls perform the scaffolding function in Wnt signaling pathways.

Although it is not clear how Dvls operate, Dvls are obligate for the signal transduction for all three Wnt pathways: canonical, planar cell polarity and non-canonical pathways. In *Drosophila*, a single Dishevelled (Dsh) is expressed and proper development requires its presence [26, 42]. Three conserved domains provide the major landmarks of Dvls: a DIshevelled and AXin (DIX) binding domain at the N-terminus; a Post-synaptic density-95, Discs-large and Zonula occludens-1 (PDZ) domain in the mid region of Dvl; and a Dishevelled, Egl-10, Pleckstrin (DEP) domain located about midway between the PDZ domain and the C-terminus of Dvl (fig. 2) [48, 49]. The DIX domain enables the possible dimerization of Dvl with other members of the Dvl family as well as with Axin, which is responsible in part for β-catenin degradation [50]. The PDZ domain provides a docking site for a large number of proteins that include protein kinases (*e.g.,* casein kinase-1 (CK1) [51], -2 (CK2) [52], and p21-activated kinase [53], phosphatases (*e.g.,* serine/threoine- protein phosphatase-2C family members [54], adaptor proteins such as β-arrestin [55], and importantly, Frizzleds with a C-terminal PDZ ligand structure [56]. An N-terminally located binding site for Diego [57] and two C-terminal regions of basic residues bracket the PDZ domain. These regions appear to bind the Par-1 kinase as well as the product of the naked cuticle gene [58, 59]. These regions also may function in targeting Dvls to the inner leaflet of the cell membrane, attracted to the negative charges there. The organization of the N-terminal basic residues in these sequences have structural similarity to the Membrane Effector Domain (MED) of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein [60], providing the possibility of a dynamic, reversible association with the lipid bilayer. Finally, the DEP domain enables protein-protein interactions between Dvl and Daam1[61], linking Dvl to the small molecular weight GTPases like RhoA, as well as between Dvl and protein kinases like the muscle-specific kinase, MuSK [53].

The three isoforms of Dvl observed in mammals display high homology in amino acid sequences, extending beyond that of the conserved DIX, PDZ, and DEP domains. It has been suggested that the 3 murine Dvl homologs may be functionally redundant isoforms, because they share more than 60% sequence identity. However, many studies reveal their different biological functions. In histological studies, Dvls are commonly found to be widely expressed. There are some notable exceptions. Dvl1 is not detectable

in spleen and Dvl2 expression is not found in liver and thymus [47]. In *Drosophila* Dsh-deficient embryos, it was shown that expression of Dvl2, but not Dvl1, can partially rescue the canonical Wnt signaling [45]. The unique functions of Dvls are determined by gene knockout studies. Mice lacking Dvl1 ($Dvl1^{-/-}$) are normal in development, but display abnormality in sensorimotor gating and social behavior [62]. Dvl2 null mice ($Dvl2^{-/-}$) show 50% lethality and the majority that do survive are female. Dvl2 deficiency presents with cardiac morphogenesis defects, including double outlet right ventricle, transposition of the great arteries, and persistent truncus arteriosis. At least for Dvl1 and Dvl2, any redundancy is far from complete and deficiency in either one alone does impact fertility, signaling, and behavior [63].

We have made use of mouse embryonic F9 teratocarcinoma cells with the capability to form the three germ layers: endoderm, mesoderm, and ectoderm as a model system for detailed analysis of Wnt signaling in development. Clones of these totipotent F9 cells expressing Frizzled-1 respond to stimulation by purified Wnt3a and go on to form primitive endoderm [34]. The Lef/Tcf-dependent transcriptional activities are monitored by Wnt reporter gene constructs. We have illuminated key facets of the role of signaling in development using these cells to probe Wnt actions on the canonical, non-canonical, and planar cell polarity pathways [24 , 28, 34, 35]. Based upon the existing knowledge of the mouse F9 cells and their utility in the study of Wnt signaling, we adopted this model for analysis of the roles of individual mammalian Dvl1, Dvl2, and Dvl3 in the Wnt/β-catenin/Lef-Tcf-sensitive gene activation or "canonical" pathway. We demonstrate both common as well as unique roles of individual Dvl isoforms in the mediation of Wnt3a-stimulated canonical signaling.

Experimental Procedures

Cell Culture- The mouse F9 teratocarcinoma (F9) cells and the mouse embryonal P19 teratocarcinoma (P19) cells (from ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Hyclone, South Logan, UT) at 37°C in a 5% CO_2 incubator. Human embryonic kidney HEK 293 cells (from ATCC, Manassas, VA) were grown in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. F9 cells were transiently transfected to express empty vector (EV), or rat Frizzled-1 (Fz1), or rat Frizzled-2 (Fz2), as described elsewhere [64, 65]. For overexpression studies, hemagglutinin (HA)- and green fluorescence protein (GFP)-tagged mouse Dvl isoforms, as well as enhanced GFP (eGFP)-tagged human Dvl isoforms were expressed by transient transfection by using LipofectAMINE Plus reagent as per the instructions of the manufacturer (Invitrogen, Calsbad, CA).

Immunoprecipitation and Immunoblotting- F9 and the other cells grown in monolayers were serum-starved for 8 hr prior to stimulation by either Wnt3a (10 ng/ml) or Wnt5a (50 ng/ml) for 6 hr. Cells were harvested in lysis buffer containing protease and phosphatase inhibitors (20 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 200 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NaF, 1mM Na_3VO_4, and 100 nM okadaic acid). Whole-cell lysates and samples from immunoprecipitation were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to nitrocellulose blots electrophoretically. The blots were blocked with 10% bovine serum albumin and probed with antibody against Dvl1, Dvl2, or Dvl3 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots of samples derived from cells that were transiently transfected to express an HA-tagged Dvl isoform were stained with anti-HA antibodies (Roche, Indianapolis, IN). Immune complexes were detected by enhanced chemiluminescence method, as per the instructions of the manufacturer (Amersham, Piscataway, NJ).

Lef/Tcf-sensitive Reporter Gene Assay- F9 clones transiently transfected with either reporter plasmid Super8XTOPFLASH or mutated reporter plasmid Super8X FOPFLASH (gifts from Dr. Randall Moon, HHMI, University of Washington, Seattle,WA); and cotransfected with 0.05-0.8 μg of expression vector harboring either Dvl1, Dvl2 or Dvl3

(dually tagged with HA- and GFP- at the C-terminus of Dvl, *i.e.*, Dvl-HA-GFP) for 24 hr. Cells were harvested in reporter lysis buffer (Promega, Madison, WI) following exposure to serum-free DMEM for 8 hr and luciferase activity was assayed. Briefly, 20 μl of cell lysates were incubated for 20 sec with 100 μl of reaction mixture containing 0.67 mM luciferin, 0.27 mM Coenzyme A, 0.1 mM EDTA, 1.1 mM $MgCO_3$, 4 mM $MgSO_4$ and 20 mM Tricine (pH 7.8) and the intensity of luminenscence was immediately measured using a luminometer (Lumat LB 9507, Berthold Technologies, Oak Ridge, TN). Samples were assayed in triplicate and the luciferase activity was normalized based to protein content of the sample.

***Knock-down of Dvl Isoforms* by si*RNA* –** At least 3 pairs of sense and antisense oligonucleotides were designed and synthesized by Ambion (Austin, TX) for each of the following molecules: mouse Dishevelled-1; mouse Dishevelled-2; and, mouse Dishevelled-3. Each pair of oligos was annealed in 200 μl of annealing buffer (100 mM potassium acetate, 2 mM magnesium acetate and 30 mM HEPES-KOH, pH 7.4) at 37°C for 1 hr. F9 cells were treated with 100 nM of annealed siRNA by using LipofectAMINE 2000. The efficiency of the knock-down for each Dvl isoform was determined by immunoblotting, staining with an antibody against each individual isoform. The sequences of siRNA oligos, which are the most effective in knock-down of targeted proteins, are listed as follows: CCAGGAUAUUGGCUUGACAtt and UGUCAAGCCAAUAUCCUGGtg target mouse Dishevelled-1, GGAAGAGAUCUCCGAUGACtt and GUCAUCGGAGAUCUCUUCCtt target mouse Dishevelled-2, and GGAAGAGAUCUCGGACGACtt and GUCGUCCGAGAUCUCUUCCtt target mouse Dishevelled-3. For the "rescue" experiments of cells treated with siRNA designed to knock-down a specific Dvl isoform, expression of human Dvl isoforms (resistant to the mouse-based siRNA) with an eGFP-tag fused to the C-terminus of each human Dvl isoform was necessary. Cells were treated with siRNA for 48 hr. Twenty four hours after the initial administration of siRNA, cells were transiently transfected with expression vector harboring eGFP-tagged human Dvl isoform by using LipofectAMINE. Cell lysates were collected and the Lef/Tcf-sensitive luciferase reporter gene activity was determined. The efficiency of the

siRNA-mediated knock down as well as the expression of exogenous Dvl-isoform (Dvl-eGFP) was established by immunoblotting.

Quantification of Stained Proteins- Exposed films were scanned by calibrated Umax 1000 absorbance scanner equipped with SilverFastAi software (LaserSoft Imaging Inc. Longboat Key, FL). The bands were quantified by use of Aida software (Raytest, Germany).

Statistical Analysis- In each experiment, triplicates were performed in the sampling. All of the data displayed are expressed as mean values ± S.E.M. for at least 3 separate experiments. Comparisons of data among groups were performed with one-way analysis of variance followed by the Newman-Keuls test. Statistical significance (p value of ≤ 0.05) is denoted with asterisks or pound symbols.

Results

Mouse Embryonal F9 Teratocarcinoma Cells Express Dvl1, Dvl2, and Dvl3 Isoforms

For these experiments we made use of mouse F9 cells that are transfected to express the rat Frizzle-1 (Fz1) and have been shown to form primitive endoderm in response to stimulation with purified Wnt3a [39]. Using the Super8XTOPFLASH luciferase reporter to assay the Lef/Tcf-sensitive transcriptional response to Wnt stimulation, we demonstrated that purified Wnt3a stimulates gene activation of the canonical pathway in F9 cells expressing Fz1 (fig. 3A). Stimulation of the canonical pathway was observed in response to purified Wnt3a, but not to purified Wnt5a, in these same cells. F9 cells transfected with either empty vector (EV) or expression vector harboring rat Frizzled-2 (Fz2), in contrast, displayed no stimulation of the canonical pathway in response to Wnt3a. For the F9 cells expressing the Frizzled-1 and stimulated with Wnt3a under these conditions, the canonical transcriptional response was stimulated robustly.

