

Regulation of Lrp6 phosphorylation

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Abstract The Wnt/ β -catenin signaling pathway plays important roles in embryonic development and tissue homeostasis, and is implicated in human disease. Wnts transduce signals via transmembrane receptors of the Frizzled (Fzd/Fz) family and the low density lipoprotein receptor-related protein 5/6 (Lrp5/6). A key mechanism in their signal transduction is that Wnts induce Lrp6 signalosomes, which become phosphorylated at multiple conserved sites, notably at PPSPXS motifs. Lrp6 phosphorylation is crucial to β -catenin stabilization and pathway activation by promoting Axin and Gsk3 recruitment to phosphorylated sites. Here, we summarize how proline-directed kinases (Gsk3, PKA, Ptk1, Grk5/6) and non-proline-directed kinases (CK1 family) act upon Lrp6, how the phosphorylation is regulated by ligand binding and mitosis, and how Lrp6 phosphorylation leads to β -catenin stabilization.

Keywords Wnt · Lrp6 · Phosphorylation · Protein kinase · Parathyroid hormone · CK1 · Gsk3 · Ccny · Cell cycle

Introduction

Wnt genes encode a family of evolutionarily conserved secreted glycoproteins that regulate many aspects of embryonic development as well as the homeostasis of adult tissues [1–3]. Wnts initiate several signaling cascades via diverse cell-surface receptors [4]. These cascades are grossly subdivided as canonical Wnt/ β -catenin signaling and non-canonical (β -catenin-independent) Wnt signaling [4–6].

Wnt/ β -catenin signaling pathway

Wnt regulates β -catenin stability and formation of a β -catenin-Tcf/Lef complex to control gene expression (Fig. 1). In the absence of Wnt, β -catenin is destabilized by a destruction complex, which contains the scaffold protein Axin, the tumor suppressor Adenomatous polyposis coli (Apc), and the protein kinases, casein kinase I α (CK1 α) and glycogen synthase kinase 3 (Gsk3). β -catenin binds to the destruction complex and is phosphorylated by CK1 α and Gsk3 [7], which marks β -catenin for ubiquitin-mediated degradation by the 26S proteasome [8, 9]. Without β -catenin, Tcf/Lef binds target gene promoters and represses gene transcription [10, 11]. Wnt stimulation suppresses Gsk3-mediated phosphorylation of β -catenin, allowing it to accumulate in the cytosol [7, 12]. Nuclear β -catenin binds Tcf/Lef, recruits transcriptional activators, and promotes target gene transcription [13–16].

Two cell-surface receptors cooperate to transmit Wnt signals across the plasma membrane to activate β -catenin signaling. Frizzled (Fzd/Fz) proteins are high affinity Wnt receptors, which are crucial for both Wnt/ β -catenin and non-canonical Wnt signaling, while low density lipoprotein receptor-related protein 5 and 6 (vertebrate Lrp5/6 and *Drosophila* Arrow) direct signaling to the Wnt/ β -catenin

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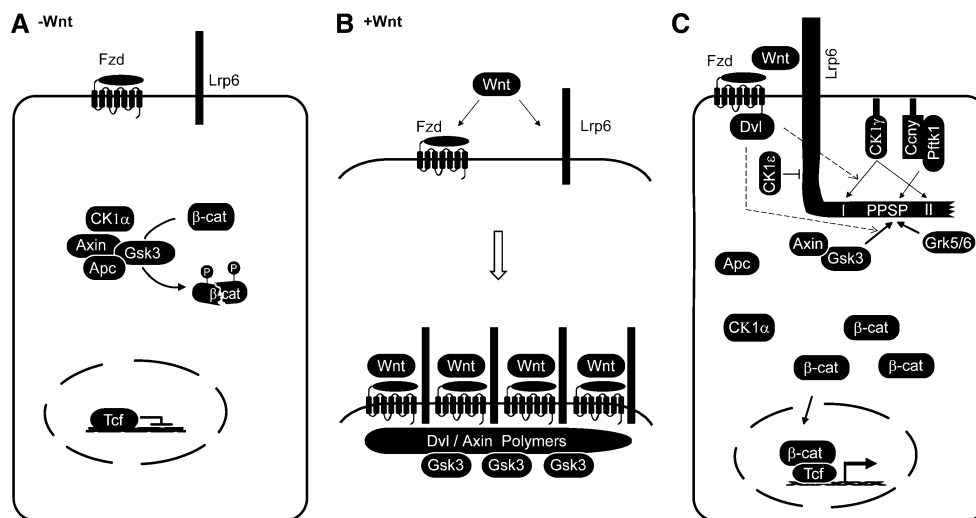


