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Keeping Wnt Signalosome in Check by Vesicular Traffic

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Abstract

Wg/Wnts are paracrine and autocrine ligands that activate distinct signaling pathways while being internalized through surface receptors. Converging and contrasting views are shaping our understanding of whether, where, and how endocytosis may modulate Wnt signaling. We gather considerable amount of evidences to elaborate the point that signal-receiving cells utilize distinct, flexible, and sophisticated vesicular trafficking mechanisms to keep Wnt signaling activity in check. Same molecules in a highly context-dependent fashion serve as regulatory hub for various signaling purposes: amplification, maintenance, inhibition, and termination. Updates are provided for the regulatory mechanisms related to the three critical cell surface complexes, Wnt-Fzd-LRP6, Dkk1-Kremen-LRP6, and R-spondin-LGR5-RNF43, which potently influence Wnt signaling. We pay particular attentions to how cells achieve sustained and delicate control of Wnt signaling strength by employing comprehensive aspects of vesicular trafficking.

Wingless (Wg, or Wnt in mammals) belongs to a family of cysteine-rich glyco- and lipoproteins functioning as paracrine and autocrine ligands (Willert et al., 2003; Willert and Nusse, 2012). Initiation of Wnt signaling is triggered by binding of Wg/ Wnt to their various 7-pass transmembrane Frizzled receptors (Fzd) (Wu and Nusse, 2002; Schulte and Bryja, 2007; Schulte, 2010). Wnt-conjugated Fzd recruits cytoplasmic protein Dishevelled (Dvl) forming Fzd-Dvl complex (Wong et al., 2003). This complex, in association with the low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6; or Arrow in *Drosophila*) induces a signal relay, often referred to as the “canonical” pathway, driving specific gene expression in a β -catenin dependent fashion (Tamai et al., 2000; Wehrli et al., 2000; He et al., 2004; MacDonald and He, 2012). The same complex is also responsible for a separate signaling cascade namely the noncanonical pathway activating the functions of small GTPases of the Rho subfamily (Eaton et al., 1996; Strutt et al., 1997; Boutros and Mlodzik, 1999; Fanto et al., 2000; Habas et al., 2001; Habas et al., 2003; Wallingford and Habas, 2005). The canonical Wnt/ β -catenin pathway in particular plays fundamental roles during fetal development and adult tissue homeostasis; inappropriate activation of this pathway in human diseases has highlighted its profound influences on cellular behavior (Reya and Clevers, 2005; Nusse et al., 2008; Angers and Moon, 2009; MacDonald et al., 2009; Clevers and Nusse, 2012). Healthy cells must employ various mechanisms to regulate Wnt pathway

activity (Nusse, 2012). That is to balance efficient signal transduction and avoidance of overactivation.

Ligand-induced receptor trafficking is one of the most important regulatory events in many signaling pathways (Sorkin and von, 2009). Internalizations of cell surface cargos, for example the various ligand-receptor complexes, through clathrin-dependent or clathrin-independent pathways are collectively known as endocytosis (Marsh and McMahon, 1999; Rappoport, 2008). In clathrin-dependent process, plasma membrane-bound cargos are first recognized by clathrin adaptor proteins (APs), by which the coat proteins—clathrins are recruited to the cargo-concentrated membrane microdomains. Clathrin adaptor complexes include AP-1, AP-2, and AP-3, which show distinct functional and structural features (Conner and Schmid, 2003; Touz et al., 2004; Gupta et al., 2006). Each AP complex is a heterotetramer consisting two large adaptin subunits, one medium, and one small subunit. By linking cargo to clathrin and other regulatory proteins, APs play key roles in coat assembly and protein trafficking (Kirchhausen, 1999; Owen et al., 2004). For endocytosis, the AP-2 complex (with $\alpha 1$, $\beta 1$, $\mu 2$, and $\sigma 1$ subunits) is particularly important for linking clathrin to plasma membrane and for cargo selection and recruitment into coated pits (Traub, 2009; Kirchhausen, 2012). Once recruited to plasma membrane, clathrin deforms the membrane into clathrin-coated pits using its specialized 3-interlocked spiral shaped structure (Marsh and McMahon, 1999). At neck of the pits, a GTPase called dynamin binds and mediates membrane fission for vesicle release (Marsh and McMahon, 1999; Praefcke and McMahon, 2004).

In clathrin-independent pathway, endocytosis is mediated through lipid rafts and caveolin family proteins (Razani et al., 2002b; Lajoie and Nabi, 2010). Lipid rafts are cholesterol and glycosphingolipid enriched membrane microdomains (Simons and Toomre, 2000; Simons and Ehehalt, 2002), where caveolin inserts hairpin loop structures to facilitate the formation of caveolae –flask-shaped membrane invaginations, roughly 50 nm in size, enriched with lipid rafts (Anderson, 1998; Parton and Simons, 2007). Membrane fission and vesicle release in caveolae-mediated endocytosis also require dynamin (Henley et al., 1999). Various classes of signaling molecules contain caveolin-binding motifs therefore this pathway may facilitate the concentration of signaling molecules. Once internalized, cargo proteins travel to membrane-bound vesicles known as early endosomes, where they are sorted: some recycled back to plasma membrane for reuse while others delivered to late endosome and lysosome for degradation (Sorkin and von, 2009; Wolfe and Trejo, 2007). Upon vesicular budding into the cytosol, coat proteins (clathrin and caveolin) quickly reassembled for recycling.

Here, we gather considerable amount of evidences to support the view that Wnt signal receiving cells utilize highly flexible and sophisticated membrane trafficking systems to balance the signal transduction. Same signaling molecules (e.g., Fzd or LRP6), in a highly context-dependent manner, can serve as regulatory hub for various signaling purposes: amplification, maintenance, inhibition, and termination. As Wnt-induced signalosome is, in our view, one of the most important pathway elements, we center our discussions on how vesicular traffic may influence signalosome function via various mechanisms. Based on current literatures, we provide updates on the vesicular trafficking mechanisms underlying

three major ligand-receptor complexes that potently control Wnt signaling: Wnt-Fzd-LRP6, Dkk1-Kremen-LRP6, and R-spondin-LGR5-RNF43. Contrasting and converging views are discussed with emphasis given to major merits of prevailing models. We tentatively conclude that Wnt engagement with surface receptors triggers a rapid biochemical response potentially within certain membrane microdomain, which, in concert with subsequent internalization of signalosome, may contribute to full and sustained pathway activation. Such pathway activation is balanced by degradation of excessive ligands and clearance of surface receptors via key endocytotic machineries and mechanisms that downplay the signal strength.

