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Regulation of Wnt Signalling by Receptor-mediated Endocytosis

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Wnts compromise a large family of secreted and hydrophobic glycoproteins that control a variety of developmental and adult processes in all metazoan organisms. Recent advances in the field of Wnt signalling have revealed that Wnt activates multiple intracellular cascades, resulting in the regulation of cellular proliferation, differentiation, migration and polarity. However, it is not clear how Wnt activates these pathways after it binds to the receptors. It has been shown that Wnt and its antagonist Dickkopf are internalized with their receptors. This review highlights distinct endocytic pathways correlate with specificity of Wnt signalling events.

Key words: endocytosis, Frizzled, low-density-lipoprotein receptor-related protein 5/6, post-translational modification, Wnt.

Abbreviations: AMF, autocrine motility factor; β_2AR, β_2-adrenergic receptor; CCVs, clathrin-coated vesicles; CK1, casein kinase 1; CME, clathrin-mediated endocytosis; CRD, cysteine-rich domain; Dkk, Dickkopf; EGF, epidermal growth factor; ER, endoplasmic reticulum; Fz, Frizzled; GPCR, G protein-coupled receptors; GPI, glycosyl-phosphatidylinositol; GSK-3, glycogen synthase kinase-3; HSPG, heparin-sulfate-modified proteoglycan; JNK, c-Jun N-terminal kinase; Krm, Kremen; Lef, lymphoid enhancer factor; LDLR, low-density-lipoprotein receptor; LRP5/6, low-density-lipoprotein receptor-related protein 5/6; PCP, planar cell polarity; PKC, protein kinase C; por, porcupine; Rho-kinase, Rho-associated kinase; sFRP2, soluble Frizzled-related protein; shi, shibire; Tcf, T cell factor; TGF-β, transforming growth factor-β; Wg, Wingless.

WNT SIGNALLING OVERVIEW

Wnts constitute a large family of cysteine-rich secreted ligands that are essential for a wide array of developmental and physiological processes (*1*). At least 19 Wnt members have been shown to be present in humans and mice until date. The members exhibit unique expression patterns and distinct functions during development. The Wnt family members can be divided into two distinct types based on their ability to induce transformation of the mouse mammary epithelial cell line C57MG (*2*). The highly transforming members include Wnt-1, Wnt-3, Wnt-3a and Wnt-7a. The intermediately transforming or non-transforming members include Wnt-2, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7b and Wnt-11. It has been thought that the two classes of Wnt signal via different intracellular pathways trigger different developmental outcomes.

The intracellular signalling pathway activated by Wnts was originally identified as a β-catenin-dependent signalling pathway that is highly conserved among various species (*1*) (Fig. 1). In the most well-understood β-catenin pathway, the binding of Wnt to its cell-surface receptor, which consists of Frizzled (Fz) and low-density-lipoprotein receptor-related protein 5/6 (LRP5/6) induces the stabilization of β-catenin and its entry into the nucleus, where it affects the transcription of target genes (*3–5*). It is thought that the Wnts showing high transforming activity in C57MG cells activate the β-catenin pathway.

Some Wnts activate a β-catenin-independent pathway such as the planar cell polarity (PCP) pathway and the Ca^{2+} pathway modulates cell movement, as initially observed during embryogenesis (*6*, *7*) (Fig. 1). The PCP pathway, which was originally identified in *Drosophila*, is mediated by some Fzs, and this pathway activates small G proteins, including Rac and Rho, c-Jun *N*-terminal kinase (JNK) and Rho-associated kinase (Rho-kinase) (*8*). Although it is conceivable that the pathway is involved in the regulation of tissue polarity, cell migration and cytoskeleton arrangement based on observations on fly genetics, the exact roles in mammals are not clear. The Ca^{2+} pathway, which is mediated by specific Wnt and Fz, can increase the intracellular Ca^{2+} concentration, probably through trimeric GTP-binding proteins, and activate calcium/calmodulin-dependent protein kinase II and protein kinase C (PKC) (*2*, *6*). Although the physiological roles of the Ca^{2+} pathway are not clear, this pathway seems to regulate cell proliferation and cell migration. It is generally believed that the intermediately transforming and non-transforming Wnts activate the β-catenin-independent pathway (*2*). However, because it has been shown that Wnt-3a can induce the accumulation of β-catenin and activate Rho and Rho-kinase (*9*), classification of Wnts according to their ability to transform C57MG cells may not always reflect distinct intracellular signalling cascades.

The interaction of Wnts with their receptors on the cell surface is the first step in transducing an extracellular signal into an intracellular response (*10*). In humans and mice, 10 Fzs, which are members of a family of seven-pass transmembrane receptors, have been identified as Wnt receptors (*1*). In addition to Fzs, the Wnt/β-catenin

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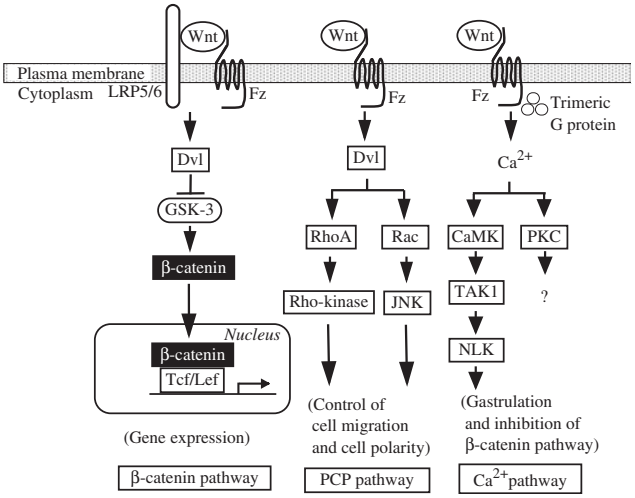

