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REVIEW

Intracellular trafficking of planar cell polarity proteins

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BACKGROUND: Planar cell polarity (PCP) is a phenomenon in which epithelial cells are polarized along the plane of a tissue. PCP is critical for a variety of developmental processes and is regulated by a set of evolutionarily conserved PCP signaling proteins. Many of the PCP proteins adopt characteristic asymmetric localizations on the opposing cellular boundaries. Currently, the molecular mechanisms that establish and maintain this PCP asymmetry remain largely unclear. Newly synthesized integral PCP proteins are transported along the secretory transport pathway to the plasma membranes. Once delivered to the plasma membranes, PCP proteins undergo endocytosis. Recent studies reveal insights into the intracellular trafficking of PCP proteins, suggesting that intracellular trafficking of PCP proteins contributes to establishing the PCP asymmetry.

OBJECTIVE: To understand the intracellular trafficking of planar cell polarity proteins in the secretory transport pathway and endocytic transport pathway.

METHODS: This review summarizes our current understanding of the intracellular trafficking of PCP proteins. We highlights the molecular mechanisms that regulate sorting of PCP proteins into transport vesicles and how the intracellular trafficking process regulates the asymmetric localizations of PCP proteins.

RESULTS: Current studies reveal novel insights into the molecular mechanisms mediating intracellular trafficking of PCP proteins. This process is critical for delivering newly synthesized PCP proteins to their specific destinations, removing the unstable or mislocalized PCP proteins from the plasma membranes and preserving tissue polarity during proliferation of mammalian skin cells.

CONCLUSION: Understanding how PCP proteins are delivered in the secretory and endocytic transport pathway will provide mechanistic insights into how the asymmetric localizations of PCP proteins are established and maintained.

Planar cell polarity and its regulators

Many epithelial cells are polarized along the plane of the epithelium, a process known as planar cell polarity (PCP). PCP is important for the polarized patterning of epithelial structures including hairs in drosophila wing, the orientation of the ommatidia unit in Drosophila eye and stereocilia bundles in mammalian inner ears (Devenport, 2014). The Drosophila wing hairs, as an example of the PCP phenomenon, are oriented distally to the Drosophila body and parallel to the wing veins with regular spacing (Wootton, 1992; Classen et al., 2005). This organization is proposed to be important for guiding air flow over the surface of the wing during flight (Wootton, 1992; Classen et al., 2005). In addition, PCP is essential for various coordinated cell behaviors, ranging from oriented cell divisions, coordinated beating of cilia in the trachea, to the process of convergent extension in developing vertebrate embryos (Devenport, 2014).

The establishment of PCP is regulated by a group of evolutionary conserved proteins. These PCP proteins can be divided into two groups: the core PCP components and the Fat-Dachsous (Ft/Ds) PCP components (Devenport, 2014). The core PCP components include transmembrane proteins Frizzled (Fzd), Van Gogh (Vang, also known as Strabismus/Stbm in Drosophila), and members in the family of cadherin EGF LAG seven-pass G-type receptor (Celsr, also known as Flamingo/Fmi in Drosophila), and cytoplasmic proteins Dishevelled (Dsh), Prickle (Pk) and Diego (Dgo). The Ft/Ds PCP components include three proteins, the atypical cadherins, Fat (Ft) and Dachsous (Ds), and the Golgi-localized transmembrane kinase, Four-jointed (Fj).

A key feature of the PCP proteins is that many of them adopt characteristic asymmetric localizations on opposing cellular boundaries. In Drosophila pupal wing, for example, Stbm and Pk are localized on the cellular boundaries that are

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proximal to the Drosophila body, whereas Fzd, Dgo and Dsh are localized distally to the body (Axelrod, 2001; Feiguin et al., 2001; Strutt, 2001; Tree et al., 2002; Bastock et al., 2003; Devenport, 2014) (Fig. 1). Fmi localizes on both sides and forms homodimers on the opposing cellular boundaries (Devenport, 2014) (Fig. 1). Similar to the core PCP proteins, Ft and Ds also reside in the opposite sides of cell surface (Ambegaonkar et al., 2012; Brittle et al., 2012) (Fig. 1). Evidence suggests that this asymmetric localization of PCP proteins is indispensable for the accurate propagation of PCP. As an example, PCP proteins in the proximal side of cell surface are proposed to recruit negative regulators of prehair formation, such as Inturned, Fuzzy and Fritz, to inhibit hair formation in Drosophila wing (Adler et al., 2004; Strutt and Warrington, 2008). Through this process, hair formation can be restricted to the distal side of the cell surface. In addition, the Drosophila casein kinase 1 γ (CK1-γ) homolog gilgamesh associates with the base of elongating prehairs at the distal side and this kinase restricts nucleation of prehair formation to a single site (Gault et al., 2012). CK1-γ/gilgamesh functions independent of the PCP pathway and regulates prehair formation through regulating polarized Rab11-mediated vesicle trafficking (Gault et al., 2012).

The molecular mechanisms that regulate the asymmetric localizations of PCP proteins remain largely unclear. One view is that the intercellular interactions among the PCP proteins at cell-cell junctions can stabilize their specific localizations on opposing cellular boundaries (Klein and Mlodzik, 2005; Chen et al., 2008; Devenport, 2014). This view is supported by evidence that Celsr/Fmi proteins on the opposing cellular boundaries form homodimers and recruit Frizzled and Vang to the cell junctions (Devenport and Fuchs, 2008; Devenport, 2014). Moreover, genetic and biochemical evidence in Drosophila suggests that Fmi exists in two forms: a Frizzled-associated form and a Vang-associated form (Chen et al., 2008). These two different forms of Fmi preferentially interact with each other (Chen et al., 2008). Through this process, the homodimer of Fmi on the cell boundaries is associated with Vang on one side of the plasma membrane and with Frizzled on the other side of the plasma membrane (Chen et al., 2008) (Fig. 1A). Similar to the core PCP proteins, Ft and Ds also interact intercellularly on the opposing cellular boundaries (Matakatsu and Blair, 2004; Ambegaonkar et al., 2012; Brittle et al., 2012) (Fig. 1B), suggesting that the interactions between these PCP proteins at cell-cell junctions may contribute to their asymmetric localizations. In addition, the Ft/Fj/Ds components are proposed to provide long-range directional information for establishing specific localizations of PCP proteins (Bayly and Axelrod, 2011).