Using immunoblotting, we probed the expression of each of the Dvl isoforms in the mouse F9 cells (fig. 3B). Antibodies against synthetic peptide fragments unique to Dvl1, Dvl2, and Dvl3 were employed to stain samples of whole-cell lysates subjected to SDS-PAGE and transfer of the resolved proteins to blots. F9 cells express all three isoforms of Dvl: Dvl1 (M_r = 75 kDa); Dvl2 (M_r = 78 kDa); and Dvl3 (M_r = 78, 80, and 82 kDa; likely differentially phosphorylated forms). We next subjected the F9 cells to treatment with siRNAs designed specifically to target each Dvl isoform individually. siRNAs designed to suppress the expression of a specific Dvl isoform successfully suppressed the targeted Dvl, while not suppressing the expression of the other non-targeted Dvls (Table1). These initial studies demonstrate the specificity of both the siRNA reagents as well as of the isoform-specific anti-Dvl antibodies employed throughout these studies. Under these conditions, the extent of the siRNA-induced suppression ranged from about 70-85% (Table 1).

Knock-down of Dvl1, Dvl2, or Dvl3 Suppresses Wnt3a-stimulated Canonical Signaling

The first and most straightforward experimental approach was to knock-down the expression of each Dvl individually and then to measure the impact of the loss of the Dvl

on the ability of purified Wnt3a to activate Lef/Tcf-sensitive gene expression (fig. 4A). Our initial experiments suggested that knock-down of either Dvl1, Dvl2, or Dvl3 is capable of attenuating the ability of Wnt3a to stimulate the canonical pathway. The reductions of Wnt3a-stimulated Lef/Tcf-sensitive gene expression for siRNA-treated F9 cells with Dvl isoform-specific knock-downs (KD) were as follows: 75% for KD-Dvl1; 60% for Dvl2; and 85% for Dvl3. Since the knock-down under these conditions provided only a single determination, we examined the effects of a dose-response to graded amounts of siRNAs on the functional read-out. We plotted the extent of the knock-down from immunoblotting data (see fig. 3B) versus the Lef/Tcf-sensitive transcriptional activation in response to Wnt3a, setting the maximal response as "100%" (fig. 4B). The results of the studies were quite revealing, as they identified differences in the character by which the loss of a specific Dvl impacted the Lef/Tcf-sensitive response to purified Wnt3a. On one extreme were the data derived from use of siRNA targeting Dvl3, in which 50% of the canonical response was lost when the amount of Dvl3 expression was suppressed by less than 20%. For Dvl2, in sharp contrast, 50% of the canonical response to Wnt3a was lost only when more than 50% of intracellular complement of Dvl2 was lost. The relationship between knock-down and canonical response to Wnt3a for F9 cells treated with increasing amounts of siRNA targeting Dvl1 was very similar to that of the same studies performed with siRNA targeting Dvl3, but not that of Dvl2. Although optimized empirically for the conditions for siRNA-induced knock-down of each Dvl isoforms (*e.g.,* sequence and concentration of siRNA employed, length of exposure to siRNA, *etc*.), we were unable to suppress the cellular content of each targeted Dvl by more than 70-85%. We speculate that some level of each individual Dvl isoform or the sum total of all three Dvls may be required for cell viability.

Estimation of the Relative Expression of Dvl1, Dvl2, and Dvl3

Although the antibodies employed to identify each Dvl isoform were specific (fig. 3), the immunoblotting approach with three different antibodies was unable to provide information on the relative level of each Dvl isoform in the total pool of Dishevelleds in F9 cells. Our attempts to produce an antibody to any one of several short regions common to all of the three Dvls (while avoiding well-known protein motifs and domains,

such as DIX, PDZ, and DEP that would not be unique to only the Dishevelleds) were unsuccessful. To obviate these obstacles, we adopted an alternative strategy that used these anti-isoform-specific antibodies in tandem with antibodies that recognize the haemagglutin antigen (HA) and green fluorescent protein (GFP)-tagged versions of each Dvl expressed exogenously (fig. 5A). F9 cells were transfected with increasing amounts of plasmid DNA of an expression vector harboring a C-terminally HA- and GFP-tagged fusion protein of each Dvl isoform. Immunoblotting of samples derived from whole-cell lysates from F9 cells were stained under the same conditions, with both an antibody specific to the individual Dvl isoform and that to the HA-tag. All three exogenously-expressed HA-tagged Dvl isoforms are recognized by the anti-HA antibody and we assume that the anti-HA antibody recognizes all three isoforms for tagged Dvls with similar affinity. Thus, we could relate the HA-tag derived expression to a comparison with the immunoblotting data for blots stained with the antibodies against individual Dvl isoforms (fig. 5A). Using this approach we were able to estimate the relative amounts of the individual Dvl isoforms expressed endogenously in F9 cells, setting the total pool (*i.e.*, Dvl1 + Dvl2 + Dvl3) to "100%".

The relative levels of expression of each Dvl isoform expressed endogenously were as follows: Dvl1, 0.7 ± 0.3%; Dvl2, 97.8 ± 0.3 %; and Dvl3, 1.5 ± 0.4% (fig. 5B). Thus, the expression of Dvl2 accounts for more than 95% of the Dvl pool, whereas at the other extreme the expression of Dvl1 contributes the least, less than 1%. We sought to probe the extent to which the rank order of Dvl isoform abundance in mouse F9 cells would approximate the same determination in other cells. The same analysis was performed using mouse P19 teratocarcinoma (P19) and human embryonic kidney (HEK) 293 cells. The rank order of endogenous expression of Dvl isoforms in P19 and HEK293 cells was found to be similar to that established in the F9 cells (fig. 5B). The relative abundance of Dvls was calculated as follows: for P19 cells, Dvl1, 3.1 ± 1.1%; Dvl2, 90.2 ± 5.1%; and Dvl3, 6.7 ± 2.1%; for HEK293 cells, Dvl1, 1.5 ± 0.5%; Dvl2, 81.2 ± 0.4%; and Dvl3, 17.3 ± 2.3%. Thus, Dvl2 is the most abundant Dvl isoform expressed in each of these mammalian cell lines, with Dvl1 and Dvl3 making up a relatively small component of the total pool. Each Dvl, however, contributes individually to the overall Wnt3a-stimulated canonical signaling (fig. 4).

Expression of Individual Dvls Activate Lef/Tcf-sensitive Transcription

By siRNA-mediated knock-down we demonstrated that Dvl1, Dvl2, and Dvl3 each contribute individually to mediating Wnt3a-stimulated activation of Lef/Tcf-sensitive transcription. We sought to analyze Dvl biology further using the converse approach, *i.e*., "over"expression of Dvl1, Dvl2, and Dvl3 individually in F9 cells. Cells were transiently transfected with increasing amounts of plasmid DNA harboring the HA-tagged fusions of Dvl1, Dvl2, or Dvl3 and then the Lef/Tcf-sensitive luciferase reporter activity measured. Overexpression of either HA-tagged Dvl1, Dvl2, or Dvl3 was observed to activate Lef/Tcf-sensitive transcription, in the absence of stimulation by Wnt3a (fig. 6B). Relative small changes in the expression of Dvl3 abundance was observed to yield a more profound enhancement of the Lef/Tcf-sensitive transcription than did substantial greater increases in either Dvl1 or Dvl2. It should be noted that under the conditions employed in which the same cells were transiently transfected with equivalent amounts of input DNA of the same expression vector, the amount of Dvl3 expressed was substantially lower than that yielded by expression vectors harboring either Dvl1 or Dvl2. We speculate that whatever the factors operating to maintain the relative levels of expression of endogenous Dvl isoforms in F9 cells (rank order Dvl2>>Dvl3>Dvl1) also are operating to regulate the amount of tagged isoform exogenously expressed under identical conditions of transfection. Whatever this/these factor(s) is/are, the data demonstrate that when expressed in the normal "background" of Dvl1, Dvl2, and Dvl3, expression of any individual isoform of Dvl promotes Lef/Tcf-dependent transcription in the absence of Wnt3a stimulation. The exogenous expression of Dvl3 yields the greatest impact on Lef/Tcf-sensitive transcriptional activity (fig. 6B).

Heterogeneity of Isoforms in Dvl-based Complexes

We sought to establish if the cellular Dvl-based complexes were heterogeneous with respect to their composition and Dvl isoforms. The approach, to perform "pull-downs" of a specific Dvl isoform followed by analysis of the presence of other isoforms in the complex, required very high quality antibodies with essentially no cross reactivity for other isoforms. To interrogate the existence of cross-reactivity among the Dvl isoform-specific antibodies, we prepared cells that overexpressed one of the three Dvl

isoforms, prepared whole-cell extracts, and performed immunoblotting of each overexpressing cell line with each of the Dvl isoform-specific antibodies (fig. 7A). No significant cross-reactivity was observed for the antibodies employed in these studies, even when overexpressing cells were used as a source of the blots. We first performed pull-downs (*i.e.*, immunoprecipitations) from F9 cells using antibody against Dvl2 (fig. 7B). Pull-downs performed with anti-Dvl2 antibodies appeared quantitative, as no immunoreactive material was detected in the supernatant from post-immunoprecipitation (post-IP, fig. 7B). Analysis of Dvl2-based complexes isolated in this manner Dvl2 (as expected) as well as Dvl3 (fig. 7B). Dvl3 immunoreactivity is detected largely in the supernatant fraction (post-IP) in Dvl2-based pull-downs, indicating that only a small portion of Dvl3 has been sequestered in the Dvl2-based complex (fig. 7B). Dvl1 was not detected under the conditions.