Fig. 1. **Wnt signalling pathways. At least three intracellular signalling pathways are activated by Wnt.** The β-catenin pathways are conserved among various species and are involved in many cellular responses. The PCP and Ca^{2+} pathways have been extensively studied in *Drosophila* and *Xenopus*, but their biological functions in mammals are not well understood.

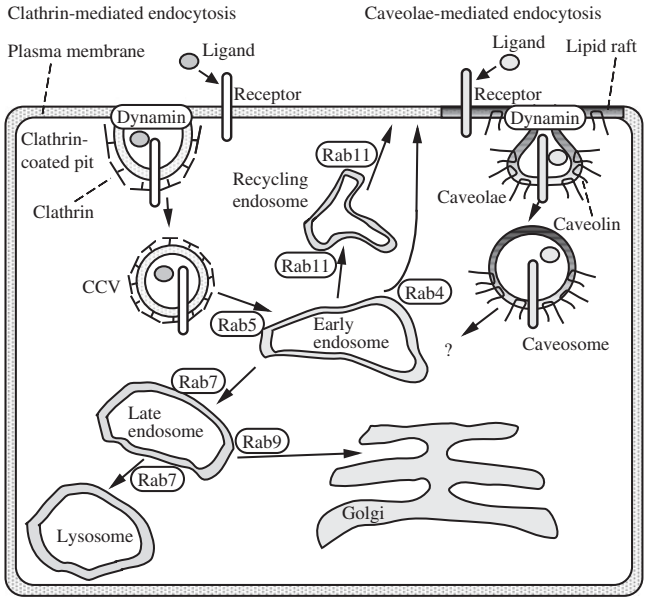

Fig. 2. **Receptor-mediated endocytosis.** There are two different types of endocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis. Clathrin and caveolin play a major role in the respective endocytosis.

pathway requires single-pass transmembrane proteins that belong to a subfamily of LRPs: LRP5 and LRP6 (*5*). Fly genetic studies indicate that arrow (a homologue of LRP5/6) is not essential for the PCP pathway (*11*). Internalization of plasma membrane proteins and lipids is mediated by clathrin-dependent and independent pathways (*12*). Clathrin-mediated endocytosis (CME) targets proteins to the early endosome and is an important pathway for downregulating many receptors through ubiquitin-dependent sorting processes involving ubiquitin-binding proteins resident in the clathrin pathway (*13*). Non-clathrin-dependent endocytosis through the lipid raft and the caveolar pathway has recently emerged as another important trafficking pathway (*14*). Lipid rafts and caveolae function in vesicular and cholesterol trafficking as well as in the internalization of toxins and SV40 virus, and they regulate the internalization of receptors for autocrine motility factor (AMF), transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) (*15*).

It has been reported that wingless (Wg) induces the internalization of *Drosophila* Fz2 (DFz2) and (*16*), that Wnt-5a induces the internalization of Fz4 and Fz5 (*17*, *18*), and that Wnt-3a induces the internalization of LRP6 and Fz5 (*19*). However, which trafficking pathway mediates the internalization of these Wnt receptors and how the internalization of the receptors mediates Wnt signalling remain uncovered. There are many reviews describing intracellular signalling cascades activated by Wnts and the relationship between Wnt signal abnormalities and development or diseases. (*1*, *4*, *6*, *20*). In this review, we highlight Wnts, their receptors, and their interaction-dependent endocytosis.

RECEPTOR-MEDIATED ENDOCYTOSIS

Clathrin-mediated Endocytosis—CME involves the concentration of high-affinity transmembrane receptors and their bound ligands into "coated pits" on the plasma membrane, which are formed by the assembly of cytosolic coat proteins, the main assembly unit being clathrin (*13*) (Fig. 2). Coated pits are encapsulated by a polygonal clathrin coat and carry concentrated receptor-ligand complexes into the cells. Clathrin is a three-legged structure, called a triskelion, formed by three clathrin heavy chains, each with a tightly associated clathrin light chain. Assembly of the clathrin cage requires other main coat constituents, the assembly proteins (AP-1, AP-2 and AP-3). Among three heterotetramer adaptor protein complexes, only AP-2 is involved in the formation of endocytic clathrin-coated vesicles (CCVs).

Dynamin, an atypical GTP-binding protein, is a master regulator of membrane trafficking events at the cell surface (*21*). At the late stages of the formation of CCVs, dynamin is thought to self-assemble into a "collar" at the necks of deeply invaginated coated pits. Dynamin acts as a "pinchase" or molecular garrote to constrict and sever invaginated pits at their necks by GTP hydrolysis-driven conformational changes for its activity. Many accessory proteins including Eps15, amphiphysin, epsin and endophilin have been implicated in CME, initially on the base of their ability to bind to clathrin, AP-2 and dynamin (*13*, *22*). They have regulatory roles in the spatial and temporal regulation of CME.

