Published in final edited form as:

Acta Physiol (Oxf). 2012 January ; 204(1): 65–73. doi:10.1111/j.1748-1716.2011.02291.x.

Dishevelled C-terminus: prolyl and histidiny motifs

Hsien-yu Wang¹ and Craig C. Malbon^{2,*}

¹Departments of Physiology & Biophysics, Health Sciences Center, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY 11794-8661 U.S.A.

²Department of Pharmacology, Health Sciences Center, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY 11794-8651 U.S.A.

Abstract

The phosphoprotein scaffold Dishevelled is an essential component of both Wnt signaling and of the signalsome that constitutes the supermolecular “*punctae*” of assembled proteins often observed in fluorescence microscopy. The C-terminal region beyond the DEP domain displays unique and interesting character, exploited herein by careful analysis of the primary structure. Human Dishevelled-1, -2, -3 and fly Dishevelled (Dsh) sequences were downloaded and interrogated *in silico*. The C-terminus of Dishevelled-3 is revealed by FoldIndex® to be rich in ordered structure. It displays primary sequence that is unique and divergent in important ways from vertebrate isoforms as well as from the fly Dsh. The region is amphipathic, high in prolyl content, and harbors polyprolines. Dishevelled-3 displays some regions where the proline content is >40%. Polyprolyl sequences (2–4 residues) likely constitute important sites of interaction with other Dishevelled isoforms. Several histidine-single amino acid repeats are notable. The 637,638/647,648 repeats of Dvl3 are essential for Wnt non-canonical, but not canonical signaling. Mutagenesis reveals that the C-terminal sequence is essential for the formation of punctae, made visible by fluorescence microscopy. These Dvl3-based signalsomes are very large (25–35 MDa MW), supermolecular complexes that display dynamic reorganization in response to Wnt stimulation. Dishevelled-3 C-terminus is rich in structure and unique motifs, worthy of detailed analysis with modern molecular tools.

Keywords

canonical; complexes; Dishevelled; Dishevelled-3; steric-exclusion chromatography; fluorescence correlation spectroscopy; proteomics; punctae; signalsome; Wnt signaling

Introduction

Wnt signaling in development remains a topic of intense research. Important new discoveries emerge (*e.g.*, roles of LRP5/6 role, heterotrimeric G proteins, trafficking of Dvl to nucleus, discovery of Ror, post-translational modifications of and purification of Wnt3a/5a, novel nuclear Tcf/β-catenin complexes, LIN-18 (Ryk), physical isolation of signalsomes

*Corresponding author Craig C. Malbon, Department of Pharmacology, Health Sciences Center, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY 11794-8651 U.S.A, craig@pharm.sunysb.edu.

Authors' contributions

The authors contributed equally to the evolution of the study design; the authors contributed equally, performed the studies, gathered the data, and outlined a draft of the manuscript. HYW wrote the manuscript; CCM edited the drafts. Both authors have read and approved the final version of this unpublished work.

Conflict of interest

The authors declare that they have no competing interests.

mediating Wnt action, *etc.*), adding new challenges to our thinking (Cadigan & Nusse 1997, Habas *et al.* 2001, He & Axelrod 2006, Bilic *et al.* 2007, Zhang *et al.* 2007, Egger-Adam & Katanaev 2008, Zeng *et al.* 2008, Angers & Moon 2009, Bikkavilli & Malbon 2010, Gao & Chen 2010, Schulte 2010). Many key questions remain unresolved and will require concerted efforts to explore. The very existence of 10 Frizzleds (Fz) and 19 Wnt ligands in mammals represents a daunting task for those studying the full complement of Wnt signaling. Which Wnts bind to each Fz? Do some Wnts act as partial agonists or antagonists of other Wnts? How many different Wnts is each Fz capable of binding? What is the role of Lrp5/6 in signaling by Fzs, other than Fz1? Epistasis studies in the fly suggest a vector-like flow of information from Wnt>Fz>Go>Dsh for the canonical and PCP pathways (Egger-Adam & Katanaev 2008), which may or may not be true in all species. What is the protein-protein signal linkage map for Wnts in mammals? Do Fz, Lrp5/6 (with Axin), G proteins, Dvls operate in a vectorial arrangement or as a multimeric network integrating signals from a variety of parallel or intersecting pathways? What is the functional significance of Dvl isoforms in vertebrates? Downstream of Dvl, how do canonical and non-canonical pathways signal and integrate Wnt signals?

Results first reported from studies conducted with the mouse totipotent embryonal teratocarcinoma F9 cells on Frizzled signaling (Liu *et al.* 2001) have been confirmed and extended in *Xenopus*, zebrafish, and more recently the fly (Egger-Adam & Katanaev 2008, for timely review, see Schulte 2010). For the canonical pathway signaling the working scheme (drawn from published work derived from flies to the mouse F9 cells) reveals Frizzled-1 signaling to Go (to Dvl) and to Gq (to PLC β) (Liu *et al.* 2001). For the non-canonical pathway, studies reveal Frizzled-2 signaling to Go (to Dvl, by KD studies and by analogy to Frizzled-1) and to Gt2 (to PDE6) and G $\beta\gamma$ to PLC β (Ahumada *et al.* 2002). Studies of regulation of intracellular concentrations of cyclic GMP and Ca²⁺ highlight the critical roles of cyclic GMP-dependent protein kinase (PKG) and the MAP kinase, p38 (Wang & Malbon 2003). To date, all Frizzled signaling (*i.e.*, canonical, non-canonical, and PCP) agrees on one basic observation, *i.e.*, Wnt action is dependent upon Dsh/Dvls (Gao & Chen 2010).