Rescue of Dvl Knock-down in F9 cells by Overexpression of Human Dvl Isoforms

We wondered if the attenuation of the Wnt3a-stimulated canonical pathway by depletion of either Dvl3 or Dvl1 could be "rescued" by expression of other Dvl isoforms? The strategy for a rescue experiment required some modifications. Since the siRNA reagents were prepared specifically to knock-down mouse Dvl1, Dvl2, or Dvl3, we could not perform the "rescue" with the mouse Dvls, but rather with the expression of the human counterparts. In addition, since overexpression of Dvl isoforms (fig. 6) can lead to activation of the canonical pathway in the absence of Wnt3a, we were restricted to employ the highest amount of exogenous expression that did not significantly increase the basal activity of the reporter gene. We achieved both parameters and were able to test the hypothesis that the expression of one Dvl isoform might rescue the function of another isoform, once depleted. As shown above, treating the Fz1-expressing F9 cells with siRNA targeting Dvl1 effectively suppressed the expression of Dvl1 as well as the ability of Wnt3a to stimulate the Lef/Tcf-sensitive transcriptional response (fig. 8A). Exogenous expression of eGFP-tagged human Dvl1 restored the Wnt3a-stimulated canonical response to approximately 75% of control levels. In sharp contrast, the expression of either human Dvl2 or Dvl3 was largely unable to rescue the Wnt3a-stimulated canonical response in the Dvl1-depleted cells. Similar experiments were

performed in Fz1-expressing F9 cells that were depleted of either Dvl2 or Dvl3 individually. Treatment of siRNA targeting either Dvl2 or Dvl3 effectively suppressed the expression of corresponding Dvl as well as the Wnt3a-induced Lef/Tcf-sensitive transcriptional response (fig. 8B, C). Exogenous expression of either human Dvl2 in the Dvl2-depleted cells or human Dvl3 in the Dvl3-depleted cells fully restores the ability of Wnt3a to stimulate the activation of the canonical pathway. In contrast to the results described above, the expression of different isoform of Dvl from that which was depleted (*i.e.*, the expression of either Dvl1 or Dvl3 in the Dvl2-depleted cells or the expression of Dvl1 or Dvl2 in the Dvl3-depleted cells), was unable to rescue the Wnt3a-stimulated response (fig. 8B, C)

Discussion

The phosphoprotein Dishevelled is a multifunctional protein harboring docking sites for more than 20 suspected binding partners [22, 23]. Prominent domains found in Dishevelled, such as DIX, PDZ, and DEP, are highly conserved from the single fly Dsh to the mammalian Dvl1, Dvl2, and Dvl3. The primary question addressed in this study is why are there three Dvls expressed in mammals and what specific role(s), if any, do the Dvl isoforms play in the well-known Wnt-stimulated stabilization of intracellular β-catenin that is responsible for activation of Lef/Tcf-sensitive transcription of genes key in development?

The current work demonstrates that several different cell lines, including mouse (F9 and P19 embryonal teratocarcinoma) and human (HEK 293), all express the three isoforms of Dvl. The next question is what are the relative levels of expression of Dvl1, Dvl2, and Dvl3? Do differences exist among the three Dvls with respect to cellular content? Through the use of both Dvl isoform-specific antibodies and antibodies targeting the protein HA-tag fused onto the C-terminus of exogenously expressed Dvl isoforms, it was possible to make a first-approximation determination of the relative levels of expression of Dvl1, Dvl2, and Dvl3. The results are clear and the expression of Dvl2 dominates the pool of Dvl isoforms expressed in these mammalian cells. The expression of Dvl2 accounts more than 95% of the Dvl pool (*i.e.,* Dvl1 + Dvl2 + Dvl3) in F9 cells. Dvl3 and Dvl1 each account for less than 2% of the Dvl pool. Similar studies performed on the mouse P19 and human HEK 293 cells suggest that same rank order of cellular abundance: Dvl2 >> Dvl3 $\geq$ Dvl1.

Little is know about the regulation of Dvl expression at levels of translation and protein stability. It is of interest that when exogenously expressed, the relative levels of Dvl isoforms mirror those among three native Dvl isoforms. Structural differences must be presenct in native Dvls to account for such disparity between the expression of Dvl2 (very high) and that of either Dvl1 or Dvl3 (very low). Considering that we employed the same expression vector to express each Dvl isoform, we speculate that the rate of protein synthesis of exogenously-expressed Dvls in F9 cells is very similar. In the future, it will be of interest to test how protein stability of individual isoform may contribute to the difference in expression among Dvl isoforms.

Considering the differences in relative abundance as well as the high-homology among the three Dvls, the ability of the siRNA-induced depletion of any one of the Dvl isoforms to impact the ability of Wnt3a to stimulate the canonical pathway was unexpected. Although studies making use of gene interruption to knock-out the expression of Dvl1, Dvl2, or both in mice suggest some level of redundancy, the mouse knock-outs do display unique differences in aspects of signaling that seem extended in the current study [62, 63, 66]. If all three mammalian Dvls were truly redundant, it seems unlikely that the knock-down of the expression of Dvl1 or Dvl3 (which together constitute ~5% of the Dvl pool in F9 cells) would have an impact on ability of Wnt3a to stimulate Lef/Tcf-sensitive transcriptional activation. Suppression of either Dvl1 or Dvl3 individually attenuated signaling in the Wnt-sensitive canonical pathway. Likewise, exogenous expression of individual Dvl isoforms increases the amount of Lef/Tcf-sensitive luciferase reporter gene [67]. Expression of either Dvl1 or Dvl3 individually yields a greater increase in the transcriptional response than that due to expression of Dvl2. The region in proximity to the C-terminal tail (extending beyond the DEP domain) of Dvl is the region with the least homology among the three Dvl isoforms (fig. 2). We speculate that this region may provide the structural basis for unique function(s) of each isoform of Dvl.

Combining the data obtained from study of Wnt3a-stimulated activation of the Lef/Tcf-sensitive transcriptional response with the relative levels of expression of either exogenously expressed (HA-tagged) or that obtained from the sum of the endogenous and exogenously expressed Dvl isoforms together, we can speculate about the relative roles of each Dvl isoform and their impact on signaling of the Wnt canonical pathway. Changes in the cellular levels of Dvl1 and Dvl3 appear to impact the function of the Wnt-sensitive canonical pathway far more than changes in Dvl2. The data obtained from the pull-downs of Dvl2-based complexes demonstrate that the complexes are not homogenous, but rather heterogeneous, with respect to composition of Dvl isoforms. The amount of Dvl3 in the Dvl2-based complexes is about 5% of the Dvl3 pool, suggesting that the majority of Dvl3 is not in complex with Dvl2. This observation is of interest as the amount of Dvl2 is nearly 20 times greater than that of Dvl3. Dvl1 was undetectable in these complexes, we suspect, due to its low abundance (only 0.7% of the Dvl pool) in F9

cells. Indeed, Dvl1 was detected by liquid chromatography in tandem with mass spectrometry (LC MS/MS) in a large-scale preparation of Dvl2-based complexes from F9 cells (data not shown). The complexation of Dvls likely involves the DIX domain, a motif that is necessary for Dvl function and canonical Wnt signaling. Recently, a Dvl2 mutant harboring Y27D substitution in the DIX domain was found to be unable to self-associate and to support Wnt-stimulated Lef/Tcf dependent gene induction [69]. This result suggests that the complexation of Dvls may be essential for Wnt signaling. If a DIX domain is obligate for Dvl complexation, a Dvl2 mutant in which the DIX domain is absent would be incapable of demonstrating normal complexation. In Dvl2-depleted F9 cells, the expression of a DIX-deficient Dvl2 mutant would be expected to be unable to restore the Wnt-induced transcription. An experiment to test this hypothesis would be worthwhile.

Attempts to rescue the Wnt3a-stimulated canonical response in Dvl1-depleted cells were successful only when performed by expression of wild-type Dvl1, and not by expression of other isoforms. Similar experiments performed in cells depleted of Dvl2 or of Dvl3 yield the same results, *i.e.*, only exogenous expression of the same Dvl isoform depleted (and no others) is able to rescue the Wnt3a-stimulated Lef/Tcf-sensitive transcriptional response. These results indicate that only those Dvl-based complexes including all three Dvl isoforms are able to transduce Wnt canonical signaling. The hypothesis may explain our results from knock-down experiment. Knock down of the least abundant Dvls (Dvl1 and Dvl3) yields the most profound effects on Wnt-stimulated canonical pathway. A knock-down of less than 20% of either Dvl1 or Dvl3 contributes to more than a 50% loss of Wnt-induced gene induction. Knock down of the most abundant Dvl2, in contrast, displayed the least effect, *i.e.*, a greater than 60% of reduction in Dvl2 impacted on Wnt-stimulated canonical response far less.

The experiments in knock-out mice, the current work in mammalian cells in culture, and recent work on the possible dynamic oligomerization of Dvl-based complexes [68, 69] suggest that each mammalian Dvl plays a critical role in the Wnt-sensitive canonical pathway, though not one that is independent of the other Dvls (fig. 9). What remains to be determined is whether the spatial and temporal localization of Dvl1,

Dvl2, and Dvl3 may be uniquely regulated by stimulation of the canonical pathway by Wnt3a.

Table 1. siRNA-induced knock down/depletion of Dvl1, Dvl2, or Dvl3 in mammalian cells. The cellular abundance of each Dvl isoform in mouse F9 cells was measured by immunoblotting. Blots were stained with Dvl isoform-specific antibodies. The abundance of the Dvl isoform in untreated cells was quantified and is set to a value of "1.0". The results are presented as mean values ± S.E.M. from three or more separate experiments. *, $p < 0.01$ for the difference from the values observed for untreated and control siRNA-treated cells.

Protein \ siRNA	Control	Dvl1	Dvl2	Dvl3
Dvl1	1.00 ± 0.09	0.21 ± 0.04*	1.09 ± 0.02	1.11 ± 0.09
Dvl2	1.00 ± 0.02	0.94 ± 0.03	0.14 ± 0.04*	0.98± 0.02
Dvl3	1.00 ± 0.03	1.11 ± 0.08	1.07 ± 0.06	0.28 ± 0.03*

Figures

Figure 1. A schematic presentation of three Wnt signaling pathways. *A*, Canonical pathway. *B*, Planar cell polarity pathway. *C*, Non-canonical pathway.

