Molecular Basis of Wnt Activation via the DIX Domain Protein Ccd1*

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The Wnt signaling plays pivotal roles in embryogenesis and cancer, and the three DIX domain-containing proteins, Dvl, Axin, and Ccd1, play distinct roles in the initiation and regulation of canonical Wnt signaling. Overexpressed Dvl has a tendency to form large polymers in a cytoplasmic punctate pattern, whereas the biologically active Dvl in fact forms low molecular weight oligomers. The molecular basis for how the polymeric sizes of Dvl proteins are controlled upon Wnt signaling remains unclear. Here we show that Ccd1 up-regulates canonical Wnt signaling via acting synergistically with Dvl. We determined the crystal structures of wild type Ccd1-DIX and mutant Dvl1-DIX(Y17D), which pack into “head-to-tail” helical filaments. Structural analyses reveal two sites crucial for intra-filament homo- and hetero-interaction and a third site for inter-filament homo-assembly. Systematic mutagenesis studies identified critical residues from all three sites required for Dvl homo-oligomerization, puncta formation, and stimulation of Wnt signaling. Remarkably, Ccd1 forms a hetero-complex with Dvl through the “head” of Dvl-DIX and the “tail” of Ccd1-DIX, depolymerizes Dvl homo-assembly, and thereby controls the size of Dvl polymer. These data together suggest a molecular mechanism for Ccd1-mediated Wnt activation in that Ccd1 converts latent polymeric Dvl to a biologically active oligomer(s).

Wnt signaling is one of the major developmental pathways regulating various cellular processes (1–3). In the canonical pathway, the extracellular Wnt ligands regulate the expression of specific target genes by modulating the amount of β-catenin. In the absence of Wnt, degradation of β-catenin is mediated by a destruction complex containing adenomatous polyposis coli, glycogen synthase kinase-3β, casein kinase 1, and Axin.2 Binding of Wnt ligands to the Frizzled and low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) receptors results in the activation of Dishevelled (Dvl), which suppresses β-catenin degradation. Thus, β-catenin is accumulated in the cytosol and subsequently translocated into the nucleus to regulate Wnt target genes.

Dvl and Axin contain a conserved region of about 100 residues, referred to as the DIX domain (4). The cytoplasmic Dvl has a tendency to form puncta via its DIX domain (Dvl-DIX), which may mediate Wnt signaling by reversible polymerization to provide an interaction platform for other Wnt components, including Axin (5–9). The DIX domain of Axin (Axin-DIX) is also required for the formation of Axin polymers and puncta (10, 11). Proper intracellular transduction of Wnt signal relies on the direct interaction between Dvl and Axin (11, 12). It is generally believed that the DIX domains play important roles in the formation of Dvl-Axin complex, although isolated Dvl-DIX and Axin-DIX do not bind to each other directly either in vivo or in vitro (9, 11–13). In addition to Dvl and Axin, the coiled-coil protein DIX domain-containing 1 (Ccd1) also contains a DIX domain that can form heteromeric complexes with Dvl and Axin and, thus, positively regulate the canonical Wnt signaling (14). Ccd1 is highly conserved in species ranging from zebrafish to human and is shown to play important roles in neurogenesis, axonal morphogenesis, and neuronal migration (15–18).

The x-ray structure of Axin-DIX was determined and revealed a helical filament in crystal and led to a head-to-tail self-association model of the DIX domain (5). However, the DIX domain of Axin cannot form hetero-complexes with either Dvl-DIX or Ccd1-DIX and is dispensable for the canonical Wnt signaling. In contrast, both DIX domains of Ccd1 and Dvl are indispensable for their hetero-interaction and the regulation of Wnt signaling. Therefore, the homomeric interaction revealed in the crystal packing of Axin-DIX might not fully define the nature for the interactions between the DIX domains. To reveal the molecular basis, we determined the crystal structures of wild type Ccd1-DIX and mutant Dvl1-DIX(Y17D) and carried out extensive biochemical studies on how the DIX domain-containing proteins can dynamically modulate each other. Despite their monomeric nature, these

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2 The abbreviations used are: Axin, axis inhibition protein; Dvl, Dishevelled; DIX, Dishevelled and Axin; Ccd1, coiled-coil-DIX1; HSQC, heteronuclear single quantum coherence.
two DIX domains are engaged in similar homo-oligomeric contacts, forming helical filaments in crystals. Structural analyses and systematic mutagenesis studies revealed that the critical residues for the DIX-DIX interaction are from three sites, two intra-filament sites (Site I and Site II) and one inter-filament site (Site III). The integrity of all three sites is required for Dvl homo-assembly, puncta formation, and Wnt signal transduction, whereas the non-conservative Site II in Axin-DIX may account for its disability to form hetero-complex with Dvl-DIX and Ccd1-DIX. In addition, we found that Ccd1 regulates Wnt signaling via association with Dvl through the head of Dvl-DIX and the tail of Ccd1-DIX. Importantly, Ccd1 depolymerizes the homo-assembly of Dvl in vitro and induces its diffuse cytoplasmic distribution in vivo. Our data together with prior work disclose the activation mechanism of Ccd1 by directly interacting with Dvl, depolymerizing the high molecular weight Dvl homopolymer, and further stabilizing Dvl in its biologically active low molecular weight state.