In epithelial cells, intracellular trafficking plays important roles in establishing apical-basolateral polarity. Interestingly, live imaging analysis indicates that Frizzled- and Dishevelled-containing vesicles are preferentially transported to the distal side of cell boundaries in Drosophila pupal wing, suggesting that polarized intracellular trafficking may contribute to establishing the PCP asymmetry (Olofsson et al., 2014; Shimada et al., 2006). Corresponding to this, the non-centrosomal microtubules are aligned along the proximal-distal axis with a subtle excess of the plus ends oriented

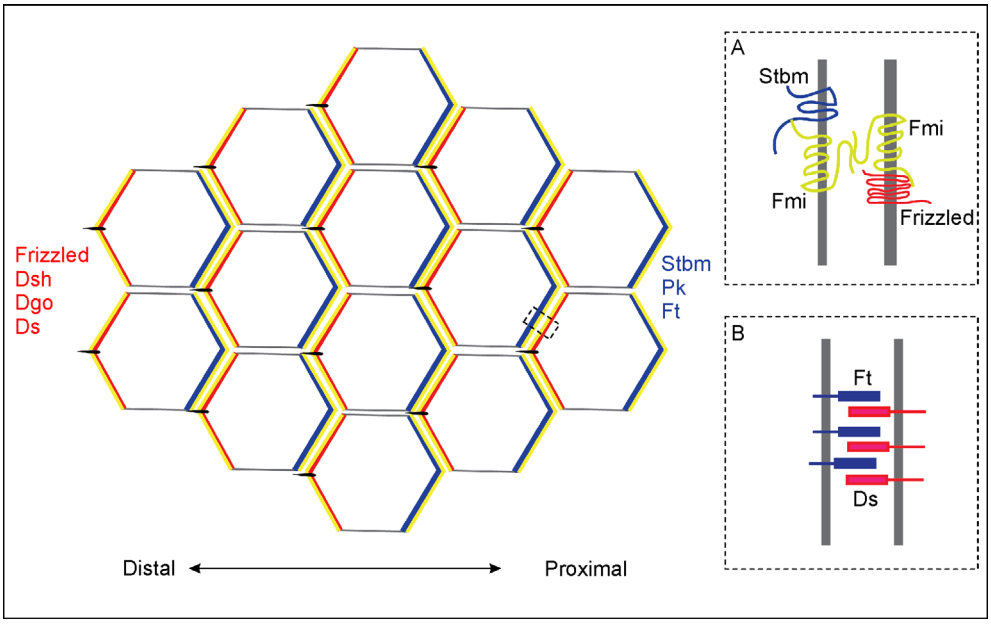

Figure 1 The asymmetric localizations of PCP proteins in Drosophila pupal wing. PCP proteins show characteristic asymmetric localizations on the opposing cellular boundaries in Drosophila pupal wing. The wing hairs shown in black are all localized at the distal side of the cells and pointing to the distal side of cell boundaries. (*inset A and inset B*) Magnified views of the asymmetric localizations of PCP proteins on the cell boundary.

distally in Drosophila pupal wing (Shimada et al., 2006; Harumoto et al., 2010; Olofsson et al., 2014). Planar polarized microtubule cytoskeleton is implicated to mediate polarized delivery of PCP proteins. This microtubule dynamics are controlled by Dachsous and Fat suggesting that Dachsous and Fat may provide a long-range directional clue for the planar polarized pattern of the non-centrosomal microtubule cytoskeleton (Harumoto et al., 2010). Planar polarized microtubule bundles are also observed within the airway epithelium and their plus ends contact membrane domains associated with the PCP proteins, Frizzled and Dishevelled (Vladar et al., 2012). Disruption of microtubules leads to defects of targeting PCP proteins to their specific destinations and misoriented cilia (Vladar et al., 2012). Some studies demonstrate that once the localizations of PCP proteins are established, microtubules are not required for the maintenance of their localizations (Sepich et al., 2011; Shi et al., 2016). These analyses suggest that polarized microtubules are important for the establishment of PCP asymmetry and, at least in some tissues, the maintenance of the established localizations of PCP proteins is independent of microtubule organization.

To gain novel insights into the molecular mechanisms that regulate the asymmetric localization of PCP proteins, it is important to understand how newly synthesized integral PCP proteins are delivered to the plasma membranes and to elucidate the endocytic transport process that mediates the internalization of PCP proteins from plasma membranes.