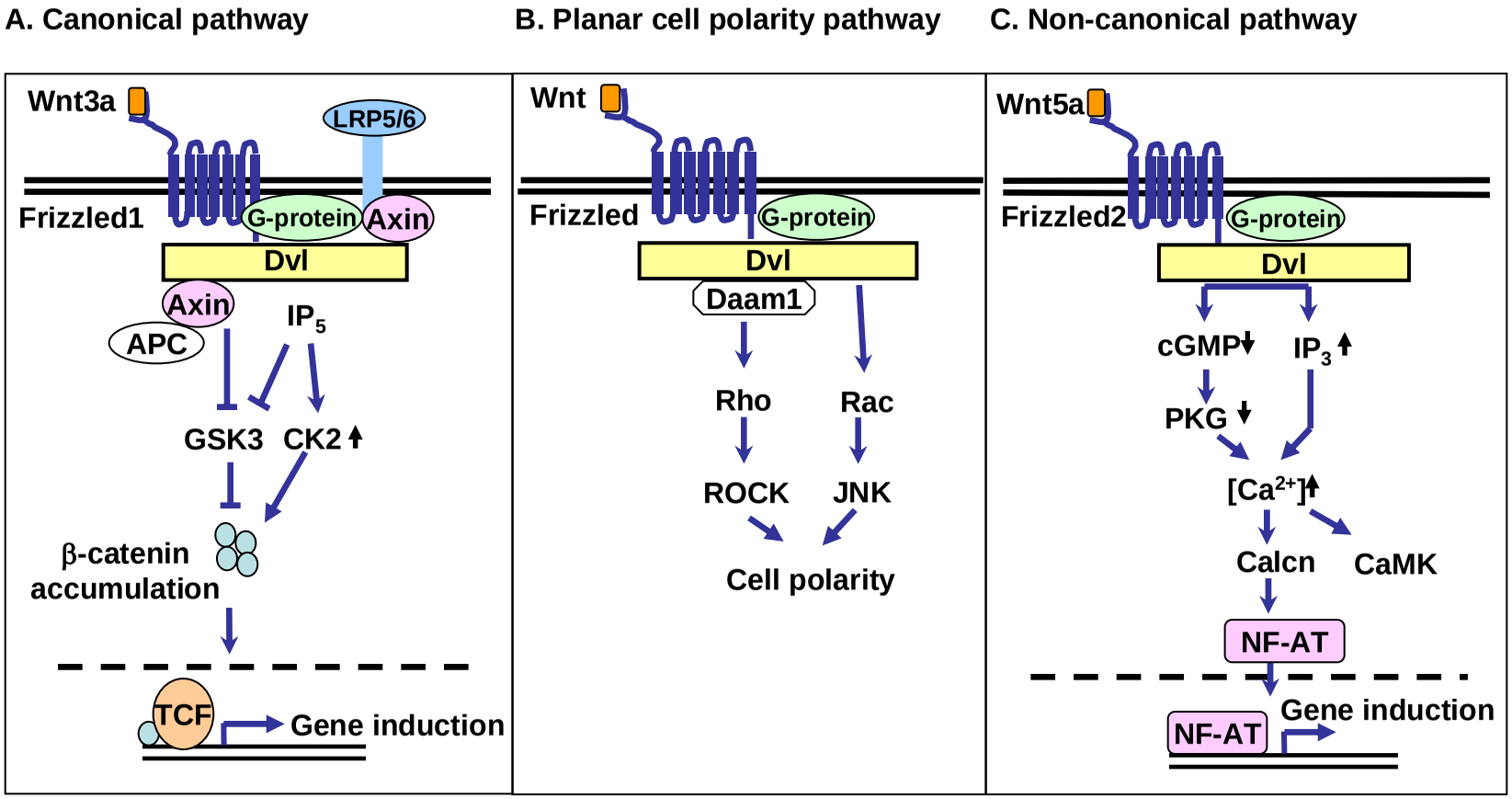

A. Canonical pathway
B. Planar cell polarity pathway
C. Non-canonical pathway
Wnt3a
LRP5/6
Frizzled1
G-protein
Axin
Dvl
Axin
APC
IP5
GSK3
CK2
β-catenin
accumulation
TCF
Gene induction
Wnt
Frizzled
G-protein
Dvl
Daam1
Rho
Rac
ROCK
JNK
Cell polarity
Wnt5a
Frizzled2
G-protein
Dvl
cGMP
IP3
PKG
[Ca2+]
Calcn
CaMK
NF-AT
NF-AT
Gene induction

Figure 2. Sequence alignment of mouse Dvl1, Dvl2 and Dvl3. Three conserved domains (DIX, PDZ and DEP) and the variable region are indicated.

