

Inhibition of GSK3 by Wnt signalling – two contrasting models

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Summary

The key read-out of Wnt signalling is a change in the transcriptional profile of the cell, which is driven by β -catenin. β -catenin levels are normally kept low by a phosphorylation event that is mediated by glycogen synthase kinase 3 (GSK3, α - and β -isoforms), which targets β -catenin for ubiquitylation and proteasomal degradation. Wnt blocks this phosphorylation event, thereby allowing β -catenin to accumulate and to co-activate transcription in the nucleus. Exactly how Wnt inhibits GSK3 activity towards β -catenin is unclear and has been the focus of intensive research. Recent studies on the role of conserved PPPSPxS motifs in the cytoplasmic tail of low-density lipoprotein receptor-related protein (LRP, isoforms 5 and 6) culminated in a biochemical model: Wnt induces the phosphorylation of LRP6 PPPSPxS motifs, which consequently access the catalytic pocket of GSK3 as pseudo-substrates, thus directly blocking its activity against β -catenin. A distinct cell-biological model was proposed more recently: Wnt proteins induce the uptake of GSK3 into multivesicular bodies (MVBs), an event that sequesters the enzyme away from newly synthesised β -catenin substrate in the cytoplasm, thus blocking its phosphorylation. This new model is based on intriguing observations but also challenges a body of existing evidence, so will require further experimental consolidation. We shall consider whether the two models apply to different modes of Wnt signaling: acute versus chronic.

Key words: Wnt, GSK3, LRP6, Multivesicular bodies, Signalosomes

Introduction

Wnt/ β -catenin signalling (also referred to as canonical Wnt signalling) is a key pathway in embryonic development and adult homeostasis (Clevers, 2006; Logan and Nusse, 2004). Its profound impact on cell behaviour is clearly demonstrated by its inappropriate activation in disease, most notably in colorectal cancer, in which mutations in the negative Wnt regulator and tumour suppressor adenomatous polyposis coli (APC) drive tumourigenesis. In the absence of Wnt proteins, the levels of the Wnt effector β -catenin are kept low by a cytoplasmic multi-protein complex comprising the Axin scaffold protein, APC and glycogen synthase kinase 3 (GSK3). APC cooperates with Axin to promote the phosphorylation of β -catenin by GSK3 [which requires priming phosphorylation by casein kinase 1, α -isoform (CK1 α)]. β -catenin phosphorylated by GSK3 is, subsequently, ubiquitylated and targeted for proteasomal degradation. Notably, the scaffolding function of Axin is essential in this process (Ikeda et al., 1998) because it enhances the efficiency of GSK3-mediated phosphorylation of β -catenin by several orders of magnitude (Dajani et al., 2003). The role of APC is less clear, but it clearly binds to both β -catenin and Axin, and could shuttle β -catenin from the plasma membrane and nucleus to the cytoplasmic Axin complex (Bienz and Clevers, 2000).

Binding of Wnt proteins to the Frizzled transmembrane receptors and their LRP co-receptors triggers activation of β -catenin. Wnt-activated Frizzled receptors recruit Dishevelled proteins to the plasma membrane (Angers and Moon, 2009) by direct binding (Wong et al., 2003). In turn, Dishevelled directly interacts with Axin (Fiedler et al., 2010) and recruits it, possibly together with its associated proteins (e.g. GSK3), to the plasma membrane, where it promotes the formation of an LRP-associated Wnt ‘signalosome’ (Bilic et al., 2007) in which GSK3-mediated phosphorylation of β -

catenin is blocked (MacDonald et al., 2009) (Fig. 1). Unphosphorylated β -catenin escapes degradation and thus accumulates in the cytoplasm and nucleus where it binds to the DNA-binding T-cell factors (TCFs) to switch on a Wnt-induced transcriptional programme, through which it specifies cell fates during normal development and adult tissue homeostasis (Clevers, 2006). Loss-of-function mutations in APC or Axin, or in the GSK3 target residues of β -catenin, prevent its phosphorylation; this mimics the inhibition of GSK3 by Wnt and, thus, causes accumulation of stabilised β -catenin, which promotes tumourigenesis in the intestine and in other tissues (Bienz and Clevers, 2000).

The molecular events that drive Wnt-induced inhibition of β -catenin phosphorylation by GSK3 have been the focus of intensive study for over a decade. These efforts, spanning the laboratories of many investigators in the field, led to a biochemical model for Wnt-dependent GSK3 inhibition, which relies on the direct interaction of GSK3 with phosphorylated LRP6 motifs as a means of suppressing GSK3 activity directed at β -catenin (Cselenyi et al., 2008; Mi et al., 2006; Piao et al., 2008; Wu et al., 2009). Recently, however, a distinct cell-biological mechanism has been proposed, in which Wnt signalling promotes internalisation of GSK3 into the lumen of multivesicular bodies (MVBs), thereby preventing this enzyme from phosphorylating newly synthesised β -catenin in the cytoplasm (Taelman et al., 2010). This new hypothesis was unexpected, and suggests a new mechanism for ligand-driven kinase inhibition – namely, the insulation of an enzyme from its substrate by two layers of internal membrane. In this Commentary, we shall put this new model into context and contrast it with the earlier biochemical model, while also attempting to identify strengths as well as weaknesses in the evidence that underpins both models.