Wg/Wnts are internalized

In *Drosophila* wing imaginal disc, Wg gene product was initially detected by an antiserum as cytoplasmic puncta in both Wg-expressing (determined by Wg mRNA) and Wg-responding cells (no Wg mRNA) (van den Heuvel et al., 1989). Disrupting endocytosis in receiving cells abolished these cytoplasmic puncta (Strigini and Cohen, 2000). The neighboring (Wg non-expressing) cells apparently received Wg molecules diffused through extracellular spaces and expressed Wg target genes such as *engrailed* (Nusse, 1997). In these receiving cells, Wg proteins were localized by electron microscopy in small membrane-bound vesicles and occasionally in multivesicular bodies (MVB) (van den Heuvel et al., 1989). MVBs are morphologically distinctive endocytotic compartments correlated to the late endocytosis process prior to the final lysosomal fusion (Piper and Katzmann, 2007; Hurley, 2008). Detection of Wg proteins in vesicles inside the Wg-receiving cells suggested that they are secreted molecules and can be internalized (Fig. 1). It is clear now that, Wg/Wnt proteins, after post-translational modification and secretion via a set of common and Wg/Wnt-specific enzymes and transporters, act as paracrine and autocrine ligands (Willert et al., 2003; Das et al., 2012; Willert and Nusse, 2012).

Activation of canonical signaling requires inactivation of a destruction complex

For signal receiving cells, when extracellular Wnts are absent, that is, the “Wnt-Off” status, cytoplasmic β -catenin is maintained at low levels by a destruction complex consisting casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3), axis inhibitor (Axin), adenomatosis polyposis coli (APC), and an E3 ubiquitin ligase β -Trcp. This complex phosphorylates β -catenin proteins by CK1 and GSK3, and delivers them to proteasome for degradation (Huang and He, 2008; Cadigan and Peifer, 2009; MacDonald et al., 2009). At “Wnt-On” condition, binding of Wnt proteins to Fzd and LRP5/6 forms a ternary complex on plasma membrane. This triggers a signal relay that inactivates the destruction complex, releasing stabilized β -catenin. Upon translocation into nucleus, β -catenin complexes with Lef/Tcf factors to form a potent transcriptional activator that drives the expression of various Wnt target genes associated with cell-cycle progression and survival (Fig. 2). For details, please also read the recent reviews (Clevers and Nusse, 2012; Lim and Nusse, 2013; Clevers et al., 2014; McCubrey et al., 2014). In order to appreciate the delicate regulations imposed by membrane trafficking on this pathway, the biochemical mechanisms behind signaling initiation at plasma membrane are important to understand.

Wnt induces rapid LRP5/6 phosphorylation and signalosome formation at plasma membrane

An immediate and probably the most remarkable molecular event stimulated by Wnt-Fzd-LRP5/6 ternary complex is the rapid phosphorylation of LRP5/6 cytoplasmic tail by GSK3 and CK1 γ at Ser1490 and Thr1479, respectively (Davidson et al., 2005; Zeng et al., 2005). As quickly as 5 min after Wnt stimulation (Kim et al., 2013), sequential phosphorylations occur at conserved PPPSPxS motifs (P stands for proline; S for serine) and are postulated to impose two fundamental biochemical responses: (1) creation of a docking site for Axin scaffold protein to bind, thereby recruiting the destruction complex (Mao et al., 2001b; Tamai et al., 2004; Davidson et al., 2005; MacDonald et al., 2008); and (2) directly inactivating GSK3 β kinase by accessing its catalytic pocket (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). These reactions provide direct connections between Wnt-induced LRP phosphorylation and β -catenin stabilization (Fig. 2). However, the mechanism behind the rapid Wnt-induced LRP6 phosphorylation was not entirely clear until the idea of “LRP6 signalosome” emerged.

Shortly after Wnt stimulation, aggregation of LRP6 proteins at or below cellular plasma membrane was first reported in HeLa, human embryonic kidney (HEK) 293, P19, mouse embryonic fibroblast (MEF), and *Xenopus* embryo cells (Bilic et al., 2007; Pan et al., 2008). These Wnt-induced LRP6 aggregates, appearing within the similar time frame as LRP6 phosphorylation does, rapidly recruit cytosolic Axin to plasma membrane while accumulating, over a time course of several hours, into large punctate structures with certain stability. Biochemically, these aggregates, after partial solubilization by Triton X-100, became ribosome-sized (20 $\times$ 30 nm) multiprotein complexes with approximate molecular weight of 670 kDa. This implied that these protein aggregates might not simply represent, at least the intact, intracellular vesicles that would presumably be largely solubilized by detergent, such as Triton X-100, treatment. Initial colocalization of these “LRP6 signalosomes” (so-called as they were detected by a phospho-LRP6 Tp1479 antibody), was indeed not observed with multiple vesicular transport markers: EEA1 (early endosome), clathrin (endocytic vesicles), calnexin (endoplasmic reticulum), and TGN38 (Golgi), suggesting that the signalosomes might not prominently arise from endo- or exocytotic processes (Bilic et al., 2007). At cellular and biochemical levels, the signalosomes comprise phosphorylated LRP6 (Tp1479), Axin, Fzd, Dvl and GSK3 β , supporting the view that CK1 and GSK3 phosphorylate LRP6 in these compartments (Fig. 2).

Importantly, formation of such signalosome strictly depends on polymerization of Dvl, a scaffold protein recruited by Fzd upon Wnt stimulation (Cong et al., 2004; Zeng et al., 2008). Dvl is an evolutionarily conserved integral component of Wg/Wnt pathway. It contains PSD95/Dlg1/ZO1 (PDZ)-, Dvl/Egl-10/ Pleckstrin (DEP)-, and Dvl/Axin (DIX)-domains, with an especially high tendency of forming homo- or hetero-polymer structures via the DIX domains. Dvl recruits Axin/GSK3 β through DIX domains thereby promoting assembly of large protein clusters at plasma membrane (Capelluto et al., 2002). As a consequence, Dvl knockdown impaired both LRP6 aggregation and phosphorylation, supporting a model where Dvl may act as a cytoplasmic platform uniting Fzd and LRP5/6.

