Review

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Signalosome assembly by domains undergoing dynamic head-to-tail polymerization

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A key mechanism for guarding against inappropriate activation of signaling molecules is their weak affinity for effectors, which prevents them from undergoing accidental signal-transducing interactions due to fluctuations in their cellular concentration. The molecular devices that overcome these weak affinities are the signalosomes: dynamic clusters of transducing molecules assembled typically at signal-activated receptors. Signalosomes contain high local concentrations of protein-binding sites, and thus have a high avidity for their low-affinity ligands that generate signal responses. This review focuses on three domains – DIX (dishevelled and axin), PB1 (Phox and Bem1), and SAM (sterile alpha motif) – that undergo dynamic head-to-tail polymerization to assemble signalosomes and similar particles that require transient high local concentrations of protein-binding sites.

Dynamic head-to-tail polymerization as a molecular principle underlying signaling

Cells communicate with each other during development through a handful of signaling pathways that determine their fates. In adult tissues, these pathways often impose cell division over quiescence or differentiation, and their inappropriate activation tends to divert cells into excess proliferation, the root cause of cancer. Therefore, cells must ensure that their signal-responding molecules do not interact with each other accidentally, for example by random fluctuations in their expression levels and/or subcellular localizations which could promote inadvertent interactions between them, causing inappropriate signaling. This is avoided through signal-responding molecules having low affinities for their effectors, and this prevents them from undergoing fortuitous interactions with these effectors at their normal cellular concentrations.

It follows that cells are in need of molecular devices that allow signal-responders to interact with their low-affinity binding partners (including signaling effectors) once the extracellular signals have engaged with their receptors, typically at the plasma membrane. One such device is the so-called signalosome [1,2], a transient co-cluster of signal transducers and transmembrane receptors that forms following the binding of transmembrane receptors to extracellular signals. The two hallmarks of signalosomes are (i) their dynamic assembly and disassembly, allowing rapid response to differences in signaling amplitude and (ii) their high local concentration of protein-binding sites resulting in a high avidity for their effectors. This avidity enables them to interact efficiently with low-affinity ligands that are present at low levels and/or dispersed in cells. Signalosomes are thus efficient and sensitive signal-responding devices that amplify incoming signals and convert them into robust responses that can be relayed from the plasma membrane to the nucleus or other target sites within signal-responding cells.

Signalosome assembly requires 'polymerizers', that is, protein domains whose primary function is to undergo dynamic head-to-tail polymerization to generate high local concentrations of linked domains that bind to signaling effectors. This is exemplified by the DIX domain [1], a domain found exclusively in the Wnt pathway, an ancient signaling pathway conserved from the most primitive animals [3,4] all the way to humans. Wnt signaling controls numerous context-dependent transcriptional switches as well as planar cell polarity during animal development and in adult tissues [5], especially in stem cell compartments, explaining why the dysregulation of this pathway often causes cancer [6].

The closest structural relative of the DIX domain is the PB1 domain, which is found in all eukaryotes. Humans have >9 different PB1-containing proteins, some with multiple paralogs. This domain was discovered through sequence similarity between two yeast proteins required for polarized budding (Bem1 and Cdc24, the only two PB1 proteins in this species), and two mammalian proteins required for superoxide production by phagocytes ($p67^{phox}$ and $p40^{phox}$) to trigger innate immune responses [7]. Additional PB1 domains were found in other proteins mediating signaling and epithelial cell polarity, including atypical protein kinase (aPKC), MEKK2/3, and MEK5 (which activate mitogen-activated protein kinase), and the signaling adaptor p62, which is involved in NF-κB signaling [8,9]. PB1 domains have been classified into three types based on their mutual heterotypic interactions [10], but a subset of the PB1 domains undergo head-to-tail polymerization, as do their DIX relatives.

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Head-to-tail polymerization was first described for the SAM domain [11], which is structurally distinct from the DIX and PB1 domains. This is one of the most common domains, and is found in eukaryotic and prokaryotic proteins participating in diverse processes including signaling, transcriptional silencing, and assembly of RNA-containing particles [12]. Most SAM domains form polymers, and these range from highly dynamic to insoluble but structured protein aggregates [13]; the former but not the latter are suitable for signaling. This review focuses on the unique molecular property of all three domains – performing dynamic head-to-tail polymerization – which constitutes a pivotal molecular principle underlying signaling, and also touches on other cellular processes that depend on phase transitions of proteins from soluble to locally concentrated [14].

Assembly of Wnt signalosomes and degradasomes by DIX domains

Dishevelled and axin have opposite roles in the Wnt signaling pathway (Figure 1): axin functions in the absence of a Wnt signal, assembling a complex with the adenomatous polyposis coli (APC) tumor suppressor and glycogen synthase kinase 3 (GSK3) to promote the ubiquitylation and proteasomal degradation of the central signaling effector β-catenin. By contrast, dishevelled functions once a Wnt signal has engaged with the transmembrane receptors frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Consequently, dishevelled interacts simultaneously with Fz and axin to recruit the axin complex to the plasma membrane and inactivate it, thus allowing β-catenin to accumulate and act as a transcriptional coactivator of TCF/LEF factors [15]. Dishevelled and axin have the typical 'beads-on-a-string' architecture of signaling proteins, being composed of multiple protein-binding domains separated by long flexible linker sequences. In addition, they each contain an N- or C-terminal DIX domain that is essential for their functions in promoting or antagonizing Wnt signaling, respectively (Figure 1).

Dishevelled and axin form striking cytoplasmic puncta if overexpressed in cells (Figure 2A). Because of their size and shape, and because dishevelled can associate with membranes via Fz binding, these puncta were widely assumed to reflect membranous vesicles. However, this was refuted by two independent studies [16,17], which concluded that these puncta reflect membrane-free protein assemblies that form as a result of high protein levels. Endogenous dishevelled puncta can also be observed in normal cells with naturally elevated dishevelled levels (e.g., [18,19]) or in colon cancer cells whose dishevelled levels are elevated due to their chronically active Wnt