EXPERIMENTAL PROCEDURES

Constructs, Mutagenesis, and Protein Purification—The DIX domains of human Ccd1 (residues 386–472), mouse Dvl1 (3–104), and mouse Dvl2 (10–122) were amplified by standard PCR procedures and inserted into pET21b or pGEX2T vectors with a C-terminal His6 tag or N-terminal GST tag. Point mutations in DIX domain were generated by overlap PCR procedures and subjected to DNA sequencing. All proteins, overexpressed in Escherichia coli BL21(DE3) cells at 20 °C were first purified over nickel-nitrilotriacetic acid (Qiagen) or glutathione-Sepharose (GE Healthcare) columns and then by ion exchange (Source-15Q/15S, GE Healthcare) followed by gel filtration chromatography (Superdex-200, GE Healthcare) at 4 °C. The proteins were stored at −80 °C and subject to crystallization trials or biochemical assays.

Crystallization, Data Collection, and Structure Determination—Crystals of Ccd1-DIX were grown using the hanging drop vapor diffusion method by mixing Ccd1-DIX protein (~10 mg/ml) with an equal volume of reservoir solution containing 0.1 M sodium cacodylate, pH 6.5, 0.3 M magnesium nitrate, 15% PEG 3350, and 4% DMSO at 4 °C. The diffraction data sets were collected at the Beijing Synchrotron Radiation Facility (Beijing, China) to a resolution of 3.0 Å. The crystal was dehydrated in 1.0 M sodium citrate for 40 min and flash-frozen without additional cryoprotectant. The data processing and refinement statistics are summarized in Table 1. All structural representations in this paper were prepared with PyMOL. The atomic coordinates and structure factors have been deposited with the Protein Data Bank under accession codes 3PZ7 for Ccd1-DIX and 3PZ8 for Dvl1-DIX(Y17D).

Gel Filtration Analysis—Size exclusion chromatography using a Superdex 200 10/300 column on an AKTA FPLC (GE Healthcare) was carried out to assess the apparent molecular weight of a protein in solution and the interaction between two proteins at 4 °C. The column was equilibrated with a buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM dithiothreitol (DTT) at a flow rate of 0.5 ml/min. All protein samples or mixtures were diluted with the equilibration buffer to indicated concentrations and incubated at 4 °C for 30 min. Each sample at a volume of 500 μl was loaded to the column, and fractions of 0.5 ml each were collected. Aliquots of relevant fractions were subjected to SDS-PAGE, and proteins were visualized by Coomassie Blue staining.

GST-mediated Pulldown Assay for Protein-Protein Interaction—0.4 mg of recombinant GST-tagged protein A was bound to 0.2 ml of glutathione-Sepharose 4B resin pre-equilibrated with a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM DTT. To remove excess unbound protein A or other contaminants, the resin was washed 5 times with 0.5 ml of buffer. Then 0.6 mg of non-tagged protein B was allowed to flow through the resin. After extensive washing, the bound proteins were eluted with 5 mM reduced glutathione and visualized by SDS-PAGE with Coomassie Blue staining.

Analytical Ultracentrifugation—The Dvl2-DIX and Ccd1-DIX proteins were purified and concentrated to ~2 mg/ml in a buffer containing 10 mM Tris and 150 mM NaCl. The sam-
Dvl2 and Dvl3 were designed and synthesized according

to standard molecular techniques. Mutation and deletion/truncation constructs were generated by overlap PCR strategy. The authenticity of all constructs were confirmed by nucleotide sequencing. HEK293T and Simian COS7 cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Sigma). Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions.

Luciferase Reporter Assay—The Wnt activities of Dvl and Ccd1 (wild type or mutants) were examined with the luciferase assay. HEK293T cells were seeded into 96-well plates, and the indicated amounts of plasmids were transfected with 15 ng of Wnt reporter SuperTOPFlash that contains eight copies of T-cell factor/Lymphoid Enhancing Factor binding sites upstream to the luciferase gene. Ectopically expressed Ccd1 resulted in Wnt activation, which was comparable to the activation induced by over-expressing Dvl (14, 28). To further assess the importance of Ccd1, we knocked down the endogenous Ccd1 by transfecting HEK293T cells with shRNA specific for Ccd1. When pSUPER-Ccd1 was co-transfected with Wnt3a, the Wnt3a-mediated transcriptional activation was strongly suppressed to ~30% of the original activation (Fig. 1A). These results demonstrate that Ccd1 indeed plays a role in the activation of Wnt signaling.