As highlighted in a 2006 Wnt meeting report, “*Dishevelled is required for multiple Wnt/Fz pathways but its function remains enigmatic*” (He and Axelrod 2006). Five years later, much new information has accumulated about Dishevelleds, but new puzzles emerge. Additionally, the presence of multiple Dishevelleds (*i.e.*, Dvl1, Dvl2, Dvl3) in mammals, *C. elegans*, zebrafish, and elsewhere in the animal kingdom versus apparently a single (fly) Dishevelled, continues to challenge current paradigms that suggest simple redundancy. The studies of knock-out (KO, by gene disruption) mice for Dvl1, Dvl2 and Dvl3 have been insightful and clearly reflect alterations in cell signaling (and behavior), although some level of redundancy appears to operate since at least the Dvl1^{−/−} KO mice develop and procreate essentially normally (Etheridge *et al.* 2008). Analysis of KO mice suggests that loss of Dvl3 may have greater impact than loss of either Dvl1 or Dvl2 (Etheridge *et al.* 2008). Knock-down studies of Dvls in mouse F9 cells clearly point to unique roles of Dvl3, in particular, that are hard to square with simple redundancy (Lee *et al.* 2008).

Careful analysis of the primary sequence and motifs of Dvl3 (Tsang *et al.* 1996) reveals several major unresolved questions. Firstly, what is the full range of Dishevelled-interacting proteins (DIPs) for those sequences of Dvl3 that are not shared among Dvl1, Dvl2, and Dsh? Thus, the highly-conserved domains among Dvls (e.g., DIX, PDZ, and DEP) are not a target in the current interrogation. Secondly, what are the structure/function implications for those regions of Dvl3, unique among Dvls? This is not to suggest that potential sites of phosphorylation, docking of other proteins, *etc.* has not been reported in a few cases for these regions (highlighted below), but that our understanding of the functional roles of these

other conserved sequences is far, far less than that of the signature DIX, PDZ, and DEP domains of Dsh/Dvls.

The three prominent and well-studied domains provide major landmarks for Dsh/Dvl: DIX, PDZ, and DEP (Fig. 1). All three are multivalent, recurrent in proteins involved in cell signaling networks, and well analyzed at the structural level. The remaining sequences of Dvl3 are for the most part, highly conserved among Dsh/Dvl, and can be segregated into several domains. A 20 amino acid, positively-charged domain (PCD) positioned between DIX and PDZ domains which displays 12 basic, one acidic, and several hydrophobic residues is homologous to the membrane effector domain (MED) found in MARCKS, EGFR, K-ras, and several A-kinase anchoring proteins 5 and 12 (AKAP, Tao *et al.* 2006). This region could function to increase local concentration of Dvls at the membrane, bind Ca^{2+} /calmodulin, and bind/sequester phosphatidylinositol 4,5-bisphosphate (PIP2) to its local domain. The dynamic spatial localization of Dvl3 may be explained, in part, by the presence of this PCD, a hypothesis worthy of investigation. Additionally, it has been shown that short, positively-charged regions may interact with phosphatidylinositol 4,5-bisphosphate/ phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the cell membrane, in a manner that amplifies the electrostatic interactions with the negatively charged PS that composes about 30% of the inner leaflet of the cell membrane (Tao *et al.* 2006). In the middle of the Dsh molecule is a Nuclear Localization Sequence (NLS, *IVLTV* at 329) critical to nuclear localization in the fly, but mutated to *ITLTV* in the mouse and human Dvl3 (Tsang *et al.* 1996). Although such a mutation leads to loss-of-function for this NLS in the fly, the mouse sequence does not conform to mammalian NLS and would likely be non-functional. Next to the “pseudo” NLS sequence is a region that has been termed a “proline-rich” domain (PRD). This region harbors a Src Homology 3 domain, SH3D, (Fig. 2), but is not nearly as rich in prolyl residues as two PRDs C-terminally to the SH3 domain (PRD1 and PRD2). Each of these domains is highly-conserved among the Dvls, although poorly characterized as to their function(s) in Wnt signaling or Dvl biology. The greatest variability among Dvls is found in the C-terminal third of the molecules. Within this region are several prominent sequences that are truly unique to Dvl3, deviating from or absent in the other Dvls/Dsh (highlighted in red, Fig. 3).

Functional features of the primary sequence of the Dvl3 C-terminus are the focus of this analysis. The sequence of each of these regions unique to Dvl3 constitute high-value targets for detailed study, displaying conservation to level of near identity (>95%) when comparing the human, *Xenopus* and zebrafish Dvl3, somewhat lesser for that in comparison to that of *C. elegans*.

Primary sequences and software

The primary sequence for human Dvl1, Dvl2, Dvl3 and fly Dsh were downloaded from the National Center of Biotechnology Information (NCBI). The sequences were interrogated, compared, and displayed using NCBI software. The relative amounts of natively ordered and unordered structure of Dishevelleds were analyzed using the *FoldIndex*® software (Prilusky *et al.* 2005).