Based on this signalosome model, Wnt-binding of Fzd and LRP6 at cell surface triggers rapid aggregation of pathway components (Fzd, LRP6, Axin, and GSK3 β) on Dvl polymerization platform beneath plasma membrane, creating highly condensed phosphorylation target sites for CK1 γ and GSK3 β (Bilic et al., 2007; Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b). As CK1 γ is constitutively resides at plasma membrane (Davidson et al., 2005), Dvl-recruited Axin-GSK3 complex to Wnt-Fzd-LRP6 complex may prime LRP6 for rapid phosphorylations (Cong et al., 2004; Zeng et al., 2008) (Fig. 2).

In keeping with this model, independent studies showed that Dvl stimulates phosphatidylinositol kinase (PI4KII and PIP5KI) activities towards production of phosphatidylinositol (4,5)-bisphosphate (PIP₂) at plasma membrane, which appears to be also indispensable for signalosome assembly and function (Pan et al., 2008). Both PI4KII and PIP5KI physically interact with Dvl to increase PIP₂ local concentration at the membrane domain, to which PIP₂ directly recruits clathrin and adaptor protein 2 (AP2), presumably creating a favorable membrane microenvironment for signaling (Kim et al., 2013). Of note, both Dvl aggregation (Nishita et al., 2010) and PIP₂ production (Grumolato et al., 2010) have also been reported for noncanonical Wnt pathway suggesting that PIP₂-participated signalosome formation might be a shared mechanism for Wnt pathways. PIP₂ recruits at least one additional scaffolding protein: Amer1 (APC membrane recruitment 1), which, in parallel to Dvl- or LRP6-dependent Axin recruitment, further engages with Axin, and presumably brings the destruction complex into close contact with CK1 γ at plasma membrane (Tanneberger et al., 2011). These factors may provide additive stabilities to Wnt signalosome and possibly amplify its function.

Taken together, Wnt-induced signalosome is a multiprotein signaling platform consisting at least LRP6, Fzd, Dvl, Axin, CK1 γ , GSK3 β , PIP₂, and Amer1 among many others still being discovered. Signalosome biochemically inactivates the cytoplasmic destruction complex, providing one of the most persuasive biochemical mechanisms for the transmitting of Wnt signal from cell surface to cytosolic destruction machinery. Nevertheless, with the long-standing observations of Wnt ligand-receptor internalizations, the question became important as of what exact role the endocytotic process plays in Wnt signal transduction.

Internalized Wg/Wnts are degraded for signal attenuation

Fzd- and/or LRP6-mediated endocytosis have been observed for both canonical and non-canonical Wnt proteins in mammalian cells; however the signaling outcomes following distinct ligand-receptor internalization events can be drastically different (Table 1). Using a conventional antibody labeling method to visualize extracellular Wg proteins, early studies in *Drosophila* first demonstrated that Wg endocytosis was likely responsible for rapid removal of extracellular ligands, thereby shaping a steep Wg morphogen gradient across the disc (Strigini and Cohen, 2000). Cells mutant for *shibire*, the *Drosophila* homolog of dynamin, which is responsible for internalizations of both clathrin- and caveolae-coated endocytic vesicles, accumulated Wg proteins on extracellular cell surface (Strigini and Cohen, 2000). Additional studies showed that internalization of Wg was, as anticipated, mediated through its receptor DFz2 and co-receptor Arrow. Internalized Wg-DFz2-Arrow

traffics through the entire endocytotic pathway to lysosome, where they are degraded in a hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) dependent manner (Rives et al., 2006) (Fig. 1). The primary consequence of Wg endocytosis at wing disc was postulated to dampen Wg signaling, a mechanism echoed by results obtained in *Drosophila* embryo. There, Wg endocytosis followed by differential lysosomal degradation capacities in anterior versus posterior signal-receiving embryonic cells contributed to the asymmetrical Wg signaling along the anterior-posterior axis (Dubois et al., 2001). These studies suggested that Wg/Wnt molecules signal at cell surface whereas they might be endocytosed primarily for ligand clearance and signal attenuation. However, mounting studies specifically designed to explore the process and mechanism of Wnt-receptor endocytosis, have suggested a much more complicated picture than we expected.

Wg/Wnt endocytosis can also promote signaling

In *Drosophila* wing disc, disruption of dynamin function affected Wg endocytosis, and simultaneously impaired Wg signaling (Seto and Bellen, 2006). Similarly, when dynamin or Rab5, a Rab small GTPase required for early endocytosis, was knocked down in cultured cells, the Wg signaling output was weakened. Conversely, Wg signaling strength could be enhanced by simply increasing endosomal transport, and this enhancement was well correlated with endosomal colocalization of Wg, Arrow, and Dvl (Seto and Bellen, 2006). If Wg/Wnt signalosome primarily signals from plasma membrane, why would internalized Wg-receptor complex strengthen the pathway? In searching for clues, early works conducted in mammalian (murine L and HeLa) cells showed that Wg and Wnt-3A were internalized possibly through the dynamin- and clathrin-dependent (Blitzer and Nusse, 2006), or caveolin-dependent (Yamamoto et al., 2006) endocytosis. These Wnt endocytotic processes appear to be necessary for substantial β -catenin stabilization (Blitzer and Nusse, 2006). Perturbing Wnt internalization by inhibitors of endocytosis or by dominant negative dynamin constructs reduced β -catenin stabilization, hinting that endocytotic process may not be solely utilized for ligand clearance, but may also be utilized for signaling enhancement at certain points. These results opened questions of whether and how Wnt induced receptor endocytosis could possibly favor signaling, and led to functional dissecting of Wnt receptor/coreceptor trafficking at molecular levels.

Wnt-induced Fzd endocytosis can promote signaling

The signaling activities of both canonical and non-canonical Wnt proteins are mediated through Fzd receptors (Schulte and Bryja, 2007; Schulte, 2010). Fzds belong to the superfamily of G-protein coupled receptors (GPCRs) also known as seven-transmembrane domain receptors.