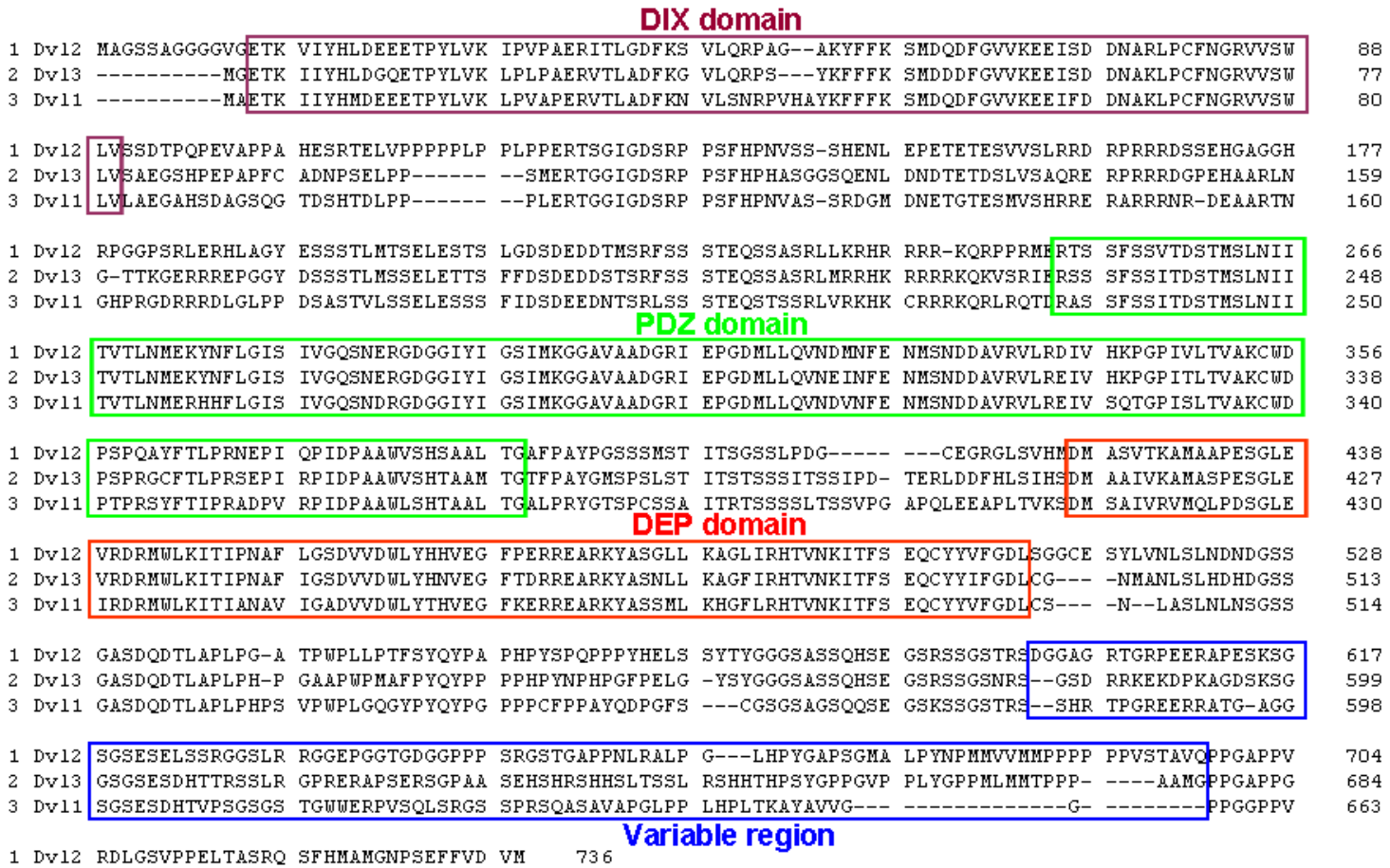

DIX domain
PDZ domain
DEP domain
Variable region
1 Dvl2 MAGSSAGGGGVGETK VIYHLDEEETPYLVK IPVPAERITLGDFKS VLQRPAG--AKYFFK SMDQDFGVVKEEISD DNARLPCFNGRVVSW 88
2 Dvl3 ----------MGETK IIYHLDGQETPYLVK LPLPAERVTLADFKG VLQRPS---YKFFFK SMDDDFGVVKEEISD DNAKLPCFNGRVVSW 77
3 Dvl1 ----------MAETK IIYHMDEEETPYLVK LPVAPERVTLADFKN VLSNRPVHAYKFFFK SMDQDFGVVKEEIFD DNAKLPCFNGRVVSW 80
1 Dvl2 LVSSDTPQPEVAPPA HESRTELVPPPPPLP PLPPERTSGIGDSRP PSFHPNVSS-SHENL EPETETESVVSLRRD RPRRRDSSEHGAGGH 177
2 Dvl3 LVSAEGSHPEPAPFC ADNPSELPP------ --SMERTGGIGDSRP PSFHPHASGGSQENL DNDTETDSLVSAQRE RPRRRDGPEHAARLN 159
3 Dvl1 LVLAEGAHSDAGSQG TDSHTDLPP------ --PLERTGGIGDSRP PSFHPNVAS-SRDGM DNETGTESMVSHRRE RARRRNR-DEAARTN 160
1 Dvl2 RPGGPSRLERHLAGY ESSSTLMTSELESTS LGDSDEDDTMSRFSS STEQSSASRLLKRHR RRR-KQRPPRMERTS SFSSVTDSTMSLNII 266
2 Dvl3 G-TTKGERRREPGGY DSSSTLMSSELETTS FFDSDEDDSTSRFSS STEQSSASRLMRRHK RRRRKQKVSRIERSS SFSSITDSTMSLNII 248
3 Dvl1 GHPRGDRRRDLGLPP DSASTVLSSELESSS FIDSDEEDNTSRLSS STEQSTSSRLVRKHK CRRRKQRLRQTDRAS SFSSITDSTMSLNII 250
1 Dvl2 TVTLNMEKYNFLGIS IVGQSNERGDGGIYI GSIMKGGAVAADGRI EPGDMLLQVNDMNFE NMSNDDAVRVLRDIV HKPGPIVLTVAKCWD 356
2 Dvl3 TVTLNMEKYNFLGIS IVGQSNERGDGGIYI GSIMKGGAVAADGRI EPGDMLLQVNEINFE NMSNDDAVRVLREIV HKPGPITLTVAKCWD 338
3 Dvl1 TVTLNMERHHFLGIS IVGQSNDRGDGGIYI GSIMKGGAVAADGRI EPGDMLLQVNDVNFE NMSNDDAVRVLREIV SQTGPISLTVAKCWD 340
1 Dvl2 PSPQAYFTLPRNEPI QPIDPAAWVSHSAAL TGAFPAYPGSSSMST ITSGSSLPDG----- ---CEGRGLSVHMDM ASVTKAMAAPESGLE 438
2 Dvl3 PSPRGCFTLPRSEPI RPIDPAAWVSHTAAM TGTFPAYGMSPSLST ITSTSSSITSSIPD- TERLDDFHLSIHSDM AAIVKAMASPESGLE 427
3 Dvl1 PTPRSYFTIPRADPV RPIDPAAWLSHTAAL TGALPRYGTSPCSSA ITRTSSSSLTSSVPG APQLEEAPLTVKSDM SAIVRVMQLPDSGLE 430
1 Dvl2 VRDRMWLKITIPNAF LGSDVVDWLYHHVEG FPERREARKYASGLL KAGLIRHTVNKITFS EQCYYVFGDLSGGCE SYLVNLSLNDNDGSS 528
2 Dvl3 VRDRMWLKITIPNAF IGSDVVDWLYHNVEG FTDRREARKYASNLL KAGFIRHTVNKITFS EQCYYIFGDLCG--- -NMANLSLHDHDGSS 513
3 Dvl1 IRDRMWLKITIANAV IGADVVDWLYTHVEG FKERREARKYASSML KHGFLRHTVNKITFS EQCYYVFGDLCS--- -N--LASLNLNSGSS 514
1 Dvl2 GASDQDTLAPLPG-A TPWPLLPTFSYQYPA PHPYSPQPPPYHELS SYTYGGGSASSQHSE GSRSSGSTRSDGGAG RTGRPEERAPESKSG 617
2 Dvl3 GASDQDTLAPLPH-P GAAPWPMAFPYQYPP PPHPYNPHPGFPELG -YSYGGGSASSQHSE GSRSSGSNRS--GSD RRKEKDPKAGDSKSG 599
3 Dvl1 GASDQDTLAPLPHPS VPWPLGQGYPYQYPG PPPCFPPAYQDPGFS ---CGSGSAGSQQSE GSKSSGSTRS--SHR TPGREERRATG-AGG 598
1 Dvl2 SGSESELSSRGGSLR RGGEPGGTGDGGPPP SRGSTGAPPNLRALP G---LHPYGAPSGMA LPYNPMMVVMMPPPP PPVSTAVQPPGAPPV 704
2 Dvl3 GSGSESDHTTRSSLR GPRERAPSERSGPAA SEHSHRSHHSLTSSL RSHHTHPSYGPPGVP PLYGPPMLMMTPPP- ----AAMGPPGAPPG 684
3 Dvl1 SGSESDHTVPSGSGS TGWWERPVSQLSRGS SPRSQASAVAPGLPP LHPLTKAYAVVG--- ----------G- --------PPGGPPV 663
1 Dvl2 RDLGSVPPELTASRQ SFHMAMGNPSEFFVD VM 736
2 Dvl3 RDLASVPPELTASRQ SFRMAMGNPSEFFVD VM 716
3 Dvl1 RELAAVPPELTGSRQ SFQKAMGNPCEFFVD IM 695

Figure 3. Fz1-expressing mouse totipotent F9 teratocarcinoma cells display canonical activation by Wnt3a and express Dv1, Dvl2, and Dvl3. *A*, mouse F9 cells transfected to express Fz1, Fz2 or empty vector (EV) were treated for 6 hr without or with purified Wnt3a (10 ng/ml) or Wnt5a (50 ng/ml). The activation of the Wnt-sensitive canonical pathway was monitored by the use of the Lef/Tcf-sensitive luciferase gene reporter. Data presented are mean values ± S.E.M. from three or more independent experiments. **, $p \leq 0.001$ for the difference from control. *B*, Whole cell lysates from prepared F9 cells either untreated (Control) or treated with siRNA individually targeting mDvl1, or mDvl2, or mDvl3. Samples of the lysates were subjected to SDS-PAGE and transfer of the resolved proteins to nitrocellulose blots. The blots were stained with primary antibodies against Dvl1, or Dvl2, or Dvl3. A blot, representative of three or more separate experiments is displayed.

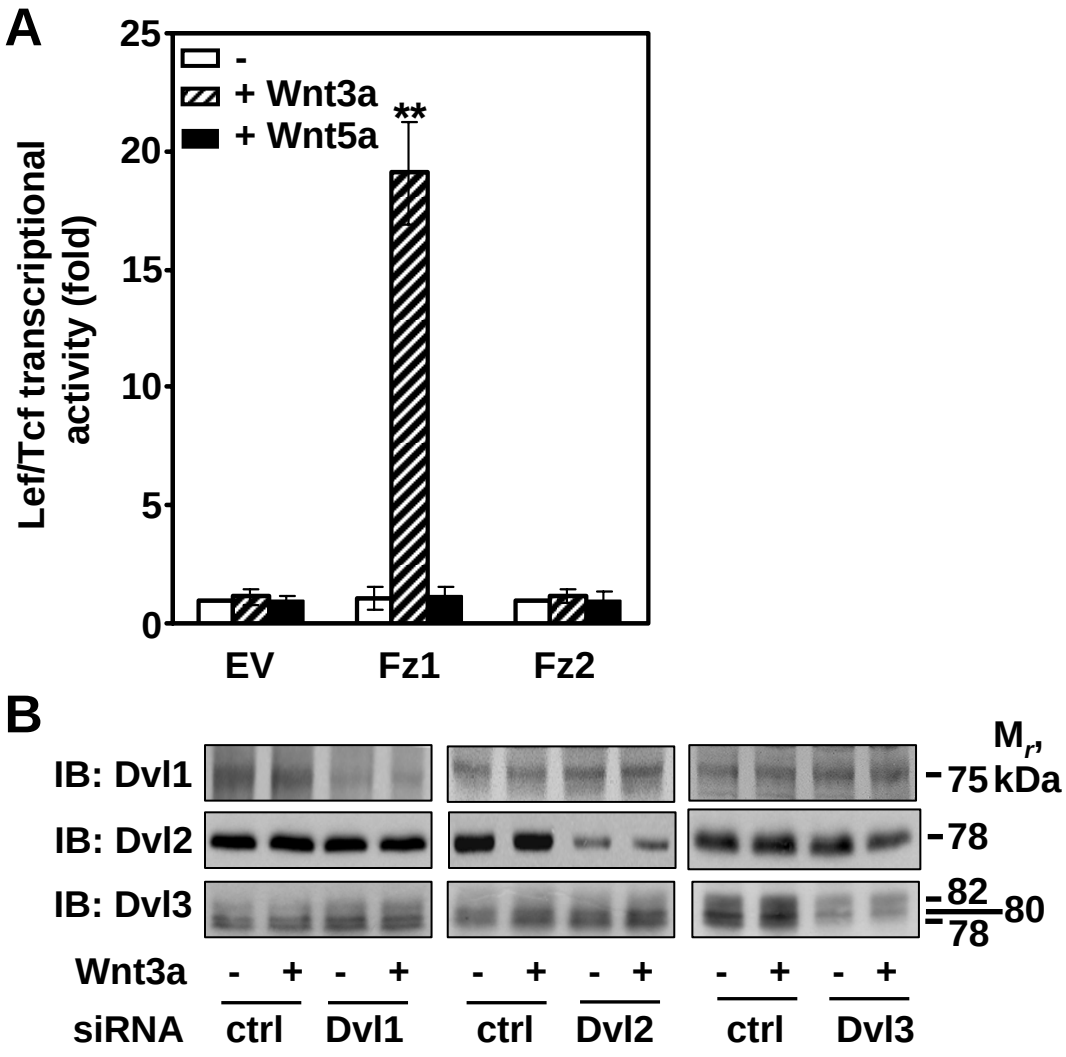
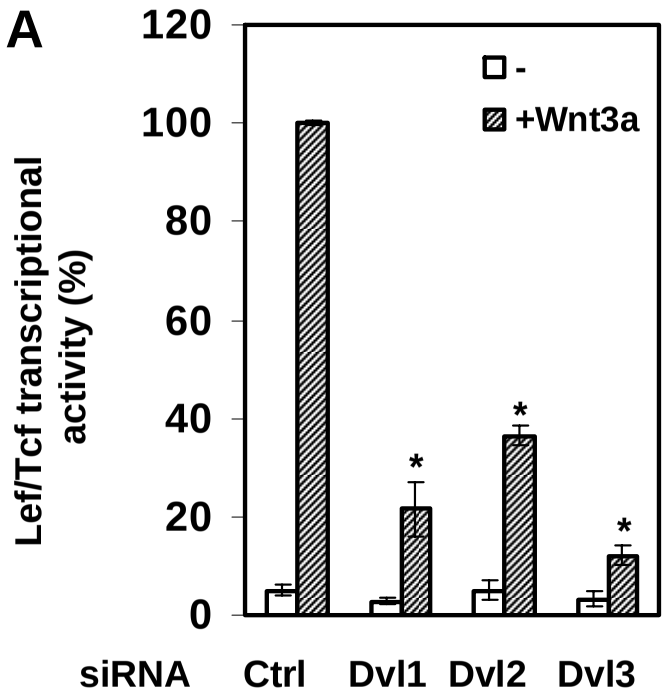