Intracellular trafficking of PCP proteins in the secretory transport pathway

The secretory transport pathway plays important roles in delivering a variety of newly synthesized proteins to their specific resident compartments. In the conventional secretory transport pathway, newly synthesized proteins are first translocated into the endoplasmic reticulum (Fig. 2). After correct folding and modification, these proteins will be packaged into transport vesicles and delivered to the Golgi apparatus to receive further modifications. The Golgi apparatus is composed of series of flattened cisterna that are aligned in parallel forming a Golgi stack. The Golgi stack is compartmentalized into *cis*-, *medial*- and *trans*- compartments. Cargo molecules enter the Golgi at the *cis* face and exit the Golgi at the *trans* face. The Golgi glycosylation enzymes acting at each step of the protein glycosylation process are sequentially enriched in *cis*-, *medial*-, and *trans*- Golgi cisternae so that newly synthesized cargo proteins can acquire the glycosylation modifications in a sequential manner when delivered across the Golgi apparatus. Cargo proteins exit the Golgi at the *trans* Golgi network (TGN). At the TGN, these proteins are packaged into transport vesicles and delivered along sophisticated post-Golgi trafficking routes to their specific destinations (Fig. 2). Elucidating the molecular mechanisms that regulate delivery of newly synthesized PCP proteins along the secretory transport pathway will provide important information on how PCP proteins are

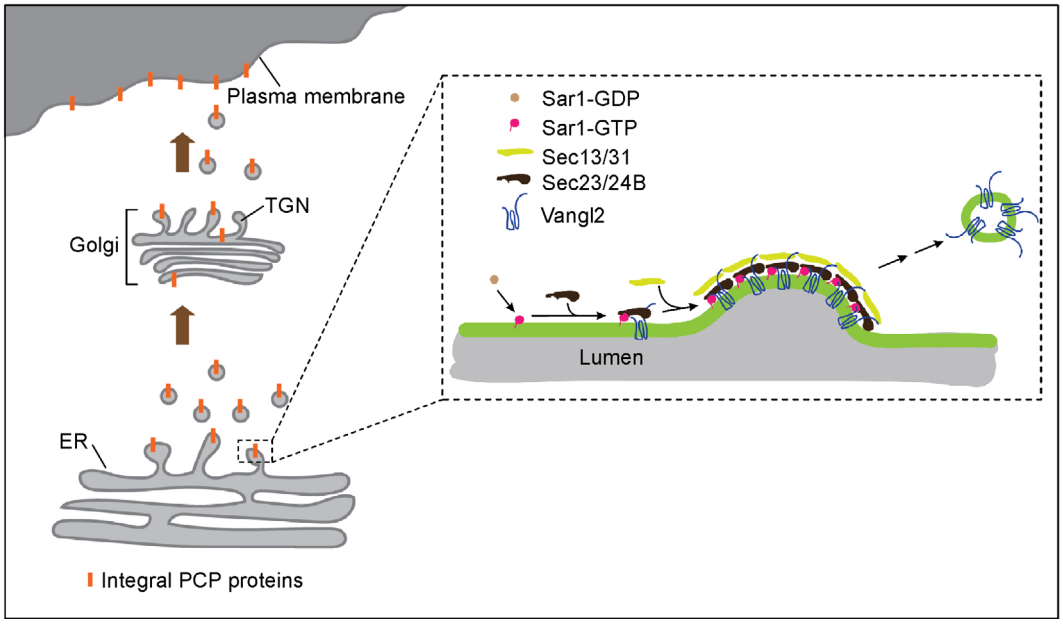

Figure 2 Delivery of integral PCP proteins along the secretory transport pathway. Newly synthesized PCP proteins are first translocated into the endoplasmic reticulum (ER). After correct folding and modification, these proteins will be delivered to the Golgi apparatus to receive further modifications. The newly synthesized proteins exit the Golgi at the *trans* Golgi network (TGN). Subsequently, these transport vesicles are packaged into transport vesicles at the TGN and delivered along sophisticated post-Golgi trafficking routes to the plasma membrane. ***(inset)*** A model for sorting of Vangl2 at the ER. The COPII coat subunit, Sar1, binds to GTP and recruits the Sec23/24B complex to the ER membranes forming pre-budding complexes. Subsequently, the Sec23/24B complex recognizes Vangl2 and recruits the Sec13/31 complex. Polymerization of the Sec13/31 complex along with its associated Sec23/24B complex and cargo proteins forms cage structures leading to membrane deformation and release of Vangl2-enriched vesicles from the ER.

delivered to the cell surface and how their asymmetric localizations are established.

Export of cargo proteins from the donor compartments is largely mediated by vesicle coat proteins, such as coat protein complex II (COPII), coat protein complex I (COPI) and clathrin. COPII mediates ER export, COPI regulates the intra-Golgi transport process, and clathrin coat is an important player regulating TGN export and endocytosis. These vesicle coat proteins, once recruited to the membranes, directly or indirectly interact with cargo molecules. In addition, vesicle coat proteins can polymerize to form electron-dense membrane coat structures. Polymerization of vesicle coat proteins provides forces to deform the lipid bilayer and also concentrates their associated cargo molecules into coated membrane patches. This process, with the help of other cellular factors, leads to formation of vesicles enriched with specific cargo proteins. In the following, we will describe our current understandings of vesicle-coat-protein-mediated ER export and TGN export of core PCP proteins in the secretory transport pathway.

Export of PCP proteins out of the endoplasmic reticulum

The ER is the first station in the secretory transport pathway. At the ER, COPII plays important roles in mediating packaging of cargo proteins into vesicles. The COPII coat is composed of the GTPase Sar1, the Sec23/24 heterodimer and the Sec13/31 heterodimer (Lee et al., 2004). Sar1 can switch from a GDP-bound status to a GTP-bound status, a process catalyzed by the guanine nucleotide exchange factor (GEF), Sec12. Upon GTP binding, Sar1 changes conformation exposing the N-terminal amphipathic motif that facilitates its association with ER membranes and recruits the Sec23/24 complex. Subsequently, the Sec23/24 complex recruits the Sec13/31 complex. Polymerization of the Sec13/31 complex along with its associated Sec23/24 complex and cargo proteins forms cage structures leading to membrane deformation and vesicle budding from the ER (Fig. 2 inset) (Lee et al., 2004).