Dishevelled-interacting proteins

The role of protein-protein interactions and of domains/motifs in signaling needs little emphasis. One notes from the *FoldIndex* plot of primary sequence information of Dvl3 (Prilusky *et al.* 2005) that more than half of Dishevelled is folded, *i.e.*, ordered structure, distributed over three large clusters, in which domains can be specified (Fig. 2). Whether they be large (*e.g.*, DIX, PDZ, DEP) or small (*e.g.*, *PXXP*), protein domains, modules, motifs (modulated by post-translational modifications, such as protein phosphorylation)

these landmarks of Dvl3 constitute the physical basis of Wnt signaling. What has become more apparent is the role of multivalency of signaling proteins, highlighted by scaffolds (*e.g.*, AKAPs, the scaffold protein JNK-interacting protein, *e.g.*, JIP1, *etc.*), and here Dsh/Dvls emerge as multiprotein, supermolecular “scaffolds”. The three best known motifs (*i.e.*, DIX, PDZ, DEP), all of which have been subjected to detailed functional and X-ray crystallographic analysis, provide docking sites for many Dishevelled-interacting proteins (DIPs, see Malbon & Wang 2006). For the DIX domain, Dsh, Dvl1, Dvl2, Dvl3, Axin, Frodo, Dapper, Pak1 are known DIPs. For the PDZ domain, docking partners include Frizzled-1, β arrestin, CK1, CK2, Dapper1, EPS8, GBP/Frat-3, Idax, Nkd1/2, PP2C, and Stbm. For DEP, Daam1, Pak1, Rac, Musk are but a few DIPs. Four “linker regions” (LNK) bridge well-known domains. The functional role(s) of these linker regions as potential docking sites remains poorly interrogated. LNK1, between DIX and PCD, may bind Pak1, Notch, and others DIPs. The positively-charged domains displaying a cluster of basic amino acids may bind the negatively-charged phosphatidylserine, lipids, and/or membrane proteins embedded in the cell membrane. LNK2, between PCD and PDZ domains, may dock Diego, PP2C, and others proteins. LNK3, between PDZ and DEP, has no known or suspected docking DIPs. Finally, LNK4, between DEP and the C-terminal region (CTM) of Dvl3, displays proline-rich domains (Fig. 2).

What stands out in this analysis is the relative paucity of Dishevelled-interacting proteins localized to the C-terminal region of the molecule. In comparison, multivalency of the well-known DIX, PDZ, and DEP domains is relatively high (Fig. 1). This is true especially for the Dvl3 sequence. Within the Dishevelled family, Dvl3 is the most unique and divergent especially in the C-terminal region (downstream of the DEP domain). The C-terminal (479–716) will be the primary focus of further in depth *in silico* analyses.

Dishevelled C-terminus

C-terminal Sequence Divergence among Dishevelleds

The unique character of Dishevelled-3, not shared with either Dvl1 or Dvl2, is displayed (see “red”-lettered sequence in Fig. 3). The bulk of the differences are found C-terminally of the DEP domain at residues 497 of Dvl3. Dvl2 has 20 additional amino acids in its sequence than does Dvl3; whereas Dvl1 has 46 fewer amino acids. Nearly 25% of this C-terminal region of Dvl3 is unique, replete with motifs of interest, and has been speculated to harbor several important functions. Dominant among the unique native substitutions found only in the Dvl3 C-terminus are those for proline (9/56) and for histidine (7/56). According to the alignment generated (Fig. 3), the similarities among the three mammalian Dvl3 and *Drosophila* Dsh can be discerned. Of the 56 native substitutions found uniquely in human Dvl3 (absent from hDvl1 and hDvl2), 29 of these native substitutions have been conserved from Dsh, although the fly Dsh is 87 amino acids shorter in its C-terminus. The dominant attention paid to the well-known DIX, PDZ, and DEP domains has resulted in relatively little attention devoted to study of the C-terminal “domain” downstream of the DEP. To fully lift up the most important structural features for analysis of their possible role in Wnt signaling we shall address: hydrophobicity/amphipathy; prolyl content and motifs; and, histidine single-amino acid repeats.

Hydrophobicity/Amphipathy of Dishevelled-3 C-terminus

The C-terminal tail of Dvl3 has a decidedly hydrophobic character, displaying more than 35% hydrophobic residues (Fig. 4). The hydrophobic residues are not localized to any one region of the C-terminus, but rather distributed throughout the 220 amino acid length of this region. Hydrophilic, polar amino acid residues constitute more than 20% of the Dvl3 C-terminus. Charged residues (positively-charged, 13%; negatively-charged, 9%) sum to more

than 20% also in the C-terminus. This abundance of polar and charged amino acyl residues suggests that the Dvl3 C-terminus should display relatively high water-solubility and exposure in an aqueous environment. We speculate that the amphipathic character of the C-terminus of Dishevelled is consistent with this region being displayed and available for docking by other molecules in its native state.