A
25
20
15
10
5
0
-
+ Wnt3a
+ Wnt5a
**
Lef/Tcf transcriptional activity (fold)
EV
Fz1
Fz2
B
IB: Dvl1
IB: Dvl2
IB: Dvl3
M$_r$, kDa
75
78
82
78
80
Wnt3a - + - + - + - + - + - +
siRNA ctrl Dvl1 ctrl Dvl2 ctrl Dvl3

Figure 4. siRNA-induced knock-down of Dvl1, Dvl2, or Dvl3 attenuated Wnt3a-stimulated canonical signaling. Mouse F9 cells expressing Fz1 were treated with maximal (panel A) or variable (panel B) amounts of siRNAs targeting one of the three Dvls (Dvl1, Dvl2, or Dvl3) and the activation of the Wnt-sensitive canonical pathway in response to Wnt3a monitored by the use of the Lef/Tcf-sensitive luciferase gene reporter. The assay of the canonical pathway was performed at 6 hr following stimulation without or with purified Wnt3a. Data presented are mean values ± S.E.M. from three or more independent experiments. *, $p \leq 0.01$ for the difference from control. In panel A, the data are plotted as % of maximal Wnt3a-stimulated canonical response versus treatment with a maximal amount of siRNA reagent. In panel B, the data are plotted as "% of maximal" of the Wnt3a-sensitive canonical response *versus* the extent of knock-down in Dvl isoform protein produced by treatment increasing amounts of siRNA targeting a given Dvl isoform.


A
120
100
80
60
40
20
0
Lef/Tcf transcriptional activity (%)
-
+Wnt3a
*
*
*
siRNA
Ctrl
Dvl1
Dvl2
Dvl3

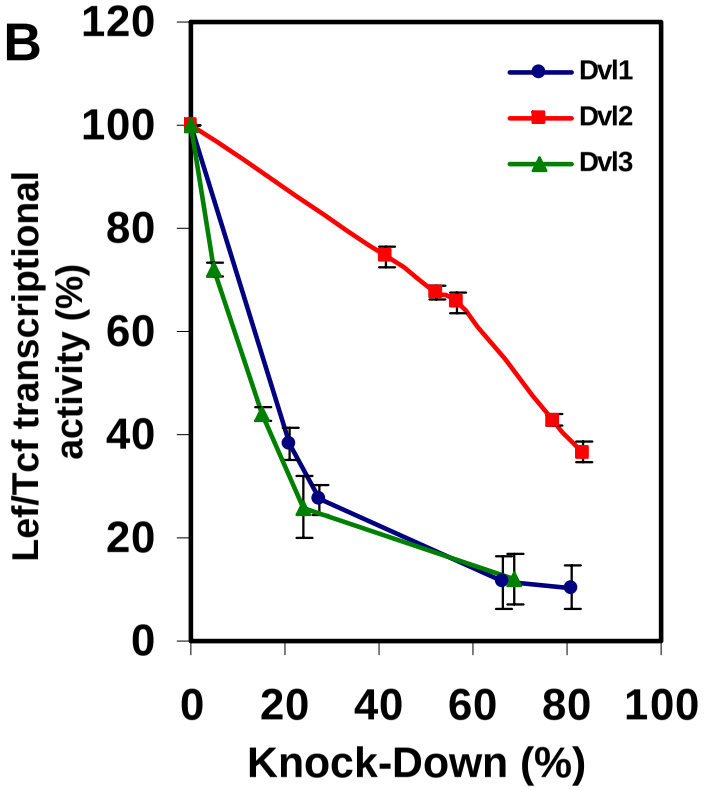
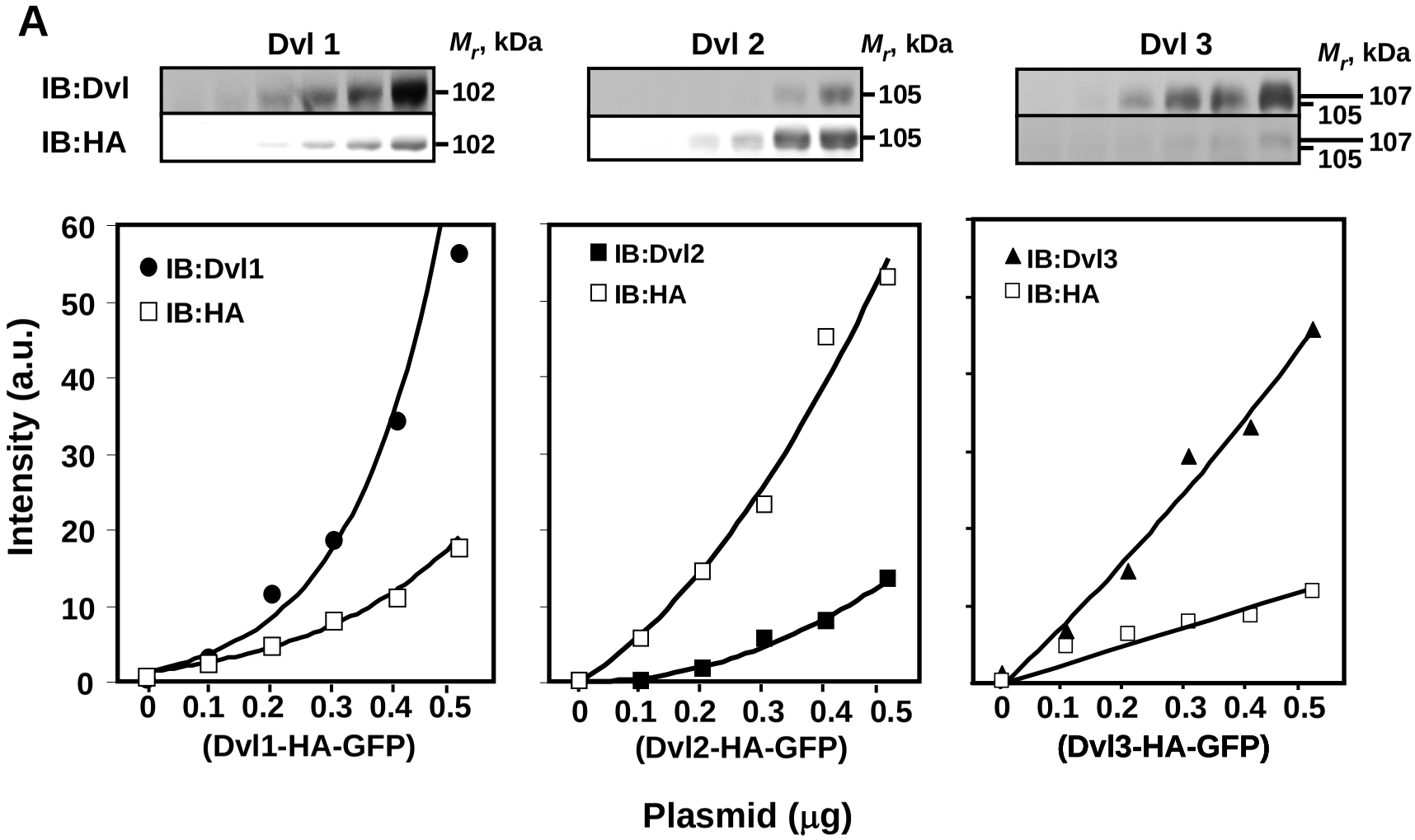

B
120
100
80
60
40
20
0
Lef/Tcf transcriptional activity (%)
Dvl1
Dvl2
Dvl3
0
20
40
60
80
100
Knock-Down (%)

Figure 5. Determination of the relative abundance of Dvl1, Dvl2, and Dvl3 in mouse F9, mouse P19, and human HEK 293 cells. A, mouse F9 cells expressing Fz1 were co-transfected without or with increasing amounts of plasmid DNA (expression vector) harboring a single Dvl isoform tagged with both HA and GFP at the C-terminus of the molecule, as described. Forty eight hours after the transfection, the F9 cells were disrupted and sample of whole-cell lysates was subjected to SDS-PAGE and transfer of the resolved proteins to nitrocellulose blots that then were stained with primary antibodies against either individual Dvls, or the HA-tag. A set of representative blots obtained from exogenously expressed Dvl isoforms (upper panels) and plots of expression of these isoforms (lower panels). B, analyses of the relative abundance of Dvl1, Dvl2, and Dvl3 in F9, P19, and HEK 293 cells. Samples (0.1 mg protein / SDS-PAGE lane) of whole-cell lysates were subjected to SDS-PAGE and transfer of the resolved proteins to nitrocellulose blots. The blots were stained with primary antibodies against Dvl1, or Dvl2, or Dvl3. A set of representative blots of endogenously expressed Dvl isoforms is displayed shown (upper panels). These data were analyzed and the relative amount of Dvl isoform expressed in each cell calculated (lower panel).