Fig. 1 Wnt/β-catenin signaling pathway. **a** In the absence of Wnt, β-catenin (β-cat) is sequentially phosphorylated by Axin-Apc-bound CK1α and Gsk3, targeting β-catenin for degradation. **b** Wnt binds to transmembrane receptors Frizzled (Fzd/Fz) and Lrp6 (or Lrp5), and recruits Dishevelled (Dvl) polymers to the plasma membrane. In turn, Dvl recruits an Axin-Gsk3 complex to the plasma membrane, promoting the formation of Lrp6 signalosomes. **c** Polymerized Lrp6 is then phosphorylated by CK1γ, in particular, at T1479 in the S/T cluster (site I). The phosphorylation of PPSP motif A by Gsk3 is

stimulated by Wnt and Dvl, and serves to prime the CK1-mediated phosphorylation of S in PPSPXS (site II), whose phosphorylation is also induced by Wnt. Doubly phosphorylated PPSPXS motifs then recruit more Axin and Gsk3, and inhibit Gsk3 by acting as direct inhibitors of this kinase. Multiple phospho-PPSPXS repeats cooperate in amplifying this signal. For simplicity only a single PPSPXS repeat is shown. β-catenin then accumulates and enters the nucleus, binds to Tcf/Lef family of transcriptional factors (Tcf) and activates gene transcription

pathway [17–22]. Wnt forms a ternary complex with Lrp6 and Fzd [22], which induces aggregation of receptor–ligand complexes into ribosome-sized Lrp6 signalosomes [23, 24]. This aggregation triggers a key step in the transmembrane transduction of Wnt signals, the phosphorylation of Lrp6 at multiple sites via distinct kinases, which initiates the downstream cascade that leads to β-catenin stabilization [22–27].

Although Lrp6 is well known to promote canonical Wnt signaling, it also plays a role in inhibiting non-canonical Wnt signaling via negative cross-talk by an unknown mechanism [28–30]. It is not clear whether the role of Lrp6 in non-canonical Wnt signaling involves regulation of Lrp6 phosphorylation.

Structure and function of Lrp6

Lrp5/6 and *Drosophila* Arrow are members of the low density lipoprotein receptor family [31, 32]. Lrp6 is a highly modular, single-pass transmembrane protein (Fig. 2a). The extracellular domain (ECD) contains 1,400 amino acids and consists of YWTD domains, EGF-like repeats, and LDL repeats [31–33]. The ECD mediates the interaction between Lrp6 and extracellular ligands such as Wnts, Sost, and Dickkopf (Dkk1) [33–35]. Wnts actually bind poorly to Lrp6 as compared to Fzd, and are probably bound as a Wnt–Fzd complex [22]. High affinity ligands are the Lrp6 antagonists Dkk1 and Sost [33–37]. Different

ligands engage different YWTD/EGF-like domains to bind Lrp6 [33, 34]. Proper folding of the YWTD domain is necessary for Lrp6 maturation and membrane localization, which is facilitated by the molecular chaperone Mesoderm Development (Mesd) in mice and its homologue Boca in *Drosophila* [38, 39]. Lrp6 lacking the ECD constitutively activates the Wnt pathway, suggesting that the ECD auto-inhibits receptor signaling [33, 40].

The Lrp6 intracellular domain (ICD) contains 215 amino acids, which is rich in proline, serine, and threonine residues. When overexpressed, the isolated Lrp6 ICD is sufficient to activate Wnt/β-catenin signaling [33, 41]. The ICD comprises five P-P-[S/T]-P (PPSP) repeats and juxtaposed casein kinase 1 (CK1) sites [25–27] (Fig. 2a). The PPSP motifs, CK1 sites and their arrangement are also conserved in Lrp5 and Arrow (Fig. 2b) [21, 25–27]. Collectively, these sites are referred to as PPSPXS motifs. Both S (or T) residues (shown in bold) are phosphorylated and this dual phosphorylation is required for β-catenin stabilization and target gene transcription [25–27]. Upstream of these PPSPXS repeats is an S/T cluster (Fig. 2a, b), which is also phosphorylated and notably T1479 phosphorylation within this cluster has been well characterized [24, 25, 42–44].

Several phospho-specific antibodies against different phosphorylation sites have been generated and are important tools to monitor Lrp6 activation, including anti-Sp1490, which recognizes the phosphorylated PPSP repeat A [25–27] and anti-Tp1572 and Sp1607, recognizing PPSP