Dvl3 C-terminus Displays High-proline Content

One of the most striking and poorly understood structural features of vertebrate Dishevelled C-termini is the abundance of prolyl residues, *i.e.*, proline-rich domains (especially PRD1 and PRD2, Figs. 2 and 4). The cyclic structure of proline provides a unique degree of rigidity. Prolines are also found as short polymers (polyprolyl stretches) that provide for a helical turn in the protein that positions the proline in the aqueous phase of solvent even though the proline has a side chain which is aliphatic. The fly Dsh has much lower proline content than do the mammalian Dvls. The content of proline in the hDvl3 C-terminus actually is not “garden-variety” for proteins, but remarkably high at 33 residues achieving >15%. Starting from the C-terminal methionine and moving N-terminally 40 residues, we encounter proline and polyproline (*i.e.*, ProPro and ProProProPro) residues that mark the overall content to that position as about 20%. If we move N-terminally another 20 residues, the content of proline now approaches 25%. Two short regions in the C-terminus contain remarkable, perhaps unprecedented, content of proline for non-structural proteins, 36% (651–692) and 43% (523–554)! The functional significance of these remarkable high-Pro content stretches in vertebrate Dishevelleds has not been reported, although worthy of thoughtful interrogation. It seems likely, although only speculation, that the polyproline regions of Dishevelleds may act as they do in structural proteins to stabilize non-covalent interactions among the three mammalian Dvls. Dvl2, which exists in nearly 50-fold greater abundance than Dvl1 or Dvl3 (Lee *et al.* 2008), may provide a structural network through which Dvl3- and Dvl1-based complexes can interact dynamically, spatially, and temporally.

Histidine Single-amino Acid Repeats in Dvl3

The content of histidine in the C-terminal regions of Dishevelleds is not remarkable. For Dvl3 C-terminus, the histidinyl content is 6%, which is average representation in proteins. What is remarkable and observed in Dvl3 alone of the Dishevelled family is the presence of two histidinyl single-amino acid repeats (Fig. 4). At positions 637,638 and again at 647,648 are His repeats that are unique to Dvl3 and shown recently to be of key functional significance. In mouse totipotent embryonal teratocarcinoma F9 cells Wnt signaling to the non-canonical pathways (*i.e.*, Wnt5a/Frizzled-2/Gt/PDE6/cGMP and Ca²⁺ mobilization/NF-AT activation) requires all three Dvls (Ma *et al.* 2010), although the signaling was most affected by reductions in the expression of Dvl3. Deletion of the C-terminus tail of Dvl3 (631–650) has little effect on the ability of Wnt3a to activate Lef/Tcf-sensitive transcription via the Wnt canonical pathway (Figs. 4 and 5A). Treating the mouse F9 cells with siRNA specified to knock-down Dishevelled-3 expression blocks the canonical pathway from activation of Lef/Tcf-sensitive gene activation (Fig. 5, Ma *et al.* 2010). This knock-down can be overcome (*i.e.*, “rescued”) with exogenous expression of the human Dvl3 (hDvl3), which is not susceptible to the siRNA. Loss of the two histidinyl single-amino acid repeats found in the C-terminus of the hDvl3 (hDvl3 Δ 631–650) had no effect on the ability of Wnt3a to signal via the Wnt/beta-catenin canonical pathway (Fig. 5A). For the non-canonical Wnt signaling pathway, the expression of Dvl3 C-terminus alone functioned as a dominant negative, suppressing Wnt5a-stimulation of NF-AT-sensitive gene expression (Fig. 5B, Ma *et al.* 2010). Deletion of the 631–650 region harboring the two His single-amino acid repeats reversed the dominant negative property of the Dvl3 C-terminus. Furthermore, histidine-to-alanine-based substitution mutagenesis of the Dvl3 C-terminal His single amino acid repeats alone (HH637,638AA and HH647,648AA, Fig. 5B), abolished the

ability of the Dvl3 C-terminus to function as a dominant negative in non-canonical pathway. Thus, the two His single-amino acid repeats unique to Dvl3 alone are essential for Wnt5a-stimulated NF-AT-sensitive gene activation by the non-canonical pathway (Fig. 5A, B).

Dishevelled C-terminal interacting proteins

Several Dishevelled-interacting proteins have been localized to the C-terminus of Dishevelleds. The Cullin-3 substrate adaptor Kelch-like 12 (KLHL12) provides the ability of an E3 ubiquitin ligase complex to dock to Dishevelled in a Wnt-dependent manner (Angers *et al.* 2006). Docking of the complex promotes poly-ubiquitination and degradation of Dishevelled, which negatively regulated the Wnt/ β -catenin/Lef-Tcf-sensitive activation of gene expression (Angers *et al.* 2006). These were the first data to implicate Dishevelled as a target for a distinct ubiquitin ligase complex, operating via a C-terminal region of the Dishevelled itself.

The K-homology splicing regulator protein (KSRP) was isolated by affinity purification using the Dishevelled-3 C-terminus as “bait” followed by identification of the “prey” by ion-trap mass spectrometry-based proteomics (Bikkavilli & Malbon 2010). This Dishevelled-interacting protein negatively regulates Wnt/ β -catenin signaling at the level of post-transcriptional mRNA stability of β -catenin. Although much investigation of the Wnt canonical pathway had been focused on the transcriptional regulation of β -catenin, prior to this study there was little appreciation for a role of post-transcriptional stability in this pathway. Thus, at the protein level GSK3 β -catalyzes phosphorylation of β -catenin that provokes degradation of β -catenin. KSRP also docks to the C-terminus of Dishevelled, but was found to operate at the level of RNA stability on β -catenin mRNA (Bikkavilli & Malbon 2010).