Signaling of typical GPCRs involves heterotrimeric guanine nucleotide-binding protein (G-protein) and the multifunctional adaptor β -Arrestin. Early studies using an artificial chimeric receptor (β_2 AR-Rfz1) comprised of the ligand-binding and transmembrane domains from the β_2 -adrenergic receptor and the cytoplasmic domains from rat Fzd 1, provided the first evidence that Fzd-mediated β -catenin-Tcf pathway might indeed utilize G-protein signaling (Liu et al., 2001). Internalization of GPCRs is typically mediated by β -Arrestin 1 and 2

(Pierce et al., 2002); ligand-activated GPCRs, upon phosphorylation by GPCR kinases, recruit β -Arrestin that binds to clathrin and adaptor proteins to promote receptor endocytosis in clathrin-coated pits (Goodman et al., 1996). This process was clearly demonstrated for Fzd 4 in HEK293 cells, where Wnt5A stimulated Fzd 4 endocytosis via sequential recruitments of Dvl 2 and β -Arrestin 2, resulting in enhanced signaling (Chen et al., 2003). In line with this, an interaction between Dvl 2 and μ 2-adaptin subunit of clathrin AP-2 complex was identified as essential for Fzd 4 endocytosis and planar cell polarity (PCP, or noncanonical Wnt) pathway (Yu et al., 2007). These observations established key connections between Dvl, Fzd endocytosis and Wnt pathway activity, extending Wnt signalosome function into the signal receiving cells.

Now, both canonical and non-canonical Wnt proteins have been shown as capable of inducing endocytosis of corresponding Fzd receptors; alterations in Fzd endocytosis or intracellular trafficking appear to impact the signaling pathways (Table 1). In HEK293 cells, Wnt3a induces rapid Fzd 5 internalization (as quickly as 30 min) through EEA1- and Rab5-positive endosomes in a clathrin-dependent manner; internalized Fzd 5 receptors are then returned through Rab11-positive vesicles back to plasma membrane after 3~4 hours of stimulation (Yamamoto et al., 2006). Remarkably, the above clathrin-mediated endocytosis of Fzd 5 happens only when it is overexpressed alone. When Fzd 5 and LRP6 are simultaneously overexpressed in HeLaS3 cells, Wnt3a stimulated the complex internalization through caveolin-mediated pathway into EEA1-positive vesicles, suggesting that incorporation of Wnt receptor into distinct endocytotic pathways might depend on differential compositions of the ligand-receptor complexes (Yamamoto et al., 2006). Likewise, in the same HeLaS3 cells, Fzd 2 was shown to mediate both canonical and noncanonical pathways activated by Wnt3a and Wnt5a, respectively. Wnt5a competes for Fzd 2 binding against Wnt3a thereby suppressing canonical signaling. Wnt5a-binding stimulates Fzd 2 endocytosis via clathrin-mediated route, inhibition of which specifically abolished Wnt5a's ability to activate Rac (the noncanonical) pathway, but not its competitive suppression on Wnt3a-induced canonical pathway (Sato et al., 2010). This hypothetic model elegantly demonstrated a special feature of Wnt5a in activating and suppressing distinct pathways through receptor endocytosis and receptor-binding competition.

In addition to Fzd internalization, the homeostatic compartmentalization of already internalized Fzd proteins and the maturation states of cellular endosome vesicles also play a role in regulating Wnt signaling. PI4-kinase type 2 β (PI4K2 β), an AP-1 and Dvl-binding protein engaging in endosomal sorting, was recently shown to colocalize with internalized Fzd 5 in late endosome, and regulates the partitioning of Fzd receptors between degradative and recycling pools (Wieffer et al., 2013). Depletion of PI4K2 β weakened canonical Wnt signaling activity in zebrafish and mammalian cells. Similarly, impaired Fzd 7 trafficking and canonical Wnt signaling was observed in Rabconnectin-3a (Rbc3a) deficient neural crest cells with an abnormality in endosomal maturation (Tuttle et al., 2014). Rbc3a is a vacuolar-type H⁺-ATPase associated protein (Sakisaka and Takai, 2005) required for lysosomal acidification (Yan et al., 2009; Sethi et al., 2010). Taken together, Wnt-stimulated internalization of various Fzd receptors, in addition to Wnt-induced plasma membrane

signalosome, also appears to be an integral component for both canonical and noncanonical signaling pathways.

Wnt-bound LRP6 may signal from lipid raft and internalized vesicle

In contrast to Fzd receptors, LRP6 coreceptor is a single transmembrane protein dedicated primarily to canonical Wnt signaling (Pinson et al., 2000). The initial colocalization of LRP6 signalosomes failed to localize them with prominent vesicular transport markers, however a small fraction of the signalosomes did colocalize with caveolin within 1 hr of Wnt treatment. This colocalization disappeared about 3.5 hr after Wnt treatment (Bilic et al., 2007), suggesting at least a transient transitioning of signalosomes through lipid rafts or internalized caveolin-coated vesicles.

Lipid rafts are regarded as highly dynamic membrane microdomains that favor stable formation of large protein clusters (Simons and Toomre, 2000; Simons and Ehehalt, 2002), such as Wnt-stimulated receptor aggregates, thus act as compartmentalized platform to promote interactions of signaling molecules. Detergent resistance is an important feature of lipid rafts, therefore cell fractionation by detergent such as Triton X-100 is widely utilized to isolate detergent resistant microdomains (DRMs) from non-DRMs, and to identify raft-enriched proteins. Several important findings were made through analyzing Wnt-stimulated lipid raft dynamics and LRP6 endocytosis. First, Wnt3a triggered the interaction of LRP6 with caveolin, causing not only Axin recruitment but also dissociation of β -catenin from Axin, supporting that LRP6 localized in caveolin-enriched rafts promotes signaling (Fig. 3B). Second, whereas Wnt3a-LRP6 binding could be detected in both DRMs and non-DRMs, Wnt3a-induced LRP6 phosphorylations at Ser1490 and Thr1479 only occurred in DRMs, indicating LRP6 localization to rafts favors its phosphorylation (or activation) by GSK3 and CK1 hence canonical signaling. Third, Wnt3a-induced LRP6 internalization depended on caveolin, and LRP6 endocytotic and recycling trafficking processes required general Rab small GTPases including Rab5 and Rab11 (Sakane et al., 2010; Yamamoto et al., 2006). Wnt3a treatment of HEK293 or HeLa cells induced intracellular localization of LRP6 in Rab5 positive early endosomes around 10 min to 2 hr after stimulation. At 3–4 hr, internalized LRP6 reappeared at plasma membrane through Rab11 positive recycling endosomes. Inhibition of caveolin, Rab5, or Rab11 all affected Wnt signaling in these conditions (Sakane et al., 2010; Yamamoto et al., 2006), observations not having been formally confirmed in mouse knockout studies (Woodman et al., 2002; Razani et al., 2002a; Yu et al., 2014a; Yu et al., 2014b; Yuan et al., 2011). Biochemically, LRP6 and caveolin formed a complex about 20 min following Wnt treatment (Yamamoto et al., 2006). The time frames of these trafficking events exhibited certain similarities to Wnt-induced LRP6 signalosome formation, implying that separate or overlapping pools of LRP6 proteins are incorporated into membrane-associated signalosome or into caveolar endocytic vesicles, in a sequential or parallel fashion.