Sec24 functions as a cargo adaptor, which directly or indirectly interacts with cargo proteins to mediate enrichment of cargo proteins into COPII vesicles (Lee et al., 2004). There are four isoforms of Sec24, termed Sec24A, Sec24B, Sec24C and Sec24D, in mammals. Among them, Sec24B is identified to be critical for PCP-regulated neural tube closure (Merte et al., 2010). Mice that are homozygous of a loss-of-function allele of Sec24B show PCP deficient phenotypes including defects in the orientation of cochlear hair cells and deficiencies in convergent extension (Merte et al., 2010). Disrupting the function of Sec24B causes abnormal subcellular localizations of Vang-like protein 2 (Vangl2) in spinal cord of mice embryos. Through an in vitro assay that reconstitutes packaging of Vangl2 into COPII vesicles, Sec24B but not other isoforms of Sec24 stimulates packaging of Vangl2 into COPII vesicles. Mice heterozygous for the mutations in Vangl2 gene, such as the D255E and S464N mutations, show a characteristic looped tail phenotype (Kibar et al., 2001a; Kibar et al., 2001b; Murdoch et al., 2001). These Vangl2 mutations are referred to as Vangl2 looptail mutations. Mice homozygous for the Vangl2 looptail mutations show severe defects in neural tube closure during early embryonic development (Kibar et al., 2001a; Kibar et al., 2001b; Murdoch et al., 2001). Mechanistic analysis indicates that Vangl2 looptail mutant protein cannot be packaged into COPII vesicles and show defects in ER export. These analyses indicate that Sec24B is critical for sorting Vangl2 into COPII vesicles to mediate delivery of Vangl2 from the ER to the Golgi (Fig. 2, inset) and this transport process is essential for the establishment of PCP (Merte et al., 2010).

Regulating the ER export process can function to regulate the surface delivery of newly synthesized signal receptors thereby mediating the intracellular signaling events. Newly synthesized Frizzled proteins are modified by glycosylation in the ER. A protein, termed Shisa, is detected in the ER and this protein interacts with the immature glycosylated form of Frizzled within the ER (Yamamoto et al., 2005). This interaction causes ER retention of Frizzled proteins thereby inhibiting the delivery of newly synthesized Frizzled proteins to the cell surface (Yamamoto et al., 2005). During early *Xenopus* embryogenesis, inhibiting the Frizzled-mediated canonical Wnt pathway leads to the inductions of a secondary head (Piccolo et al., 1999). Correspondingly, Shisa inhibits the activity of Frizzled and promotes head formation in *Xenopus* embryos (Yamamoto et al., 2005). Inhibition of Shisa function elicits sensitization to the canonical Wnt signaling and suppresses head formation during gastrulation (Yamamoto et al., 2005). These analyses indicate that Shisa functions as a cell autonomous factor that regulates cell surface level of Frizzled thereby regulating Frizzled-mediated canonical Wnt signaling events. PCP is regulated by the non-canonical Wnt signaling pathway. A similar ER retention mechanism can also function to regulate PCP signaling.

Export of PCP proteins out of the *trans* Golgi network

The *trans*-Golgi network (TGN) is an essential transport hub in the secretory transport pathway, where various cargo sorting machineries are assembled to package specific cargo proteins into transport vesicles (Guo et al., 2014). These transport vesicles shuttle cargo proteins to specific downstream compartments. Defects in TGN sorting cause protein mistargeting and induce defects in various physiological processes (Guo et al., 2014). The key players that mediate TGN sorting include the Arf family of small GTPases and cargo adaptors. The Arf family proteins include Arf proteins, Arf-like proteins and Arf-related proteins (Gillingham and

Munro, 2007; Donaldson and Jackson, 2011). Sar1 also belongs to the Arf family proteins but it is remotely related. The Arf family proteins, once binding to GTP, undergo conformational changes, exposing their N-terminal lipid binding motifs to bind TGN membranes and GTP binding also causes conformation changes of their switch domains to recruit various cytosolic cargo adaptors (Gillingham and Munro, 2007; Donaldson and Jackson, 2011). Following recruitment at the TGN, cargo adaptors directly or indirectly interact with specific sorting motifs on cargo molecules to enrich them into nascent vesicles (Guo et al., 2014). Some cargo adaptors recruit clathrin and polymerization of clathrin causes deformation of the TGN membranes. This process with the help of other factors eventually causes vesicle budding from the TGN.

In mammalian cells, there are at least eight known Arf family proteins that are localized at the Golgi. Through an siRNA knockdown screen, one of the TGN-localized Arf family proteins, Arfrp1, is demonstrated to be required for TGN export of Vangl2 (Guo et al., 2013). GTP-bound Arfrp1 specifically interacts with the clathrin-associated adaptor complex-1 (AP-1), a major TGN-localized cargo adaptor. Knockdown of subunits of AP-1 causes defects in TGN export of Vangl2. Mechanistic analysis indicates that the C-terminal cytosolic domain of Vangl2 contains a conserved tyrosine sorting motif that directly interacts with AP-1. Arfrp1 recruits AP-1 to synthetic liposome membranes in a GTP-dependent manner. These analyses suggest a model that Arfrp1 mediates recruitment of AP-1 to the TGN membranes where AP-1 recognizes the tyrosine sorting motifs on Vangl2 to package Vang2 into transport vesicles (Fig. 3 inset A) (Guo et al., 2013).