Another recently identified signaling protein that docks to the C-terminus of Dishevelled is the receptor tyrosine kinase Ror2 (Witte *et al.* 2010). It is of interest that the functional role of Ror2, like ubiquitination of Dvl and destabilization of β -catenin mRNA, is to negatively regulate Wnt signaling, both the β -catenin/Lef-Tcf-sensitive gene expression of the canonical pathway as well as the Wnt5a/Ror2, ATF2 pathway (Witte *et al.* 2010). Further, in this regard, the central role of phosphorylation and hyperphosphorylation (in which Dvl displays a “shifted” electrophoretic mobility on gel electrophoresis) of Dishevelled sequentially activates and later inactivates the scaffold protein (Bernatik *et al.* 2011). The hyperphosphorylation of Dishevelled (mediated by Dvl C-terminus) deactivates and attenuated the ability of Dishevelleds to interact with each other and form the large punctae (Bernatik *et al.* 2011). These are very important observation as they provide a strong linkage between Dishevelled phosphorylation/function and the formation of the large punctae, visible by fluorescence microscopy. It has been proposed that such Dishevelled-based punctae are essential for Wnt signaling (Wang & Malbon 2011). Bernatik *et al.* (2011) provide compelling evidence that phosphorylation by casein kinase 1 δ/ϵ sequentially regulates activation and then de-activation of Dishevelled.

Discussion

Wnt signaling continues to challenge existing models, as we begin to discern large-scale punctae (*i.e.*, Dishevelled-based supermolecular complexes) through which Dishevelleds, DIPs, and key molecules of intersecting pathways may interact. The earliest insights gained about these large scale assemblies came from studies of Dishevelleds by immunolocalization/immunofluorescence studies which revealed “punctate” structures of Dvls (Schwarz-Romond *et al.* 2005, 2007). These “punctae” were shown to be assemblies of proteins (absent any liposome attachment) whose size and cellular distribution were

influenced by Wnt. Phosphorylation is a central mechanism of regulating docking of signaling proteins to the Dishevelled scaffold (Yokoyama & Malbon 2007, Yokoyama *et al.* 2007, Yokoyama & Malbon 2009, and reviewed by Witte *et al.* 2010). Later studies revealed that these dynamic multiprotein assemblies were operating in Wnt signaling (Yokoyama *et al.* 2010), hence termed by analogy as “signalsomes” (Luttrell 2005, Kendall & Luttrell 2009).

The work of Bernatik *et al.* (2011) reported that phosphorylation by CK1 ϵ of the C-terminus of Dishevelled regulates function and punctae formation, highlighting the need for careful physical analysis of Dishevelled-based punctae (*i.e.*, supermolecular complexes also known as signalsomes). Recent physical, fluorescence, and proteomic data both *in vitro* and *in situ* in live cells reveals much more about the nature, size, and localization of these signalsomes, as Dvl3-based supermolecular complexes (Yokoyama *et al.* 2010). According to steric-exclusion chromatography (SEC) on wide-bore gel packed in very long columns, Dvl3-based signalsomes isolated biochemically from F9 and HEK293 cells minimally are assemblies displaying M_r of 1–2 MegaDa (MDa). Upon activation of canonical pathway in these cells with Wnt3a, these assemblies reorganize and display M_r of much larger dimensions, 5–7 MDa. This is a very large upfield shift in apparent size, occurring early, in advance of the activation of the canonical transcriptional pathway. Fluorescence correlation spectroscopy, *fcs*, allows measurement of the diffusion coefficient of the such assemblies (eGFP-Dvl3-based signalsomes) *in situ* from which an MW can be calculated (Yokoyama *et al.* 2010, Wang & Malbon, 2011). By *fcs* the size (MW) of the Dvl3-based signalsomes in live cells was estimated at 25–35 MDa. *Fcs*, like SEC, reported a 5–7 MDa upfield shift in MW when the *fcs* was performed in cells treated with Wnt3a. The difference in apparent size of the Dvl3-based signalsomes in untreated cells deduced by *fcs* may well represent the effects of SEC shear force on larger scale (25–35 MDa) complexes, disrupting weaker binding of components or oligomeric partners to the Dvl3-based complex. The take home message is that Dvl3-based punctae observed by fluorescence microscopy have been revealed to be very large signalsomes by two independent measures, one physical, the other optical. Additionally, Dvl3-based assemblies/signalsomes are provoked by Wnt3a treatment of the cells to even larger scale assemblies, displaying an upfield shift of 5–7 MDa, observed for both SEC and *fcs*.

Proteomics of the SEC-isolated signalsomes from peaks of 2 and 7 MDa- M_r peak fractions, performed by liquid chromatography electrospray ionization tandem mass spectrometry (LQ-ESI-MS-MS), reveals more about the nature of these Dvl3-based supermolecular complexes (Wang & Malbon 2011). Such signalsomes include all three Dvl isoforms, consistent with earlier studies. The signalsomes include many key molecules presumed to be present in such assemblies (*e.g.*, Axin, glycogen synthase kinase 3- β , CK1, CK2), not unlike the cartoon of DIPs offered on websites, such as the Wnt home page (<http://www.stanford.edu/group/nusselab/cgi-bin/wnt/>). We now envision such complexes in different terms. New physical evidence enables us to integrate facts already in evidence, *i.e.*, that Dvl-based supermolecular complexes are large punctae which dock protein kinases, phosphatases, adaptor molecules, etc. critical to Wnt signaling. Studies of mutations of Dvl2/Dvl3 that block punctae formation likewise display functional consequences; they block the ability of the canonical pathway to function (Schwarz-Romond *et al.* 2005, 2007, Ma *et al.* 2010, Bernatik *et al.* 2011). Thus, although very new and perhaps heretical, our new working model of Dvl-based signalsomes accommodates important physical and functional properties of these large signaling complexes (Fig. 6). The assembly of very large complexes about Dishevelleds and organizing in a manner that is essential for Wnt signaling has become a new paradigm, one worthy of intense interrogation. Of interest is the C-terminal region of Dishevelled-3, whose continued study will likely provide a rich new source of understanding how its unique sequence motifs couple to function.