CCVs are uncoated after internalization from the plasma membranes and then fused with the early endosomes. Early endosomes are multifunctional organelles that regulate membrane transport between the plasma membrane and various intracellular components (*23*). After arriving at early endosomes, membrane proteins and lipids in uncoated vesicles are either returned to the plasma membrane, as in the case

of recycling receptors and bulk membrane constituents, or transported to late endosomes and lysosomes for degradation. Another transport route connects early endosomes to the trans-Golgi network. Rab GTP-binding proteins exert a regulatory function in both exocytic and endocytic transports, through the recruitment of specific effector proteins to the membrane on which they are localized (*24*). Among the Rab family members Rab5 organizes a membrane domain defining the site of entry into early endosomes. Rab4 and Rab11, which are implicated in the fast and slow recycling pathways, respectively, are localized to separate membrane domains on early endosomes that are distinct from those harbouring Rab5. This sorting and recycling of the vesicles is fundamental to proper cellular function and growth.

Caveolae-mediated Endocytosis—Caveolae are flask-shaped invaginations of the plasma membrane and demarcate cholesterol and sphingolipid-rich microdomains of the plasma membrane, in which many diverse signalling molecules and membrane transporters are concentrated (*12*, *14*) (Fig. 2). The shape and structural organization of caveolae are conferred by caveolin, a dimeric protein that directly binds cholesterol. Caveolin self-associates to form a striated caveolin coat on the surface of the membrane invaginations. Caveolae mediate the internalization of sphingolipid binding toxins such as cholera toxin and shiga toxin, glycosyl-phosphatidylinositol (GPI)-anchored proteins and the receptors for AMF, TGF-β and EGF.

Caveolae-mediated endocytosis shares many common features with clathrin-dependent pathways, including the same dynamic requirement of molecular machinery such as actin, dynamin, intersectin, cortactin and epsin in budding and fission process. However, there are clearly striking differences between the dynamic association of clathrin with the plasma membrane and the stable association of caveolin. Unlike clathrin, which forms a transient association with the plasma membrane, caveolin forms a highly stable microdomain with a slow rate of turnover. Caveolin is an integral membrane protein with the usual topology; a 33-amino acid intramembrane domain is believed to insert into the inner leaflet of the plasma membrane to form a hairpin loop with both N and C terminal regions of the protein cytoplasmic domain (*14*). Caveolin directly interacts with cholesterol and cholesterol depletion of the plasma membrane causes caveolae to flatten. Although the mechanism of caveolae formation is becoming clear, the trafficking pathway after the internalization of caveolae has not yet been clarified.

LIGANDS AND RECEPTORS IN WNT SIGNALLING

Wnt—In addition to the 19 mammalian *Wnt* genes, there are eight *wingless* (*Wg*, Wnt homologue) genes in *Drosophila* (*1*). All of Wnts contain a signal sequence followed by a highly conserved distribution of cysteines. Although it has been long difficult to purify active Wnts, it is now possible to purify Wnt-3a, Wnt-5a and DWnt-8 from conditioned medium (*9*, *18*, *25–27*). Purified Wnt-3a stabilizes β-catenin and activates Rho-kinase (*9*, *25*), and purified Wnt-5a inhibits the transcriptional activity of T cell factor/lymphoid enhancer factor (Tcf/Lef) and stimulates cell migration (*18*, *26*, *28*). These activities are consistent with the observations made in the experiments involving the overexpression of plasmids and conditioned medium. However, other Wnts have not yet been purified. Clarification of the biological activities of purified Wnts is important for understanding the molecular details of Wnt signalling.

Once secreted, Wnts interact with glycosaminoglycans in the extracellular matrix and bind tightly to the cell surface (*29*, *30*). Wnts are indeed associated with the plasma membrane or extracellular matrix in cultured cells and are hard to extract from these fractions. Responses to Wg require cell-surface heparin sulphate, a glycosaminoglycan component of proteoglycans (*30*) and disruption of dally, which encodes a cell-surface heparin-sulfate-modified proteoglycan (HSPG), produces phenotypes comparable to those found with loss of function of Wg or *Drosophila* Fz1 (DFz1)/Dfz2 (*31*). Therefore, glycosaminoglycans can modulate the extracellular localization of Wg and plays a role in the movement of Wg between cells. The interaction of Wnt with HSPG might increase the local concentration of Wnt in the lipid-raft domains present on the cell surface and bring Wnt into close proximity of Fz and LRP5/6 receptors. Furthermore, it has been reported that Wg associates with lipoprotein particles (*32*). Lipoprotein particles consist of phospholipids monolayers that surround a core of esterified cholesterol and triglycerides, scaffolded by members of the apolipoprotein family. In *Drosophila*, the formation of a Wg-lipoprotein particle complex is required for long-range signalling in the wing imaginal disc, but does not influence short-range effects.