Because Ccd1 can interact with Dvl, we asked whether Ccd1 functions through Dvl. To address this issue, we depleted endogenous Dvl in HEK293T cells by RNAi and assessed the effect on Wnt activation by ectopically expressed Ccd1. In higher organisms (including mammals), there are three Dvl isoforms, Dvl1, Dvl2 and Dvl3, and they were reported to function similarly and cooperatively in positively mediating the canonical signaling (29–34). The abundance of endogenous Dvl1 was extremely low whereas Dvl2 and Dvl3 constituted over 99% of the total pool in HEK293 cells, and a mixture of Dvl2/3 siRNAs was thus used to knockdown endogenous Dvl proteins (27, 35). As shown in Fig. 1B, depletion of Dvl significantly down-regulated Wnt3a-mediated activation. Similarly, the stimulation of Wnt signaling by Ccd1 was substantially suppressed, indicating that Dvl is required for Ccd1-mediated Wnt activation. Remarkably, coexpression of Ccd1 and Dvl dramatically stimulated the T-cell factor/
Lymphoid Enhancing Factor-mediated transcription, whereas Ccd1 or Dvl alone yielded a modest activation of the reporter (Fig. 1C). Therefore, these data demonstrated that Ccd1 and Dvl synergistically stimulate Wnt signaling.

Crystal Structure of Ccd1-DIX—Ccd1 directly interacts with Dvl via their DIX domains, and we, thus, cloned and purified these DIX domains (Fig. 2A). A notable property of Dvl and Axin is their ability to form via their conserved DIX domains. Ctd, control. C, synergistic effects of Dvl and Ccd1 on Wnt activation. Some 10 ng of Myc-Ccd1 (wild type or truncation/mutations as indicated) were transfected into HEK293T cells with or without Dvl2 (10 ng).

FIGURE 1. Ccd1 activates canonical Wnt pathway through Dvl. A, knockdown of endogenous Ccd1 attenuates Wnt-3a mediated T-cell factor transcriptional activity. HEK293T cells were transfected with Wnt-3a (0 or 2 ng) and pSUPER-Ccd1 (0 or 30 ng) as indicated. Some 36 h later the activation of Wnt signaling was quantified by luciferase activity. Asterisks denote p < 0.001 when compared with the corresponding samples only transfected with Wnt-3a. B, knockdown of endogenous Dvl suppresses Ccd1 function. HEK293T cells were cotransfected with 100 nm Dvl2/3 siRNAs together with Wnt-3a (2 ng) or Ccd1 (10 ng). *, p < 0.05; **, p < 0.005 compared with the corresponding samples transfected with control siRNAs. Ctr, control.

FIGURE 2. Crystal structures of Ccd1-DIX and Dvl-DIX(Y17D). A, sequence alignment of the DIX domains from human Ccd1, mouse Dvl1–3, and mouse Axin. Secondary structural elements of Ccd1-DIX are indicated in blue, and the hydrophobic core residues are boxed. Residues at Site I and Site II are indicated, respectively, by triangles and asterisks, and residues at the head and tail of the DIX domain are colored, respectively, by orange and cyan. Residues at Site III are indicated by green diamonds. B, oligomerization states of Dvl-DIX, Axin-DIX, Ccd1-DIX, and their swap mutants are shown. The size exclusion chromatography was performed to determine the apparent molecular weight of the DIX domains/mutants at a concentration of 50 μM. C and D, schematic representations of Ccd1-DIX and Dvl-DIX(Y17D) are shown. Both structures adopt the ubiquitin-like fold, and the β-strands are labeled. E, superposition of three DIX structures, Ccd1-DIX (blue), Dvl1-DIX(Y17D) (magenta), and Axin-DIX (green, PDB code 1WSP). The head and tail essential for DIX-DIX interaction are also indicated.
mains large protein assemblies. Consistently, recombinant Dvl2-DIX and Axin-DIX were eluted similarly as homopolymers in the gel filtration analysis (Fig. 2B). However, the retention time of Ccd1-DIX corresponded to an apparent molecular mass of 11 kDa, in reasonable agreement with its calculated monomeric molecular mass of 10 kDa. The oligomerization states of Dvl2-DIX and Ccd1-DIX were also characterized by analytical ultracentrifugation (see Fig. 7B). The sedimentation coefficient of the polymeric Dvl2-DIX was determined to be 4.7 S, comparable with previous result (5), whereas that of Ccd1-DIX was 1.5 S, consistent with its monomeric nature. To elucidate the molecular mechanism of DIX domain in Wnt signaling, we carried out crystallization attempts to crystallize the wild type DIX domains of all three DIX domain-containing proteins and other structurally related proteins (supplemental Fig. S1). All of these residues are invariant or highly conserved throughout three DIX domains and other structurally related proteins (Fig. 2A). The structure of Ccd1-DIX is readily superimposable to the previously determined structure of Axin-DIX (5) with an overall root mean square deviation of 1.6 Å over 80 core Cα atoms. The major structural deviations were mapped to the loop regions (Fig. 2E). In particular, the subtle conformational change of the β3-β4 loop is critical to the interactions between these DIX domains (see below).