A
Dvl 1
Dvl 2
Dvl 3
M_r, kDa
IB:Dvl
IB:HA
102
105
107
Intensity (a.u.)
IB:Dvl1
IB:HA
IB:Dvl2
IB:Dvl3
(Dvl1-HA-GFP)
(Dvl2-HA-GFP)
(Dvl3-HA-GFP)
Plasmid (μg)

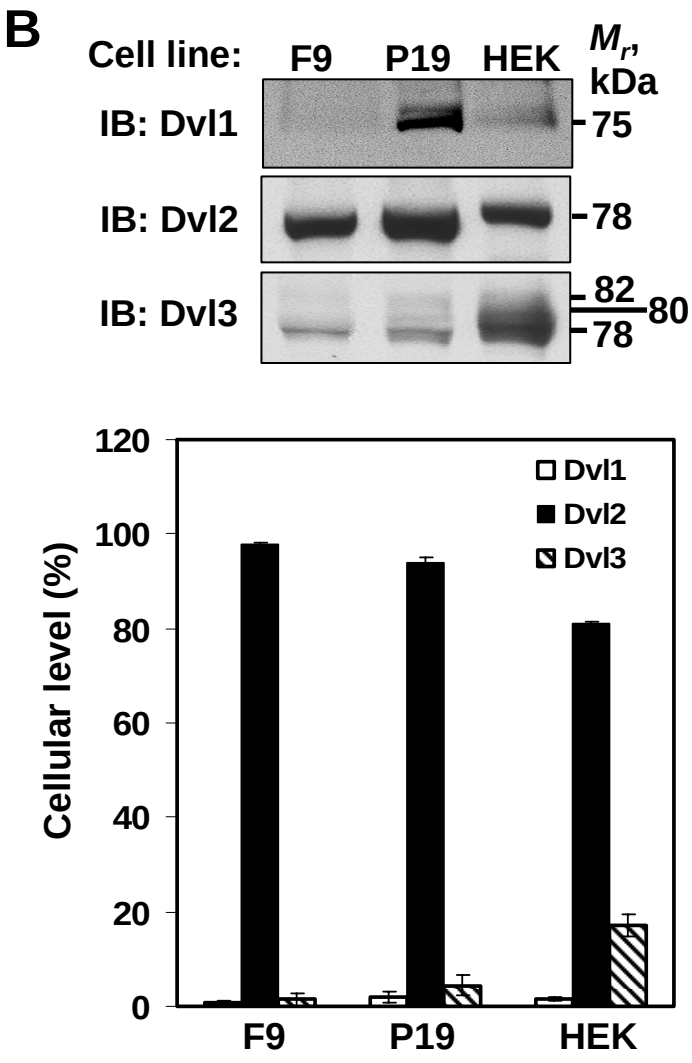

B
Cell line:
F9
P19
HEK
M_r, kDa
IB: Dvl1
75
IB: Dvl2
78
IB: Dvl3
82
80
78
Cellular level (%)
120
100
80
60
40
20
0
Dvl1
Dvl2
Dvl3
F9
P19
HEK

Figure 6. Effects of "over"expression of Dvl1, Dvl2, or Dvl3 on the activation of Lef/Tcf-sensitive transcriptional activation, in the absence of Wnt3a stimulation. Fz1-expressing F9 cells were transiently co-transfected with Super8XTOPFLASH reporter gene in the absence or presence of increasing amounts of input plasmid DNA (expression vector) harboring either Dvl1, Dvl2, or Dvl3 which had been tagged on the C-terminus with HA. Forty eight hours after the transfection, cells were disrupted and whole-cell lysates prepared. *A,* sample of whole-cell lysates was subjected to SDS-PAGE and transfer of the resolved proteins to nitrocellulose blots. The blots were stained with primary anti-HA antibody and their relative levels of expression compared. *B*, the activation of the Lef/Tcf-sensitive transcription was assayed by using Super8XTOPFLASH luciferase gene reporter. The luciferase reporter activities were plotted versus the amount of immunoreactive staining for the HA-tag (in arbitrary unit; a.u.) quantified in the blots obtained from the same cell lysates. Data presented are mean values ± S.E.M. from three or more independent experiments. *, $p \leq 0.01$ for the difference from the "Control" cells that were treated with empty vector.

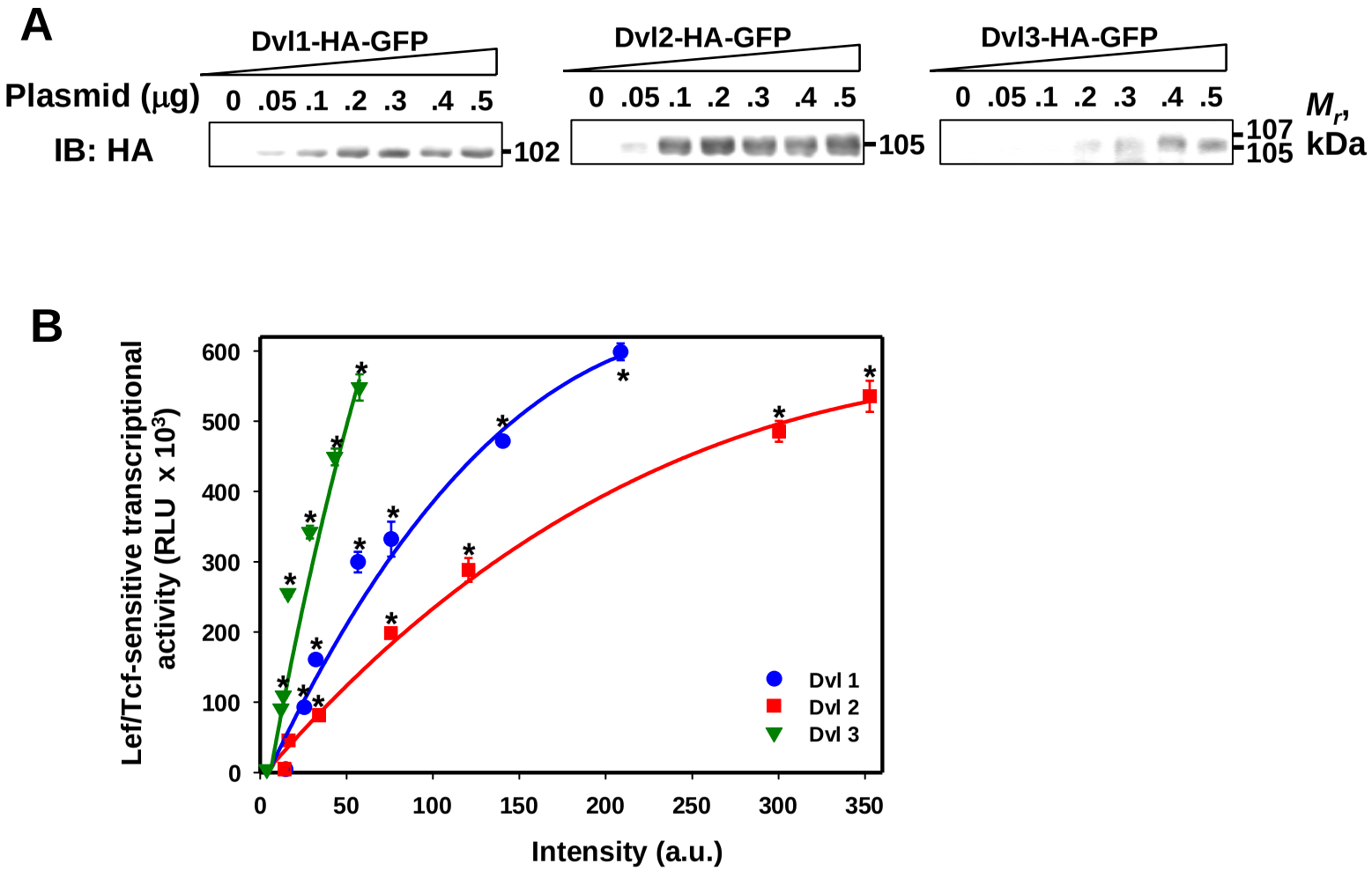
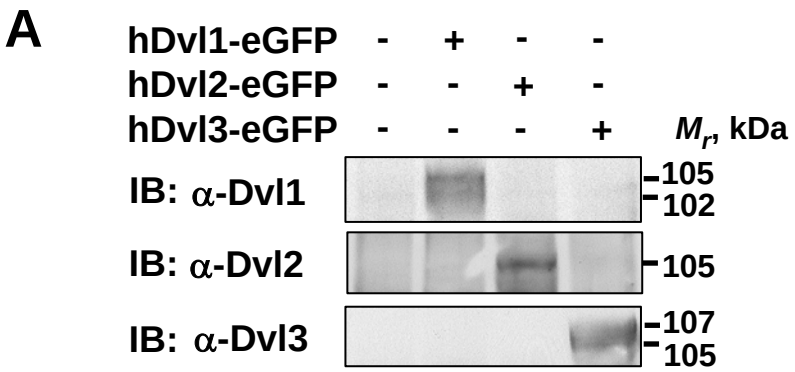

A
Dvl1-HA-GFP
Dvl2-HA-GFP
Dvl3-HA-GFP
Plasmid (μg)
0 .05 .1 .2 .3 .4 .5
0 .05 .1 .2 .3 .4 .5
0 .05 .1 .2 .3 .4 .5
IB: HA
102
105
107
105
Mr, kDa
B
Lef/Tcf-sensitive transcriptional activity (RLU x 10^3)
600
500
400
300
200
100
0
0
50
100
150
200
250
300
350
Intensity (a.u.)
Dvl 1
Dvl 2
Dvl 3

Figure 7. Dvl-based complexes are heterogeneous with respect to Dvl isoforms. *A*, Dvl isoform-specific antibodies were tested for cross reactivity by immunoblotting of whole-cell lysates obtained from cells "over"expressing a single Dvl isoform. Immunoblots were stained with antibodies against individual isoform of Dvl. *B*, Immune precipitation-based "pull-downs" from whole-cell lysates of F9 cells were performed using antibodies that recognize only Dvl2. The Dvl-based complexes isolated in the pull-downs (IP complex), whole-cell lysates (Total lysates), and supernatant from post-immunoprecipitated fractions (Sup. Post IP) were subjected to SDS-PAGE and the resolved proteins transferred to nitrocellulose blots. The blots were stained with antibodies specific for each Dvl isoform. The data presented are blots representative of three or more independent analyses.