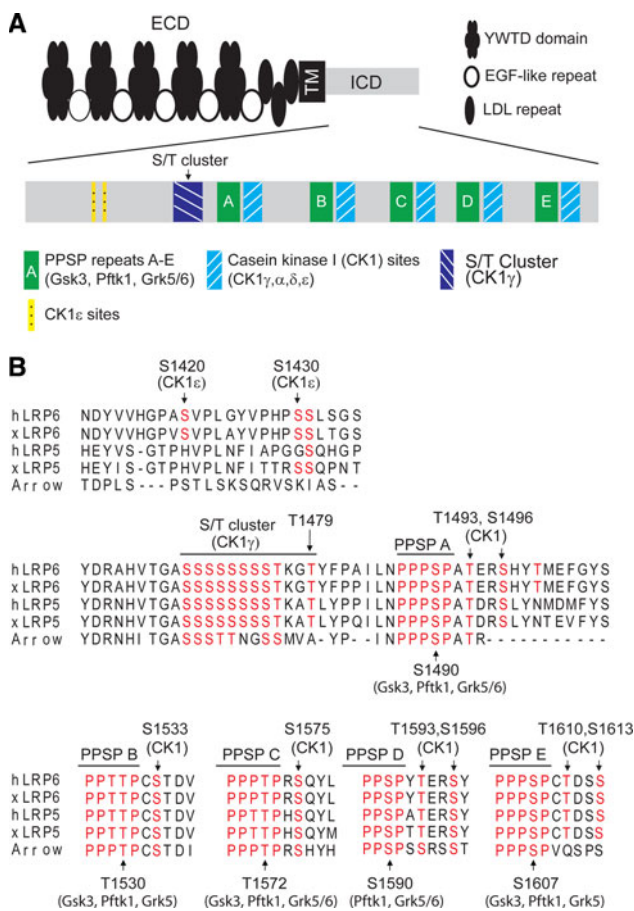


Fig. 2 Lrp6 structure and phosphorylation sites. **a** Lrp6 is a single-pass transmembrane protein. It has a modular extracellular domain (ECD), consisting of YWTD domains, EGF-like repeats, and LDL repeats. The intracellular domain (ICD) consists of PPSP repeats (A–E), each juxtaposed with CK1 sites. Protein kinases promoting Lrp6 signal transduction are indicated. Note that N-terminal phosphorylation by CK1ε is inhibitory to Wnt/Lrp6 signaling. **b** Alignment of the ICD sequences from human (*h*) and *Xenopus* (*x*) Lrp6, Lrp5, and *Drosophila* Arrow. The S/T cluster, PPSPXS motifs, and CK1ε sites are highlighted in red. The target sites and corresponding protein kinases are indicated by arrows. Residues are numbered according to the human Lrp6 sequence

repeats C and E [45] (Fig. 2b). The PPSP sites are phosphorylated in a Wnt-dependent fashion but also show constitutive phosphorylation [25–27, 42, 45–49]. This distinction is important because the PPSP kinases involved in acute Wnt-induced or constitutive PPSP phosphorylation appear to be different [25–27, 45–47]. Anti-Tp1479 recognizes the phosphorylated T1479 in the S/T cluster and anti-Tp1493 recognizes the phosphorylated T1493 in PPSPXS motif A (Fig. 2b). T1479 and T1493 phosphorylation are both strictly Wnt-dependent [25, 26].

Since Lrp6 phosphorylation is crucial for Wnt signal transduction, it is important to characterize the kinases involved. So far, five classes of protein kinases are known to phosphorylate Lrp6, which fall into two groups: proline-

directed kinases which phosphorylate PPSPXS sites, namely Gsk3, Protein kinase A (PKA), Ptk members, and G protein-coupled receptor kinases (Grk5/6), and non-proline directed kinases, namely members of the CK1 family, which phosphorylate PPSPXS, the S/T cluster and additional N-terminal sites (Fig. 2a, b; Table 1).

Gsk3 mediates PPSP phosphorylation in response to acute Wnt signaling

Gsk3 is a serine/threonine protein kinase, which is encoded by *shaggy* in *Drosophila* and by *Gsk3α* and *Gsk3β* in vertebrates. Gsk3 has a plethora of substrates and participates in numerous cell signaling cascades [50–52]. In Wnt/β-catenin signaling, Gsk3 is a master negative regulator of β-catenin. In spite of this negative role, Gsk3 phosphorylates Lrp6 and thereby positively regulates Wnt/β-catenin signaling [26]. In vitro, Gsk3 directly binds Lrp6 ICD and phosphorylates PPSP repeats [26, 53]. In cultured cells, overexpression of Gsk3 promotes PPSP phosphorylation at S1490 [26]. When artificially anchored to the plasma membrane, Gsk3 activates Wnt/β-catenin signaling in cultured cells and *Xenopus* embryos [26]. Wnt induces Lrp6 PPSP phosphorylation at repeats A, C, and E (S1490, T1572, S1607) and most likely also at repeats B and D (T1530, S1590). Wnt-induced PPSP phosphorylation is inhibited by pharmacological Gsk3 inhibitors or genetic ablation of Gsk3 [26, 45, 47]. Gsk3 substrates share the unique phosphorylation motif [S/T]-X-X-X-p[S/T], which typically must first be primed by phosphorylation at the +4 S/T site [51]. Gsk3 phosphorylation of Lrp6 PPSP repeats is unusual, as there is no priming residue at +4 positions in the PPSPXS repeats. The results indicate that Gsk3 mediates PPSP phosphorylation in response to acute Wnt signaling. However, its physiological relevance in vivo remains to be demonstrated.

Protein kinase A mediates Lrp6 PPSP phosphorylation in response to parathyroid hormone

Protein kinase A (PKA) is a cyclic AMP (cAMP)-dependent serine/threonine protein kinase. PKA phosphorylates transcription factor CREB and hundreds of other targets [54]. A recent report showed that parathyroid hormone (PTH) can signal through Lrp6 by forming a ternary complex with its receptor PTH1R and Lrp6 to promote osteoblast differentiation [48]. PTH treatment induces rapid Lrp6 S1490 phosphorylation and activates PTH and β-catenin signaling. A pharmacological PKA inhibitor blocks PTH-induced S1490 phosphorylation, suggesting that this kinase is involved in Lrp6 phosphorylation. In