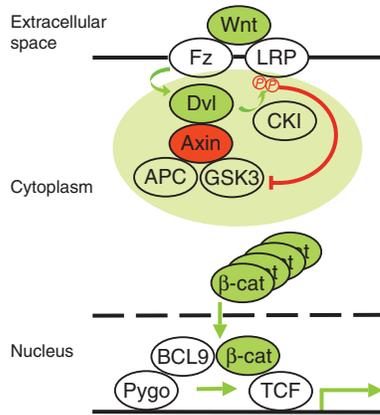


Fig. 1. Signalosome-based Wnt/ β -catenin signalling. According to the signalosome hypothesis, a Wnt ligand binds simultaneously to Frizzled (Fz) and LRP, clustering these transmembrane receptors and, thereby, triggering the recruitment and DIX-dependent polymerisation of Dishevelled (Dvl). Owing to an increase in avidity, polymerisation of Dishevelled enables it to bind to Axin (through direct heterotypic DIX interactions) and to recruit Axin together with GSK3 to LRP. LRP thus becomes a substrate for GSK3 and CK1 (γ - and ϵ -isoforms), which then phosphorylate the PPPSPxS motifs in the LRP cytoplasmic tail, enabling this motif to bind to the catalytic pocket of GSK3 (see Fig. 3), thereby blocking its activity towards β -catenin (β -cat). Unphosphorylated β -catenin is released and escapes ubiquitylation and proteasomal degradation, which allows it to accumulate in the cytoplasm and nucleus. There, it functions as a co-activator of TCF to promote Wnt-induced transcription – which is facilitated by the chromatin-binding Pygo–BCL9 complex.

Signalosome assembly

Wnt-induced signalosome formation at the plasma membrane is the key step that precedes inhibition of GSK3. Dishevelled and, specifically, its highly conserved DIX domain (Bilic et al., 2007), have a pivotal role in the assembly of the signalosome. The DIX domain has a remarkable property in that it mediates reversible head-to-tail polymerisation, which is crucial for Dishevelled signalling activity (Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2005). DIX-dependent homo-polymerisation of Dishevelled allows it to assemble large, yet highly dynamic, protein clusters that are detectable by immunofluorescence in live and fixed cells as distinct cytoplasmic puncta (Fig. 2Ai). These DIX-dependent protein assemblies were initially identified as membrane-containing vesicle-like structures, and a putative phospholipid-binding VKEEIS motif was found to be required for formation and activity of Dishevelled puncta (Capelluto et al., 2002). Subsequently, however, two independent studies using live imaging and photobleaching discovered that Dishevelled puncta represent dynamic protein assemblies and ruled out that they are endocytic vesicles by demonstrating that they failed to colocalise with a large number of endocytic markers, membrane lipids and endocytic cargo (Schwarz-Romond et al., 2005; Smalley et al., 2005). A more recent study, which focused on non-canonical Wnt signalling, similarly failed to colocalise Dishevelled puncta with various vesicle and membrane markers (Nishita et al., 2010). Indeed, the VKEEIS motif turned out to be located on the DIX–DIX interaction surface, as revealed by X-ray crystallography; this interface is essential for DIX-dependent homo-polymerisation of Dishevelled in vitro, and Dishevelled puncta formation and signalling in vivo (Liu et al., 2010; Schwarz-Romond et al., 2007a). The same interface also mediates heterotypic interaction with the

DIX domain of Axin, which closely mimics the homotypic interaction (Fiedler et al., 2010); this heterotypic interaction between the two domains mediates recruitment of Axin by Dishevelled into the signalosome and, consequently, results in the activation of β -catenin (Schwarz-Romond et al., 2007b).

Notably, the affinity of Dishevelled for its PDZ- and DEP-domain-binding partners, including Frizzled, is weak, typically with a K_d in the low micromolar range (Simons et al., 2009; Wong et al., 2003; Yu et al., 2010). It was, therefore, proposed that the DIX-dependent polymerisation of Dishevelled results in a high binding avidity for its signalling partners, owing to a transient high local concentration of protein interaction sites, which allows Dishevelled to bind efficiently to its low-affinity interacting partners (Fiedler et al., 2010; Schwarz-Romond et al., 2007a). DIX–DIX interactions themselves are even weaker (with a K_d in the mid-micromolar range) (Fiedler et al., 2010; Schwarz-Romond et al., 2007a), and are, thus, unlikely to occur spontaneously at physiological concentrations. Therefore, DIX-dependent polymerisation is likely to require an event triggered by Frizzled and is possibly amplified by Frizzled-dependent regulatory factors, as outlined in the next paragraph.

The exact sequence of events during signalosome formation is still unclear but analysis of a non-canonical signalling pathway (involving Wnt5a, Fz7, Ror2 and Dishevelled) suggested that engagement (and possibly also the clustering) of Wnt receptors and co-receptors by their Wnt ligands triggers the polymerisation of Dishevelled (Nishita et al., 2010). Dishevelled polymers therefore acquire the necessary avidity to co-polymerise with Axin (and associated proteins), thereby overcoming the low affinity between the two DIX domains (Fiedler et al., 2010). Clustered Dishevelled then promotes the polymerisation-dependent phosphorylation of LRP6 by CK1 [ϵ - and γ -isoforms (Davidson et al., 2005; Zeng et al., 2005)], which is crucial for the functioning of the signalosome (Bilic et al., 2007; Zeng et al., 2008).

The stability of Wnt signalosomes is essential for their signalling activity, and appears to depend on the interaction of additional proteins with the cytoplasmic tail of LRP6 (Metcalf et al., 2010). Such ‘stability’ factors might include phosphatidylinositol (4,5)-bisphosphate, production of which is stimulated by Dishevelled acting on phosphatidylinositol kinases (Pan et al., 2008); in mammalian cells, this phospholipid is recognised by the signalosome-promoting protein AMER1 (also known as Amer1/WTX) (Tanneberger et al., 2011). DEP-domain-mediated interactions of Dishevelled with peripheral membrane proteins such as clathrin adaptors [in the case of non-canonical signalling (Yu et al., 2010)], and/or with lipid head groups of the plasma membrane itself (Simons et al., 2009), might also contribute to signalosome stability, although this has not been tested explicitly. Additional potential signalosome-stabilising factors have been reviewed elsewhere (Wu and Pan, 2010).