**Determinant for the Monomeric Nature of Ccd1-DIX**—Consistent with the polymeric state in solution, Axin-DIX was found to engage in homomeric head-to-tail interactions in crystals (5). Interestingly, inspection of crystal packing in the structure of Ccd1-DIX revealed the presence of a similar helical filament (Fig. 3A), although this crystallographically derived model may not be physiologically relevant. In the crystallographic dimers of both Axin-DIX and Ccd1-DIX, the head (strands β1 and β2 and the C terminus of α-helix) of Molecule A interacts with the tail (strands β3 and β4) of the adjacent Molecule B. The protein-protein interfaces are centered at the intermolecular backbone hydrogen bonds between strand β2 of Molecule A and strand β4 of Molecule B that run approximately perpendicular to each other.

This intermolecular interaction involves two potential sites. As shown in Fig. 3D, there is a cluster of hydrophobic residues on the Axin-DIX interface composed of Tyr 766 from strand β2 of Molecule A and Tyr-793 and Phe-807 from strands β3 and β4 of Molecule B (5). These residues are conserved in Dvl-DIX (Fig. 2A), and the side chains of the corresponding Tyr-27, Phe-56, and Lys-68 of Dvl2 likely preserve this hydrophobic patch as the methylene group of Lys would provide the hydrophobic contact as well. However, the hydrophobic Tyr/Phe is replaced by a polar residue His-432 in Ccd1-DIX (Fig. 3B), which may account for its monomeric nature. To assess this structure-based hypothesis, we individually swapped the residues of Ccd1-DIX or Dvl-DIX with the corresponding amino acids and analyzed their oligomeric states in solution by gel filtration (Fig. 2B). Indeed, mutating His-432 of Ccd1-DIX into hydrophobic Phe led to an increase of the elution volume, suggestive of a change in the oligomer-monomer equilibrium. In turn, when Phe-56 of Dvl2 was replaced by histidine, the mutant protein formed low molecular weight oligomers compared with the wild type Dvl2. As expected, the conserved substitutions, mutation of Phe on strand β2 to Tyr or vice versa, had no impact on the oligomerization states of Ccd1-DIX and Dvl-DIX (data not shown). These data clearly indicate that this hydrophobic interface plays an important role in the homomeric DIX-DIX interactions of Dvl assemblies and that the presence of a basic residue histidine leads to the monomeric nature of Ccd1-DIX.

**Additional Contacts in DIX-DIX Self-association of Dvl**—Further analysis of the crystal packing of Ccd1-DIX has enabled us to identify additional contacts between each two adjacent molecules (Fig. 3C). Phe-440 from the heavily twisted β4-strand at the tail of Molecule B inserts into a hydrophobic pocket on the head of Molecule A, lined by aliphatic side chains of Leu-393 and the hydrophobic methylene groups of Lys-391 and Lys-460. In addition, the positively charged Lys-391 on head strand β1 interacts with two acidic amino acids, Asp-437 and Glu-439, from the tail β3-β4 loop. Sequence alignment between Ccd1 and Dvl reveals a high degree of conservation within the residues that comprise this binding site (Fig. 2A), suggesting an augmentative role of this region in the formation of Dvl homo-assemblies and the hetero-interaction between Dvl and Ccd1. However, Axin-

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**TABLE 1**

Data collection and refinement statistics

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* Both data sets were collected from single crystal.

* The highest resolution shell is shown in parentheses.
DIX does not seem to possess an interactive conformation in Site II, as the corresponding Phe-801 in Axin shifts outwards more than 6 Å (Fig. 3E). For simplicity, the previously defined hydrophobic patch essential for Dvl and Axin homopolymerization is hereafter referred to as Site I, and the new patch as Site II.

Schwarz-Romond et al. (5) have proposed a model for Dvl2-DIX oligomer based on the Axin-DIX filament structure, which demonstrated the importance of the hydrophobic Site I in mediating Dvl homo-oligomerization. To verify the importance of the new Site II on Dvl self-assembly, we generated a series of point mutations on Dvl2-DIX and examined their effect on its polymerization. Remarkably, the Site I mutations, Y27D at the head and K68A at the tail, led to two monomers, and mutant F56A was eluted as the low molecular weight oligomer (Fig. 3F). When the corresponding interface residues at Site II were individually mutated, most of the Dvl2-DIX mutants were markedly retained on the size exclusion column, indicating a dissociation of Dvl polymer (Fig. 3G). In the analytical ultracentrifugation analyses, the sedimentation coefficient of the monomeric Site I mutant Y27D was approximately 1.7 S and that of wild type Dvl2-DIX was ~4.7 S as reported (5). Notably, the Site II mutant F64A showed an intermediate sedimentation value of 2.4 S, confirming that this mutant exhibits impaired polymerization ability (supplemental Fig. S2). Together, these results indicated that Site I indeed plays a significant role in Dvl homopolymerization, and Site II exerts an auxiliary function.