Post-translational Modification of Wnt—The experiments using tunicamycin (an inhibitor of *N*-linked glycosylation) have shown that Wnt-1, Wnt-3a, Wnt-5a, Wnt-5b, Wnt-6 and Wnt-7b enter the endoplasmic reticulum (ER) and are glycosylated (*33*). Wnt-3a is glycosylated at both Asn87 and Asn298, and Wnt-5a is modified with *N*-glycans at Asn114, Asn120, Asn311 and Asn325 (*18*, *27*) (Fig. 3). Glycosylation of Wnt-3a and Wnt-5a plays an important role in their secretion. Recently, it was reported that Wntless binds to Wnt-3a and stimulates the secretion of Wnt-3a (*34*), but glycosylation of Wnt-3a is not required for the binding to Wntless.

One of the reasons for the difficulty of purifying Wnts is their hydrophobic insolubility, resulting from their acylation. Wnt-3a and Wnt-5a are modified with palmitate at Cys77 and Cys104, respectively (*18*, *25*) (Fig. 3). These cysteine residues are conserved among Wnt family proteins, but whether other Wnts are palmitoylated at this cysteine residue is not known. Palmitoylation at the cysteine residue is important for determining the hydrophobicity of Wnt-3a and Wnt-5a and is also important for the actions of these Wnts but not essential for their secretion. The Wnt-3a mutant, which lacks palmitoylation at Cys77, does not bind to its receptors, Fz8 and LRP6, or to soluble Frizzled-related protein 2 (sFRP2), and does not induce the internalization of LRP6, and therefore, has lost the ability to induce β-catenin

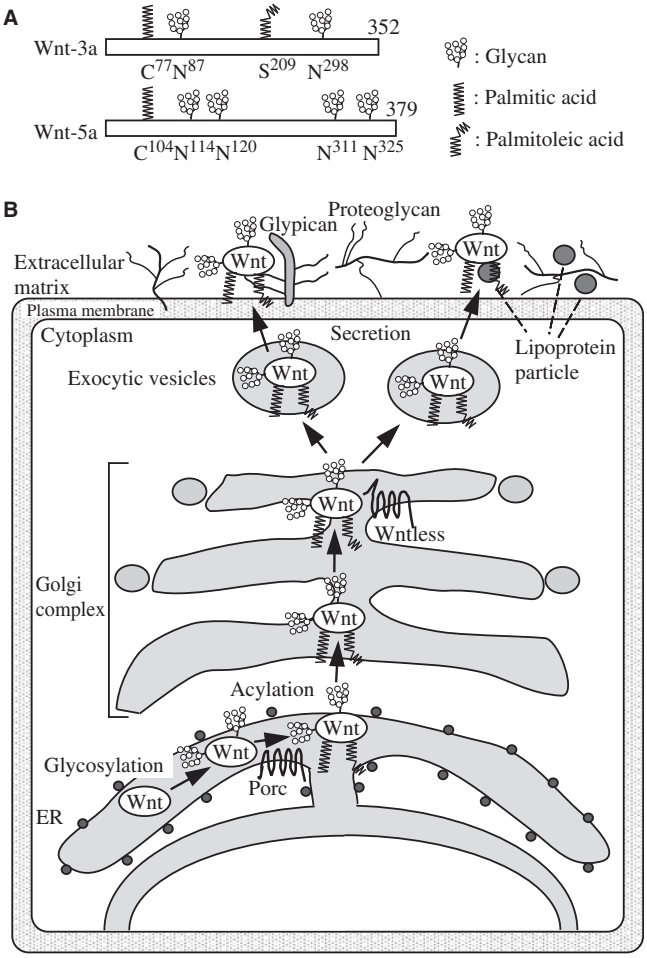

Fig. 3. **Post-translational modifications of Wnt.** (A) Glycosylation and acylation sites of Wnt-3a and Wnt-5a. (B) Maturation of Wnt. Wnts enter into ER, where initial glycosylation and acylation occur, and are then transported into the Golgi apparatus, where further glycosylation processes occur, and are finally secreted from the cell surface in a mature and active form. After secretion, Wnts are associated with proteoglycans, lipoprotein particles or other extracellular matrix components, and are transported to the target cells.

accumulation (*27*). The Wnt-5a mutant, which lacks palmitoylation at Cys104, neither binds to Fz5 nor induces the internalization of Fz5, and therefore, lacks the abilities to inhibit Tcf activity and to stimulate cell migration (*18*). In addition to Cys77, Wnt-3a has been shown to be modified with another kind of lipid at Ser209 (*35*). Unlike palmitoylation at Cys77, Ser209 is modified with a monounsaturated fatty acid, palmitoleic acid (C16:1). The bent structure of the unsaturated fatty acid might be advantageous for packing fatty acid chains into the interior of a small lipid particle. This would fit with the observation that lipoprotein particles serve as extracellular transporters of Wnts (*32*). Acylation of Wnt-3a at Ser209 is essential for its secretion and the Wnt-3a mutant that lacks acylation at Ser209 is retained in the ER. Therefore, multiple lipidation at different amino acids has distinct roles in exerting the actions of Wnts.

The protein (Porc) encoded by the *porcupine* gene (*porc*) in *Drosophila* has been suggested to be a membrane-bound acyltransferase (*36*). It has been reported that porc is required for Wg-producing cells to generate the fully functional protein signal (*37*) and for secreting Wg (*38*). Four different types of mouse Porc (Mporc A, B, C and D) are generated from a single gene by alternative splicing (*38*). Since knockdown of Porc in fibroblasts reduces the acylation of Wnt-3a (*35*), it is thought that Porc acylates Wnt-3a in mammalian cells. Glycosylation of Wnt-3a is required for acylation, but acylation is not necessary for glycosylation (*27*, *35*). Therefore, glycosylation of Wnt-3a may be necessary for proper folding, enabling the protein to bind to the machinery required for acylation.