AP-1 has a closed conformation in which its cargo binding site is blocked and an open conformation in which its cargo binding site is exposed (Heldwein et al., 2004; Lee et al., 2008; Ren et al., 2013). A Golgi-localized Arf family protein, Arf1, interacts with AP-1 and induces AP-1 to switch from the closed confirmation to the open confirmation to allow AP-1 to directly interact with cargo proteins (Ren et al., 2013). The interaction between cargo proteins with AP-1 can stabilize membrane association of AP-1. Thus, cargo sorting motifs on synthetic membranes are shown to stimulate Arf1-mediated AP-1 recruitment (Lee et al., 2008; Ren et al., 2013). Interestingly, Vangl2 cytosolic domain can stimulate Arfrp1-mediated AP-1 recruitment to synthetic membranes, whereas Arf1-mediated AP-1 recruitment cannot be stimulated by Vangl2 cytosolic domain (Guo et al., 2013). These analyses suggest that: Arfrp1 can also function to stimulate conformational changes of AP-1 from a closed conformation to an open conformation (Fig. 3 inset B); the tyrosine sorting motif on Vangl2 binds a novel cargo binding site on AP-1; AP-1 has a least two open conformations, one exposed the cargo binding site that interacts with the canonical AP-1 binding motif and the other exposed the cargo binding site that interacts with the tyrosine sorting motif on Vangl2 (Guo et al., 2013).

Interestingly, TGN-export of another PCP protein, Frizzled6, is independent of the Arfrp1/AP-1 machinery (Guo et al., 2013; Ma et al., 2018), suggesting that sorting of Frizzled6 is mediated by another cargo sorting mechanism. Differential sorting will cause packaging of cargo proteins into distinct transport vesicles. Consistent with this, Vangl2 and Frizzled6 are shown to be enriched in separate vesicles through an in vitro assay that reconstitutes vesicular release from the TGN (Ma et al., 2018). Further analysis indicates that TGN export of Frizzled6 depends on another clathrin adaptor, epsinR (Ma et al., 2018). EpsinR forms a stable complex with clathrin and this complex interacts with the polybasic sorting motif on the C-terminal cytosolic domain of Frizzled6 to mediate packaging of Frizzled6 into transport vesicles (Fig. 3 inset B). EpsinR directly interacts with AP-1. Interestingly, binding of Frizzled6 to epsinR causes dissociation of epsinR from AP-1 which presumably allows epsinR and AP-1 to perform distinct cargo sorting functions (Ma et al., 2018).

Disrupting the function of AP-1 induces defects in PCP signaling processes in the Drosophila wing and disrupts the polarized pattern of PCP proteins (Carvajal-Gonzalez et al., 2015). Arf1 regulates PCP-mediated processes in Drosophila and Zebrafish (Carvajal-Gonzalez et al., 2015). Expressing the GTP-restricted form of Arf1 reduces the enrichment of core PCP proteins at the plasma membrane and knockdown of Arf1 blocks the newly synthesized Frizzled to be delivered to the plasma membrane (Carvajal-Gonzalez et al., 2015). These analyses indicate that AP-1 and Arf1 are important mediators of trafficking of PCP proteins and regulate PCP in vivo (Carvajal-Gonzalez et al., 2015). Membrane recruitment of epsinR is blocked by Brefeldin A, an Arf GEF inhibitor, suggesting that an unknown Arf family protein mediates membrane associations of epsinR (Hirst et al., 2003) thereby regulating sorting of Frizzled6 at the TGN (Fig. 3 inset B). A possible role for Arf1 in regulating trafficking of Frizzled is that Arf1 can function to recruit epsinR to the TGN membranes to allow epsinR to mediate packaging of Frizzled into transport vesicles. The Drosophila homolog of epsinR, *lqfR*, is shown to regulate wing hair patterning indicating epsinR regulates planar cell polarity (Lee et al., 2009). However, the majority of the defects in *lqfR* mutants can be recused by exon 6 of the *lqfRa* gene, encoding a domain that is homologous to Tel2, a protein that regulates DNA repair and telomere maintenance (Lee and Fischer, 2012). The role of epsinR in PCP remains to be further investigated in vivo.

All together, these analyses indicate that Vangl2 which represents PCP proteins that are targeted to the proximal side of cell boundaries and Frizzled6 which represents PCP proteins that are targeted to the distal side of cell boundaries are sorted differentially at the TGN by different cargo adaptors (Fig. 3). Differential sorting will cause packaging of distal and proximal PCP proteins into distinct transport vesicles. Polarized delivery of these vesicles along the planar