Direct catalytic inhibition of GSK3 in the LRP signalosome: the biochemical model

How signalosomes lead to a block of β -catenin phosphorylation by GSK3 remains an open question. A major advance towards answering this question was the discovery that, following Wnt signalling, the multiple conserved PPPSPxS motifs within the cytoplasmic tail of LRP – which are crucial for Wnt signal transduction (Mao et al., 2001; Tamai et al., 2004) – become dually phosphorylated by CK1 (γ - and ϵ -isoforms) following priming by GSK3 (Davidson et al., 2005; Zeng et al., 2005) (this dual

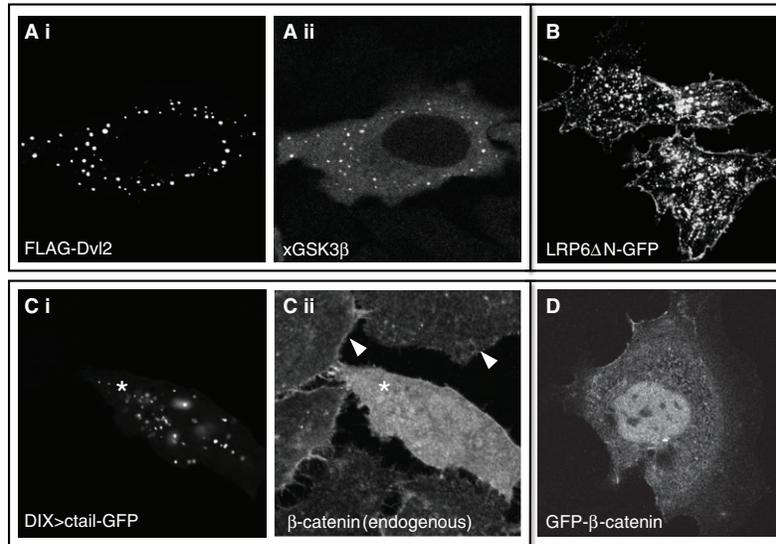


Fig. 2. Protein assemblies capable of activating β -catenin signaling in the absence of Wnt. (A–C) HeLa cells transfected with various Wnt signalling components after fixation and antibody staining (except for GFP-tagged proteins). (A*i*) FLAG-Dvl2, stained with anti-FLAG antibody, revealing the round and regularly shaped cytoplasmic Dishevelled puncta, co-expressed with (A*ii*) *Xenopus* GSK3 β (stained with anti-GSK3 β antibody). Only a small fraction of the total GSK3 β is recruited into the Dvl2 puncta, and much of it remains diffuse. GSK3 recruitment to Dvl2 is probably limited by the low levels of endogenous Axin, whose DIX domain interacts directly with the Dvl2 DIX domain and, thus, facilitates the Dvl–GSK3 interaction (see main text). (B) LRP6 Δ N-GFP, forming irregularly shaped and variably sized speckles in the cytoplasm, which reflects its clustering and, also, its internalisation into cytoplasmic vesicles. Some of these vesicles are expected to be positive for caveolin (Yamamoto et al., 2006; Yamamoto et al., 2008), whereas others are likely to be destined for lysosomal degradation (only a small fraction of this abnormal transmembrane protein truncation is retained at the plasma membrane). (C*i*) DIX>ctail-GFP puncta (reminiscent of the Dvl2 puncta) that stabilise endogenous β -catenin in the transfected cell (C*ii*, asterisk), as the read-out of the potent signalling activity of these cytoplasmic DIX>ctail ‘signalosomes’. (D) COS-7 cell transfected with GFP- β -catenin (image provided by Carolina Mendoza-Topaz, MRC Laboratory of Molecular Biology, Cambridge, UK), which shows a predominantly diffuse distribution (the high nuclear level of GFP- β -catenin is a consequence of the GFP tag, which stabilises this chimera in the nucleus) with barely detectable puncta (perhaps reflecting the endogenous Axin complex) superimposed on the diffuse fluorescence. A similar pattern is seen with endogenous β -catenin in the untransfected HeLa cells (arrowheads in C*ii*). This contrasts the pattern observed by Taelman and co-workers, who reported predominantly punctate GFP- β -catenin (see Taelman et al., 2010, and supplementary figure 3D therein).

phosphorylation event is the opposite of that acting on β -catenin, mentioned above, in which CK1 α acts as a priming kinase for GSK3). CK1-mediated phosphorylation of LRP6 depends on polymerisation by the Dishevelled DIX domain (Bilic et al., 2007; Metcalfe et al., 2010). It has been proposed that the phosphorylated PPPSPxS motifs constitute a docking site for Axin (Mao et al., 2001), thereby promoting a crucial protein interaction for the functioning of the signalosome, possibly by synergising with the DIX-dependent Dishevelled–Axin interaction, in order to stabilise and sustain the signalosome during the ‘amplification’ phase (Zeng et al., 2008).

A subsequent landmark discovery arose from four independent studies, each of which was aimed at addressing different questions: these studies ascribed a direct role of phosphorylated PPPSPxS motifs in the Wnt-dependent inhibition of GSK3 (Cselenyi et al., 2008; Mi et al., 2006; Piao et al., 2008; Wu et al., 2009). Mi and colleagues showed that GSK3 β binds directly to, and can phosphorylate, the cytoplasmic tail of LRP6, and were the first to propose that this downregulates the activity of GSK3 towards β -catenin (Mi et al., 2006). It was subsequently found that LRP6 depends on its phosphorylated PPPSPxS motifs to inhibit GSK3 (Cselenyi et al., 2008). Crucially, dually phosphorylated PPPSPxT peptides are sufficient to inhibit GSK3 kinase activity directed at β -catenin and other physiological GSK3 target sites (including non-Wnt targets, such as tau and glycogen synthase) *in vitro* and *in vivo* (Piao et al., 2008; Wu et al., 2009). Indeed, Piao and co-

workers discovered that an LRP6 peptide containing the phosphorylated PPPSPxT motif is a direct competitive inhibitor of GSK3, with a K_i of 13 μ M, and they proposed that this phosphorylated LRP peptide blocks the phosphorylation of β -catenin and other substrates by accessing the catalytic pocket of GSK3 as a ‘pseudo-substrate’ (Fig. 3) (Piao et al., 2008).