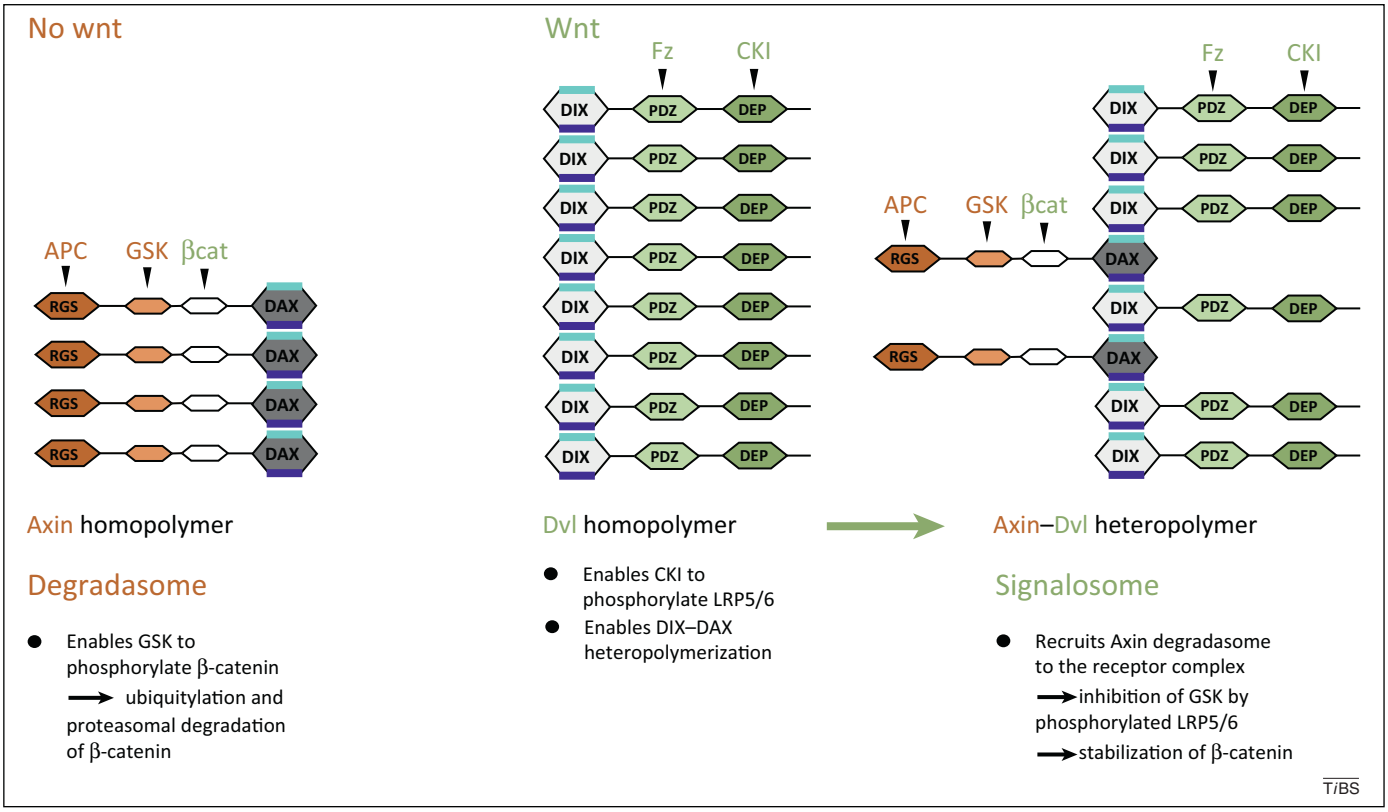

Figure 1. The signalosome hypothesis. Left, in the absence of Wnt, axin homo-polymerizes through its DIX domain (named DAX, to distinguish it from dishevelled DIX), to assemble degradasomes that bind to adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK), to promote the phosphorylation of the Wnt effector β-catenin (βcat), thereby targeting it for ubiquitylation and proteasomal degradation; RGS, regulator of G protein signaling domain (binding to APC). Right, Wnt binding to the frizzled (Fz) receptor and low-density lipoprotein receptor-like protein 5/6 (LRP5/6) coreceptor induces their clustering, which enables dishevelled (Dvl) to bind to Fz and to homo-polymerize through its DIX domain. Dvl polymers thus bind to casein kinase 1ε/γ (CKI) to promote the phosphorylation of the cytoplasmic tail of LRP5/6 (which thus inhibits GSK by competitive binding to its catalytic cleft [67]), and to axin through a heterotypic DIX–DAX interaction, mediating recruitment into the receptor complex [15]. The DIX–DAX interaction also has a dominant-negative effect on axin homo-polymerization, thus blocking its function in assembling degradasomes, which contributes to the signaling activity of dishevelled [24]; PDZ, postsynaptic density protein, disc large tumor suppressor, and zonula occludens-1 protein domain (binding to Fz); DEP, dishevelled, Egl-10, and pleckstrin domain (binding to CKI).