Crystal Structure of Dvl1-DIX(Y17D)—Because the crystalization trials for wild type Dvl-DIX were unsuccessful, we turned to solve the structure of Dvl-DIX mutants to validate the Dvl homopolymerization model. We successfully generated diffractable crystals of a Dvl-DIX mutant, i.e., Dvl1-DIX(Y17D) containing residues 3–104, and solved the structure by molecular replacement using Ccd1-DIX as searching model (Table 1).

FIGURE 3. Crystal structure of Ccd1-DIX reveals additional contacts for head-to-tail DIX-DIX interaction. A, helical filaments in crystals of Ccd1-DIX (blue and slate) and Axin-DIX (green and lemon) are shown. The top molecule of each filament is superimposed. Site I and Site II that mediate the head-to-tail interactions are indicated on the superposed hetero-dimers on the right. B–E, close-up views of Site I and Site II at the interfaces of Ccd1-DIX and Axin-DIX heterodimers. The molecules are colored the same as in panel A, and critical residues from the head and tail are highlighted in orange and cyan, respectively. F and G, oligomerization states of Dvl2-DIX Site I and Site II mutants determined by gel filtration analyses. The protein concentration used was approximate 50 μM.
and supplemental Fig. S1). Because of the introduction of a charged side chain into the hydrophobic interface, mutant Dvl1-DIX(Y17D) was a monomer in solution regardless of the protein concentration (supplemental Fig. S3); however, it packs into a head-to-tail polymer in the crystal (Fig. 4A). The arrangements of the crystallographic helical filaments of 3 DIX domains are slightly different; Ccd1-DIX, like Axin-DIX, assembles into a compact helical structure of 6 molecules per turn and with a pitch of approximately 80 Å, whereas Dvl1-DIX(Y17D) packs into a loose polymer with 8 molecules per turn and a pitch of ~140 Å.

Intriguingly, the eight Dvl1-DIX(Y17D) molecules within an asymmetric unit polymerize into a pair of intertwined right-handed helical filaments (Fig. 4B). A close-up view of the crystallographically intermolecular interface reveals that Dvl possesses the hydrophobic patch as in Axin despite the Y17D mutation (Fig. 4C). Notably, the intermolecular Site II newly identified in Ccd1-DIX is highly conserved in the crystallographic assembly of Dvl1-DIX(Y17D), reaffirming its role in DIX-DIX interaction (Fig. 4D). Further inspection of the crystal packing of Dvl1-DIX revealed that a novel symmetric interface (hereafter referred to as Site III) between two adjacent molecules form distinct filaments in addition to the two intra-filament sites (Fig. 4E). Met-52 and Val-58 from the hairpins of Dvl1 make hydrophobic contacts to the equivalent residues on the adjacent molecule. Notably, two glycine residues (Gly-57) are positioned closely at the center of this hydrophobic interface. The polar side chain of Asn-74 from the loop to strand of one molecule hydrogen bonds to the side-chain carboxyl group of Asp-55 and the carbonyl oxygen of Gln-54 on the loop of the adjacent molecule. Notably, two glycine residues (Gly-57) are positioned closely at the center of this hydrophobic interface. The polar side chain of Asn-74 from the loop to strand of one molecule hydrogen bonds to the side-chain carboxyl group of Asp-55 and the carbonyl oxygen of Gln-54 on the loop of the adjacent molecule. This interface is largely conserved in Axin-DIX, but the Thr substitution on strand β4 of Ccd1-DIX is likely to disturb the hydrophobic contacts (Fig. 2A). To investigate the importance of this site, corresponding mutants of Dvl2-DIX were generated, and their abilities to form oligo-

FIGURE 4. Crystal structure of Dvl1-DIX(Y17D) indicates interfilament contacts crucial for Dvl polymerization. A, different arrangements of the helical filaments in crystals of Ccd1-DIX (blue and slate), Dvl1-DIX(Y17D) (magenta and pink), and Axin-DIX (green and lemon) are shown. The top molecule of each filament is superimposed, and the helical pitches of three filaments are indicated. B, eight Dvl1-DIX(Y17D) molecules within an asymmetric unit packed into two intertwined helical filaments. The molecules forming two filaments are colored in magenta/pink and sand/yellow, respectively. Three sites for Dvl homopolymerization are indicated. C and D, close-up views of Site I and Site II at the intrafilament interface of Dvl1-DIX(Y17D) are shown. The orientations are the same as in Fig. 3, B and C, respectively. Residues from the head and tail are highlighted in orange and cyan, respectively, and corresponding Dvl2 residues are labeled in parentheses. E, close-up view of Site III at the interfilament interface of Dvl1-DIX(Y17D). Residues from two adjacent molecules are highlighted in green and slate sticks. F, oligomerization states of Dvl2-DIX Site III mutants (~50 μM) are characterized by gel filtration analyses. The profiles for wild type Dvl2-DIX and mutant Y27D were also shown for comparison.
Wnt Activation by Ccd1