Abbreviations

AKAP	A-kinase anchoring proteins
DIPs	Dishevelled-interacting proteins
Dvl1	Dishevelled-1
Dvl2	Dishevelled-2
Dvl3	Dishevelled-3
Dsh	Fly Dishevelled
F9	mouse totipotent embryonal teratocarcinoma F9 cells
HEK	human embryonic kidney 293 cells
<i>fcs</i>	fluorescence correlation spectroscopy
JIP	scaffold protein JNK-interacting proteins
LQ-ESI-MS-MS	liquid chromatography electrospray ionization tandem mass spectrometry
PCD	positively-charged domains
SEC	steric-exclusion chromatography

Acknowledgments

We would like to thank Mr. Paul Stern (Department of Pharmacology, Stony Brook University, NY) for his invaluable assistance in the computational aspects of the *in silico* analysis. This research was supported by United States Public Health Services grant GM69375 from the NIGMS (to HYW) and grant DK30111 from the NIDDK (to CCM), National Institutes of Health. The New York STEM Cell program of NYSTEM provided critical support for the work (HYW).

References

- Angers S, Moon RT. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Nat Rev Mol Cell Biol.* 2009; 10:468–477. [PubMed: 19536106]
- Ahumada A, Slusarski DC, Liu X, Moon RT, Malbon CC, Wang HY. Signaling of rat Frizzled-2 through phosphodiesterase and cyclic GMP. *Science.* 2002; 298:2006–2010. [PubMed: 12471263]
- Bikkavilli RK, Malbon CC. Dishevelled-KSRP complex regulates Wnt signaling through post-transcriptional stabilization of beta-catenin mRNA. *J Cell Sci.* 2010; 123:1352–1362. [PubMed: 20332102]
- Bilic J, Huang YL, Davidson G, Zimmermann T, Cruciat CM, Bienz M, Niehrs C. Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science.* 2007; 316:1619–1622. [PubMed: 17569865]
- Bernatik O, Ganji RS, Dijksterhuis J, Konik P, Cervenka I, Polonio T, Krejci P, Schulte G, Bryja V. Sequential activation and inactivation of dishevelled in the Wnt/b-catenin pathway by casein kinases. *J Biol Chem.* 2011; 286 in the press.
- Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. *Genes Dev.* 1997; 11:3286–3305. [PubMed: 9407023]
- Egger-Adam D, Katanaev VL. Trimeric G protein-dependent signaling by Frizzled receptors in animal development. *Front Biosci.* 2008; 13:4740–4755. [PubMed: 18508542]
- Etheridge SL, Ray S, Li S, Hamblet NS, Lijam N, Tsang M, Greer J, Kardos N, Wang J, Sussman DJ, Chen P, Wynshaw-Boris A. Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. *PLoS Genet.* 2008; 4:e1000259.

- Gao C, Chen YG. Dishevelled: The hub of Wnt signaling. *Cell Signal*. 2010; 22:717–727. [PubMed: 20006983]
- Habas R, Kato Y, He X. Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell*. 2001; 107:843–854. 2001. [PubMed: 11779461]
- He X, Axelrod JA. WNTer wonderland in Snowbird. *Development*. 2006; 133:2597–2603. [PubMed: 16794030]
- Kendall RT, Luttrell LM. Diversity in arresting function. *Cell Mol Life Sci*. 2009; 66:2953–2973. [PubMed: 19597700]
- Luttrell LM. Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. *J Mol Neurosci*. 2005; 26:253–264. [PubMed: 16012199]
- Lee YN, Gao Y, Wang H-Y. Differential mediation of the Wnt canonical pathway by mammalian Dishevelleds-1, -2, and -3. *Cell Signal*. 2008; 20:443–452. [PubMed: 18093802]
- Liu T, DeCostanzo AJ, Liu X, Wang H, Hallagan S, Moon RT, Malbon CC. G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. *Science*. 2001; 292:1718–1722. [PubMed: 11387477]
- Ma L, Wang Y, Malbon CC, Wang H-Y. Dishevelled-3 C-terminal His single amino acid repeats are obligate for Wnt5a activation of non-canonical signaling. *J Mol Signal*. 2010; 5:19. [PubMed: 21092292]
- Malbon CC, Wang H-Y. Dishevelled: a mobile scaffold catalyzing development. *Curr Top Dev Biol*. 2006; 72:153–166. [PubMed: 16564334]
- Prilusky J, Felder CE, Zeev-Ben-Mordehai T, Rydberg EH, Maa O, Beckmann JS, Silman I, Sussman JL. FoldIndex©: a simple tool to predict whether a given protein sequence is intrinsically unfolded. *Bioinformatics*. 2005; 21:3435–3438. [PubMed: 15955783]
- Schulte G. International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors. *Pharmacol Rev*. 2010; 62:632–667. [PubMed: 21079039]
- Schwarz-Romond T, Fiedler M, Shibata N, Butler PJ, Kikuchi A, Higuchi Y, Bienz M. The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nat Struct Mol Biol*. 2007; 14:484–492. [PubMed: 17529994]
- Schwarz-Romond T, Merrifield C, Nichols BJ, Bienz M. The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles. *J Cell Sci*. 2005; 118:5269–5277. [PubMed: 16263762]
- Tao J, Shumay E, McLaughlin S, Wang H-Y, Malbon CC. Regulation of AKAP-membrane interactions by calcium. *J Biol Chem*. 2006; 281:23932–23944. [PubMed: 16762919]
- Tsang M, Lijam N, Yang Y, Beier DR, Wynshaw-Boris A, Sussman DJ. Isolation and characterization of mouse dishevelled-3. *Dev Dyn*. 1996; 207:253–262. [PubMed: 8922524]
- Wang H-Y, Malbon CC. Wnt signaling, Ca²⁺, and cGMP: visualizing Frizzled functions. *Science*. 2003; 300:1529–1530. [PubMed: 12791979]
- Wang H-Y, Malbon CC. Probing the physical nature and composition of signalsomes. *J Mol Signaling*. 2011; 6:1.
- Witte F, Bernatik O, Kirchner K, Masek J, Mahl A, Krejci P, Mundlos S, Schambony A, Bryja V, Stricker S. Negative regulation of Wnt signaling mediated by CK1-phosphorylated Dishevelled via Ror2. *FASEB J*. 2010; 24:2417–2426. [PubMed: 20215527]
- Yokoyama N, Golebiewska U, Wang H-Y, Malbon CC. Wnt-dependent assembly of supermolecular Dishevelled-3-based complexes. *J Cell Sci*. 2010; 123:3693–3702. [PubMed: 20940260]
- Yokoyama N, Malbon CC. Phosphoprotein phosphatase-2A docks to Dishevelled counterregulates Wnt3a/beta-catenin signaling. *J Mol Signal*. 2007; 2:11. [PubMed: 17961224]
- Yokoyama N, Yin D, Malbon CC. Abundance, complexation, and trafficking of Wnt/beta-catenin signaling elements in response to Wnt3a. *J Mol Signal*. 2007; 2:12. [PubMed: 17961225]
- Yokoyama N, Malbon CC. Dishevelled-2 docks and activates Src in a Wnt-dependent manner. *J Cell Sci*. 2009; 122:4439–4451. [PubMed: 19920076]