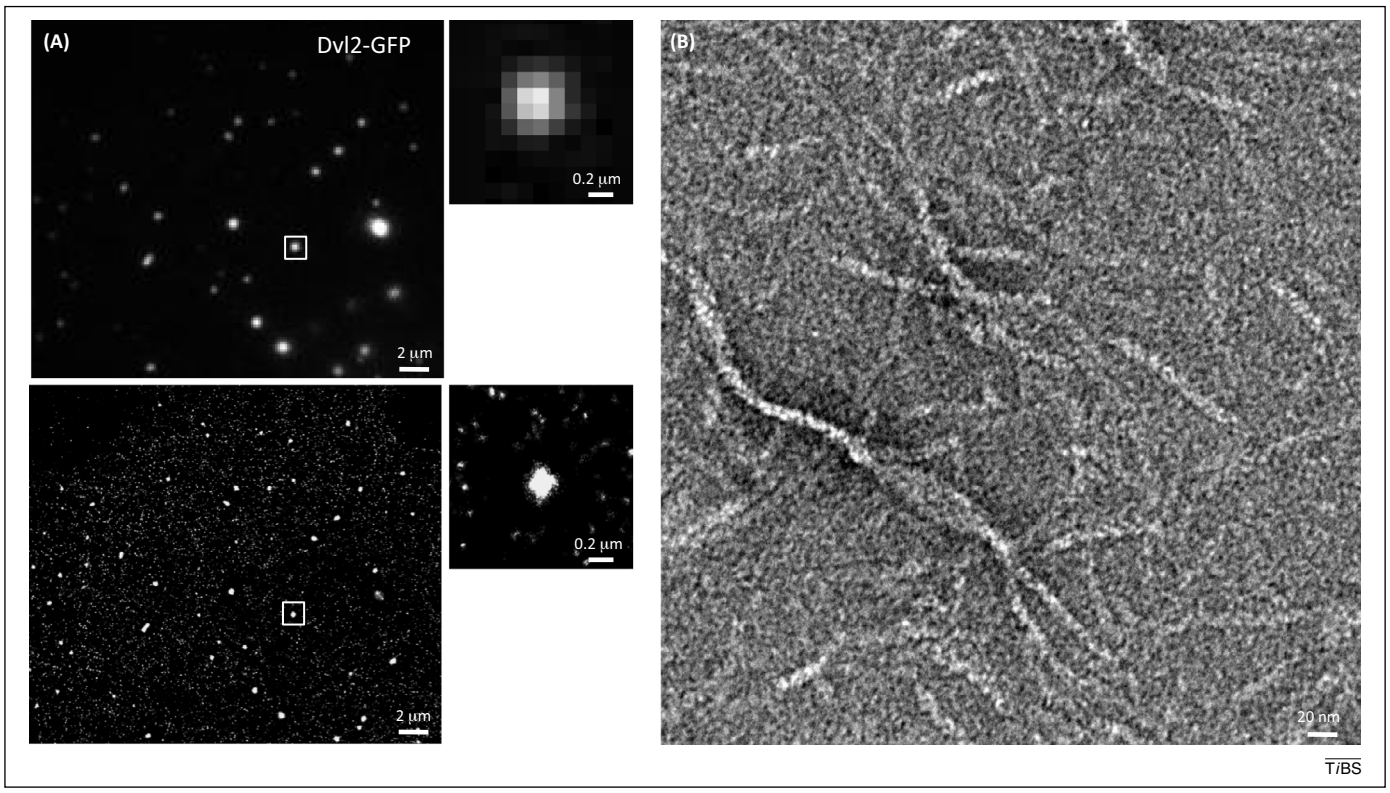

Figure 2. Dishevelled signalosomes and DIX filaments. **(A)** Top, confocal image of a COS7 cell transfected with Dvl2–GFP, forming Wnt-independent signalosomes with polymerization-dependent signaling activity (see also [1,24,25]); magnified view on the right shows a Dvl2 polymer. Bottom, the same cell imaged by high-resolution stochastic optical reconstruction microscopy; magnified view reveals a substructure of a large Dvl2 polymer (apparently composed of smaller puncta following collisions), surrounded by numerous small speckles (putative DIX monomers). Images were provided by Melissa Gammons. **(B)** Electron micrograph revealing filaments formed by purified Dvl2 DIX domain, visualized by negative staining ([1] and Madrzak, J. *et al.* unpublished); note the tendency of protofilaments to collate into superhelical fibers. Image provided by Julia Madrzak.

pathway [20]. Endogenous axin puncta can be observed in colon cancer cells after tankyrase inhibition, which stabilizes axin [21]. Importantly, fluorescence recovery after photobleaching (FRAP) experiments with overexpressed green fluorescent protein (GFP)-tagged proteins revealed that the dishevelled and axin puncta are highly dynamic and reversible, with a half-time of recovery of 10–40 s (dishevelled) or >3 min (axin) after photobleaching [16]. Thus, these puncta correspond to highly concentrated protein assemblies that are in rapid equilibrium with their respective diffuse cytoplasmic pools. Estimates have indicated that an average-sized punctum contains $\sim 10^6$ molecules, achieving a >1000× higher local concentration than their diffuse pools [16]. The dynamicity of these puncta distinguishes them from irreversible protein aggregates, such as those forming in neurodegenerative disease [22], which do not redisperse (thus barely recovering after photobleaching [23]) and continue to grow over time.

Subsequent biochemical and structural work revealed that puncta formation depends on the DIX domain, which self-associates through two complementary surfaces named 'head' and 'tail' (Figure 3A). Purified DIX domains form structurally ordered filaments that can collate into higher-order fibers [1] (Figure 2B), and they form head-to-tail polymers in crystals (Figure 3B). Polymerization has also been monitored biochemically, namely by gel filtration chromatography, analytical ultracentrifugation (AUC) or multi-angle light scattering (MALS) [1,24,25]. Crucially, equilibrium AUC demonstrated that these DIX polymers are reversible protein assemblies, consistent with the FRAP experiments, and that the degree of polymerization is concentration-dependent [1].

Instrumental for linking these insights from the *in vitro* behavior of purified DIX domains to the cellular functions of dishevelled and axin were point mutations in the head and tail surfaces of these domains (termed M2–5) [1,24]. M2 was originally identified in a genetic screen in *Drosophila* [26], but the others were designed, based on the crystal structure, in the head and tail surfaces of DIX, based on its crystal structure [1,24]. These mutations block the self-association of purified DIX domains *in vitro* [as was shown by pull-down assays, MALS, electron microscopy (EM), or nuclear magnetic resonance (NMR) spectroscopy]; if introduced into full-length protein, they block puncta formation and the activity of dishevelled and axin in cell-based assays [1,24,25] and in rescue assays of null mutants in *Drosophila* [24,27]. M2 and M4 proved to be the most effective polymerization-blocking mutants of dishevelled. Because they act as mild dominant-negatives, interfering with the polymerization of endogenous protein, they were used to demonstrate the importance of head-to-tail polymerization in assembling Wnt-induced signalosomes at the plasma membrane [2]. These mutants have since been used widely to block the signaling function of dishevelled in diverse cellular contexts (e.g., [28–31]).

The signalosome hypothesis envisages that the head-to-tail polymerization by dishevelled serves to generate a transient high local concentration of protein-binding