omers were examined by gel filtration and velocity ultracentrifugation analyses. As shown in Fig. 4F, mutant V66D formed low molecular weight oligomers, whereas mutation of Asn-82 or Met-60 to Asp exhibited little effect on Dvl polymerization. Moreover, substitution of Gly-65 with any other amino acid would introduce steric clashes into the tightly packed inter-filament Site III, and mutant G65D was, thus, defective in polymerization (Fig. 4F and supplemental Fig. S2D). These data suggested that, in addition to the filament formation via Sites I and II, Dvl may further assemble into higher-order aggregates via Site III.

DIX-DIX Assembly of Dvl Is Important for Puncta Formation and Wnt Signaling—Because the polymerization of Dvl is believed to be crucial for its physiological function, we next examined the effect of these mutations in full-length Dvl2 on puncta formation and Wnt signaling. As shown in Fig. 5A, mutant R84A of Dvl2 that retains the ability of polymerization gave rise to puncta formation in a pattern indistinguishable from that of wild type Dvl2. However, mutants Y27D and K68A that behaved as monomers in vitro as well as the DIX domain truncation (Dvl2-ΔDIX, residues 95–736) were evenly distributed in the cytoplasm of transfected simian COS7 cells. Interestingly, mutants F56A, D61A, and G65D from three distinct sites, which only polymerized into low molecular weight oligomers, formed somewhat smeared puncta, indicating that these mutants possess a modest tendency to polymerize at high concentrations. Remarkably, the ability of these mutants to stimulate Wnt signaling, assessed by using TOP-Flash reporter assays, was completely defective in signaling as that observed for the DIX truncation mutant (ΔDIX). Partial activations were detected for mutants with impaired ability of self-assembly and puncta formation. Thus, these results strongly suggested that Dvl homopolymerization via its DIX domain is a critical process during the initiation of Wnt signaling.

![Figure 5](https://www.jbc.org/content/286/10/8604/F5)

**FIGURE 5. Oligomerization via DIX domain is required for Dvl puncta formation and Wnt stimulation. A**, puncta formation of full-length Dvl2 wild type and representative mutants is shown. COS7 cells were transfected with 1 µg of HA-tagged Dvl2, and the localization of Dvl2 was visualized by indirect immunofluorescence. Scale bar, 10 µm. B, Wnt activities of full-length Dvl2 wild type and mutants. Some 20 ng of HA-Dvl2 mutants were transfected into HEK293T, and TOPFLASH reporter assays were performed to determine the Wnt activities after 36 h. The bottom panel shows the expression levels of all Dvl2 mutants detected by Western blotting using anti-HA antibodies.

Heteromeric Interaction between the Head of Dvl-DIX and the Tail of Ccd1-DIX—To elucidate the molecular mechanism underlying how Ccd1 regulates the intracellular Wnt signal transduction through hetero-interaction with Dvl, we first examined this hetero-interaction using purified Dvl2-DIX and Ccd1-DIX. When Ccd1-DIX was applied to GST-Dvl2-DIX preimmobilized to glutathione resin (Fig. 6A, first lane) or vice versa (Fig. 6C, first lane), these two DIX domains formed a stable hetero-complex as resolved by SDS-PAGE. In view of the high sequence similarity, structure homology, and similar crystal packing mode, we hypothesized that Dvl-DIX and Ccd1-DIX form a hetero-complex in a similar head-to-tail pattern.

To investigate the exact interface on Ccd1-DIX, we performed NMR titration studies. The addition of non-labeled Dvl2-DIX into 15N-labeled Ccd1-DIX resulted in changes of the peaks in the 1H-15N HSQC spectra, and peak disappearance was observed due to the size increase or slower exchange rate of the protein complex at the NMR timescale (supplemental Fig. S4). Residues displaying significant peak intensity changes were mostly clustered on strands β3 and β4, the tail of Ccd1-DIX, distributing over both sites (Fig. 6D). To complement the NMR analysis, we carried out systematic GST-mediated pulldown assays. Some of the Ccd1-DIX mutants, K444A on Site I and D437A and F440A on Site II, abolished the formation of Ccd1-Dvl2 hetero-complex, indicating that both Sites I and II of Ccd1 are required for its interaction with Dvl (Fig. 6A). The NMR data also suggested the involvement of the β1-β2 loop, but mutation of those indicated residues had little effect on in vitro binding analysis (data not shown). Notably, all three effective mutations are located at the tail of Ccd1-DIX, whereas none of the head mutants disrupted the hetero-assembly (Fig. 6D). Next, we generated various mutations in the backbone of full-length Ccd1 and determined their effects using the Wnt reporter assay. Consistent with their affinities to Dvl, the tail mutants D437A, F440A, and
K444A as well as the DIX domain truncation mutant (ΔDIX) were unable to activate the Wnt reporter, whereas mutants that retain the heteromeric interaction with Dvl mostly reserved the ability of Wnt activation (Fig. 6B). In addition, Ccd1 mutants D437A and ΔDIX that are unable to bind to Dvl failed to synergize with Dvl in stimulating Wnt signaling (Fig. 1C). Taken together, these data demonstrated that the tail of Ccd1-DIX interacts with Dvl, and that this heteromeric interaction is indispensable for Ccd1-mediated Wnt activation.