- Zhang X, Zhu J, Yang GY, Wang QJ, Qian L, Chen YM, Chen F, Tao Y, Hu HS, Wang T, Luo ZG. Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat Cell Biol.* 2007; 9:743–754. [PubMed: 17558396]
- Zeng X, Huang H, Tamai K, Zhang X, Harada Y, Yokota C, Almeida K, Wang J, Doble B, Woodgett J, Wynshaw-Boris A, Hsieh JC, He X. Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development.* 2008; 135:367–375. [PubMed: 18077588]

Dishevelled-3 (mouse): Dishevelled-interacting proteins (DIPs)

Figure1.

Linear diagram of mouse Dishevelled-3 known structural domains and Dishevelled-interacting proteins (DIPs) known or suspected (not exhaustive). The three key landmarks for orientation on Dishevelled-3 are as follows: DIX, Dishevelled and AXin (DIX) binding domain at the N-terminus; PDZ, a Post-synaptic density-95, Discs-large and Zonula occludens-1 domain in the mid region of Dvl; and a DEP, Dishevelled, Egl-10, Pleckstrin (DEP) domain located about midway between the PDZ domain and the C-terminus of Dvl. Additional domains include the Positively-Charged Domain (PCD), a Src Homology-3 domain (SH3D), a putative Nuclear Localization Site (NLS), a putative Nuclear Export Sequence (NES), and two Proline-Rich Domains (PRD1 and PRD2). The docking proteins are identified by their common abbreviation. For additional details and original references, please see Malbon & Wang 2006, from which the figure is adapted.

Figure 2.

FoldIndex® analysis of human Dishevelled-3. The sequence was analyzed by *FoldIndex®* (Prilusky *et al.* 2005). The DIX, PDZ, and DEP domains are indicated, as are the positively-charge domains (PCD), Src-homology domain 3 (SH3D), proline-rich domains (PRD), a unique domain (UNQ), and four linker domains (LNK1–4), and lastly the C-terminal region (CTM). For details, please see the text.