If speculating from a “sequential” point of view, Wnt-induced LRP6 signalosomes may assemble in lipid raft plasma membrane, get activated by phosphorylation inside rafts, and undergo caveolar endocytosis in a linear time course. This scenario has been somewhat allayed by the observation that Wnt3a-induced LRP6 phosphorylation and internalization

events, instead of sequentially taking place, can occur independently of each other, whereas both events are necessary for β -catenin stabilization (Yamamoto et al., 2008). These observations suggest an existence of multiple LRP6-driven signaling enhancement pathways.

The preferential activation of LRP6, i.e., phosphorylation by GSK3 and CK1, in raft membrane microdomain has been particularly favored by several independent studies. For example, the Ly6 family protein LY6/PLAUR domain-containing 6 (Lypd6), a protein anchored to lipid raft membrane domains by glycosylphosphatidylinositol (GPI), physically interacts with Fzd 8 and LRP6, and promotes LRP6 phosphorylation in rafts hence enhancing canonical signaling (Özhan et al., 2013). Conversely, the secreted Dickkopf1 (Dkk1) protein inhibits canonical signaling by extracting LRP6 away from the lipid raft domains (more detailed discussion below) (Yamamoto et al., 2008) (Fig. 3A). On the other hand, the importance of caveolar endocytosis of LRP6 has also been supported by independent studies screening for Wnt pathway trafficking modulators. This screen identified a vertebrate conserved Rab small GTPase, RAB8B, which interacts with LRP6 and CK1 γ , and promotes LRP6 caveolar endocytosis as well as canonical Wnt signaling in *Xenopus laevis*, *Danio rerio* and mammalian cells (Demir et al., 2013).

Truncated or chimeric LRP6 receptors that are generated to forcefully alter their cellular and membrane localizations have also offered useful information regarding LRP6 location-dependent contributions to the signaling. In contrast to wild type LRP6 largely localized to cell surface at a static condition, LRP6^N, lacking extracellular ligand binding domain, localizes to cytoplasmic puncta and strongly stabilizes β -catenin. This constitutively active LRP6^N protein is associated with caveolin and is phosphorylated (Yamamoto et al., 2008). However, LRP6C, containing only the cytosolic tail with no membrane attachment, becomes diffused in cytoplasm with a total loss of β -catenin-stabilization capacity. Forced attachment of LRP6C to plasma membrane by the strong membrane-targeting motif of Ras “CAAX” (LRP6C-CAAX) fails to activate the receptor, whereas forced targeting LRP6C to lipid rafts by the EGF receptor transmembrane domain (EGFR581–668)-LRP6C rescues its β -catenin-stabilization activity (Yamamoto et al., 2008). These observations collectively suggested that raft incorporation of LRP6 might be key for its signaling activity.

Nevertheless, the relative extent and importance of LRP6 caveolar endocytosis discussed above may also depend on the particular cell types used in the studies. For example, Wnt stimulation of mouse F9 cells induced casein kinase 2 (CK2)-mediated phosphorylation of LRP6 at Ser1579, driving LRP6 interaction with the endocytic adaptor disabled-2 (Dab2) protein and internalization with clathrin rather than caveolin (Jiang et al., 2012). Furthermore, super-resolution fluorescence microscopic studies indicated that LRP6 internalization, if exists in HEK293, MEF and BS-C-1 cells, only occurs at very low levels, whereas WNT3A-induced LRP6 signalosomes are primarily localized to large clathrin-coated plaques (>200 nm) at plasma membrane. At least in certain cell types, Wnt treatment does not appear to incur substantial LRP6 endocytosis in cell biology and biochemical assays (Kim et al., 2013); instead, rapid LRP6 internalization in Wnt-treated cells could be artificially triggered by inducing PIP₂ hydrolysis, a process necessary for clathrin-mediated endocytosis (Krauss and Haucke, 2007; McMahon and Boucrot, 2011). These studies, in

contrast to caveolar endocytosis model, argue that Wnt-induced PIP₂-mediated recruitment of clathrin and AP-2 primarily facilitates LRP6 signaling from the plasma membrane. Whether LRP6 is internalized or not may highly depend on cell specific membrane environment such as differential PIP₂ production levels. It is also possible that only the LRP6 proteins that fail to be incorporated into the signalosomes at plasma membrane get internalized. This returns to the above point that multiple LRP6-driven pathways may exist and be activated in response to different ligand abundances. Together, caveolin-dependent raft dynamics or endocytosis appear to favor LRP6 activity (Yamamoto et al., 2006; Bilic et al., 2007; Yamamoto et al., 2008; Taelman et al., 2010; Glinka et al., 2011; Ohkawara et al., 2011; Demir et al., 2013) whereas clathrin-mediated coat assembly at membrane or endocytosis is also important for Wnt signaling (Blitzer and Nusse, 2006; Kikuchi et al., 2009; Jiang et al., 2012; Hagemann et al., 2014).

GSK3 sequestration model: possible mechanism for sustained & reversible signal maintenance

Membrane-associated signalosomes discussed above are likely sufficient to inactivate the destruction complex for signal transduction. What benefits do cells possibly gain by utilizing endocytotic pathways? Interestingly, recent studies have proposed a MVB-mediated GSK3 β sequestration model (Fig. 3A). In this elegant model, Wnt-conjugated receptor complexes including GSK3 β , namely the entire signalosome, is internalized into the intraluminal vesicles of MVBs. By doing so, GSK3 β gets efficiently insulated from cytoplasmic β -catenin thereby favors Wnt signaling (Taelman et al., 2010). Aside from the biochemical mechanism discussed above, this model offered a novel endocytosis-based mechanism for GSK3 β inactivation. Depending on cellular conditions, MVB-capsulated GSK3 β can undergo either degradation through MVB-lysosomal fusion or rejuvenation via recycling to vesicular and plasma membranes via back-fusion (Taelman et al., 2010; Dobrowolski et al., 2012; Dobrowolski and De, 2012). It appears that dissociation of Wnt signalosome from p120-catenin/cadherin complex on cell membrane facilitates MVB sequestration (Vinyoles et al., 2014). Thus, signalosome-captured MVB may serve as a regulatory unit subjecting to reversible and sustained trafficking regulation, hence providing means for long-term signal maintenance or clearance (Taelman et al., 2010; Dobrowolski et al., 2012; Dobrowolski and De, 2012). Such mechanism may be particularly favored in situations when extracellular Wnts are less abundant. However, how do cells precisely control the balance between GSK3 β degradation and recycling, and how GSK3 β sequestration may impact other signaling pathways require further investigations (Dobrowolski et al., 2012; Metcalfe and Bienz, 2011).