The notion that GSK3 might be inhibited by the binding of a phosphorylated LRP motif to its active site was particularly persuasive, as it mimics the well-established regulation of GSK3 by growth factors including, most famously, insulin – a separate regulation of GSK3 β that was discovered long before its regulation by Wnt signalling emerged, and that is thought to occur in parallel to Wnt signalling, without apparent crosstalk (Ding et al., 2000; Ng et al., 2009). The N-terminus of GSK3, following phosphorylation at serine residue 9 by growth-factor-activated protein kinase B/Akt (Stambolic and Woodgett, 1994), creates a pseudo-substrate (phospho-GSK3-S9) that is thought to fold back on GSK3 itself and insert into its catalytic pocket (Dajani et al., 2001). Indeed, a detached phospho-GSK3 β -S9 peptide acts as a pseudo-substrate inhibitor, with a K_i of 700 μ M (Dajani et al., 2001) – which is considerably lower (>50 times) than the K_i of the phosphorylated PPPSPxT peptide from LRP6 (Piao et al., 2008). In both cases, however, the potency of GSK3 inhibition is likely to be much greater in the context of full-length proteins and/or within the functional protein complexes – through cis-linkage in the case of phospho-GSK3 β -S9, and through avidity provided by

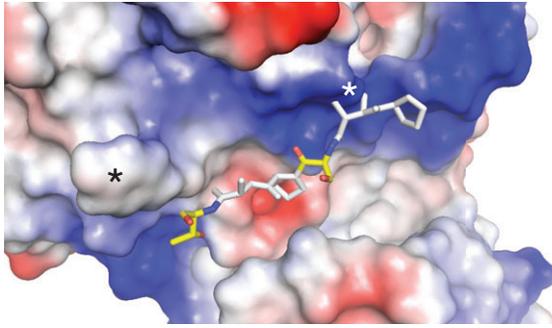


Fig. 3. Direct inhibition of GSK3 by phosphorylated LRP – the biochemical model. Electrostatic surface representation of the area surrounding the catalytic pocket of GSK3 β , overlaid stick representation shows the engaged LRP6 peptide. The model was generated by Tom Miller (MRC Laboratory of Molecular Biology, Cambridge, UK) and is based on 1H8F (see Dajani et al., 2001). The sequence of the glycogen synthase peptide previously modeled by Dajani et al. (Dajani et al., 2001) was mutated to PPSPAT, reflecting the membrane-proximal PPPSPxT motif from LRP6, with its two phosphorylation target residues serine and threonine highlighted in yellow (red, oxygen atoms; blue, nitrogen atoms). The threonine residue (left) is the target for phosphorylation by CK1, a process primed by phosphorylation of the serine residue (in the –3 position) by GSK3. Note that the precise position of this peptide is hypothetical, as this image has been created by superimposing it with a different kinase–substrate complex (see Dajani et al., 2001); in particular, the N-terminus of the peptide is likely to be mispositioned, creating a steric clash of the second proline with the GSK3 β pocket (white asterisk). Note also that the rotamer conformation of the putative activation segment tyrosine 126 (black asterisk) (see Dajani et al., 2001) was changed, to avoid a steric clash with the downstream threonine phosphorylation target of the PPSPAT peptide.

the multiple PPPSPxS motifs within the LRP cytoplasmic tail, most of which can inhibit GSK3-mediated phosphorylation of β -catenin and other substrates (Wu et al., 2009). Direct binding of GSK3 to phosphorylated PPPSPxS motifs could promote the binding of Axin to LRP and reinforce retention of GSK3 within the LRP signalosome (Piao et al., 2008; Zeng et al., 2005). Again, the efficiency of this (weak) interaction might be increased significantly by DIX-dependent clustering.

Together, these studies culminated in a structurally plausible biochemical model of Wnt-mediated GSK3 inhibition through phosphorylated LRP (Cselenyi et al., 2008; Mi et al., 2006; Piao et al., 2008; Wu et al., 2009) (Fig. 3), which provided a conceptually satisfactory answer to a long-standing question, indicating that similar molecular mechanisms underlie GSK3 inhibition by both Wnt and insulin signalling. Notably, this model can readily explain the rapid Wnt-induced block of GSK3-mediated phosphorylation of β -catenin, and of Axin – both of which are detectable within 5–15 minutes after Wnt stimulation (Luckert et al., 2011; Willert et al., 1999). Altogether, this biochemical model is highly persuasive, although some questions remain unanswered. For example, for the inhibition of GSK3 to be sustained, phosphorylated LRP might need to remain associated with the enzyme and it is currently not entirely clear how this would be achieved. However, it is also possible that the phosphorylated LRP-mediated inhibition of GSK3 operates in a hit-and-run fashion during the immediate-early phase of Wnt signalling, which then triggers longer-lasting secondary consequences: notably, blocking the GSK3-mediated phosphorylation of Axin lowers its affinity for β -catenin (Willert et al., 1999), which causes the release of β -catenin from the

membrane-associated Axin complex (Kishida et al., 1999). Furthermore, the high affinity of APC for β -catenin also depends on its phosphorylation by GSK3 (Ha et al., 2004; Rubinfeld et al., 1996; Xing et al., 2003). In other words, the acute Wnt-induced inhibition of GSK3 directed against negative regulators of β -catenin might further amplify and sustain the immediate-early block of β -catenin phosphorylation through GSK3, by promoting the disassembly of the Axin complex.