Table 1 Summary of Lrp6 protein kinases

Kinase	Sites	Features; effect on Wnt signaling (+, -)	References
Gsk3	S1490 T1530 T1572 S1590 S1607	Wnt-inducible; Axin-, Dvl-, and Fz-dependent; promotes Lrp6 signaling; PI4KII α - and PIP5KI-dependent; (+/-)	[26,42,45,47,110]
PKA	S1490	PTH-inducible, Wnt-independent; Dkk1-sensitive; (+)	[48]
Grk5/6	S1490 T1530 T1572 S1590 S1607	Wnt-inducible; Dkk1-sensitive; (+)	[66]
Pftk1/ Ccnv	S1490	likely Wnt-independent; Dkk1-independent; Dvl-independent; (+)	[71]
CK1 γ	T1479 T1493	Wnt-inducible; Dvl-dependent; PI4KII α - and PIP5KI-dependent; (+)	[24,25,42,71,101]
CK1 α,δ,ϵ	T1493	Wnt-inducible; (+)	[26,101]
CK1 ϵ	S1420 S1430	(-)	[102]

vitro, PKA can directly phosphorylate Lrp6, but the sites phosphorylated have not been analyzed [48]. PKA also mediates a Wnt-mediated myogenic pathway during mouse development [55], suggesting that PKA may be more widely involved in Lrp6 phosphorylation. In the osteoblastic cell line Saos-2, PTH/PKA signaling can also induce Ser9 phosphorylation and inhibition of Gsk3, thereby promoting canonical Wnt signaling [56], raising the possibility that PTH/PKA effect phosphorylation at two different levels which synergize to activate canonical Wnt signaling. Like Lrp6, PKA has also been implicated in regulating Wnt/Planar Cell Polarity (PCP) signaling during convergent extension movements [57, 58]. It is unknown if the role of Lrp6 in PCP signaling involves PKA.

Grk5/6 mediate PPSP phosphorylation in response to acute Wnt signaling

Grk5/6 are membrane-associated protein kinases belonging to the G protein-coupled receptor kinase (Grk) family that specifically recognize and phosphorylate agonist-activated G protein-coupled receptors (GPCR). GPCR phosphorylation leads to GPCR desensitization, endocytosis, intracellular trafficking, resensitization, and modulation of intracellular GPCR signaling [59]. However, Grks also phosphorylate non-GPCR substrates, such as Synuclein and Tubulin [60–64]. In Wnt/ β -catenin signaling, Grk2 binds Apc and inhibits the signaling [65]. Conversely, Grk5/6 can promote both Lrp6 S1490 phosphorylation and Wnt signaling [66]. Grk5 is necessary for S1490 phosphorylation in response to acute Wnt stimulation. Grk5/6 knock down phenocopies loss of Wnt signaling in zebrafish. In vitro, Grk5/6 can directly phosphorylate PPSP repeats A–E. Therefore, Grk5/6 functions as an Lrp6 PPSP kinase in

response to acute Wnt signaling. Interestingly, parathyroid hormone, whose receptor itself is phosphorylated by Grk5 [67], also regulates Lrp6 PPSP phosphorylation [48].

Pftk1/cyclin Y mediate PPSP phosphorylation during mitosis

The cyclin-dependent protein kinase (Cdk) superfamily of serine/threonine protein kinases plays important roles in eukaryotic cell cycle regulation. Eukaryotic cell division is an ordered process that consists of four distinct phases: G1 phase, S phase, G2 phase (collectively known as interphase), and M phase (mitosis). Cdks are dependent on associations with their activating subunits, the cyclins, to be enzymatically active [68]. The levels of regulatory cyclins typically oscillate throughout cell cycle so that Cdks can initiate phase-specific events and control transitions of specific phases [68]. Apart from directly activating Cdks, cyclins contain regions that can restrict Cdks to specific subcellular locations or targets [69, 70].

Recently, a Cdk member was identified as a PPSP kinase, namely *Drosophila* L63 and its vertebrate homologue PFTAIRE protein kinase 1 (Pftk1) [71]. Homologues of Pftk1/L63 are PFTAIRE protein kinase 2 (Pftk2) and PCTAIRE protein kinase 1-3 (Pctk1-3) [72, 73]. Cyclin Y (Ccnv) is a partner of L63/Pftk1 [71, 74, 75] and regulates Pftk1 activity at multiple levels [71]. In the presence of Ccnv, Pftk1 phosphorylates Lrp6 PPSP repeat A both in vivo and in vitro, while the kinase on its own is inactive towards Lrp6. Ccnv contains an N-terminal myristoylation signal and is predominantly enriched at the inner surface of plasma membrane [71, 75], where it binds the receptor Lrp6 and recruits Pftk1 [71]. Importantly, overexpressed Ccnv promotes Pftk1 phosphorylation of Lrp6, while

disruption of Ccny membrane association abolishes this ability [71]. Ccny is regulated by ubiquitin-mediated proteolysis and its level along with Pftk1 peaks during G2/M phase of cell cycle [71]. Remarkably, Lrp6 PPSP phosphorylation and Wnt/ β -catenin signaling are also under cell cycle control and peak during G2/M phase in cultured cells and in *Xenopus* embryos [71]. Ccny inhibition in *Xenopus* embryos induces antero-posterior patterning defects, which phenocopy loss of Wnt/ β -catenin signaling. Intriguingly, a reduction of Lrp6 phosphorylation and Wnt signaling provoked by Lrp6 knock down is partially rescued by knock down of cdc25 [71]. Cdc25 is a protein tyrosine phosphatase, which plays a key role in regulating entry into mitosis, by dephosphorylating cyclin B-bound Cdk1 [76]. Cdc25 knock down arrests cells in G2 phase, thereby promoting Lrp6 phosphorylation and downstream signaling. The surprising finding that Lrp6 phosphorylation and Wnt/ β -catenin signaling peak during mitosis is also supported by previous findings that β -catenin levels peak between G2/M [77, 78] and that *Cdc25* mutants in mice and in *Drosophila* show enhanced Wnt signaling [79, 80]. Taken together, Pftk1/Ccny represents a mitotic Cdk, which functions as Lrp6 PPSP kinase.