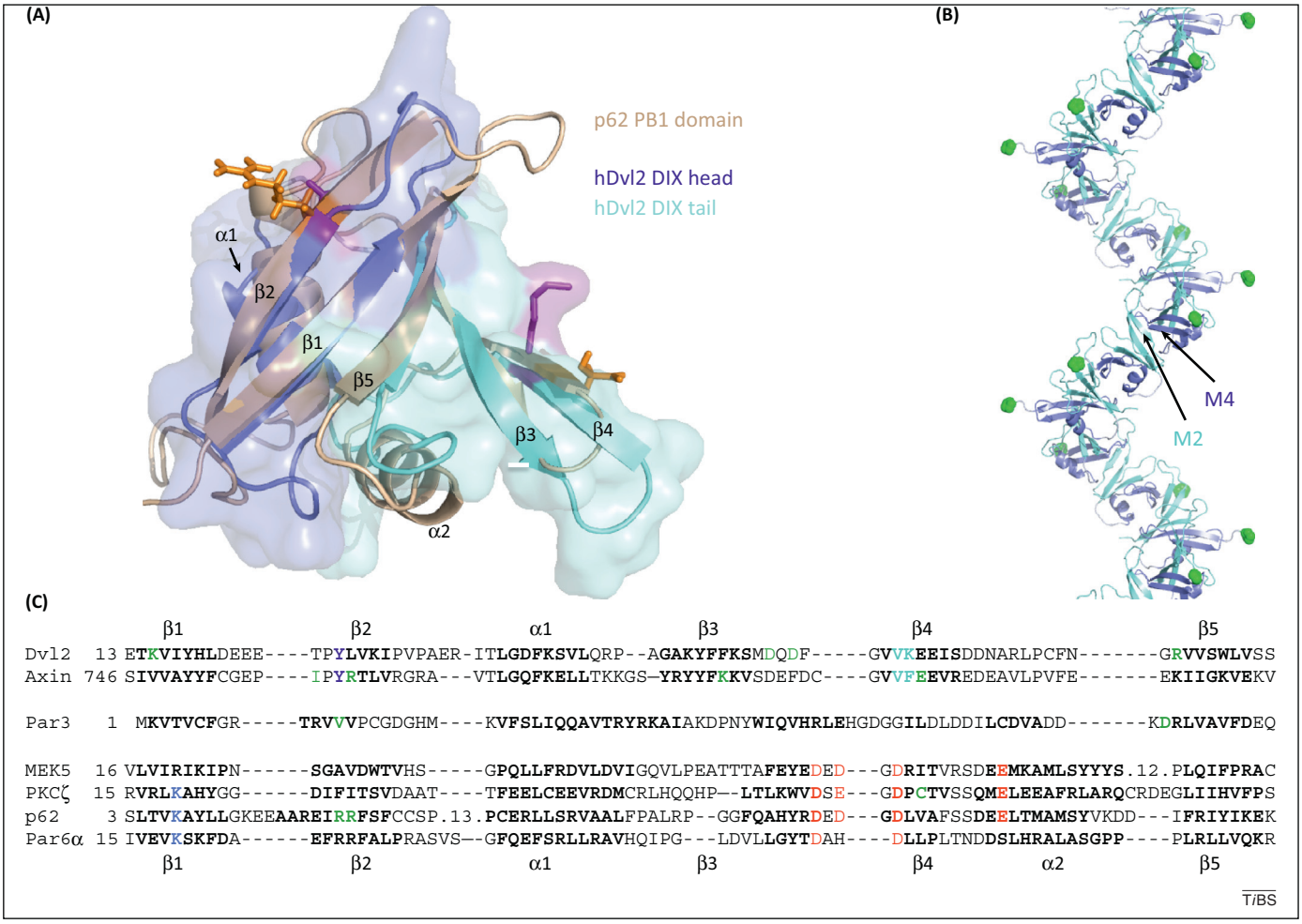

Figure 3. DIX and PB1 domain structures and sequences. **(A)** Superimposition of the crystal structures of the Dvl1 DIX [25] (purple, head; cyan, tail) and p62 PB1 [68] (wheat) monomers (RMSD 2.2 Å), revealing their closely related ubiquitin-like fold; in stick representation are residues that were substituted in Dvl2 M4 (Y27, magenta) and M2 (K68, magenta) and their topological equivalents in p62 (R21 and L74, orange); β-strands and α-helices are labeled; note the additional α-helix (α2) in p62 PB1, present also in other PB1 domains (C), in place of an extended loop between DIX β4 and β5, which distinguishes the two types of domains. **(B)** Homotypic filament of the axin DIX domain as seen in the axin DIX crystal [1], with M2 and M4 in the monomer interfaces; green blobs at the C-termini represent the body of Dvl2 (including its ligand-binding PDZ and DEP domains; see also Figure 1). Structural images were provided by Marc Fiedler. **(C)** Sequence alignments of DIX and PB1 domains from human Dvl2 and axin (top), Par3 (middle), and MEK5 (type I, with OPCA motif), p62, aPKCζ (type I/II, with OPCA and β1-K motifs), and Par6 (type II, with β1-K motif); the register between these domains was obtained by mutual superimpositions of their structures [25,46,68]. Cyan, purple, green: residues substituted to block polymerization of DIX and PB1 domains (purple, M4 defining DIX head surface, Y27D in Dvl2, Y760D in axin; cyan, M2 defining DIX tail surface, V67A K68A in Dvl2, V800A F801A in axin; green, M3, I758A R761D in axin head surface; M5, K789A E802L in axin tail surface; DR21A, R22A in p62 'back' surface; V13D in Par3 'back' surface, D70K in Par3 'front' surface; C68Y in aPKCζ 'front' surface); OPCA motifs, red (incomplete in Par6α); β1-K motifs, blue; also colored are topologically equivalent acidic (D61, D63, red) and basic (K15, R84, blue) residues in Dvl2 ('site II' residues) whose alanine substitutions attenuate its polymerization [25]; bold, residues forming β-strands (β1-5) or α-helices (α1; α2 in PB1 domains only); sequence gaps are indicated by dashes, numbers refer to additional residues in loop 6 (MEK5) or loop 2 (p62).

domains. As a result, dishevelled polymers attain a high avidity for low-affinity signaling partners [1] including regulatory proteins that control Wnt signaling (e.g., Fz [32]), kinases such as CK1ε/γ [33,34], phosphatidylinositol-4-phosphate 5-kinase 1 [35], and receptor-interacting protein kinase 4 (RIPK4) [30], the E3 ubiquitin ligase Itch [36], and a clathrin adaptor [37]. Some of these are required selectively for planar polarity, for example in *Drosophila* pupal imaginal discs [38] or in gastrulating *Xenopus* embryos [37]. Notably, these ligands typically bind to their cognate domains in dishevelled (Figure 1) with low-affinity (K_d low- to mid-micromolar) [32,39,40] and thus cannot interact efficiently with dishevelled at its normal physiological concentration (in the sub-micromolar range) [41]. However, owing to its vastly increased concentration in the punctate pool, dishevelled attains a high avidity for these low-affinity binding partners (presumably due to a decreased off-rate, although this has not been determined experimentally), which enables it to interact efficiently with them.

Likewise, axin depends on its DIX domain to assemble the β-catenin destruction complex [24], also known as the degradasome [27], possibly to interact efficiently with its β-catenin substrate whose cytoplasmic concentration is exceedingly low in unstimulated cells [41,42]. However, axin also depends on its DIX domain to bind to dishevelled during Wnt signaling, through a direct heterotypic DIX–DIX interaction involving the same head and tail residues that mediate homo-polymerization [24]. The affinity between the two DIX domains is also low (K_d mid-micromolar) [24], perhaps explaining why dishevelled needs to polymerize to bind to axin. Notably, this heterotypic interaction has a dominant-negative effect on axin, which could contribute to the signaling activity of dishevelled (Figure 1): the cellular concentration of dishevelled tends to be >10× higher than that of axin [41,42], and the

dishevelled polymers therefore have the potential to break up axin polymers, thereby attenuating the function of axin in assembling β-catenin degradasomes [24] (note that the DIX–DIX auto-affinity for axin is not known, but is likely to be in the mid-micromolar range, as is the auto-affinity of dishevelled DIX [1]). A similar dominant-negative effect could also be exerted by the dishevelled-like protein Ccd1 [25], which may attenuate the homo-polymerization of axin through a heterotypic DIX–DIX interaction, although this has not been studied in depth. These insights illustrate the versatility of the double-faced DIX domain, which can undergo both homotypic and heterotypic interactions, with different signaling outcomes, depending on the cellular concentrations of their protein bearers. The following sections will outline how the same principles also apply to other double-faced domains that participate in signaling events.