Sequestration of GSK3 from its substrates by internal membranes: the cell-biological model

Taelman and colleagues have recently published a study that proposed an entirely different model describing how GSK3 activity might be blocked by Wnt signalling (Taelman et al., 2010). They presented multiple lines of evidence showing that Wnt pathway activity is accompanied by the internalisation of GSK3 β , together with other components of the signalosome, into MVBs (see Fig. 4 for an outline of the endocytosis pathway). GSK3 is therefore sequestered away and insulated from newly synthesised β -catenin – which, therefore, remains unphosphorylated. The authors state that their study was prompted by their inability to recover Wnt-induced inhibition of GSK3 in Wnt-treated cell extracts that had been prepared in the presence of the detergent Triton X-100. They surmised that Wnt stimulation leads to protection of GSK3 by internal membranes that are dissolved during detergent-mediated lysis of cells. Indeed, digitonin-mediated permeabilisation of Wnt-stimulated L cells, which solubilises cholesterol-rich patches in the plasma membrane while leaving intracellular vesicles and organelles intact, allowed the authors to demonstrate an ~66% reduction in GSK3 activity. Even more strikingly, they went on to show that Wnt stimulation renders GSK3 β protease resistant in digitonin-permeabilised cells [and protease sensitivity of GSK3 β was recoverable by treatment with Triton X-100 (Taelman et al., 2010)]. Together, these data provide key evidence for the model of GSK3 sequestration by intact internal membranes. A caveat is that the selective permeabilisation of plasma versus internal membranes by digitonin was not monitored, and it was not tested whether blocking MVB maturation (see below) reverses the protective effect of internal membranes on GSK3.

Before considering additional evidence by Taelman and co-workers in support of their model (Taelman et al., 2010), we offer an alternative explanation on why Triton X-100 might eliminate the Wnt-induced inhibition of GSK3 in cell extracts: loss of signalosome function could be due to a detergent sensitivity of the inhibitory complex itself. As we have outlined above, the direct inhibition of GSK3 by phosphorylated LRP relies on a weak affinity between the two proteins, whose successful interaction might depend on a high local concentration within the signalosome. Furthermore, the signalosome itself depends on multiple weak and transient protein interactions. For example, the association of Dishevelled with LRP signalosomes is highly dynamic (Schwarz-Romond et al., 2007b) and barely stable enough to survive biochemical fractionation (Bilic et al., 2007). Weak protein interactions are notoriously difficult to preserve during cell lysis using detergents. Along similar lines, Simons et al. demonstrated that the plasma membrane microenvironment (specifically its pH and charge) promotes the retention of Dishevelled at the plasma membrane through an electrostatic interaction between the negatively charged lipid head groups and a positively charged patch in its DEP domain (Simons et al., 2009), and thus enables Dishevelled to interact directly with Frizzled (Wong et al., 2003).

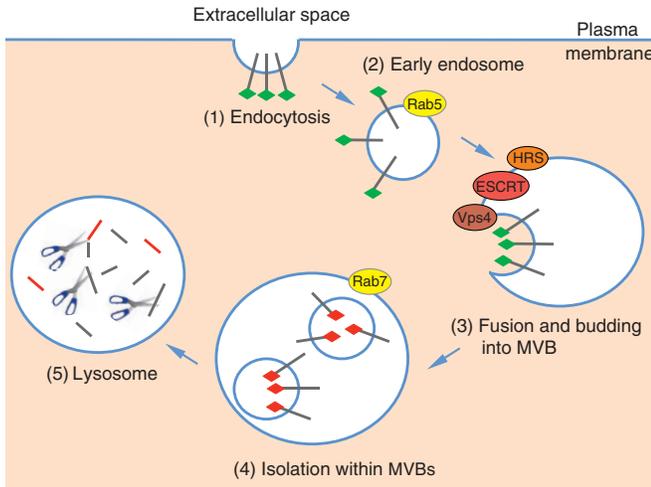


Fig. 4. MVB-mediated isolation of transmembrane receptor-bound proteins from the cytoplasm during late endocytic stages. Simplified sketch of the endocytosis pathway (five successive steps) through which transmembrane proteins (grey sticks; e.g. Frizzled and LRP) and associated cytoplasmic cargo (green diamonds; e.g. signalosome components such as Axin and GSK3; see also Fig. 1) are internalised from the plasma membrane and targeted to lysosomes for degradation. Arrows indicate the default flux through the pathway (although retrograde and branching-off retrieval fluxes may also occur). Also indicated are the factors used by Taelman et al. to manipulate the flux through the pathway (Taelman et al., 2010), and the steps regulated by these factors (see also text; ESCRT, ESCRT-I–III; HRS functions prior to, and Vps4 subsequent to, ESCRT-I–III). Note that an essential feature of the cell-biological model proposed by Taelman and colleagues is that Wnt and LRP promote the internalisation of GSK3 into intraluminal vesicles of MVBs (red diamonds, insulated from the cytoplasm) rather than merely into endosomes (green diamonds, exposed to the cytoplasm), to achieve insulation of GSK3 from de-novo-synthesised β -catenin. According to this model, β -catenin escapes phosphorylation by GSK and subsequent proteasomal degradation under these conditions and, therefore, accumulates and translocates into the nucleus to co-activate transcription (see Taelman et al., 2010, and figure 7 therein).

Specifically, the application of sphingosine, a cationic lipid that reduces the negative charge at the inner membrane surface, substantially impairs Frizzled-mediated recruitment of Dishevelled to the cell membrane (Simons et al., 2009). It is, therefore, conceivable that the weak protein interactions that underlie GSK3 inhibition in the signalosome (e.g. phospho-PPPSPxS–GSK3 or DIX–DIX interactions) are sensitive to treatment with Triton X-100, although this has not been tested directly.