Mitotic Wnt signaling suggests that Wnt signaling may play a broader role beyond transcriptional regulation. In fact, Apc is localized to kinetochores, centrosomes, and the +end of microtubules [81, 82] and regulates spindle orientation, cell fate specification, chromosome alignment, chromosome stability, and the mitotic checkpoint [81–86]. Likewise, β -catenin binds to centrosomes and regulates their separation and normal spindle formation [87, 88]. Similarly, Gsk3 and Axin also have direct functions in the mitotic apparatus [89–91]. Another prominent example for mitotic Wnt/ β -catenin signaling is *Caenorhabditis elegans*, where it is essential for orienting the mitotic spindle during asymmetric cell division in embryonic development [92–94]. Taken together, this suggests that Pftk1/Ccny phosphorylates Lrp6 at G2/M to induce Wnt/ β -catenin signaling for orchestrating a mitotic program beyond transcriptional regulation.

Casein kinase 1 family

While all other kinases mentioned are proline-directed kinases involved in PPSP phosphorylation, members of the CK1 family are responsible for phosphorylation of PPSPXS sites as well as the S/T cluster. The CK1 family of serine/threonine protein kinases is highly conserved from yeast to humans [95]. The family comprises CK1 α , δ , ϵ (discs overgrown/*dco* in *Drosophila*) and γ 1–3 isoforms (gilgamesh/*gish* in *Drosophila*). Consensus CK1 recognition motifs are (1) p[S/T]-X₁₋₂[S/T] and (2) [D/E]-X₁₋₂[S/T],

where the S/T is the CK1 phosphorylation site. Phosphorylated S/T or the negatively charged D/E residue primes the phosphorylation [96, 97]. Additional consensus recognition sites have also been documented [98]. In Wnt/ β -catenin signaling, CK1 members phosphorylate multiple pathway components, including β -catenin, Dishevelled (Dvl/Dsh), Apc, Axin and Tcf/Lef to negatively and/or positively regulate signaling [99, 100]. In Lrp6 phosphorylation, multiple CK1 members act to positively and negatively regulate the receptor as discussed below.

CK1 γ promotes Wnt-induced Lrp6 phosphorylation

CK1 γ was identified in a screen for proteins that covalently modify Lrp6 [25]. Overexpression of CK1 γ induces Lrp6 T1479 phosphorylation in vitro and in vivo. CK1 γ is unique within the CK1 family, because it harbors a C-terminal lipid modification signal and accordingly is bound to the plasma membrane [25, 95], where it interacts directly with Lrp6 [25]. Disruption of CK1 γ membrane association abolishes Lrp6 binding and its ability to promote Wnt signaling. CK1 γ is required for Wnt signaling during *Xenopus* antero-posterior patterning, where its knock down phenocopies loss of Wnt signaling. CK1 γ phosphorylates CK1 sites T1479 (in the S/T cluster) and T1493 (Fig. 2b). Phosphomimetic amino acid substitutions at these sites activate Wnt/Lrp6 signaling, while alanine substitutions are inhibitory, demonstrating that these sites are essential for signal transduction. CK1 γ probably also phosphorylates PPSPXS sites B–E, since serial deletions of these sites progressively inhibits Lrp6/CK1 γ signaling [25]. CK1 γ phosphorylation of Lrp6 occurs in response to acute Wnt signaling and can be detected after 10 min. Taken together, CK1 γ is an Lrp6 kinase, which positively regulates Wnt/ β -catenin signaling. T1479 phosphorylation by CK1 γ is widely used to monitor acute Wnt signaling via a Tp1479-specific antibody, and it was essential for the discovery of Lrp6 signalosomes and to the study on Lrp6 endocytosis [24, 43].

CK1 α , δ , ϵ promote Wnt-induced Lrp6 phosphorylation

Similar to CK1 γ , the non-lipid modified CK1 α , δ , ϵ isoforms may also jointly phosphorylate CK1 sites in the PPSPXS motifs [26]. In vitro, CK1 ϵ directly phosphorylates T1493 in PPSPXS repeat A [26]. CK1 α , δ , ϵ , γ overexpression induces phosphatase-sensitive mobility shifts of Arrow in gel electrophoresis [101]. In cultured cells, Wnt induces Lrp6 T1493 phosphorylation, which can be inhibited by combined loss of CK1 α , δ , ϵ [26]. Furthermore, CK1 γ /Gish and CK1 ϵ /Dco synergize in promoting Wingless signaling during wing patterning in *Drosophila* [101]. Thus, CK1 α , δ , ϵ and CK1 γ may have partially redundant roles in

jointly phosphorylating the CK1 sites in PPSPXS repeats [26]. Whether there is also a role for CK1 α,δ,ϵ in phosphorylation of the S/T cluster and other PPSPXS motifs has not been documented.

CK1 ϵ may negatively regulate Lrp6 signaling

While most CK1-mediated phosphorylation promotes Wnt signaling via phosphorylation of PPSPXS sites, CK1 ϵ may also have a negative role by phosphorylating the more N-terminal sites S1420 and S1430 [102]. Alanine substitution of S1420 and S1430 enhances Lrp6 activity in Wnt reporter assays, suggesting that S1420/S1430 phosphorylation negatively regulates Wnt/ β -catenin signaling [102]. The physiological significance of this phosphorylation remains to be established.