Dkk1-Kremen-LRP6 complex: distinct LRP6 internalization pathways govern signal outcome

In addition to several secreted extracellular Wnt- or Fzd-binding proteins, such as the Wnt inhibitory factors (WIFs) (Hsieh et al., 1999) and the secreted frizzled-related proteins (SFRPs) (Rattner et al., 1997) that modulate Wnt signaling, vertebrates express a highly special Dkk family of secreted proteins (Dkk-1, -2, -3, and -4) that antagonize Wnt

signaling. Dkk-1 was identified as a potent head inducer in *Xenopus* embryos, binds LRP6 through a domain that differs from Wnt/ Frizzled-interacting domains, and strongly inhibits Wnt signaling (Glinka et al., 1998; Niehrs, 1999; Bafico et al., 2001; Mao et al., 2001a; Semenov et al., 2001).

Prior to inception of the Wnt signalosome idea, a cDNA library-based screening for Dkk-1 binding partners identified two transmembrane receptors: Kremen 1 and Kremen 2. Both bind Dkk-1 with high affinities and such interactions physiologically occur during Wnt signaling (Mao and Niehrs, 2003). Whereas neither Kremen protein inhibited Wnt reporter activity by itself, coexpressing either with Dkk-1 inhibited Wnt signaling, an effect replicated in the developing *Drosophila* wing with ectopically expressed Kremen and Dkk-1. Of note, Dkk and Kremen are only found in vertebrates and neither is in *Drosophila*, suggesting that this complex might be an add-on regulatory unit arising late into vertebrate evolution. Biochemical experiments suggested that Dkk-1 interacts with Kremen extracellular domain and forms a Dkk-Kremen-LRP6 ternary complex that undergoes rapid endocytosis (Fig. 3B). Thus, it was proposed that Dkk-1 binding to LRP6 and Kremen triggers the internalization and removal of LRP6 from cell surface, inhibiting Wnt-signaling (Mao et al., 2002).

The above model was further developed in great details with subsequent studies (Yamamoto et al., 2006, 2008; Sakane et al., 2010). First, Wnt3a and Dkk-1 are proposed to regulate distinct LRP6 internalization pathways mediated by caveolin and clathrin, respectively (Yamamoto et al., 2008) (Fig. 3A). Both ligands induce LRP6 internalization into Rab5 endosomes, from where the internalized LRP6 is recycled through Rab11 endosomes back to cell membrane while internalized Dkk-1 traffics into Rab7 late endosome presumably for degradation (Sakane et al., 2010). This causes either LRP6 activation in the case of Wnt3a or signal attenuation by Dkk-1. Dkk-1-induced clathrin endocytosis of LRP6 is consistent with the observation that Kremen1 co-localizes with clathrin but not caveolin on cell surface, and its cytosolic tail participates in clathrin-mediated endocytosis by engaging AP-2 complex, with the D⁴⁶⁶XXXLV motif identified being the responsible sorting element for Dkk-1 mediated LRP6 down-regulation (Mishra et al., 2012). Second, in contrast to Wnt3a that does not alter LRP6 membrane distribution between DRM and non-DRM fractions, Dkk-1 removes LRP6 specifically away from detergent resistant lipid rafts (Sakane et al., 2010; Yamamoto et al., 2008). Third, inhibition of clathrin- but not caveolin-mediated endocytosis abolishes Dkk-1's inhibitory effect on LRP6 and Wnt-signaling; whereas fusion of LRP6C with caveolin is sufficient to stabilize β -catenin (Yamamoto et al., 2008). These data collectively suggested that distinct LRP6 endocytotic routes exist, probably in order to transduce different extracellular signal messages into the cells.

R-spondins-LGR5-RNF43 and RNF43-Fzd complexes: cell surface Fzd concentration determines signal strength

The R-spondin family contains 4 secreted proteins, R-spondin 1~4, each with 2 N-terminal furin domains and a thrombospondin domain (Kazanskaya et al., 2004; Kim et al., 2005). R-spondins are vertebrate-specific Wnt agonists that robustly increase LRP6 phosphorylation and β -catenin stabilization when added with Wnt3a (Wei et al., 2007). Early studies in

searching for R-spondin receptor suggested potential candidates as LRP6 (Wei et al., 2007), Kremen (Binnerts et al., 2007), syndecan 4 (Ohkawara et al., 2011), and Fzd (Nam et al., 2006), with various mechanisms proposed to interpret their Wnt signaling promoting actions.

Recently, multiple structural and genetic studies confirmed that R-spondins enhance Wnt signaling by binding to the leucine-rich G protein-coupled receptors (LGR), a subgroup of eight within the superfamily of Rhodopsin GPCRs (Carmon et al., 2011; Glinka et al., 2011; de Lau et al., 2011; Ruffner et al., 2012). Structural features divide them into three classes, with the class-B LGR subfamily consists three members: LGR4, LGR5, and LGR6. LGR5 has been proven to be a bone fide marker of adult stem cells in mammalian gastrointestinal epithelia (Barker et al., 2007), hair follicle (Jaks et al., 2008), and numerous other tissues (Clevers and Nusse, 2012). Each of the four R-spondins can bind to the above three LGR receptors, and removal of LGR receptors abrogates R-spondin induced Wnt signal enhancement (de Lau et al., 2011). Depletion of LGR4 by siRNA abolished R-spondin-induced β -catenin signaling, which could be restored by overexpressing LGR5, indicating they are functional homologs (Ruffner et al., 2012). Independent studies suggested that LGR4 and LGR5 mediate the impact of R-spondin on both β -catenin and PCP signaling pathways (Carmon et al., 2011; Glinka et al., 2011). In mouse intestine, genetic ablation of *Lgr5* and *Lgr4* impairs Wnt target gene expression and causes rapid demise of intestinal crypts where stem cells reside (de Lau et al., 2001; Kinzel et al., 2014).