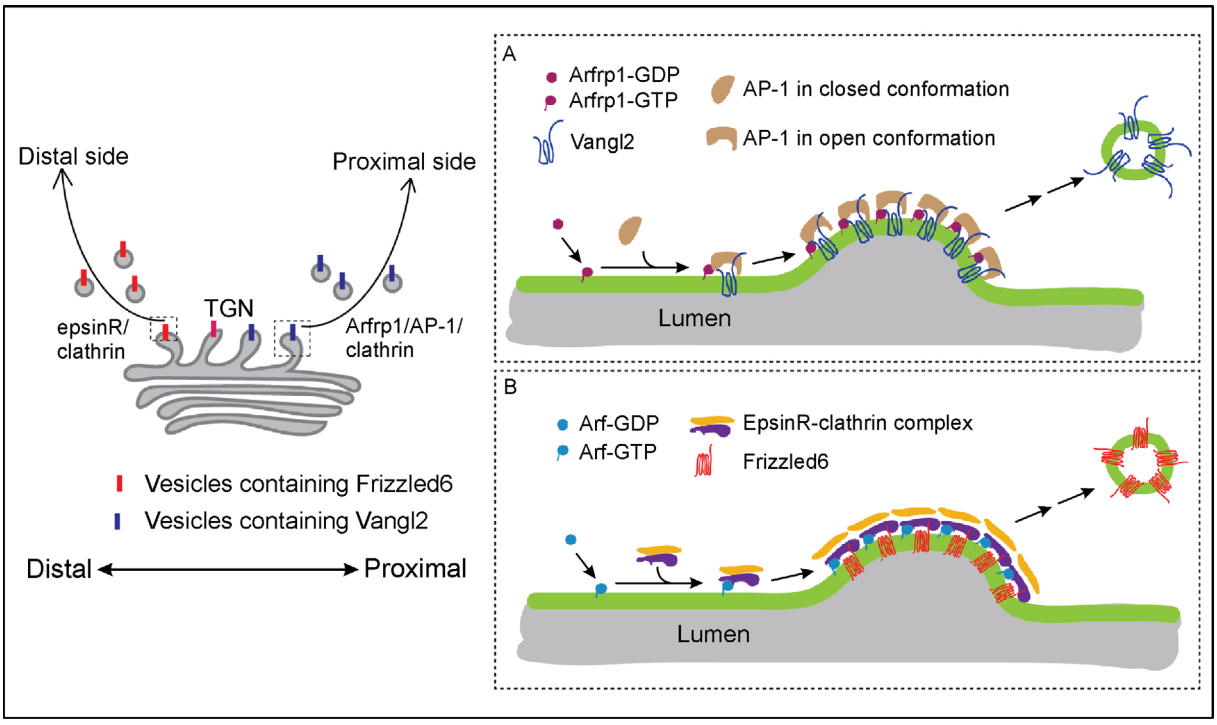

Figure 3 Sorting of PCP proteins at the TGN. Vangl2 which represents PCP proteins that are targeted to the proximal side of cell boundaries and Frizzled6 which represents PCP proteins that are targeted to the distal side of cell boundaries are sorted differentially at the TGN by different cargo adaptors. Differential sorting will cause packaging of distal and proximal PCP proteins into distinct transport vesicles. Polarized delivery of these vesicles along the planar polarized microtubule cytoskeleton may contribute to establishing the PCP asymmetry. ***(inset A)*** A model demonstrating sorting of Vangl2 at the TGN. GTP-bind Arfrp1 recruits AP-1 to the TGN membrane and promotes a conformational change of AP-1 to allow AP-1 to binds the tyrosine sorting motif on Vangl2 thereby enriching Vangl2 into nascent transport vesicles. ***(inset B)*** A model demonstrating sorting of Frizzled6 at the TGN. An unknown Arf family protein, once binding to GTP, recruits the epsinR/clathrin complex to the TGN. At the TGN, the epsinR/clathrin complex binds the polybasic sorting motif on Frizzled6 cytosolic domain to mediate packaging of Frizzled6 into transport vesicles.

polarized microtubule cytoskeleton may contribute to establishing the PCP asymmetry.

Trafficking of PCP proteins in the endocytic transport pathway

Endocytosis plays important roles in mediating turnover of mislocalized components as well as in controlling the level of plasma membrane-localized signaling receptors for extracellular ligands. Following endocytosis, the internalized signaling receptors are normally delivered to lysosomes for degradation or recycled back to the plasma membrane. Recent evidence suggests that endocytic trafficking regulates the localization of PCP components and the PCP signaling process.

Many plasma membrane-localized signaling receptors are internalized by clathrin-mediated endocytosis. A core PCP component, Dishevelled2 (Dvl2) interacts with μ2-adaptin, a subunit of the clathrin-associated adaptor complex-2 (AP-2) (Yu et al., 2007). Mutating the μ2-adaptin binding site on Dvl2 impairs Wnt5a-induced endocytosis of Frizzled4 in mammalian cells (Yu et al., 2007). Mutating the μ2-adaptin binding site of Xdsh, the *Xenopus* ortholog of Dvl2, compromises the activity of Xdsh in PCP-regulated cell movements during gastrulation (Yu et al., 2007). Thus, Dvl2 mediates endocytosis of Frizzled4 through linking Frizzled4 to the clathrin/AP-2 endocytic machinery and this process is required for PCP signaling. In addition, Dvl2 also interacts with β-arrestin 2, an essential adaptor for clathrin-mediated endocytosis (Chen et al., 2001; Chen et al., 2003). This interaction links β-arrestin 2 to Frizzled4 on the plasma membrane to promote Wnt5a-stimulated endocytosis of Frizzled4 (Chen et al., 2003). In *Xenopus*, Frizzled7 and Dishevelled undergo endocytosis during convergent extension movements and this internalization process is regulated by the atypical receptor related tyrosine kinase (Ryk) (Kim et al., 2008).

Studies in Drosophila pupal wing indicate that the stability of Flamingo/Celsr at cellular junctions requires the proximal core PCP components, Stbm/Vangl and Prickle and the distal PCP component, Frizzled, at the adherens junctions (Strutt and Strutt, 2008). Loss of Frizzled or Stbm increases the level of Flamingo in endocytic compartments (Strutt and Strutt,

2008). Endocytosis of the unstable Flamingo is mediated by Dishevelled-dependent and Dishevelled-independent mechanisms (Strutt and Strutt, 2008) and this process is at least partially dynamin- and Rab5- dependent (Strutt et al., 2011). Rabenosyn-5 (Rbsn-5), an effector of Rab5, is localized at the apical cell cortex preferentially aligned along the proximal-distal boundaries upon the onset of PCP signaling (Mottola et al., 2010). This localization pattern of Rbsn-5 is dependent on Flamingo (Mottola et al., 2010). Loss of Rbsn-5 leads to intracellular accumulations of Flamingo and mistargeting of Flamingo to a late Hrs-positive compartment (Mottola et al., 2010). These analyses indicate that endocytosis regulates removal of Flamingo that is not stably associated with other PCP proteins from the cell junctions which may contribute to establishing the PCP asymmetry.

Many core PCP components are modified by ubiquitination. This modification process has profound effects on regulating the level as well as endocytic trafficking of PCP proteins thereby mediating the propagation of PCP. Double knockout of Smurf E3 ubiquitin ligases, Smurf1 and Smurf2, in mice causes severe defects in PCP including convergent extension and neural tube closure (Narimatsu et al., 2009). Smurf2 specifically binds phosphorylated Dvl2 and this interaction is proposed to recruit Smurfs to the polarity protein, Par6. The Par6-Dvl-smurf complex promotes the localized ubiquitination and degradation of Prickle. Double knockout of Smurf1 and Smurf2 causes defects in the asymmetric distribution of Prickle in the Cochlea and neuroectoderm cells during neurulation in mice embryos (Narimatsu et al., 2009). This localized ubiquitination and degradation process can be an effective way to establish the asymmetric distribution of Prickle that is opposite to Dishevelled during planar cell polarity signaling (Narimatsu et al., 2009).