Dickkopf—The Dickkopf (Dkk) family of proteins comprises four members (Dkk-1 to Dkk-4), which antagonize Wnt signalling (*39*). Dkks contain two characteristic cysteine-rich domains (CRD-1 and CRD-2) separated by a linker region of variable length. The characteristic developmental function of Dkk-1 is its head-inducing activity. Dkk-1 is expressed in the anterior endomesoderm of the Spemann organizer of *Xenopus* embryos. Dkk-1-knockout mice lack anterior head structures, indicating that Dkk-1 is essential for head formation. Furthermore, knockout of Dkk-1 in mice increases the bone mass and expression of Dkk-1 reduces the hair and feather follicles.

Dkk-1 prevents activation of the Wnt signalling pathway by binding to LRP5/6 rather than to Wnts (*40*). The C-terminal domains of Dkk-1 and Dkk-2, which contain the CRD-2 region, are necessary and sufficient for association with LRP6. There are two possible mechanisms by which Dkk-1 might inhibit the β-catenin pathway. One possibility is that Dkk-1 functions by preventing Fz-LRP6 complex formation (*41*). The other possible mechanism is different from this, although not mutually exclusive. Dkk-1 interacts with another class of receptor, the single-pass transmembrane proteins Kremen1 (Krm1) and Krm2 in addition to LRP5/6 (*42*). Dkk-1 forms a ternary complex with LRP5/6 and Krm and promotes endocytosis and removal of LRP5/6 from the cell-surface membrane, which inhibits the Wnt signalling. The details of these actions are described subsequently.

Frizzled—Genes for the *Fz* family of the superfamily of G protein-coupled receptors (GPCR) have been found in many animal species. They encode seven-pass transmembrane proteins that act as receptors for secreted Wnt (*43*). All Fzs (there are 10 Fzs in humans and mice) share the following structural similarities: a signal peptide sequence at the amino terminus; a conserved region of ~120 amino acids in the extracellular domain containing 10 invariantly spaced cysteines (called the cysteine-rich domain, CRD); a seven-pass transmembrane region, in which the transmembrane segments are well conserved; and a cytoplasmic domain with little homology among members of the family. The CRD has been shown to be necessary and sufficient for the binding of Wnt ligand to the surface of expressing cells (*44*).

The Wnt-Fz ligand-receptor relationship is best characterized in *Drosophila*. Two members of the Fz family,

Dfz1 and Dfz2, bind to Wg with a dissociation constant (*Kd*) value of 10^{-8} M and 10^{-9} M, respectively (*45*). Fly mutants lacking both *Dfz*1 and *Dfz2*, but not mutants lacking either, have severely defective Wg signalling (*46*), providing evidence that Dfz1 and Dfz2 are partially redundant Wg receptors in many contexts. A *Dfz1* mutant, but not a *Dfz2* mutant, shows the PCP phenotypes, indicating that Dfz1 mainly regulates the PCP pathway. Therefore, different Fzs may provide intracellular signalling specificity.

Although it is not well understood how Fzs transmit the signals to downstream molecules, the following evidence supports the notion that Fzs are coupled with trimeric G proteins. The Wnt-stimulated pathway is sensitive to inactivation by pertussis toxin (*47*), an ADP-ribosyltransferase that inactivates only members of the Gi/Go family of G proteins. β_2-Adrenergic receptor (β_2AR)/RFz1 and β_2AR/RFz2 chimeras display a GTP-dependent and agonist-specific shift in receptor affinity, which demonstrates the direct receptor-G protein interaction (*47*). The *Drosophila* homologue of human Go is essential in the activation of β-catenin and PCP pathways (*48*). However, how G proteins activate the downstream signal in the Wnt pathway remains to be clarified.

The Dvl protein family is a critical component of the Wnt signalling pathway (*49*). All members of this family contain three highly conserved domains: a DIX domain, a PDZ domain and a DEP domain. Dvl transmits the Wnt signal to at least two distinct pathways (the β-catenin and PCP pathways), and thereby organizes pathway-specific subcellular signalling complexes, signal amplification and dynamic control through feedback regulation (*1*). The cytoplasmic domains of the Fz family reveal a very high level of homology among the 10 gene products. The highest level of homology is confined to the first 25 residues of the N-terminal region of the C-terminal tail. The KTXXXW motif, which is located two amino acids C-terminal to the seventh transmembrane domain, is required for the activation of the β-catenin pathway and for membrane relocalization and phosphorylation of Dvl (*50*). In fact, chemical-shift perturbation of nuclear magnetic resonance spectroscopy reveals that the 12-amino acid peptide containing this sequence of Fz7 directly binds to the PDZ domain of Dvl1 (*51*). Although the DEP domain of Dvl does not bind to the Fz7 peptide, the DEP domain plays a role in the membrane localization of the protein (*52*). Therefore, signal transduction between Fz and Dvl may require the membrane-targeting function of the DEP domain to bring the two proteins into close proximity to one another.