Given the head-to-tail model, the head of Dvl-DIX was, therefore, thought to be involved in the hetero-association with Ccd1-DIX, and we carried out pulldown assays with Dvl2-DIX mutants (Fig. 6C). Mutant Y27D at Site I of Dvl2-DIX ablated the hetero-interaction by disrupting the hydrophobic interaction, and substitutions of Ile-17 and Arg-84 with alanine in Dvl2-DIX that might weaken the interactions at Site II also led to severe compromise in the formation of hetero-complex. These critical residues are indeed clustered.
at the head of Dvl2-DIX. In addition, mutations at Site III that interfere with the interfilament interactions retained the ability to form hetero-complex with Ccd1 (data not shown). These data demonstrated that Dvl-DIX and Ccd1-DIX interact with each other through two distinct interfaces, the head of Dvl and the tail of Ccd1 (Fig. 6D).

**Ccd1 Converts Polymeric Dvl to an Oligomer of Lower Order**—In an attempt to analyze the interaction between Dvl-DIX and Ccd1-DIX using gel filtration assays, it was found that the addition of Ccd1-DIX converted the high molecular weight Dvl2-DIX polymer to complexes of lower order (Fig. 7A). Surprisingly, Dvl-DIX and Ccd1-DIX likely formed a 3:1 hetero-oligomer, but not a 1:1 heterodimer, even in the presence of 5-fold excess Ccd1-DIX. The Ccd1-DIX mutant D437A that is unable to bind to Dvl failed to attenuate the formation of Dvl2-DIX homopolymers regardless of the protein concentration. These data indicated that the hetero-interaction between Dvl-DIX and Ccd1-DIX is weaker than the homopolymerization of Dvl-DIX, which may result from the presence of a polar His side chain within the hydrophobic Site I of Ccd1-DIX (Fig. 3B). The depolymerization of Dvl by Ccd1 was confirmed by analytical ultracentrifugation (Fig. 7B). The sedimentation profile of the mixture of Dvl-DIX with wild type Ccd1-DIX was distinguished from those of the respective DIX proteins, and the calculated sedimentation value of the complex was approximate 3.2 S, lower than that of Dvl2-DIX ho-
mopolymer (~4.7 S). These data indicated that the Dvl2-Ccd1 complex exhibits an apparent molecular weight smaller than that of Dvl2 polymer. On the contrary, the mixture of Dvl-DIX with the non-interactive Ccd1-DIX(D437A) exhibited two separate peaks corresponding to the isolated proteins. Thus, wild type Ccd1 is able to depolymerize Dvl into lower order oligomers via the direct interactions between their DIX domains.

To explore the biological significance of this low molecular weight hetero-oligomer in vivo, we assessed the effect of Ccd1 on the puncta formation of Dvl (Fig. 7C). Coexpression with wild type Ccd1 caused a dramatic change in Dvl2 localization; Dvl2 could no longer form puncta and became colocalized with Ccd1, evenly distributed throughout the cytoplasm. The same diffuse appearance was observed when Dvl2 was coexpressed with Ccd1-DIX alone, indicating that the DIX domain of Ccd1 is sufficient to disrupt the polymerization of Dvl in vivo. In contrast, coexpression of mutant Ccd1-DIX(D437A) that is defective in interacting with Dvl-DIX had little effect on the puncta formation of wild type Dvl2. These results suggested that Ccd1 is able to attenuate the homo-assembly of Dvl in vitro and induce a diffuse cytoplasmic distribution in vivo.

DISCUSSION

Although Ccd1 was shown to positively regulate the Wnt pathway (14, 36), the precise mechanism of Ccd1-mediated Wnt activation remains unsolved. We found that Ccd1 regulates Wnt signaling through the direct interaction with Dvl. Our study has further elucidated the structural basis for complex formations among the DIX domain proteins and demonstrated that the capability of Ccd1 to convert latent polymeric Dvl to biologically active oligomer underlies its positive role on Wnt signaling.