Several models have been proposed for R-spondin-mediated Wnt signaling amplification. Initial studies suggested that R-spondin and Wnt3a co-stimulation induces formation of a supercomplex consisting R-spondin-LGR5 and Wnt-Fzd-LRP6. This supercomplex gets internalized, and the LGR5 cytosolic tail appears to mediate endocytosis and signaling (Carmon et al., 2012; Snyder et al., 2013b). Indeed, there is a constitutive internalization of LGR5 through Rab7-, Rab9-positive endosomes, and Vps26-positive retromer, reaching a steady state distribution in trans-Golgi network (Snyder et al., 2013b). Structure analysis of *Lgr5* pointed out potential domain, in particular a tri-serine motif at 873–875 within the C-terminal tail, with ability to recruit β -Arrestin 2 (Snyder et al., 2013a). These observations centered on LGR5 endocytosis set the stages for its important actions on 2 stem cell enriched transmembrane E3 ubiquitin ligases: RING finger 43 (RNF43) and Zinc RING finger 3 (ZNRF3) (Fig. 4).

RNF43 and its homolog ZNRF3 contain an extracellular N-terminal protease-associated domain, a single pass transmembrane helix, and an intracellular C-terminal RING domain with E3 ligase activity (Hao et al., 2012; Koo et al., 2012). Both E3 ligases mediate multiubiquitination of lysines in the cytoplasmic loops of Fzd receptors, leading to rapid endocytosis and degradation of Fzd (Fig. 4). RNF43 accomplishes Fzd membrane clearance by directly binding to Fzd and delivering the ubiquitin-tagged Fzd into Rab5 endosome and CD63-positive lysosome (Koo et al., 2012). In normal small intestine, RNF43 and ZNRF3 are predominantly expressed in LGR5+ stem cells, where they negatively regulate Wnt signaling (Hao et al., 2012; Koo et al., 2012). Both are proposed as genuine tumor suppressors, mutations of which have been identified in several types of human cancers, such as gastric adenocarcinoma (Zhou et al., 2013), pancreatic ductal adenocarcinoma (Jiang

et al., 2013), liver fluke-associated cholangiocarcinoma (Ong et al., 2012), and mucinous ovarian tumors (Ryland et al., 2013).

Double knockout of Rnf43 and Znf3 in mouse intestinal epithelium leads to development of paracrine Wnt-dependent adenomas, which can be arrested in culture by inhibitors of Wnt secretion (Koo et al., 2012), supporting the notion that they act at Wnt receptor level. Remarkably, when R-spondin-bound LGR4–6 interacts with RNF43/ZNRF3, Fzd ubiquitination is blocked hence Wnt signaling is enhanced (Hao et al., 2012). Structural explorations indicated that ZNRF3 interacts with R-spondin through its ectodomain; whereas R-spondin1 binds ZNRF3 through its β -hairpins 1–2 of the Furin 1 domain with Furin 2 exhibiting domain flexibility in the absence of LGR4/5. Superposition of ZNRF3-R-spondin complex with R-spondin-LGR5 shows that ZNRF3 overlaps with the dimeric partner LGR5 in R-spondin-LGR5 complexes, suggesting that binding of R-spondin-LGR5 with ZNRF3 would likely block or disrupt the quaternary arrangement (Peng et al., 2013). According to this model, R-spondin binding to ZNRF3 induces the association between LGR4/5 and ZNRF3, leading to endocytotic clearance of ZNRF3 from the membrane and Wnt signaling enhancement (Fig. 4). Thus, R-spondin promotes Wnt signaling by inhibiting RNF43/ZNRF3, two negative regulators of the pathway.

Although R-spondin is a vertebrate-specific Wnt agonist, LGR receptor such as Lgr2 can be found in *Drosophila* (Luo et al., 2005; Nishi et al., 2000). Likewise, the *C. elegans* PLR-1 E3 ligase has been reported to mediate a similar mechanism for regulating Fzd receptor density in plasma membrane, thus being proposed as an orthologue of RNF43/ZNRF3 (Moffat et al., 2014). Furthermore, balanced ubiquitylation and deubiquitylation of Fzd have long been proposed as a mechanism in both mammalian cells and *Drosophila* to regulate cellular responsiveness to Wg/ Wnt (Mukai et al., 2010). Gain or loss of function of a deubiquitylating enzyme UBPY/ubiquitin-specific protease 8 (USP8) led to up- or down-regulation of canonical Wnt signaling, respectively (Mukai et al., 2010). Taken together, modulation of cell surface Wnt receptor and coreceptor levels via endocytosis appear to be critical for either enhancing (e.g., R-spondin-LGR4/ 5) or dampening (e.g., Dkk1-Kremen, RNF43/ZNRF3) Wnt signal strength.

Closing remarks

From above discussion of vesicular modulation of Wnt signaling, it appears that Wnt receptor endocytosis, degradation, and recycling play important roles in signal initiation, maintenance, and attenuation. Cells possibly utilize multiple endocytotic mechanisms to comply with different extracellular Wnt molecule densities, either clearing or retaining the signalosomes. Meanwhile, parallel and convergent endocytotic trafficking routes exist in preparation for surface signals triggered by distinct ligand-receptor complexes. Almost all regulatory platforms receive complicated positive and negative influences; whereas the outcomes of most vesicular trafficking processes are highly context-dependent. More sophisticated regulatory ligand-receptor complexes and mechanisms arise during vertebrate evolution. The exact physiological functions of a number of key trafficking modulators in Wnt signaling pathway are still pending for verification using distinct model organisms by molecular genetics.

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Fig. 1.

Internalization of DFz2 and Arrow in response to Wg. In *Drosophila*, Wg binds DFz2 and Arrow, inhibits cytoplasmic destruction complex, and induces β -catenin (Arm) dependent canonical signaling. Wg-DFz2-Arrow complex is internalized and transported through the entire endocytotic pathway.

Fig. 2.

Wnt induces LRP5/6 phosphorylation and signalosome formation. Under the Wnt-Off status, the destruction complex phosphorylates and degrades β -catenin (Left Panel). At Wnt-On condition, Wnt binds Fzd and LRP5/6 co-receptors. A multiprotein complex, the signalosome, assembles on a Dvl aggregation platform, in which LRP6 is phosphorylated by GSK3 and CK1 leading to inactivation of the destruction complex. Dvl also stimulates PI4KII and PIP5KI activity towards production of PIP₂ at plasma membrane. PIP₂ recruits Amer1 and other factors to enhance signalosome function, resulting in β -catenin stabilization (Right Panel).