Homotypic and heterotypic interactions by PB1 domains

The PB1 domain is the closest structural relative of the DIX domain [1]. Both exhibit a ubiquitin-like fold, typically with five β-strands and an α-helix, which supports the mixed β-sheet formed between β2, β1, and β5, while the β-sheet formed by β3 and β4 is supported by a second α-helix (PB1) or a long loop (DIX) located between β4 and β5 (Figure 3A). The PB1 domain was identified through a highly conserved acidic motif [the OPCA motif (from 'OPR, PC, and AID'), D-X-D/E-G-D-X8-E/D] [10] flanking β4, which is required for heterotypic interaction with a highly conserved lysine in β1 (β1-K) in the 'back' surface of another PB1 domain, opposite the acidic 'front' bearing the OPCA motif [7,9]. On the basis of these motifs, PB1 domains were classified as type I (OPCA motif but no β1-K; including Cdc24, $p40^{phox}$, MEK5), type II (β1-K but no OPCA motif; including Bem1, $p67^{phox}$, Par6, MEKK2/3), or type I/II (both; including p62, aPKC), and numerous studies have shown the importance of these residues in specifying heterotypic PB1 interactions [9]. In a seminal study, Lamark *et al.* [43] established a PB1-interation code by systematically monitoring these interactions via yeast two-hybrid and pull-down assays, which confirmed previously reported heterotypic interactions but also uncovered new ones that conform to the predictions from the PB1 classifications. They also identified residues outside OPCA and β1-K that are essential for heterotypic interactions, including p62 residues R21 and R22 whose substitutions with alanine abrogated p62 binding to aPKC. Interestingly, these residues correspond to the DIX residues that were mutated in M4 and M3, respectively (Y27 and L28 of Dvl2, Y760 and R761 in axin; Figure 3C). Furthermore, a recent study used a cysteine-modifying compound to reveal that C68 within the PB1 domain of aPKCζ is crucial for its interaction with p62 PB1. This cysteine corresponds to the axin DIX domain glutamate that was mutated in M5 [24], which abuts M2 [1] (Figure 3C). Unbiased screens such as these revealed the importance of the topological PB1 equivalents of M2/5 and M3/4 in mediating PB1 interactions, similarly to their OPCA and β1-K motifs which dominated the functional analysis of this domain. As in the DIX domain, the PB1 residues corresponding to M2/5 and M3/4 are located centrally in the interacting surfaces, flanked by the OPCA and β1-K motifs. Although the DIX domains lack these motifs, they contain several charged residues peripherally in their head and tail surfaces (K15, D61, D63, R84; 'site II residues' in Dvl2) whose substitution with alanine attenuates or blocks DIX-dependent polymerization [25] (Figure 3C).

Interestingly, Lamark *et al.* [43] also discovered that type I/II PB1 domains (including that of p62) can form homotypic interactions leading to oligomerization. Furthermore, they showed that interaction-blocking substitutions in p62 prevent it from forming puncta (e.g., R22A was diffuse), similarly to the M2–5 substitutions, which block puncta formation by dishevelled and axin. Subsequent FRAP experiments demonstrated that these p62 puncta are highly dynamic, with a half-time of recovery of 1–2 minutes [44]. These PB1-dependent puncta are therefore akin to the DIX-dependent puncta, and are likely to reflect signalosome-like structures. Notably, p62 assemblies can also be enclosed by membranes following internalization into autophagosomes, which occurs upon binding to the ubiquitin-like LC3 adaptor on pro-phagocytic membranes to the p62 LC3-interacting region (LIR) motif [45]. These p62-containing 'cytoplasmic inclusions' look virtually indistinguishable from the dynamic p62 puncta, but they are stable structures that do not recover after photobleaching [44]. FRAP is therefore a crucial test for the classification of puncta as dynamic signalosome-like assemblies.

A landmark paper on the structural analysis of Par3 uncovered a previously unrecognized PB1 domain in its N terminus that undergoes head-to-tail polymerization [46]. Indeed, this domain was difficult to purify, as are the DIX and p62 PB1 domains, because it formed high molecular weight oligomers during gel filtration, which prompted the authors to conduct a systematic screen for point mutations that block polymerization. They thus discovered two monomeric mutants, one of which they used for structure determination by NMR. Strikingly, the resulting substitution (V13D) is identical to M4 (Figure 3C) [1]. The two self-interaction surfaces of Par3 defined by NMR correspond to the well-known negatively charged 'front' (tail in DIX) and positively charged 'back' (head in DIX) surface of the PB1 domain, in whose center V13D is localized (Figure 3C). Furthermore, the purified Par3 PB1 domain forms filaments that can be observed by EM, whose diameter of ~70 Å corresponds to that predicted from its structural model, similar to the diameter of the DIX filaments in the crystal (~65 Å) [1,25] (Figure 3B). The affinity for self-association is low (K_d ~100 μM), similar to that of DIX (K_d 20–80 μM) [1,24], suggesting that the Par3 polymers are dynamic and reversible, like the DIX polymers, although this has not been tested explicitly [46].

Importantly, Feng *et al.* [46] showed that V13D also blocks the targeting of Par3 to the plasma membrane, where the wild type protein forms discrete puncta that colocalize with the tight-junction marker zonula occludens-1, which is crucial for Par3 function in determining apicolateral cell polarity [8,9]. They therefore proposed that Par3 polymerizes at the apical membrane of epithelial cells to generate a high local concentration of protein-binding sites, thereby attaining an increased avidity for

its ligands [46], very much paralleling the signalosome hypothesis proposed for dishevelled [1,2].

PB1 domains are therefore divided into those that undergo only heterotypic interactions (e.g., Par6, MEKK2/3, MEK5) and those that can also undergo homotypic interactions leading to polymers (e.g., p62, Par3). High affinities (low- to mid-nanomolar) are typical for heterotypic pair-wise interactions specified by domains with OPCA and/or β1-K motifs owing to potent electrostatic interactions between these motifs [47–49]. The homotypic interaction by the p62 PB1 domain is similarly strong [49], owing to its OPCA and β1-K motifs. However, the Par3 PB1 domain, which lacks these motifs, has low auto-affinity despite several charged residues in its 'back' and 'front' surface, one of which was found to be crucial for interaction (Figure 3C) [46]. Indeed, the distinction between PB1 domains on the basis of OPCA and β1-K motifs may be somewhat artificial because domains without them nevertheless contain charged residues in their interfaces that contribute to their mutual interactions. For example, the aPKC PB1 domains contain both motifs but do not seem to undergo homotypic interactions [43,49], and should thus be reclassified as type I PB1 domains [49].

In summary, the charged residues flanking the centrally located key interaction residues (defined by M2–5) in PB1 and DIX interfaces are crucial for the specificities and affinities of mutual interactions. These affinities determine the dynamicity of the resulting signaling assemblies, which are tuned to their cellular functions: highly dynamic signalosomes are suitable for transient signaling events that need to be highly responsive to temporal changes in signaling amplitude (e.g., Wnt signaling in the early *Drosophila* embryo that lasts for only ~3 h [5], or NF-κB signaling in response to microbial infections [8]), whereas signaling events that need to be maintained for days (such as planar or apico-basal polarity of epithelial cells [5,8]) are likely to require more stable signalosomes. Importantly, these affinities also determine the outcome of competitions between homotypic and heterotypic interactions, which are crucial for specifying the signaling outcomes. Typically, heterotypic interactions are stronger than homotypic interactions, and thus out-compete the latter by acting as 'natural' dominant-negatives [24,25,47–49] (also Figure 1).