Internalisation of GSK into MVBs following activation of the Wnt pathway

Wnt stimulation of mammalian cells causes the internalisation of LRP6 into endosomal vesicles, probably through a caveolin-dependent pathway (Yamamoto et al., 2006; Yamamoto et al., 2008). Likewise, Wg and Arrow – the *Drosophila melanogaster* homologue of LRP (isoforms 5 and 6) – are internalised into the same vesicles in *Drosophila* tissues (Rives et al., 2006; Seto and Bellen, 2006). Given the association between GSK3 and phosphorylated LRP in signalosomes, it was reasonable for Taelman and colleagues to presume that GSK3 is internalised along with LRP following Wnt signalling (Taelman et al., 2010). However, for their insulation model to be valid, it was crucial to demonstrate

that GSK3 is internalised not only into endosomes, whose topology would still allow GSK3 to access cytosolic β -catenin, but also into the lumen of MVBs (Fig. 4), in which GSK3 would be effectively isolated from newly synthesised β -catenin in the cytoplasm. They achieved this using cryo-immunoelectron microscopy, which revealed the presence of GSK3 β within MVBs of 3T3 mouse fibroblasts following exposure to Wnt3a (Taelman et al., 2010). The group also provided evidence that the bulk of overexpressed (and endogenous) GSK3 β is relocated into cytoplasmic puncta following pathway activation by Wnt itself (i.e. by a chimera between *Xenopus laevis* Wnt8 and Venus, a derivative of yellow fluorescent protein) or, more typically, by overexpression of a constitutively active truncation of LRP6 (CA-LRP6), which lacks its extracellular N-terminal domain but still contains its transmembrane domain [also referred to as LRP6 Δ N (Mao et al., 2001; Tamai et al., 2004)]. The cytoplasmic puncta were identified as vesicles using markers – predominantly LysoTracker (a dye that marks acidic vesicles, most notably lysosomes), but also Rab7 (tagged with GFP) as a late endosomal marker, and Vps4 (marking the final stages of vesicle formation in MVBs) (Fig. 4). Perhaps the most compelling evidence was obtained by the overexpression of a dominant-active mutant of Rab5 – a small GTPase that regulates vesicle docking and fusion in the early endocytic pathway – which causes the formation of giant endosomes and whose membranes clearly enclose co-overexpressed GSK3 β following activation of the Wnt pathway (which is driven by overexpression of LRP6 Δ N; see below).

Relocalisation of the bulk of GSK3 β into punctate structures was also observed following co-expression with Dishevelled, or with a fusion protein derived from the Dishevelled DIX domain and the cytoplasmic tail of LRP6 (DIX>ctail) (Taelman et al., 2010). DIX>ctail is one of the most potent activators of β -catenin signalling in mammalian cells and in *Drosophila*; it completely bypasses the requirements for Wnt, LRP and Dishevelled (Metcalf et al., 2010). DIX>ctail forms numerous cytoplasmic puncta (Fig. 2Ci), similar to those formed by overexpressed Dishevelled (Fig. 2Ai) but distinct from the irregular LRP6 Δ N speckles (Fig. 2B), which tend to be positive for endosomal vesicle markers such as caveolin (Yamamoto et al., 2006; Yamamoto et al., 2008). Indeed, the puncta formed by DIX>ctail (which lacks the transmembrane domain of LRP6) fail to colocalise with a variety of endosomal vesicle markers (Metcalf et al., 2010) and, thus, are likely to represent protein assemblies, similar to Dishevelled puncta. Importantly, in that study, DIX>ctail puncta recruit apparently stoichiometric amounts of co-expressed Axin – probably through direct DIX–DIX interactions – but only small amounts of co-expressed GSK3 β (Metcalf et al., 2010). This is similar to Dishevelled 2 (Dvl2), which only recruits a fraction of co-expressed GSK3 β (Fig. 2Aii) – probably because this recruitment is mediated by endogenous Axin (Metcalf et al., 2010) and is, thus, limited by the low expression levels of Axin [(Lee et al., 2003); notably, only 3–5% of the endogenous GSK3 β pool is associated with Axin (Ng et al., 2009)]. These findings contrast those of Taelman et al., who observed relocalisation of the bulk of co-expressed GSK3 β into Dishevelled and DIX>ctail puncta – a niggling inconsistency that needs to be resolved (Taelman et al., 2010).

Ubiquitin modifications are a key requirement for the targeting of membrane proteins, such as epidermal growth factor receptor (EGFR) to MVBs, and also for their subsequent internalisation into the MVB internal compartment (Huang et al., 2006; Raiborg et al., 2003). Given that Taelman et al. observed efficient targeting of GSK3 β into MVBs when co-expressed with LRP6 Δ N (Taelman

et al., 2010), one might expect LRP6 Δ N aggregates to be ubiquitylated – indeed, one might expect to see ubiquitylation of GSK3 itself; this would earmark it for direct recognition by the ESCRT (endosomal sorting complex required for transport; a series of cytosolic protein complexes called ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) machinery, which depends on ubiquitylation of client proteins. The ubiquitylation status of GSK3 or LRP has not been examined so far the demonstration that GSK3 is ubiquitylated in response to Wnt signalling would provide powerful evidence in support of the MVB internalisation hypothesis. The identification of key ubiquitylation sites on signalosome-associated proteins would also potentially allow the design of specific mutants to test directly the functional consequences of trafficking to MVBs – as outlined in the next section.