In Drosophila, knockdown of Smurf does not cause defects in PCP (Cho et al., 2015). Another E3 ubiquitin ligase, the Cul1 E3 ligase complex, is demonstrated to function as a regulator of trichome polarity patterning in the Drosophila wing (Cho et al., 2015). Further analysis indicates that disrupting the functions of the Cul1 complex elevates the levels of core PCP proteins at the cell junctions (Cho et al., 2015). Additional evidence suggests that the Cul1 complex can function to ubiquitinate Prickle to target Prickle for degradation (Cho et al., 2015). Overexpression of Prickle induces co-clustering of core proteins, Flamingo and Stbm, at cell junctions (Tree et al., 2002; Bastock et al., 2003; Cho et al., 2015) and also stimulates endocytosis of Prickle, Flamingo and Stbm (Cho et al., 2015). Forcing accumulations of the distal PCP protein, Frizzled, at the cell boundaries induces the Prickle-dependent removal of the proximal protein, Stbm, within the same cell (Cho et al., 2015). Interestingly, Stbm is shown to recruit Prickle to proximal cell boundaries and Stbm also promotes proteasomal degradation of excess Prickle (Strutt et al., 2013b). These analyses suggest that there is a feedback loop between Prickle and Stbm which contributes to the asymmetric localizations of core PCP proteins.

In addition to Prickle, other PCP components are also shown to be regulated by ubiquitination. Levels of Dishevelled at cell junctions are regulated by a Cullin-3-Diablo/Kelch ubiquitin ligase complex (Strutt et al., 2013a). Furthermore, the deubiquitinating enzyme Fat facets is proposed to prevent Flamingo from lysosomal degradation (Strutt et al., 2013a). These enzymes, presumably through regulating the ubiquitination and deubiquitination processes, regulate levels of core PCP components at the cell junctions, and their dysfunctions reduce core protein asymmetry and cause disruptions in PCP (Strutt et al., 2013a).

In addition to the non-canonical Wnt signaling, the canonical Wnt signaling can also be regulated by ubiquitination. In Drosophila, the deubiquitinating enzyme dUBPY facilitates canonical Wnt signaling and overexpression of dUBPY enhances cell surface level of Frizzled (Mukai et al., 2010). Mechanistic analysis suggests a model that dUBPY deubiquitinates Frizzled thereby suppressing lysosomal trafficking and enhancing recycling of Frizzled to the plasma membrane. The deubiquitinating enzyme CYLD and the ubiquitin ligase KLHL12-Cullin-3 regulate ubiquitination and degradation of Dishevelled homologs thereby interfering with the canonical Wnt signaling pathway (Angers et al., 2006; Tauriello et al., 2010). It remains to be further investigated whether these processes also regulate PCP.

PCP mediates hair follicle polarization and orientation in mammalian skin. The core PCP components are asymmetrically localized in the epidermal basal layer of embryonic skin coincides with hair follicle initiation (Devenport and Fuchs, 2008). Basal epidermal progenitor cells frequently undergo proliferation. Interestingly, the core PCP components are shown to be selectively internalized through clathrin-mediated endocytosis in basal epidermal cells during mitosis (Devenport et al., 2011). In contrast, other integral proteins associated with cell junctions including E-Cadherin and β4 integrin are localized at the plasma membranes throughout the cell cycle (Devenport et al., 2011). The internalized PCP components are distributed equally into two daughter cells and then recycled back to the plasma membrane following cell division (Devenport et al., 2011). Internalization of the PCP protein, Celsr1, depends on its juxtamembrane dileucine motif and mutating this motif causes defects in epidermal planar polarity *in vivo* (Devenport et al., 2011). These analyses suggest that the mitotic internalization is critical for the PCP signaling.

Mitotic control of the internalization of PCP proteins provides a mechanism to prevent the unequal inheritance of the asymmetrically localized PCP components into the daughter cells thereby preserving tissue polarity during proliferation (Devenport and Fuchs, 2008; Devenport et al., 2011). Internalization can also function to block the mitotic cells from the PCP signaling process (Devenport et al., 2011). It is also worth mentioning that the mitotic control of

internalization does not take place in the dividing Drosophila wing blade and in Drosophila sensory-organ precursors (Gho and Schweisguth, 1998; Lu et al., 1999;Aigouy et al., 2010; Bellaiche et al., 2004).

Mitotic internalization of PCP components needs to be coordinated with cell-cycle progression. Evidence suggests that the mitotic kinase Polo-like Kinase 1 (Plk1) interacts with Celsr1 and phosphorylates several S/T phosphorylation sites adjacent to the Celsr1 endocytic motif (Shrestha et al., 2015). Plk1-mediated phosphorylation of Celrs1 is critical for Celsr1 mitotic endocytosis presumably through regulating recruitment of Celsr1 into clathrin-coated vesicles (Shrestha et al., 2015). Inhibition of Plk1 blocks mitotic internalization of Celsr1 and induces defects of the asymmetric localization of Celsr1 in mouse embryonic skin explants grown ex vivo (Shrestha et al., 2015). These analyses indicate that Plk1 is an important factor that coordinates mitotic internalization of Celsr1 and mitotic entry.