Versatile homo- and heteropolymeric assemblies by the SAM domain

The SAM domain was the first protein module to be recognized for its ability to undergo head-to-tail polymerization. This domain was discovered through a sequence similarity between yeast 'sterile' proteins involved in sexual differentiation and *Drosophila* polycomb-group (Pc-G) proteins involved in transcriptional silencing, and was suspected to mediate signaling [50]. The SAM domain turned out to be very common, with representatives in all eukaryotic phyla and in some bacteria, and is found in proteins involved in diverse biological contexts and mechanisms [12].

One of the early structural studies of a SAM domain (from an ephrin receptor tyrosine kinase, EphB2) revealed an oligomeric filament in the crystal, suggesting that this domain could undergo head-to-tail polymerization [51], albeit with a low auto-affinity (K_d ~1 mM) [51,52]. Bowie and colleagues subsequently defined two complementary surfaces within the SAM domain of TEL (termed ML, for mid-loop; and EH, for end-helix) through which this transcriptional repressor forms head-to-tail polymers [11]. The auto-affinity of this SAM domain turned out to be high (K_d ~2 nM), which meant that this domain was difficult to study biochemically (owing to its tendency to form insoluble and heterogeneous aggregates during purification). As for some of the DIX and PB1 domains, solving its structure depended crucially on polymerization-disabling amino acid substitutions in the interaction surfaces, isolated in a systematic screen for soluble SAM monomers [11].

Auto-affinities between different SAM domains range from strong (K_d low nanomolar) to weak (K_d high micromolar to millimolar), as confirmed in a recent systematic study of the human complement of SAM domains [13]. More than half of the human SAM domains (41) self-associated weakly (and are thus likely to form reversible polymers) while the remaining 31 formed relatively stable aggregates. Notably, the latter proved to be structurally ordered, as shown by EM [13], which distinguishes them from unspecific insoluble aggregates due to protein unfolding [22]. As expected, the dynamic SAM polymers tend to be involved in signaling (e.g., Eph2B, tankyrase, DGKδ) [51,53,54], while transcriptional silencing depends on rather more stable SAM polymers, possibly because SAM-containing Pc-G components such as polyhomeotic (Ph) and sex-combs-on-midleg (Scm) need to assemble stable repressive complexes that can be propagated through cell divisions and/or mediate chromatin condensation [55,56]. Indeed, some of these SAM domains are localized in histologically visible particles and protein densities, such as RNA-containing P-bodies (bicaudal-C1) [57] and pre- and post-synaptic protein meshworks (caskin and SHANK2/3, respectively) [58,59].

Similarly to their DIX and PB1 counterparts, SAM domains not only form homotypic polymers but also undergo heterotypic interactions mediated by the same surfaces [60,61]. For example, in the case of the *Drosophila* transcriptional repressor Yan (the ortholog of TEL), the homotypic SAM–SAM interaction (K_d 7–11 μM) is >1000× weaker than the heterotypic interaction with the SAM domain from Mae (K_d ~11 nM); therefore, Mae caps the Yan polymer and depolymerizes it, which leads to derepression of Yan and Mae target genes *in vivo* [60]. By contrast, the affinity between Scm and Ph SAM is comparable to the homotypic SAM–SAM interaction of Scm (mid-nanomolar); in both cases, their auto-affinities differ 5–20× between the two pairwise surface combinations (EH–ML versus ML–EH) [61]. These affinities determine the composition of a mixed copolymer of Scm and Ph, which is crucial for the assembly of the repressive complex formed by these proteins together with other Pc-G proteins [61].

Notably, FRAP experiments with wild type and polymerization-deficient SAM mutants revealed that Ph forms dynamic nuclear puncta (half-time recovery of ~5 minutes), which correspond to repressive polycomb complexes at PcG target genes [56]. Furthermore, the SAM-containing transmembrane protein stromal interaction molecule 1 (STIM1) forms SAM-dependent dynamic puncta in the

endoplasmic reticulum in response to Ca^{++} depletion, which enables STIM1 to interact with calcium channels in the plasma membrane [62].

In summary, SAM domains exhibit the same intrinsic property of head-to-tail polymerization as the DIX and PB1 domains, despite being structurally unrelated. However, SAM domains appear more versatile than the latter, being capable of generating ultra-stable assemblies, perhaps explaining why some SAM domains have been coopted into distinct functional contexts beyond signaling. This versatility is also reflected in the locations and arrangements of SAM domains within proteins: although most are N- or C-terminal, as in DIX and PB1, some SAM domains are found in the bodies of proteins, interspersed with other domains. Furthermore, some proteins (e.g., caskin) contain 2–3 tandem SAM domains that can adopt different modes of polymerization [59], in contrast to DIX and PB1, which are never found in tandem arrays. Finally, some SAM domains form structural units with other domains (e.g., in STIM1) that impact upon their polymerization [63].

Regulation of head-to-tail polymerization

As outlined above, polymerization by SAM, PB1, and DIX domains increases the local concentration of their bearers, and thus their avidity for their ligands. If SAM/PB1/DIX domain bearers (or their ligands) have kinase or other enzymatic activities, these enzymatic functions are likely to be activated by the polymerization and directed towards themselves or their substrates. For example, the SAM-dependent polymerization of the ephrin receptors is expected to promote their autophosphorylation [51,52], which in turn triggers signal transduction. Furthermore, the dishevelled DIX domain promotes phosphorylation of the cytoplasmic LRP6 tail in a polymerization-dependent fashion [2,64], a key event required for the transduction of the Wnt signal. Therefore, the polymerization of these domains must be tightly regulated to avoid accidental signaling.

Little is known about the regulatory mechanisms that trigger polymerization by these domains. In the cases where these domains assemble signalosomes that embrace the cytoplasmic tails of transmembrane receptors and/or coreceptors, it is conceivable that their polymerization is triggered by the clustering of these receptors and coreceptors following their binding to extracellular signals [2]. Another likely device is phosphorylation, either of the polymerizing domain itself, which could increase its auto-affinity, or of a linked domain, which could increase its affinity for its ligand. Phosphorylation could thus trigger polymerization, or stabilize a weak polymer, thereby increasing its lifetime and enabling it to undergo productive interactions with signaling partners and effectors. Intriguingly, Par6 PB1 contains a serine in place of the glutamate seen in PB1 domains with OPCA motifs (Figure 3C), whose phosphorylation could conceivably increase its auto-affinity and thus trigger its polymerization. Conversely, phosphorylation of p62 S24 in its positively charged 'back' surface [44] might block its polymerization, although this has not been tested experimentally. Phosphorylation of dishevelled outside its DIX domain by CK1 or RIPK4 contributes to signalosome assembly [29,30], but the mechanisms by which these phosphorylations control polymerization at a distance remain obscure. Finally, a striking example of a polymerization-activating device is the Ca^{++}-binding EF hands (named from regions E+F in prototypical Ca^{2+}-binding proteins) that form an integral unit with the SAM domain of STIM1. Loss of Ca^{++}-binding appears to cause local unfolding of the EF hands, thus exposing the polymerizing SAM surfaces; this triggers its polymerization and assembly of a signalosome encompassing Ca^{++} channels at the plasma membrane, which activates Ca^{++} entry into the cell [63].