Sequestration of GSK3 by MVBs during Wnt/ β -catenin signalling

The GSK3 sequestration hypothesis predicts that proteins that are required for MVB maturation – as, for example, hepatocyte growth-factor-regulated tyrosine kinase substrate (HRS) – are also essential for Wnt signal transduction. Localised to the cytoplasmic surface of the early endosome, HRS forms part of the molecular machinery that regulates the sorting of transmembrane proteins into the intraluminal vesicle of MVBs (Raiborg et al., 2003). Taelman and colleagues found that siRNA-mediated depletion of HRS blocks the accumulation of β -catenin in Wnt-exposed mammalian cells, as well as its transcriptional activity (Taelman et al., 2010). The same siRNA treatment also blocked LRP6 Δ N-mediated relocalisation of GSK3 β into vesicle-like structures, which was accompanied by an increase in levels of endogenous GSK3 β , thereby suggesting that sequestration of GSK3 in MVBs eventually leads to its degradation. This is expected, given that MVBs are destined to fuse with lysosomes, resulting in the degradation of vesicle cargo by lysosomal hydrolases (Raiborg et al., 2003) (Fig. 4). Taelman and co-workers confirmed their observations by overexpressing a dominant-negative form of Vps4 (Taelman et al., 2010) – an ATPase that is required for the pinching off of vesicles towards the lumen of MVBs (Raiborg et al., 2003). They provide evidence that the half-life of overexpressed GSK3 β increases approximately twofold (from ~1.5 to ~3 hours) following co-expression with dominant-negative Vps4, which further supports the notion that GSK3 is normally degraded in the MVB–lysosome pathway (although the half-life of endogenous GSK3 β was not determined under these conditions, and its steady-state levels remained the same following Wnt stimulation). These are interesting observations, and their physiological relevance was demonstrated by monitoring axis duplication (which serves as a readout of activated β -catenin during early development) in *Xenopus* embryos after blocking MVB maturation (Taelman et al., 2010).

The demonstration that Wnt signalling requires MVB maturation in mammalian cells and *Xenopus* embryos is in contrast to evidence that has been obtained in *Drosophila* tissues, which shows that β -catenin signalling is not suppressed but, if anything, enhanced, following a block in MVB maturation, e.g. in *hrs*-null mutant clones of cells (Rives et al., 2006; Seto and Bellen, 2006). Indeed, various genetic manipulations that attenuate or block MVB maturation, e.g. loss-of-function of HRS and of other ESCRT components, cause cell-autonomous hyperactivation of several developmental signalling pathways – most notably signalling by tyrosine kinases and Notch – owing to a failure in the downregulation of these receptors (Lloyd et al., 2002; Thompson et al., 2005; Vaccari and Bilder, 2005). In particular, overexpression

of dominant-negative *Drosophila* Vps4 (dVps4) results in the formation of enlarged endosomes and in the accumulation of ubiquitylated substrates (probably membrane receptors), causing pleiotropic effects including dysregulation of the actin cytoskeleton and loss of apico-basal polarity (Rodahl et al., 2009a). Notably, loss-of-function of dVps4 also promotes apoptosis, owing to cell-autonomous hyperactivation of Jun kinase signalling; restoration of cell viability by double-deficiency of dVps4 and Jun kinase activity revealed prominent cell overgrowth, analogous to neoplastic tumour formation (Rodahl et al., 2009a). ESCRT proteins have, thus, been widely viewed as tumour suppressors because they broadly attenuate signalling by growth factors and their receptors and, in particular, that of EGFR (Raiborg et al., 2003; Rodahl et al., 2009b; Tanaka et al., 2008). This provides a conceptual problem with the model proposed by Taelman and co-workers, which envisages that MVB maturation promotes β -catenin signalling (at least in the presence of Wnt), therefore implying a tumour-promoting role of ESCRT proteins, given the potent oncogenic properties of activated β -catenin (Taelman et al., 2010). Implicit in this model is that the regulation of β -catenin signalling by the MVB–lysosome pathway is opposite to that of other growth factor signalling pathways.

Another conceptual problem arises from the observation by Woodgett and colleagues, based on their GSK3 knock-out studies in mice. The total GSK3 pool (i.e. α - plus β -isoforms) needs to be depleted to 25% of its normal level before β -catenin activation can be detected (Doble et al., 2007). This suggests that cells can tolerate large fluctuations of GSK3 levels without activating their Wnt/ β -catenin pathway. Accordingly, it would be necessary to sequester the bulk of the cellular GSK3 β and GSK3 α for sequestration to have an effect on Wnt/ β -catenin signalling.

Perhaps the most puzzling result of the study by Taelman was their inability to detect a reduction in the endogenous GSK3 β levels following Wnt stimulation (Taelman et al., 2010) – which is in agreement with another study reporting that the steady-state levels of GSK3 β did not change following Wnt stimulation (Blitzer and Nusse, 2006). Clearly, one would expect to see this if Wnt were to induce the internalisation of GSK3 into MVBs, which are destined to fuse with lysosomes (Fig. 4). This poses a significant problem with the sequestration model, as acknowledged by the authors. They offer various explanations, one of which is that endogenous GSK3 might have a long half-life – although this does not appear to apply because their own data indicate a half-life of ~1.5 hours (albeit of overexpressed GSK3 β). This should allow the detection of GSK3 downregulation after the 2 hours of Wnt stimulation that was employed in this experiment. Alternative explanations, which might reconcile this negative result with the MVB sequestration model are also provided by Taelman and colleagues [see the discussion section in their article (Taelman et al., 2010)]. For example, it is conceivable that the cell employs a Wnt-specific factor that regulates MVB maturation, or that Wnt signalling imposes a Wnt-specific block of the MVB–lysosome progression, which might allow retrieval of GSK3 into the cytoplasm before it reaches the lysosomal compartment for degradation. There are currently no known precedents for such a highly specialised retrieval mechanism dedicated to a single pathway.

A new function of β -catenin in the uptake of GSK3 into MVBs?