The structures of Ccd1-DIX and Dvl1-DIX(Y17D) belong to the ubiquitin-like fold, the same β-grasp topology as the previously determined Axin-DIX structure. Regardless of the distinct monomeric/oligomeric nature of the proteins used for structural studies, all three DIX domains form helical filaments in crystals, involving similar head-to-tail packing. It is noteworthy that a number of proteins form the head-to-tail complexes via association of their homotypic domains and, thus, exert widespread roles in the precise transduction of cellular signaling pathways (37–39). In addition to the previously identified hydrophobic interface (Site I), the crystal packing of Ccd1 and Dvl indicates additional contacts (Site II) between the N terminus of strand β1 or β5 of one molecule and the twisted β3-β4 loop of the adjacent molecule within the filament. Interestingly, the eight molecules of Dvl1-DIX(Y17D) within each asymmetric unit pack into two intertwined helical filaments, which enables us to identify a symmetric interfilament interface (Site III) for the assembly of higher order aggregate. Recently, some residues at Sites II and III were predicted to have potential roles in mediating DIX-DIX interaction by comparative modeling (40). Our structure-guided mutagenesis studies demonstrated that the integrity of the interface, including the primary Site I, the auxiliary Site II, and the interfilament Site III, is indispensable for Dvl homo-oligomerization, puncta formation, and Wnt signaling. In addition, Dvl and Ccd1 directly interact with each other through the head of Dvl-DIX and the tail of Ccd1-DIX, involving residues from Sites I and II. However, Axin-DIX may not possess an active Site II and is, therefore, incapable to form hetero-complex with Dvl-DIX and Ccd1-DIX (9, 11, 12, 26, 36). The hydrophobic pocket at Site II of Axin-DIX is partially deformed; a small Ala-756 on the head substitutes the bulky Leu/Ile in Ccd1/Dvl, and concomitantly the aromatic ring of Phe-801 on the tail of Axin shifts outwards more than 6 Å (Fig. 3E). Moreover, the charged residues forming salt bridges in Ccd1/Dvl are replaced by Ser-798 and Val-754 in Axin, which may lead to the disruption of the hydrophilic interactions at Site II. Therefore, Site II may be unique to Dvl-DIX and Ccd1-DIX.

Overexpressed Dvl has a tendency to form high molecular weight protein assemblies, visualized as puncta in cytosol, and the polymerization of Dvl is believed to be crucial for the initiation of Wnt signaling (5–9). Recently, the endogenous Dvl proteins were shown to display a diffuse or small punctate pattern in contrast to the heavy puncta of ectopically expressed Dvl (9, 41, 42). Bienz and co-workers (5, 8) have demonstrated that Dvl-DIX can undergo reversible polymerization/dem polymerization and that endogenous Dvl2 forms far smaller oligomers, likely trimers. Importantly, the membrane-associated Dvl after Wnt stimulation appears to form smaller punctates than those in cytosol, suggesting that the large Dvl polymer undergoes a transient disassociation and subsequent translocation to the plasma membrane as low molecular weight oligomers (6, 7, 9, 43–45). Moreover, casein kinase Iε, a positive regulator of Wnt signaling, is able to bind to the PDZ and DEP domains of Dvl, phosphorylates it within a basic region, and induces a relatively even distribution within the cytoplasm in response to Wnt signal (46–48). Together, these results have indicated that the biologically active Dvl assemblies are in fact low molecular weight oligomers but not large punctate polymers as suggested by overexpression systems.

In the present study we observed that the positive Wnt regulator Ccd1, upon binding to Dvl, attenuates the homo-assembly of Dvl in vitro and leads to diffused cytoplasmic distribution of Dvl in vivo. Of a particular note, we have resolved a paradox between the observations that Dvl needs to homopolymerize for Wnt activation and that Ccd1 acts to depolymerize Dvl in Wnt activation. It is clear that a prior requirement for Dvl to exert its positive role in Wnt signaling is to be able to form a homo-assembly of at least a dimer, in fact, as aforementioned a trimer. In the resting state, Dvl proteins accumulate in cytosol and form large punctate polymers that may preclude the access of other Wnt components, including Axin, due to the tight self-association. The Dvl assemblies are dynamic, and Wnt stimulation triggers a membrane translocation of the low molecular weight Dvl oligomers by direct interaction with Frizzled receptors. Recently, the activation of Wnt signaling was found to coincide with the increase of Ccd1 protein level due to a decrease in its ubiquitin-mediated degradation (49). Thus, in response to Wnt stimulation, Ccd1 is somehow accumulated in cytosol, then recruited to the Dvl
Wnt Activation by Ccd1

polymer and concomitantly depolymerize it, yielding the biologically active trimer. Under this conjecture, Dvl, by virtue of this reversible homo-assembly, is able to both keep the Wnt activity in check and to stimulate Wnt signaling, depending on the availability of Wnt signal.

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