Celsr1 on the opposing cellular boundaries interacts with each other across cell junctions and links other core PCP proteins to the plasma membranes of the adjacent cells. Interestingly, it is demonstrated that the Celsr1 homodimers remain intact during mitotic internalization, causing an uptake of the PCP proteins, Celsr1, Frizzled6 and Vangl2, from the adjacent interphase cells (Heck and Devenport, 2017). Trans-internalized Celsr1 appears to be destined for degradation (Heck and Devenport, 2017). Frizzled6 is shown to be *cis*- and *trans*-internalized in mitotic cells (Heck and Devenport, 2017). In contrast, Vangl2 is internalized mostly in *trans* during mitosis (Heck and Devenport, 2017). Vangl2 in the dividing cell remains plasma membrane-associated, which can function to instruct the establishment of PCP in daughter cells (Heck and Devenport, 2017). Within the dividing cell, the plasma-membrane localized Vangl2 stabilizes Celsr1 and impedes its internalization, suggesting that endocytosis of Celsr1 requires dissociation of Vangl2 from Celsr1 (Heck and Devenport, 2017). Thus the PCP proteins can undergo intercellular exchange between cells through this trans-internalization process.

Concluding remarks

In summary, the secretory transport pathway and the endocytic transport pathway have been shown to be important for delivery of PCP proteins to their specific destinations, for removing unstable or mistargeted PCP proteins from the plasma membranes, and for preserving tissue polarity during proliferation. The fidelity of the intracellular trafficking process relies on accurate sorting of PCP proteins into transport vesicles mediated by interactions between cargo sorting machineries and specific sorting motifs on PCP proteins. These analyses provide novel mechanistic insights into how the asymmetric localization of PCP proteins are established and maintained. We hypothesize that the secretory transport pathway plays important roles in the delivery of newly synthesized membrane-signaling proteins such as Vangl2 and Frizzled6 to their specific destinations. During this process, the non-centrosomal microtubules are aligned along the proximal-distal axis with more plus ends on the distal side of cell boundaries (Fig. 4). We propose that vesicles enriched with the distally or proximally localized PCP proteins are preferentially delivered to the plus or minus end of the microtubules respectively (Fig. 4). This process is an important initial step to establish the PCP asymmetry. The asymmetric localizations of PCP proteins are then reinforced by association with PCP proteins at the opposing cellular junctions. The mis-targeted PCP proteins can be removed

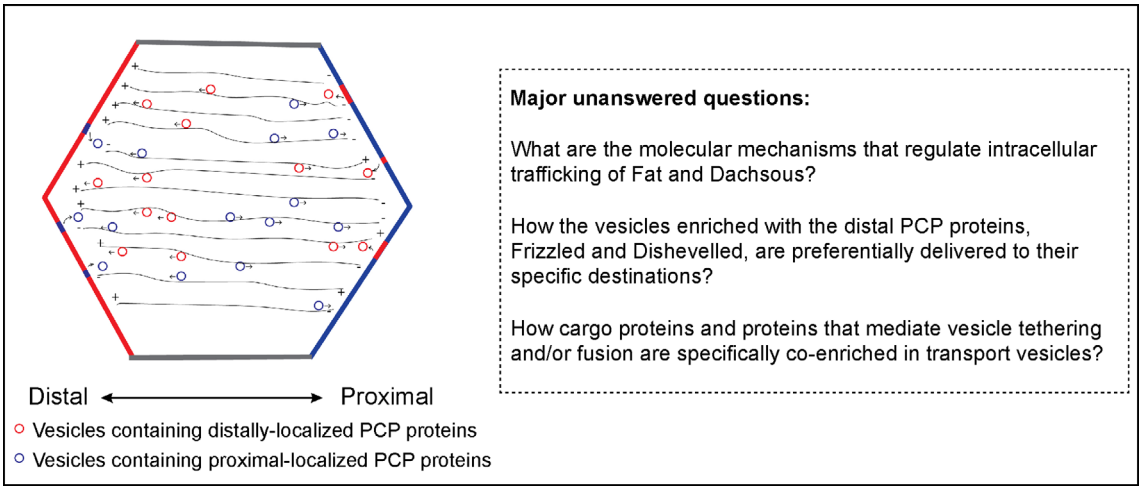

Figure 4 The proposed model showing how intracellular trafficking contributes to the asymmetric localizations of PCP proteins. The non-centrosomal microtubules are aligned along the proximal-distal axis with more plus ends on the distal side of cell boundaries. Vesicles enriched with the distally or proximally localized PCP proteins are preferentially delivered to the plus or minus end of the microtubules respectively. The mis-targeted PCP proteins can be removed from the plasma membrane through the endocytic transport process. (*inset*) Major unanswered questions regarding intracellular trafficking of PCP proteins.

from the plasma membrane through the endocytic transport process (Fig. 4). During mitosis, PCP proteins are selectively endocytosed and this process ensures the equal inheritance of the asymmetrically localized PCP components into the daughter cells.

Although significant progresses have been achieved, several important aspects of intracellular trafficking of PCP proteins remain to be further investigated (Fig. 4, inset). What are the molecular mechanisms that regulate intracellular trafficking of Fat and Dachsous? How the vesicles enriched with the distal PCP proteins, Frizzled and Dishevelled, are preferentially delivered to their specific destinations? A transport vesicle should not only contain the correct cargo protein but also proteins that mediate transport and/or targeting of vesicles to their specific destinations. How these proteins that medicate different transport processes are specifically co-enriched with PCP proteins in transport vesicles?

Several experimental approaches can be utilized to investigate these aspects. The in vitro assay that reconstitutes the vesicular release of PCP proteins (Ma et al., 2018) will be a powerful approach to reveal novel factors that regulate intracellular trafficking of PCP proteins and to reveal the protein components that are co-enriched with a specific PCP protein in transport vesicles. Moreover, live imaging analysis using light-sheet microscope will provide a better understanding of the directionality of the intracellular trafficking of PCP proteins.

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