Hierarchy of Lrp6 phosphorylation at S/T cluster and PPSPXS motif A

With multiple Lrp6 kinases, their effects may be hierarchical, additive, or mutually exclusive [103]. Certain Lrp6 protein kinases including Gsk3 and CK1 family members are known to be primed by pre-phosphorylation at adjacent sites [104]. Since Lrp6 contains multiple Gsk3 and CK1 phosphorylation sites, this suggests that there may be a hierarchy of phosphorylation events. Analysis of this hierarchy faces two main difficulties. First, each Lrp6 phosphorylation site is phosphorylated by more than one kinase. This may explain why, using the same phospho-specific antibodies, different researchers arrive at different conclusions [25, 26], because they may in fact monitor distinct phosphorylation events. For example, S1490 is phosphorylated Wnt-dependently by Gsk3 and Grk5/6, and Wnt-independently by Pftk1 and PKA. Second, while in vitro kinase assays with partially purified or recombinant kinases are a clean and informative way to reduce complexity to study Lrp6 phosphorylation, the physiological relevance of such studies may be compromised. This is because, in in vitro assays, kinases tend to be more promiscuous than in in vivo.

With these caveats, three hierarchies of Lrp6 phosphorylation have been proposed which, however, are not mutually exclusive (Fig. 3). There is good evidence that T1493 phosphorylation is dependent on S1490 phosphorylation in cultured cells and in vitro [25, 26]. This would be consistent with the established CK1 consensus phosphorylation site p[S/T]-X₁₋₂-[S/T] [25, 71, 105]. It seems reasonable to extrapolate this hierarchy to all PPSPXS sites (Fig. 2b). However, while pre-phosphorylation is necessary for CK1 phosphorylation, it seems not sufficient in vivo. For example, in various cell lines, Lrp6

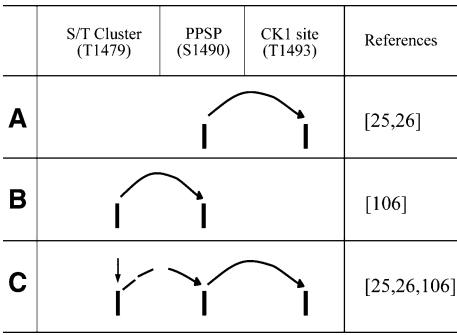


Fig. 3 Hierarchy of Lrp6 phosphorylation at S/T Cluster and PPSPXS motif A. **a** S1490 primes T1493 phosphorylation. **b** Phosphorylation of the S/T cluster promotes S1490 phosphorylation. **c** Sequential phosphorylation (“ping-pong” mode): S/T cluster (including T1479) phosphorylation by CK1 γ occurs independently of S1490 and T1493 phosphorylation (vertical arrow) and may promote S1490 phosphorylation by Gsk3 (dashed arrow), which primes CK1 phosphorylation at T1493

shows constitutive phosphorylation at S1490 but not at CK1 sites [25, 71]. Instead, CK1 site phosphorylation requires Wnt-mediated receptor clustering and signalosome formation [24]. For another example, Pftk1/Ccny-mediated S1490 phosphorylation triggers CK1 phosphorylation only in the presence of Wnt [71]. Furthermore, Wnt-dependent CK1 phosphorylation of the S/T cluster (including T1479) is independent of S1490 phosphorylation [25, 106]. It is unclear if S/T cluster phosphorylation involves another priming event or not, since CK1 can also phosphorylate non-primed sites [96]. There is evidence that S/T cluster phosphorylation stimulates PPSP phosphorylation [106] (Fig. 3b). This is consistent with the direct binding of Gsk3 to the phosphorylated S/T cluster [107].

Taken together, this suggests a sequential priming model, which starts from the S/T cluster and progresses C-terminally (Fig. 3c). If this model is correct, it would imply that CK1 and Gsk3 act in an alternating fashion (“ping-pong” mode). It will be important to systematically address the hierarchy of Lrp6 phosphorylation, for example, by using phospho-specific antibodies against the relevant sites, by determining the kinetics of phosphorylation following Wnt stimulation, and by combining this with specific knock down of relevant protein kinases.

Processive or distributive phosphorylation of multiple PPSPXS sites

Phosphorylation is referred to as processive if a protein kinase binds once to a substrate and phosphorylates all of the available sites before dissociating. Alternatively, phosphorylation may be distributive, where each

phosphorylation requires a separate binding event between the kinase and substrate [103]. With multiple phosphorylation sites, mixed forms of processive and distributive phosphorylation may also occur.

As an example of processive phosphorylation, Gsk3 phosphorylates sites that contain a phospho-Ser at the +4 position, and once Ser656 of glycogen synthase has been phosphorylated by CK2, Gsk3 β sequentially phosphorylates S652, S648, S644, and S640. It has been shown that each phosphorylation depends on prior phosphorylation at the +4 position [108, 109]. The modular Lrp6 structure initially may suggest a processive phosphorylation mechanism, where the kinases scan along the cytoplasmic domain to phosphorylate Lrp6. For Gsk3, however, the PPSP sites are spaced too far apart to be priming in a conventional processive mode. Furthermore, Gsk3 is inhibited via end product inhibition by phosphorylated PPSP sites [53, 107, 110, 111] (see below). This suggests that, upon Wnt stimulation, at least Gsk3 may phosphorylate Lrp6 in a distributive mode. For any of the other Lrp6 kinases, we do not know whether they scan Lrp6 in a processive mode.