LRP5/6 and Arrow—LRP5, LRP6 and arrow, which are type-I single-pass transmembrane proteins, constitute a subfamily of the low-density-lipoprotein receptor (LDLR) family, whose members play diverse roles in metabolism and development (*5*). The roles of arrow and LRP6 in Wnt signalling were discovered in genetic studies. *Drosophila* mutants lacking *arrow* phenotypically resemble the Wg mutant (*11*), and mutant mice lacking *Lrp6* exhibit combined phenotypes similar to those caused by mutations of several individual Wnt genes (*53*). Unlike Fz, which is required for multiple Wnt pathways, arrow and LRP6 appear to be specifically required for Wnt/β-catenin signalling. In *Drosophila*, arrow mutants exhibit a normal PCP (*11*), indicating that arrow is not required for the PCP regulated by Dfz1. Similarily, in *Xenopus*, blocking LRP6 function has little effect on gastrulation movements (*41*).

LRP5, LRP6 and arrow have similar structures, a long extracellular region (~1300 amino acids) and a short intracellular region (~300 amino acids). There are four YWTD β-propeller domains, three LDLR type A domains and four EGF-like domains in the extracellular region, and the intracellular region contains an Axin binding site, which is essential for the activation of the β-catenin pathway (*5*). An LRP6 mutant lacking the intracellular domain is completely inactive, and blocks the Wnt signalling in a dominant negative fashion (*54*). Conversely, LRP5/6 mutants that lack the extracellular domain activate the β-catenin pathway constitutively in mammalian cells (*55*) and *Xenopus* embryos (*54*).

Kremen—Kremen (Krm) was originally isolated as a novel type of kringle-containing protein (*56*). The kringle domain, a repeating homologous triple-disulfide-linked peptide region, is conserved in diverse proteins, including prothrombin, tissue plasminogen activator, hepatocyte growth factor and Ror1/2. Krm is a type-I single-pass transmembrane protein, and it has WSC and CUB domains in addition to the kringle domain in the extracellular region and a short (64 amino acid) intracellular domain, which has no known homology to other proteins. Although the functions of Krm have not been clarified, it has been found that Dkks bind to Krm1 and Krm2 (*42*). In mammalian cells, either Krm1 or Krm2 can cooperate with Dkk-1 in the inhibition of the β-catenin pathway. *Drosophila* has no Dkk or Krm homologs, although ectopic expression of vertebrate Dkk-1 and Krm2 together results in inhibition of Wg signalling. Dkk-1 induces the internalization of LRP6 from the cell surface by binding to both LRP6 and Krm, thereby attenuating the Wnt signalling.

INTERNALIZATION OF LIGANDS AND RECEPTORS IN WNT SIGNALLING

Wg-DFz-Arrow in Flies—The best evidence for the role of endocytic trafficking in Wnt/Wg signalling regulation *in vivo* comes from works in *Drosophila* (Fig. 4). In the embryonic epidermis and the wing imaginal disc, Wg can be detected in cytoplasmic puncta within Wg-responsive cells (*57*). Such punctuate structures are not observed when endocytosis is compromised (*58*). Furthermore, endocytosis-defective cells accumulate extracellular Wg on their surfaces, suggesting that Wg is normally internalized and trafficked through the endocytic pathway.

Endocytosis of ligand is the first step in its transport to the lysosome, where signalling may be attenuated by degradation of an active receptor complex. Lysosomal degradation of Wg in the embryonic epidermis is required for restricting the range of Wg signalling posterior to the source (*59*). When endocytosis and subsequent lysosomal degradation are compromised in this tissue, excess Wg levels cause elevated signalling and misspecification of epidermal cell fate.

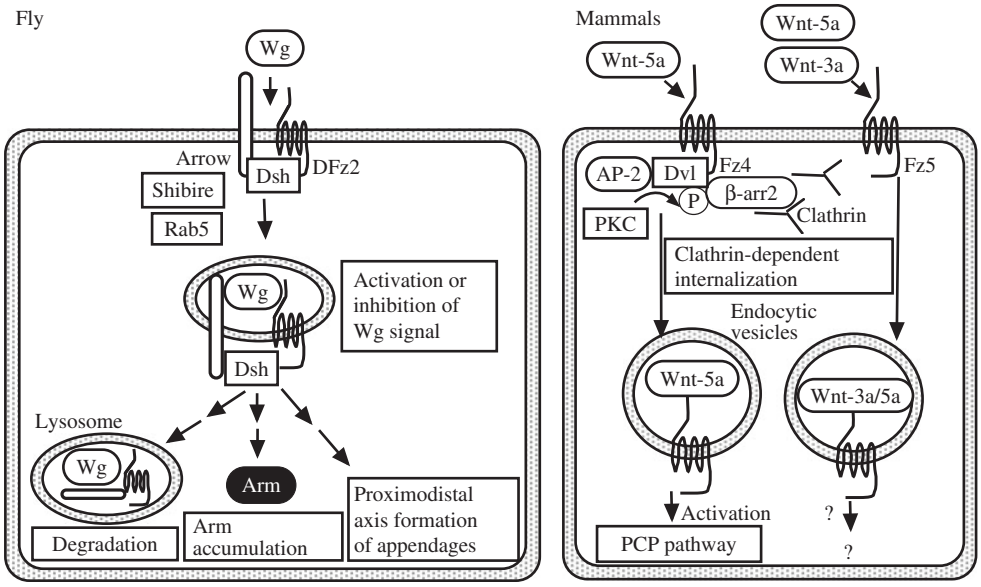

Fig. 4. **Internalization of Fz in response to Wnt.** In flies, the internalization of the Wg and Dfz complex triggers the regulation of the β-catenin pathway and degradation of Wg in lysosomes. In mammals, Wnts induce the internalization of Fzs. Combination of Wnt and Fz may determine the specific route of the endocytic pathway. Arm, armadillo; β-arr, β-arrestin2.