Likewise, attenuation or termination of signaling requires downregulation of polymerization, or de-polymerization. Capping of polymers by heterotypic interactions is one such mechanism [60], but many others are likely to be in use. For example, ubiquitylation of polymerizing domains could block their mutual interactions: the Wnt-dependent ubiquitylation of dishevelled DIX [28] blocks its ability to polymerize (Madrzak, J. *et al.*, unpublished), and this is likely to downregulate dishevelled-dependent signaling. Indeed, ubiquitylation or ubiquitin-binding have been exploited as an indirect device for terminating polymerization-dependent signaling: phosphorylation of the ubiquitin-binding domain of p62 increases its affinity for ubiquitin; this stabilizes the highly dynamic p62 signalosome, thereby causing its internalization into autophagosomes and targeting for lysosomal degradation [44]. Likewise, DIX-dependent signalosomes are disposed by autophagy, which attenuates Wnt signaling [65].

Caveats

The somewhat unusual property of head-to-tail polymerization by SAM, PB1, and DIX domains has been the cause for two types of experimental artefacts. First, because polymerization increases the avidity for ligands, this has led to the identification of non-physiological partners of these domains or their bearers. Indeed, comparative mass spectrometry of proteins co-immunoprecipitating with wild type versus M2-mutant dishevelled identified numerous proteins whose association with dishevelled was polymerization-dependent (M. Graeb and M.B., unpublished), including *bona fide* direct and indirect ligands (such as axin and GSK3, respectively), but possibly also polymerization-dependent artefacts (e.g., protein trapped in the polymer meshwork during the sample preparation, such as transcription factors or centrosomal proteins). These mutants are therefore crucial tools for evaluating ligands of polymerizing proteins and domains, for example the reported non-SAM ligands of SAM domains [12].

Second, the puncta formed by polymerizing proteins appear indistinguishable from membranous structures (e.g., endocytic vesicles, autophagosomes), large subcellular particles (e.g., P-bodies, P-granules, stress granules [66]) and unstructured aggregates (e.g., those leading to neurodegeneration [22]). Moreover, these puncta can be heterogeneous, reflecting fundamentally different classes of structures (e.g., p62 forms signalosomes, but is also engulfed by autophagosomes [44]). Testing their dynamicity by FRAP experiments can be crucial to distinguish signalosome-like assemblies from stable structures and aggregates.

Concluding remarks and future directions

There are at least two fundamentally different folds of head-to-tail polymerizing domains (SAM, and PB1/DIX) that can form dynamic protein assemblies. Evidently, this molecular device has evolved at least twice, resulting in distinct protein folds whose only common feature is the opposing electric charges of their complementary surfaces that contribute to (or specify) mutual interactions. The dynamicity of the resulting protein assemblies makes them highly responsive to changes of signaling amplitude, and their avidity for ligands enables them to undergo rapid and efficient interactions with dispersed low-affinity signaling partners. The latter implies a need for tight control, to avoid fortuitous signaling interactions due to accidental polymerization. The molecular mechanisms that trigger and limit polymerization of these domains remain largely unexplored, and will no doubt be a major focus for future studies.

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References

1 Schwarz-Romond, T. *et al.* (2007) The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nat. Struct. Mol. Biol.* 14, 484–492
2 Bilic, J. *et al.* (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* 316, 1619–1622
3 Ringrose, J.H. *et al.* (2013) Deep proteome profiling of *Trichoplax adhaerens* reveals remarkable features at the origin of metazoan multicellularity. *Nat. Commun.* 4, 1408
4 Pang, K. *et al.* (2010) Genomic insights into Wnt signaling in an early diverging metazoan, the ctenophore *Mnemiopsis leidyi*. *Evodevo* 1, 10
5 Cadigan, K.M. and Nusse, R. (1997) Wnt signaling: a common theme in animal development. *Genes Dev.* 11, 3286–3305
6 Clevers, H. and Nusse, R. (2012) Wnt/beta-catenin signaling and disease. *Cell* 149, 1192–1205
7 Ito, T. *et al.* (2001) Novel modular domain PB1 recognizes PC motif to mediate functional protein-protein interactions. *EMBO J.* 20, 3938–3946
8 Moscat, J. *et al.* (2006) Cell signaling and function organized by PB1 domain interactions. *Mol. Cell* 23, 631–640
9 Sumimoto, H. *et al.* (2007) Structure and function of the PB1 domain, a protein interaction module conserved in animals, fungi, amoebas, and plants. *Sci. STKE* 2007, re6
10 Ponting, C.P. *et al.* (2002) OPR, PC and AID: all in the PB1 family. *Trends Biochem. Sci.* 27, 10
11 Kim, C.A. *et al.* (2001) Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. *EMBO J.* 20, 4173–4182
12 Kim, C.A. and Bowie, J.U. (2003) SAM domains: uniform structure, diversity of function. *Trends Biochem. Sci.* 28, 625–628
13 Knight, M.J. *et al.* (2011) A human sterile alpha motif domain polymerizome. *Protein Sci.* 20, 1697–1706
14 Sear, R.P. (2008) Phase separation of equilibrium polymers of proteins in living cells. *Faraday Discuss.* 139, 21–34
15 MacDonald, B.T. *et al.* (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26
16 Schwarz-Romond, T. *et al.* (2005) The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles. *J. Cell Sci.* 118, 5269–5277
17 Smalley, M.J. *et al.* (2005) Dishevelled (Dvl-2) activates canonical Wnt signalling in the absence of cytoplasmic puncta. *J. Cell Sci.* 118, 5279–5289
18 Yanagawa, S. *et al.* (1995) The dishevelled protein is modified by wingless signaling in *Drosophila*. *Genes Dev.* 9, 1087–1097
19 Miller, J.R. *et al.* (1999) Establishment of the dorsal–ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol.* 146, 427–437
20 Metcalfe, C. *et al.* (2010) Dvl2 promotes intestinal length and neoplasia in the ApcMin mouse model for colorectal cancer. *Cancer Res.* 70, 6629–6638
21 de la Roche, M. *et al.* (2014) LEF1 and B9L shield beta-catenin from inactivation by Axin, desensitizing colorectal cancer cells to tankyrase inhibitors. *Cancer Res.* 74, 1495–1505
22 Goedert, M. *et al.* (2010) The propagation of prion-like protein inclusions in neurodegenerative diseases. *Trends Neurosci.* 33, 317–325
23 Roberti, M.J. *et al.* (2011) Confocal fluorescence anisotropy and FRAP imaging of alpha-synuclein amyloid aggregates in living cells. *PLoS ONE* 6, e23338
24 Fiedler, M. *et al.* (2011) Dishevelled interacts with the DIX domain polymerization interface of Axin to interfere with its function in down-regulating beta-catenin. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1937–1942
25 Liu, Y.T. *et al.* (2011) Molecular basis of Wnt activation via the DIX domain protein Ccd1. *J. Biol. Chem.* 286, 8597–8608
26 Penton, A. *et al.* (2002) A mutational analysis of dishevelled in *Drosophila* defines novel domains in the dishevelled protein as well as novel suppressing alleles of axin. *Genetics* 161, 747–762
27 Mendoza-Topaz, C. *et al.* (2011) The adenomatous polyposis coli tumour suppressor is essential for Axin complex assembly and function and opposes Axin's interaction with Dishevelled. *Open Biol.* 1, 110013
28 Tauriello, D.V. *et al.* (2010) Loss of the tumor suppressor CYLD enhances Wnt/beta-catenin signaling through K63-linked ubiquitination of Dvl. *Mol. Cell* 37, 607–619
29 Yokoyama, N. *et al.* (2012) Assembly of Dishevelled 3-based supermolecular complexes via phosphorylation and Axin. *J. Mol. Signal.* 7, 8
30 Huang, X. *et al.* (2013) Phosphorylation of Dishevelled by protein kinase RIPK4 regulates Wnt signaling. *Science* 339, 1441–1445
31 Kim, I. *et al.* (2013) Clathrin and AP2 are required for PtdIns(4,5)P2-mediated formation of LRP6 signalosomes. *J. Cell Biol.* 200, 419–428
32 Wong, H.C. *et al.* (2003) Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell* 12, 1251–1260
33 Davidson, G. *et al.* (2005) Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867–872
34 Zeng, X. *et al.* (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438, 873–877
35 Pan, W. *et al.* (2008) Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science* 321, 1350–1353
36 Wei, W. *et al.* (2012) The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. *Mol. Cell. Biol.* 32, 3903–3912
37 Yu, A. *et al.* (2007) Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. *Dev. Cell* 12, 129–141
38 Axelrod, J.D. (2001) Unipolar membrane association of Dishevelled mediates Frizzled planar cell polarity signaling. *Genes Dev.* 15, 1182–1187
39 Hino, S. *et al.* (2003) Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. *J. Biol. Chem.* 278, 14066–14073
40 Yu, A. *et al.* (2010) Structural analysis of the interaction between Dishevelled2 and clathrin AP-2 adaptor, a critical step in noncanonical Wnt signaling. *Structure* 18, 1311–1320
41 Lee, E. *et al.* (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol.* 1, E10
42 Tan, C.W. *et al.* (2012) Wnt signalling pathway parameters for mammalian cells. *PLoS ONE* 7, e31882
43 Lamark, T. *et al.* (2003) Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. *J. Biol. Chem.* 278, 34568–34581
44 Matsumoto, G. *et al.* (2011) Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Mol. Cell* 44, 279–289