A
hDvl1-eGFP - + - -
hDvl2-eGFP - - + -
hDvl3-eGFP - - - +
M_r, kDa
IB: α-Dvl1
105
102
IB: α-Dvl2
105
IB: α-Dvl3
107
105

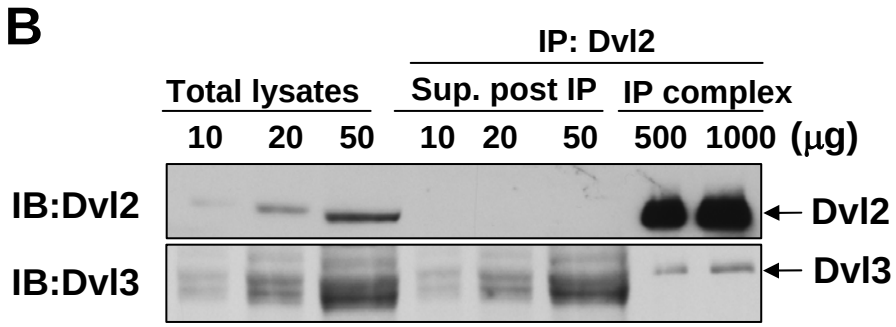
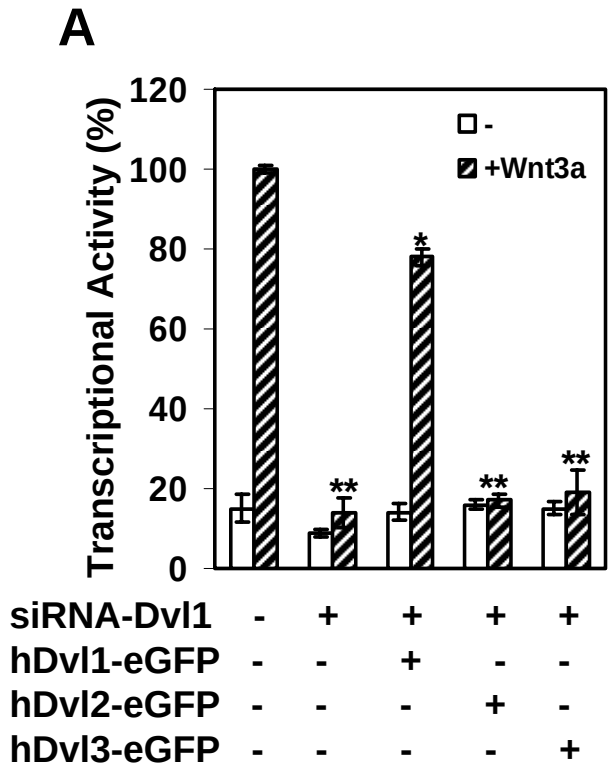
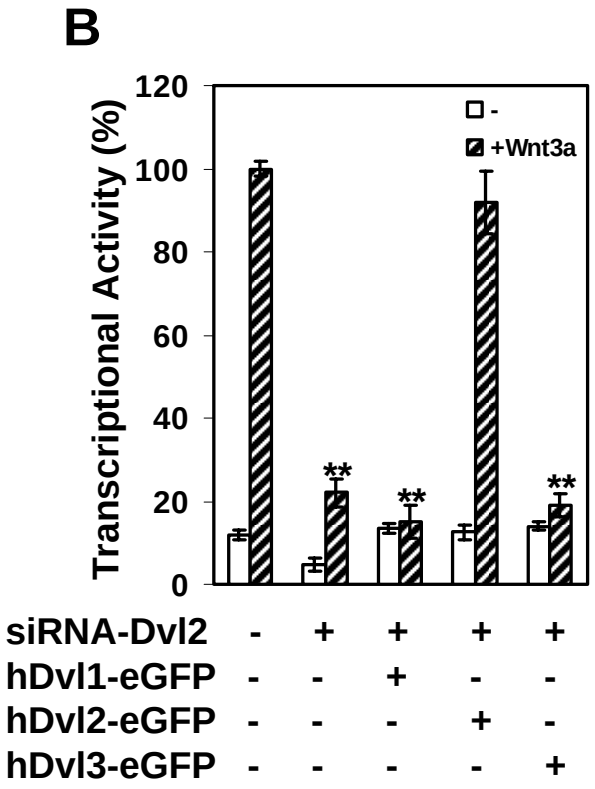

B
IP: Dvl2
Total lysates
Sup. post IP
IP complex
10 20 50 10 20 50 500 1000 (μg)
IB:Dvl2
Dvl2
IB:Dvl3
Dvl3

Figure 8. Rescue of Wnt3a-stimulated Lef/Tcf-sensitive transcriptional activation in Dvl3- or Dvl1-depleted mouse F9 cells using exogenous expression of hDvl1, hDvl2, or hDvl3. Fz1-expressing mouse F9 cells were treated with siRNA targeting mouse Dvl1 (A), Dvl2 (B), or Dvl3 (C). Twenty four hours after the administration of siRNA, cells were co-transfected with the Super8XTOPFLASH luciferase reporter gene construct and an expression vector harboring one isoform of hDvl that was tagged with eGFP at its C-terminus. Cells were cultured for additional 24 hr. The assay of the Wnt3a-sensitive canonical pathway was performed by assay of the activity of luciferase after cells were stimulated without or with purified Wnt3a for 6 hr. A, rescue of Wnt3a-stimulated activation of the canonical pathway in Dvl1-depleted F9 cells by exogenous expression of human Dvl1, Dvl2, or Dvl3. B, rescue of Wnt3a-stimulated activation of the canonical pathway in Dvl2-depleted F9 cells by exogenous expression of human Dvl1, Dvl2, or Dvl3. C, rescue of Wnt3a-stimulated activation of the canonical pathway in Dvl3-depleted F9 cells by exogenous expression of human Dvl1, Dvl2, or Dvl3. Data presented are mean values ± S.E.M. from three or more independent experiments. *, $p \leq 0.01$; **, $p \leq 0.001$ for the difference from control, +Wnt3a group.


A
Transcriptional Activity (%)
120
100
80
60
40
20
0
-
+Wnt3a
*
**
**
**
siRNA-Dvl1 - + + + +
hDvl1-eGFP - - + - -
hDvl2-eGFP - - - + -
hDvl3-eGFP - - - - +


B
Transcriptional Activity (%)
120
100
80
60
40
20
0
-
+Wnt3a
**
**
**
siRNA-Dvl2 - + + + +
hDvl1-eGFP - - + - -
hDvl2-eGFP - - - + -
hDvl3-eGFP - - - - +

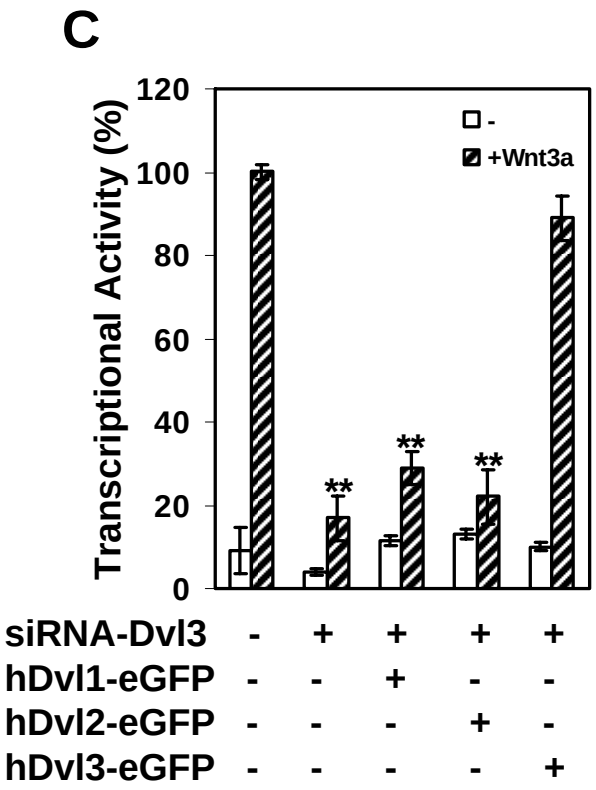
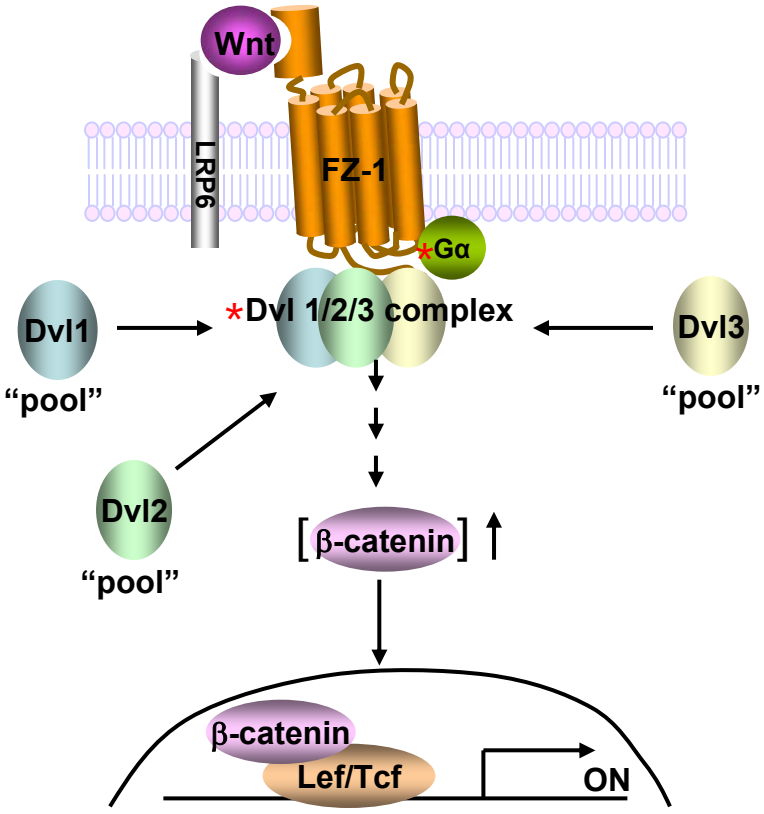

C
Transcriptional Activity (%)
120
100
80
60
40
20
0
-
+Wnt3a
**
**
**
siRNA-Dvl3 - + + + +
hDvl1-eGFP - - + - -
hDvl2-eGFP - - - + -
hDvl3-eGFP - - - - +

Figure 9. A schematic presentation of Dvl complex containing Dvl1, Dvl2 and Dvl3 in Frizzled-1/β-catenin signaling pathway. *, denotes “activation” in response to Wnt3a.


Wnt
LRP6
FZ-1
*Gα
*Dvl 1/2/3 complex
Dvl1
"pool"
Dvl3
"pool"
Dvl2
"pool"
[β-catenin]
β-catenin
Lef/Tcf
ON

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