Therefore, normal embryonic patterning requires lysosomal trafficking to downregulate Wg levels. Wg, arrow and DFz2 are trafficked to the lysosome in the wing imaginal disc (*16*). In contrast, signalling in the developing wing is not attenuated by lysosomal targeting (*16*).

The endocytic vesicles are cleaved from membranes by the function of dynamin (*21*), a protein encoded by *shibire* (*shi*) in *Drosophila*. In *shi* mutant embryos, Armadillo staining is reduced but not eliminated (*60*), suggesting the facilitation of Wg signalling by dynamin. Furthermore, knockdown of dynamin or Rab5 reduces Wg reporter activity, suggesting that internalization and endosomal transport facilitate signalling (*61*). However, it has also been reported that Wg signalling is negatively regulated by Rab5 (*62*). The variation in results may be attributed to differences in the experiments, in which particular parts of the body or cells of a tissue were used.

Wnt-Fz in Mammals—Internalization of GPCRs has been extensively studied in the field of mammalian receptor studies. β-Arrestin1/2 are critical for mediating the endocytosis of a number of GPCRs (*63*). Typically, when receptors are activated, they are rapidly phosphorylated by G protein-coupled receptor kinases and then bind to β-arrestin1/2. β-Arrestins desensitize second-messenger generation by sterically blocking the receptor-G protein interaction and mediate endocytosis of the receptors in clathrin-coated pits by binding to clathrin, AP-2 and other elements of the endocytic machinery (*64*). In addition, β-arrestins serve as scaffolds linking GPCRs to other signalling proteins, such as the src family kinases and members of the mitogen-activated protein kinase cascade (*63*).

Since Fz belongs to the GPCR family, β-arrestin is implicated in the internalization of Fz (*17*, *65*) (Fig. 4). Wnt-5a triggers endocytosis of Fz4 mediated by β-arrestin2. This internalization of Fz4 requires the activation of PKC. Dvl2 recruits β-arrestin2 but not β-arrestin1 to the plasma membrane, and this recruitment also requires the phosphorylation of Dvl2 by PKC. Dvl2 also interacts with μ2-adaptin of AP-2 through its DEP domain, and this interaction is required for Wnt-5a-induced internalization of Fz4 (*66*). Furthermore, in *Xenopus* embryos, the Dvl mutant that lacks the binding site to AP-2 interferes with gastrulation mediated by the PCP pathway (*66*). Therefore, Wnt-5a induces the internalization of Fz4 by binding to Dvl2, probably via the β-arrestin2- and AP-2-dependent pathway, thereby activating the β-catenin-independent pathway. Wnt-5a also induces the internalization of Fz5, but the activation of PKC is not required (*18*) (Fig. 4). Furthermore, Wnt-3a triggers endocytosis of Fz5 and this internalization is mediated by clathrin (*19*). Thus, it is conceivable that Fz is internalized by the clathrin-dependent pathway in response to Wnt.

Wnt-LRP5/6 in Mammals—LRP5 or LRP6 is a key signalling receptor for the β-catenin pathway (Fig. 5). This receptor has been shown to bind to Axin through its intracellular domain (*55*). Axin acts as a scaffold protein to degrade β-catenin by forming a complex with glycogen synthase kinase-3 (GSK-3), β-catenin, and adenomatous polyposis coli gene product (*3*, 67, 68). The binding between the LRP5/6 intracellular domain and Axin is directly linked to the stabilization of β-catenin. This stabilization depends on the phosphorylation of several motifs containing Ser/Thr residues in the cytoplasmic domain of LRP5/6 (*54*, 69, 70). The motifs contain two clusters of Ser/Thr residues or a PPPSP site. Casein kinase 1 (CK1) phosphorylates the cluster regions and GSK-3 phosphorylates the PPPSP sites followed by the phosphorylation by CK1. Wnt promotes rapid