45 Johansen, T. and Lamark, T. (2011) Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 7, 279–296
46 Feng, W. *et al.* (2007) The Par-3 NTD adopts a PB1-like structure required for Par-3 oligomerization and membrane localization. *EMBO J.* 26, 2786–2796
47 Wilson, M.I. *et al.* (2003) PB1 domain-mediated heterodimerization in NADPH oxidase and signaling complexes of atypical protein kinase C with Par6 and p62. *Mol. Cell* 12, 39–50
48 Hu, Q. *et al.* (2007) Insight into the binding properties of MEKK3 PB1 to MEK5 PB1 from its solution structure. *Biochemistry* 46, 13478–13489
49 Ren, J. *et al.* (2014) Structural and biochemical insights into the homotypic PB1-PB1 complex between PKCzeta and p62. *Sci. China Life Sci.* 57, 69–80
50 Ponting, C.P. (1995) SAM: a novel motif in yeast sterile and *Drosophila* polyhomeotic proteins. *Protein Sci.* 4, 1928–1930
51 Thanos, C.D. *et al.* (1999) Oligomeric structure of the human EphB2 receptor SAM domain. *Science* 283, 833–836
52 Stapleton, D. *et al.* (1999) The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. *Nat. Struct. Biol.* 6, 44–49
53 De Rycker, M. and Price, C.M. (2004) Tankyrase polymerization is controlled by its sterile alpha motif and poly(ADP-ribose) polymerase domains. *Mol. Cell. Biol.* 24, 9802–9812
54 Harada, B.T. *et al.* (2008) Regulation of enzyme localization by polymerization: polymer formation by the SAM domain of diacylglycerol kinase delta1. *Structure* 16, 380–387
55 Kim, C.A. *et al.* (2002) The SAM domain of polyhomeotic forms a helical polymer. *Nat. Struct. Biol.* 9, 453–457
56 Isono, K. *et al.* (2013) SAM domain polymerization links subnuclear clustering of PRC1 to gene silencing. *Dev. Cell* 26, 565–577
57 Maisonneuve, C. *et al.* (2009) Bicaudal C, a novel regulator of Dvl signaling abutting RNA-processing bodies, controls cilia orientation and leftward flow. *Development* 136, 3019–3030
58 Baron, M.K. *et al.* (2006) An architectural framework that may lie at the core of the postsynaptic density. *Science* 311, 531–535
59 Stafford, R.L. *et al.* (2011) Tandem SAM domain structure of human Caskin1: a presynaptic, self-assembling scaffold for CASK. *Structure* 19, 1826–1836
60 Qiao, F. *et al.* (2004) Derepression by depolymerization; structural insights into the regulation of Yan by Mae. *Cell* 118, 163–173
61 Kim, C.A. *et al.* (2005) Structural organization of a Sex-comb-on-midleg/polyhomeotic copolymer. *J. Biol. Chem.* 280, 27769–27775
62 Liou, J. *et al.* (2007) Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9301–9306
63 Stathopulos, P.B. *et al.* (2008) Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell* 135, 110–122
64 Metcalfe, C. *et al.* (2010) Stability elements in the LRP6 cytoplasmic tail confer efficient signalling upon DIX-dependent polymerization. *J. Cell Sci.* 123, 1588–1599
65 Gao, C. *et al.* (2010) Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. *Nat. Cell Biol.* 12, 781–790
66 Hyman, A.A. and Simons, K. (2012) Beyond oil and water – phase transitions in cells. *Science* 337, 1047–1049
67 Stamos, J.L. *et al.* (2014) Structural basis of GSK-3 inhibition by N-terminal phosphorylation and by the Wnt receptor LRP6. *Elife* 3, e01998
68 Saio, T. *et al.* (2010) PCS-based structure determination of protein–protein complexes. *J. Biomol. NMR* 46, 271–280