Fig. 3.

Ligand-dependent LRP6 endocytosis. (A) Wnt induces caveolar endocytosis of LRP6, activating β -catenin pathway; whereas DKK1 shifts LRP6 away from rafts into the clathrin-dependent endocytotic and degradative pathway thereby inhibiting β -catenin pathway. Note that MVB is proposed to sequester the signalosome for sustained pathway activation. (B) DKK1 binds Kremen and LRP6 to induce internalization of the newly formed ternary complex, removing surface LRP6 hence inhibiting β -catenin signaling pathway.

Fig. 4. Modulation of surface Fzd density by R-spondin-LGR5-RNF43. Cell surface Fzd is ubiquitinated and cleared by stem cell enriched E3 ubiquitin ligases RNF43/ZNRF3 for dampening the signaling (Left Panel). R-spondin enhances Wnt/β-catenin signaling pathway by binding to RNF43/ZNRF3 and LGR5, triggering internalization of ternary complex consisting RNF43/ZNRF3 thereby increasing surface Fzd density (Right Panel).

TABLE 1

Recent studies of vesicular regulation of Wnt signaling components.

Cargos	Endocytic Proteins	Functions	Wnt Pathway Activity	Refs
Wg		Wingless protein is degraded in lysosome.	↓	(Dubois et al., 2001)
Wg		Arrow-Frizzled2-Wingless complexes internalized and degraded in lysosome.	↓	(Piddini et al., 2005)
Wnt3a	Clathrin/dynamin	Wg/Wnt proteins are rapidly endocytosed by a clathrin- and dynamin-mediated process.	↑	(Blitzer and Nusse, 2006)
LRP6	Caveolin/Clathrin	Wnt3a induces the caveolin-dependent internalization of LRP6; Dkk1 induced the internalization of LRP6 with clathrin.	↑↓	(Yamamoto et al., 2008)
LRP6	Caveolin	Wnt-3a triggers the interaction of LRP6 with caveolin and promotes recruitment of Axin to LRP6.	↑	(Yamamoto et al., 2006)
LRP6	Rab5/Rab11	Dkk1 internalized LRP6 in a Rab5-dependent mechanism. The internalized LRP6 was recycled back in a Rab11-dependent mechanism.	↓	(Sakane et al., 2010)
LRP6	Disabled-2 (Dab2)	Dab2 selectively recruits LRP6 to the clathrin-dependent endocytosis.	↓	(Jiang et al., 2012)
LRP6	RAB8B	RAB8B is required for caveolin mediated endocytosis of LRP6.	↑	(Demir et al., 2013)
LRP6	Kremen1/2	Kremen2 forms a ternary complex with Dkk1 and LRP6, and induces rapid endocytosis.	↓	(Mao et al., 2002)
LRP6	PI4KII/ PIP5KI	PI4KII and PIP5KI are required for Wnt3a-induced LRP6 phosphorylation at Ser1490 and Dvl directly interacts with and activates PIP5KI	↑	(Pan et al., 2008)
LRP6	PtdIns (4,5) P2	PtdIns(4,5)P(2) promotes the assembly of LRP6 signalosomes via the recruitment of AP2 and clathrin.	↑	(Kim et al., 2013)
LRP6	Amer1/WTX	Amer1 is recruited to the membrane by PtdIns(4,5)P(2). It binds CK1 γ , recruits Axin and GSK3 β , thus acts as a scaffold protein to stimulate phosphorylation of LRP6.	↑	(Tanneberger et al., 2011)
LRP6	Waif1a	Waif1a binds to LRP6 and inhibits Wnt-induced LRP6 internalization into endocytic vesicles.	↓	(Kagermeier-Schenk et al., 2011)
Dvl2	AP2 μ 2	Ap2 μ 2 is essential for Dvl2 stability and signalosome formation.	↑	(Hagemann et al., 2014)
Kremen1	Clathrin	Kremen1 is internalized from the cell surface in a clathrin- dependent manner.	–	(Mishra et al., 2012)
Frizzled 2	Clathrin	Fz2 is internalized through a clathrin-mediated route and required for Wnt3a-dependent accumulation of β -catenin.	↑	(Sato et al., 2010)
Frizzled 4	β -arrestin 2/Dvl2	Dvl2 and β -arrestin 2 play an important role in the endocytosis of Fz4 on Wnt5a stimulation.	–	(Chen et al., 2003)
Frizzled 4	Micro2-AP2/Dvl2	Direct interaction of Dvl2 with AP-2 is important for Frizzled internalization.	↑	(Yu et al., 2007)
Frizzled 4/5	PI4K2 β	PI4K2 β binds to Dvl and regulates endosomal recycling of Fz receptors.	↑	(Wieffer et al., 2013)

Cargos	Endocytic Proteins	Functions	Wnt Pathway Activity	Refs
Frizzled 5	RNF43/ZNRF3	RNF43 and ZNRF3 selectively ubiquitinate and target frizzled receptors to lysosome for degradation.	↓	(Koo et al., 2012)
Frizzled 7	Rabconnectin-3a (Rbc3a)	Rbc3a promote endosomal maturation to coordinate intracellular trafficking of Wnt receptors.	↑	(Tuttle et al., 2014)
Lgr4/Lgr5	Clathrin	R-spondins bind to LGR4 and LGR5 and trigger β -catenin signaling through clathrin mediated endocytosis.	↑	(Glinka et al., 2011)
Lgr5	Clathrin	With the ligands R-spondin1 and Wnt3a, LGR5 interacts and forms a supercomplex with LRP6 and Fzd5 which is rapidly internalized through a dynamin- and clathrin- dependent pathway.	↑	(Carmon et al., 2012)
Lgr5	Dynamin	LGR5 rapidly internalizes into EEA1- and Rab5-positive endosomes through dynamin dependent pathway.	–	(Snyder et al., 2013b)
Lgr5	β -arrestin-2	Lgr5 recruits β arr2 and the “SSS” amino acids (873–875) in Lgr5 are critical to this process.	–	(Snyder et al., 2013a)
GSK3	Multivesicular Bodies (MVBs)	Wnt signaling triggers the sequestration of GSK3 from the cytosol into MVBs.	↑	(Taelman et al., 2010)
GSK3	p120-catenin/cadherin	The internalization of the GSK3-containing Wnt-signalosome complex into MVBs is dependent on the dissociation of p120-catenin/cadherin from this complex.	↑	(Vinyoles et al., 2014)