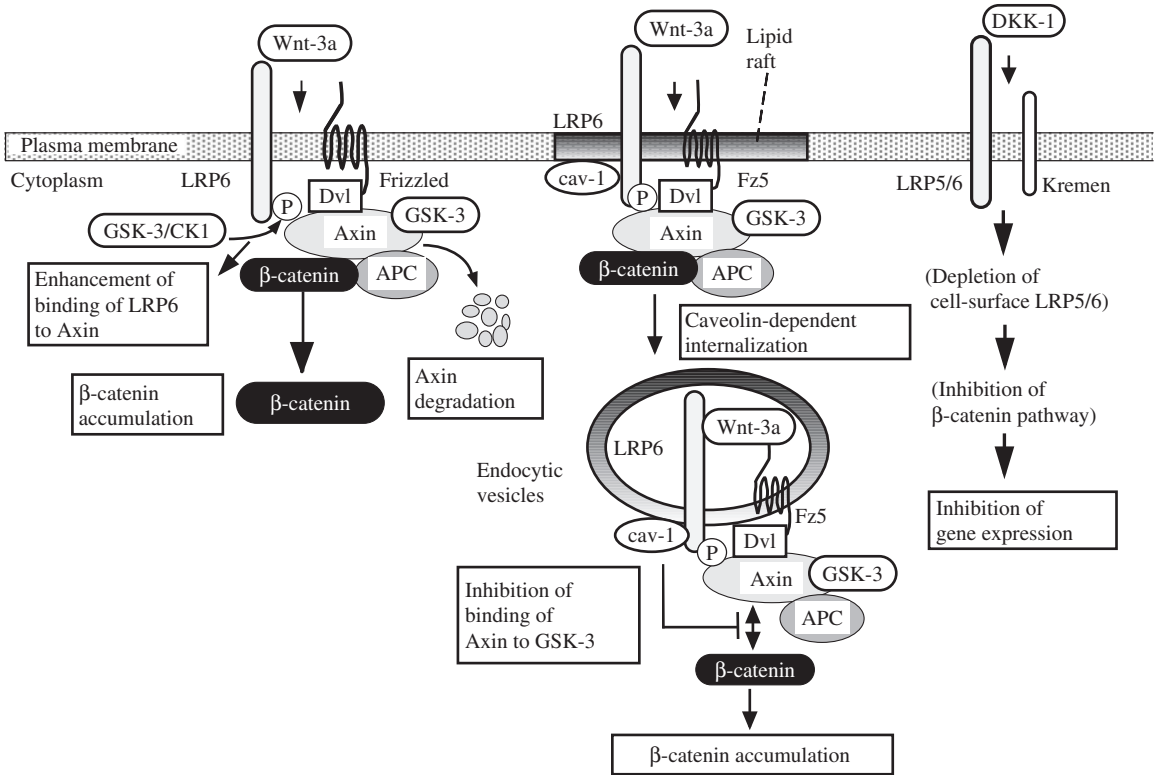

Fig. 5. **Wnt- and Dkk-dependent internalization of LRP6 and regulation of the stability of β-catenin.** Distinct endocytic pathway of LRP6 in response to Wnt or Dkk may correlate with the specificity of the activation of the β-catenin pathway. cav-1, caveolin-1.

phosphorylation at GSK-3 and CK1 sites, which results in recruitment of Axin to LRP5/6 on the plasma membrane, where Axin is inactivated and/or targeted for degradation. It has been reported that Wnt stimulation promotes Axin degradation in cultured mammalian cells (*71*, *72*) and fly embryos (*73*). This may result in the stabilization of β-catenin because Axin is an essential component of the degradation complex (*3*) (Fig. 5).

An important insight into the binding of Axin and LRP6 came from the finding that LRP6 binds to caveolin in response to Wnt and is then internalized (*19*) (Fig. 5). Wnt-3a stimulation induces the binding of LRP6 and caveolin, which allows caveolin to form a complex with Axin through LRP6. Expression of the cytoplasmic region of LRP6, although it contains an Axin binding site, neither binds to Axin nor stabilizes β-catenin, while a fusion protein of the LRP6 cytoplasmic region with caveolin is highly phosphorylated, associates with Axin, and induces the stabilization of β-catenin (Yamamoto, H., unpublished data). Since formation of the complex of LRP6 and caveolin inhibits the binding of β-catenin and Axin, β-catenin may consequently be stabilized (Fig. 5). Thus, caveolae-mediated endocytosis plays critical roles in inducing the internalization of LRP6 and activating the β-catenin pathway. Furthermore, dominant negative forms of Rab5 and dynamin suppress both the Wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin. Three to four hours after Wnt-3a stimulation, internalization of LRP6 is recycled to the plasma membranes through Rab11 (*19*). A dominant negative form of Rab11 inhibits the recycling of LRP6 but not the stabilization of β-catenin. Therefore, the process from fission of the plasma membrane by dynamin to the caveolin-coated vesicle formation is important for the stabilization of β-catenin.

Wnt-3a induces the internalization of Fz5 in a clathrin-dependent manner (*19*) and Wnt-3a also activates both the β-catenin and PCP pathways (*9*). Taken together with the observation that the AP-2-dependent internalization of Fz may activate the PCP pathway (*66*), these findings raise the possibility that distinct endocytic pathways may correlate with specificity of Wnt signalling pathways.

Dkk-Krm—Binding of Dkk-1 to LRP5/6 and Krm results in the formation of a ternary structure and induces rapid endocytosis and removal of LRP5/6 from the plasma membrane, and thereby attenuating the Wnt signalling (Fig. 5). However, since the Krm intracellular domain is neither conserved nor required for any of these functions (*42*), the roles of Krm in the internalization of LRP6 is not well understood. Dkk-1 induces the internalization of LRP6 with Krm2 in a clathrin-dependent manner, and knockdown of clathrin blocks Dkk-1-dependent inhibition of Wnt-3a-β-catenin signalling, although it does not affect Wnt-3a-dependent activation of the β-catenin pathway (Yamamoto, H., unpublished data). Therefore, it is possible that Dkk induces the internalization of the complex of LRP6 and Krm by

endocytic pathway different from Wnt-induced internalization of LRP6.

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