Multiple PPSPXS motifs function cooperatively to transduce Wnt signaling

Each PPSPXS motif on its own, for example, when artificially anchored to the plasma membrane, is able to activate Wnt/ β -catenin signaling and recruit Axin [25–27, 45]. Progressive deletion of Lrp6 PPSPXS motifs incrementally reduces their ability to promote Wnt signaling [25, 45, 46]. However, in the context of full length Lrp6, a single PPSPXS is insufficient to transduce Wnt signaling [45, 46]. Instead, multiple PPSPXS motifs function cooperatively to transduce Wnt signaling. Consistently, in response to Wnt stimulation, Lrp6 is phosphorylated at multiple PPSPXS motifs [25–27, 45]. A higher stoichiometry of Lrp6 phosphorylation may promote higher affinity binding to Axin and Gsk3 and hence promote downstream signaling. This highlights that it will be important in the future to determine the contribution of individual kinases in regard to their contribution to stoichiometric Lrp6 phosphorylation, which has not been analyzed either *in vivo* or *in vitro*.

Lrp6 phosphorylation and signaling in signalosomes

Real-time imaging showed that within minutes of Wnt treatment, cytoplasmic Axin colocalizes with Lrp6 in punctate structures below the plasma membrane. Further analysis showed that these structures represent Lrp6

aggregates, or signalosomes, which are phosphorylated, ribosome-sized multiprotein complexes containing Fzd, Dvl, Gsk3, and Axin. Signalosomes presumably represent some sort of endocytic vesicles, which, however, are negative for most endocytic markers, except for occasional colocalisation with caveolin. This would be consistent with the fact that Wnt stimulation requires caveolin mediated endocytosis of Lrp6 [43]. The scaffold protein Dvl is required for Lrp6 aggregation and Lrp6 phosphorylation at T1479 [24, 42] and S1490 [24, 42, 47]. Dvl itself forms cytoplasmic polymers, which can be visualized as microscopic punctae and which are recruited to the plasma membrane upon Wnt stimulation, presumably via binding to activated Fzd [112]. DIX domain mediates Dvl polymerization, which probably increases the avidity for Axin [113, 114]. The ability of Dvl to polymerize is essential for Lrp6 phosphorylation and Wnt signaling [24, 114]. Lrp6 signalosome formation also requires the action of phospholipids and lipid kinases. Wnt stimulates activities of phosphatidylinositol 4-kinase type II α (PI4KII α) and phosphatidyl-4-phosphate 5-kinase type I (PIP5KI) through Fzd and Dvl. This in turn induces formation of PtdIns(4,5)P2 [42], which stimulates Lrp6 phosphorylation through an unknown mechanism. Phospholipid is known to be necessary for Wnt-induced Dvl phosphorylation and Wnt signaling [115, 116].

Taken together, these results suggest a signalosome model for Lrp6 phosphorylation and signaling (Fig. 1b), where Wnt induces the formation of a ligand–receptor complex which recruits Dvl, Axin, and Gsk3. Dvl polymers promote Lrp6 aggregation, which triggers the phosphorylation by CK1 γ and Gsk3, presumably providing a high density of phosphorylation sites (Fig. 1c). The phosphorylation is a consequence but not the cause of aggregation, since Lrp6 signalosomes also form in the presence of dominant-negative CK1 γ , which blocks phosphorylation [24]. The key prediction of this model is that any kind of Lrp6 polymerization should induce phosphorylation and Wnt signaling. Results obtained with Lrp6 fused to artificial oligomerization domains are consistent with this [23]. The net result of Lrp6 signalosome formation is the recruitment and inhibition of Gsk3 by phosphorylated Lrp6 (see below), which derepresses β -catenin [24].

According to an initiation–amplification model, Lrp6 phosphorylation happens in two phases [47]. In the initiation phase, Wnt induces an Lrp6–Fzd complex, which recruits the Axin–Gsk3 complex through Fzd-bound Dvl to initiate Lrp6 phosphorylation. In the amplification phase, initial phospho-Lrp6 serves as high affinity docking site for more Axin–Gsk3, so that Gsk3 acts *in cis* and possibly *in trans* to phosphorylate Lrp6 [47]. During the amplification phase, there is a positive feedback loop: phospho-Lrp6 will

recruit more Axin–Gsk3 complex, which may phosphorylate more Lrp6 and inhibit more Gsk3 because of product inhibition, thereby amplifying Wnt signaling. The signalosome model and dynamic polymerization-phosphorylation are consistent with this proposed amplification. In contrast, it appears there is neither a need nor compelling evidence for signaling happening in a distinct initiation phase.