HDv12	PERREARKYASGLLKAGLIRHTVNKITFSEQCYIVFG-----DLSGGCESYLVNLSLNDN	524
HDv13	TDRREARKYASNLLKAGFIRHTVNKITFSEQCYIIFG-----DLCG----NMANLSLHDH	509
HDv11	KERREARKYASSLLKHGFLRHTVNKITFSEQCYIVFG-----DLCs----NLATLNLNS-	486
DshDros	QDRREARRIVSAMLRSNYIKHTVNKLTFSEQCYIVVNEERNPNLLG--RGHLHPHQPLPHG	498
	:*****: . * : * : . : :*****:*****:.. : * . : . *	
HDv12	DGSSGASDQDTLAPLP--GATPWPLLPTFSYQYP-APHPYSPQPPPYHELSSYTYGGGSA	581
HDv13	DGSSGASDQDTLAPLPHPGAAPWPMMA--FPYQYPPPHPYNPHPG-FPELG-YSYGGGSA	565
HDv11	-GSSGTSQDTLAPLP-HPAAPWPLGQGYPYQYPGPPPCF--PPAYQDPG-FSYGSGST	540
DshDros	HGGHALSHADTESITS--DIGPLPNP---PIYMP-----YSATYNPSHGYQPI	541
	*. . * . ** : . * * . * : : * .	
HDv12	SSQHSEGSRSSGSTRSDGGAGRTGRPEERAPE SKSG-SGSESEPS SRGGS LRRGGEASGT	640
HDv13	SSQHSEGSRSSGSNRS--GSDRKEKD PKAGD SKSGSGSES DHTTR-SSLRGPRERAPS	622
HDv11	GSQQSEGSKSSGSTRS---SRRAPGREKEKERRAAGAGGSGSES DHTAP-----SGVGS	589
DshDros	QYGIAERHISSGSSSS---DVLTSKDTSASQSDITSVIHQANQLTI-----AAHGS	589
	: * ****. * : : : : : :	
HDv12	SDGGPPPSRGSTGG----APNLRAHPGLHPYGP PPGMALPYNPMMVVMPPPPPPVPPAV	696
HDv13	ERSGPAASEHSHRS HHS LASSLRSHHTHP SYGPP-GVPPLYGPPMIMMPPPP-----AAM	676
HDv11	SWRERPAGQLSRGS----SPRSQASATAPGLPPPHPTTKAYT-----VVG	630
DshDros	NKSSGS SNRGGGGG-----	603
		
HDv12	QPPGAPPVRDLGSVPPELTASRQSFHMAMGNPSEFFVDVM	736
HDv13	GPPGAPPGRDLASVPPELTASRQSFHMAMGNPSEFFVDVM	716
HDv11	GPPGGPPVRELAAVPPELTGSRQSFQKAMGNPCEFFVDIM	670
DshDros	GGGGNNTNDQ-----DVSVSNYVL-----	623
	*. . : : : * . :	

Figure 3.

C-terminal region of human Dishevelled-1, -2, and -3 as well as *Drosophila* Dsh: homology alignment. The primary sequence of each is displayed, commencing with the C-terminal boundary of the DEP domain (light grey font color) to the carboxy-terminal region (adapted from PubMed).

Hydrophobic, hydrophilic, positive-, and negatively-charged amino acids

```

481 vnkitfseqc yyifgdlcgn manlslhdhd gssgasdqdt laplphpgaa pwpmafpyqy
541 pppphhpynph pgfpelgysy ggsassqhs egsrssgsnr sgsdrrkekd pkagdsksgg
601 sgsesdhtttr sslrgprera psersgpaas ehshrshhsl asslrshhth psygppgvpp
661 lygppmlmmp pppaamgppg appgrdlasv ppeltasrqs frmamgnpse ffvdvm

```

Prolyl amino acid residues

```

481 vnkitfseqc yyifgdlcgn manlslhdhd gssgasdqdt lappphpgaa pwrmafpyqy
541 pppphpynph pgfpelgysy ggsassqhs egsrssgsnr sgsdrrkekd pkagdsksgg
601 sgsesdhtttr sslrgprera psersgpaas ehshrshhsl asslrshhth psygppgvpp
661 lygppmlmmp pppaamgppg appgrdlasv ppeltasrqs frmamgnpse ffvdvm

```

Histidines and histidinyl single-amino acid repeats

```

481 vnkitfseqc yyifgdlcgn manlslhdhd gssgasdqdt laplphpgaa pwpmafpyqy
541 pppphhpynph pgfpelgysy ggsassqhs egsrssgsnr sgsdrrkekd pkagdsksgg
601 sgsesdhtttr sslrgprera psersgpaas ehshrshhsl asslrshhth psygppgvpp
661 lygppmlmmp pppaamgppg appgrdlasv ppeltasrqs frmamgnpse ffvdvm

```

Figure 4.

C-terminal region of human Dsihevelled-3: structural analysis. The primary sequence of hDvl3 is displayed, commencing with the C-terminal boundary of the DEP domain (underlined) to the carboxy-terminal region, (adapted from *PubMed*). Hydrophobic residues are highlighted in green; hydrophilic residues are highlighted in blue; positively-charged residues are highlighted in purple; negatively-charged residues are highlighted in red. For details of the analysis of primary sequence and color-coding, please see the text.

Figure 5.

Histidine single-amino acids repeats found in the C-terminus of Dishevelled-3 at 637,638 and 647,648 are essential for Wnt non-canonical signaling, but non-essential for Wnt canonical signaling. The read-outs of the analysis of the deletion and substitution mutagenesis of the C-terminal tail of Dvl3 are as follows: Lef/Tcf-sensitive transcriptional activation in response to the absence (–) or presence (+) of purified Wnt3a (20 ng/ml for 6 hr, panel A); and, NF-AT-sensitive transcriptional activation in response to absence (–) or presence (+) of Wnt5a (50 ng/ml for 6 hr, panel B). Adapted from data presented elsewhere (Ma *et al.* 2010).

Dishevelled-3 signalsome subunit:
unstimulated cells
(2-5 MDa- M_r)

Figure 6.

Depiction of a Dvl3-based supermolecular complex also known as signalsome. Note that Dvl2-based polymers are acting as dynamic tethers to the signalsome subunit, acting via Dvl3. This Dvl3-based signalsome subunit is assumed to be the dominant form of Dvl3-based complexes that is resolved by SEC, whereas these same subunits may form large 25–35 MDa-MW signalsomes detected in live cells using *fcs*. Please see the text for detailed description of the Dishevelled-3 signalsome subunit.