One particularly surprising finding by Taelman and colleagues was that β -catenin itself is required for GSK3 sequestration into MVBs

(Taelman et al., 2010). This suggests an intriguing new mechanism of how soluble β -catenin (the Wnt-transducing pool of cellular β -catenin) might act outside the nucleus, the location where it normally executes its function in Wnt-induced transcription. Given the association of Axin with LRP signalosomes, it was reasonable for the authors to presume that Axin is required for GSK3 uptake by MVBs following LRP6 Δ N co-expression [as shown (Taelman et al., 2010)]; however, it was somewhat unexpected that β -catenin is also necessary. In fact, they found that overexpression of an activated (non-phosphorylatable) GFP-tagged form of β -catenin is sufficient to relocate GSK3 β to MVBs. GFP- β -catenin appears to achieve this by being internalised itself into MVBs (for which the authors provide evidence by inducing giant endosomes with dominant-active Rab5). This is in apparent contrast to earlier findings, mentioned above, that β -catenin is released from the membrane-recruited Axin complex following Wnt signalling (Kishida et al., 1999; Willert et al., 1999). Curiously, when Taelman and co-workers merely overexpressed GFP- β -catenin, without stimulating the Wnt pathway, they observed prominent LysoTracker-positive green-fluorescent puncta but only very little diffuse green fluorescence (Taelman et al., 2010). Other groups (including ours), however, have reported endogenous β -catenin and overexpressed GFP-tagged β -catenin to be predominantly diffuse (Fig. 2Cii, arrowheads; Fig. 2D), as would be expected for a well-folded soluble cytoplasmic protein.

Taelman et al. also provide evidence that β -catenin uptake into MVBs is crucial for its axis-inducing activity in *Xenopus* animal cap explants (Taelman et al., 2010), as its transcriptional read-outs are blocked by RNAi-mediated depletion of HRS and by expression of a dominant-negative form of Vps4. There are obvious concerns regarding cell viability under these conditions and also regarding other pleiotropic effects, such as those uncovered in the genetic studies in *Drosophila*, as mentioned above. However, the authors allayed these concerns by conducting various control experiments (including the overexpression of a β -catenin–TCF chimera that is directly targeted to Wnt target genes, and overexpression of Siamois itself, the product of a pivotal β -catenin target gene in early *Xenopus* embryos). Further investigations are needed to unravel the mechanism of how β -catenin prompts internalisation of GSK3 into MVBs, and how its sequestration in MVBs promotes β -catenin-dependent transcription in the nucleus – which depends on newly synthesised β -catenin (Willert et al., 2002).

Insulation between different growth factor pathways that result in GSK3 inhibition

As mentioned already, GSK3 is a multi-faceted kinase that is regulated by different growth factor signalling pathways and that affects a diverse range of physiological processes. Although crosstalk between signalling pathways is a feature of development, homeostasis and disease, it is widely accepted that the Wnt pathway is insulated from other signalling pathways: in Wnt-stimulated cells, inhibition of GSK3 has a profound influence on β -catenin and Axin phosphorylation but does not substantially affect phosphorylation of other GSK3 target substrates, such as tau and glycogen synthase (Ding et al., 2000). Indeed, Axin is thought to have the key role in this insulation mechanism through its association with both enzyme and substrate, and by providing extensive scaffolding (Dajani et al., 2003). The biochemical model of signalosome-mediated GSK3 inhibition (Figs 1, 3) implies that only the small fraction of GSK3 that is associated with the Axin complex [3–5% (Ng et al., 2009)] is recruited to – and inhibited

by – phosphorylated LRP, leaving a significant amount (>95% of the total pool) of free cytoplasmic GSK3 that is unaffected. Despite this, Taelman et al. find, by using protein labelling and pulse-chase experiments, that inhibition of GSK3 by Wnt affects the half-life of 20% of all cellular proteins (including β -catenin), which leads them to propose that Wnt signalling controls global protein half-life (Taelman et al., 2010). This is difficult to reconcile even with the MVB sequestration model because this model implies pathway insulation much like the biochemical model (Figs 1, 3) – both models depend on Axin having a crucial role in blocking GSK3 activity towards β -catenin.

Conclusions and Perspectives

Intensive efforts in the Wnt field have, in recent years, generated substantial insight into the events that precede LRP phosphorylation, a step that is widely recognised to be crucial for Wnt signal transduction to β -catenin. Many groups have tackled the question of how phosphorylated LRP translates into the inhibition of β -catenin phosphorylation by GSK3. Here, we have commented on two contrasting models that describe how this might occur: a biochemical model that suggests direct catalytic inhibition of Axin-associated GSK3 by phosphorylated LRP peptides, and an indirect cell-biological model that is based on LRP- and Axin-mediated GSK3 sequestration by MVBs. The rapid block of GSK3-mediated phosphorylation of β -catenin following acute Wnt stimulation is explained more readily by the former rather than the latter, which was derived largely from experiments that depended on extensive periods of pathway stimulation. Indeed, the experimental design by Taelman et al. involved typically overexpression of an abnormal LRP truncation that accumulates to a high concentration (Taelman et al., 2010, and figure 3B therein). Truncated LRP at high levels, might itself be targeted to MVBs and for subsequent lysosomal degradation (like other abnormal transmembrane proteins). It is, therefore, conceivable that these rather artificial conditions of chronic Wnt hyperactivation could have triggered a ‘damage-limitation pathway’ that is usually not operational during periods of physiological Wnt stimulation as they occur during development and in adult tissues (Clevers, 2006; Logan and Nusse, 2004). However, it is also conceivable that both mechanisms are in operation, and complement each other; for example, catalytic inhibition of GSK3 might occur during acute Wnt stimulation, whereas the membrane-mediated insulation of GSK3 might come into effect during extensive periods of chronic Wnt signalling. Careful time courses are needed to support this idea. Indeed, both models are still relatively new and, naturally, require further consolidation. This is especially true for the new cell-biological model, which has emerged from a single thought-provoking study that could not possibly have addressed all potential concerns. Here, we have highlighted a number of key issues that require further investigation before complete understanding may be reached of how Wnt signalling activates β -catenin through the blocking of GSK3.

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