Downstream of Lrp6 phosphorylation: Axin–Gsk3 recruitment and Gsk3 inhibition

Lrp6–Axin interaction

One of the key consequences of Lrp6 phosphorylation is recruitment of two β -catenin inhibitors, Axin and Gsk3. Axin is present at very low levels and it is a rate-limiting component in Wnt/ β -catenin signaling [117, 118]. Wnt stimulation rapidly results in Lrp6 phosphorylation and Axin recruitment [41]. Axin binding to Lrp6 is very likely direct, since truncated Axin interacts with the Lrp6 cytoplasmic domain in yeast two-hybrid assays [41, 118]. Full-length Axin does not interact with Lrp6/Arrow, because the N-terminal half of Axin prevents interaction [118]. However, full-length Axin interacts with Lrp6 in response to Wnt, as measured by fluorescence resonance energy transfer, which requires very close proximity of partner proteins [41].

Lrp6–Axin interaction requires phosphorylation of both PPSP repeats and CK1 sites [25–27]. Phosphomimetic mutation of Lrp6 CK1 sites promotes Lrp6–Axin interaction. Similarly, alanine substitution of CK1 sites (and PPSP repeat) abolishes Lrp6–Axin interaction [25]. As expected, Lrp6 kinases modulate Lrp6–Axin interaction: dominant-negative CK1 γ abolishes Lrp6–Axin interaction [25]; dual phosphorylation by Gsk3 and CK1 enables Lrp6 to bind Axin, while neither Gsk3 nor CK1 alone can promote binding [26]; and PKA agonists promote Lrp6–Axin interaction [48]. Conversely, CK1 ϵ phosphorylation of S1420 and S1430 diminishes Lrp6–Axin interaction [102]. Taken together, it appears that phospho-Lrp6 directly signals to Axin to activate downstream signaling.

Lrp6–Gsk3 interaction

Direct binding has also been shown between Gsk3 β and Lrp6 ICD [26, 53, 107]. This binding is stimulated by Wnt [24]. Consistently, phospho-Lrp6 binds Gsk3 preferentially over non-phospho-Lrp6 in vitro, which is mediated by the S/T cluster [107]. Gsk3 then recruits Axin to phospho-Lrp6 [107]. Hence Gsk3 may be recruited to Lrp6 both directly and indirectly via Axin.

Phospho-Lrp6 inhibits Gsk3 phosphorylation of β -catenin

Wnt stimulation suppresses Gsk3-phosphorylation of β -catenin [7], possibly by reducing Gsk3 enzymatic activity [119]. However, it is not clear how exactly Wnt regulates Gsk3 activity. It has been shown that phosphorylation of an N-terminal serine (Ser9 in Gsk3 β and Ser21 in Gsk3 α) inactivates Gsk3 [120]. However, homozygous knock-in mice carrying Ser9Ala-Gsk3 β and Ser21Ala-Gsk3 α develop and grow normally [121], confirming that Wnt-regulation of Gsk3 β is independent of Ser9/Ser21 phosphorylation [119]. This raises the possibility that Gsk3 activities are regulated by other inhibitors, for example, Frat [122]. However, compound knock-out of all three *Frat* genes does not affect Wnt/ β -catenin signaling in mice, which argues against this scenario [123]. More recent evidence indicates that phospho-Lrp6 directly inhibits Gsk3. Lrp6 ICD directly binds Gsk3 and inhibits Gsk3 phosphorylation of β -catenin [53, 111]. Synthetic phosphopeptides containing the PPSPXS motifs strongly inhibit Gsk3 [107, 110]. Phospho-peptides containing different PPSPXS motifs vary in their ability to inhibit Gsk3 phosphorylation of β -catenin, with PPSP repeat A and E being the most potent [110]. Furthermore, not only PPSPXS motifs but also the phosphorylated S/T cluster binds Gsk3 and enhances the interaction between Lrp6 and Gsk3 [107]. Thus, unexpectedly, the Lrp6 sites which Gsk3 phosphorylates appear to inhibit Gsk3 activity by a product inhibition mechanism.

Conclusions

In recent years, significant advances have been made in understanding how the Wnt co-receptors Lrp6 and Fzd transduce Wnt stimuli. Wnt signaling occurs on Lrp6 signalosomes, which contain receptor complexes bound to Dvl polymers. In response to signalosome formation, Lrp6 phosphorylation is triggered on an S/T cluster and on multiple PPSPXS sites by Gsk3 and multiple CK1 members, notably CK1 γ . Phospho-Lrp6 recruits Axin and additional Gsk3, whose activity is inhibited by product inhibition. This derepresses β -catenin and promotes Wnt signaling. In addition to Gsk3, other kinases have been implicated in PPSP phosphorylation. Grk5/6 phosphorylate Lrp6 to mediate acute Wnt signaling. PKA phosphorylates Lrp6 to mediate parathyroid hormone signaling via β -catenin. A cyclin-dependent protein kinase complex, Ptk1/Ccny, phosphorylates Lrp6, which explains why Wnt signaling is under mitotic control. Thus, there is context-dependent PPSP phosphorylation by different kinases (Wnt-, PTH-, or mitosis-dependent PPSP phosphorylation).

The multitude of kinases involved indicates that Lrp6 phosphorylation represents an important regulatory node and is an opportunity for signaling cross-talk, suggesting that there may be additional kinases yet to be identified. Open questions are how different kinases cooperate in Lrp6 phosphorylation, and whether they act additively, sequentially, or mutually exclusively. Furthermore, the stoichiometry of Lrp6 phosphorylation by individual PPSP kinases and their combinations is unclear. Lastly, the physiological significance for most kinases *in vivo* needs to be analyzed. However, the tools and conceptual framework are now at hand to address these questions.

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