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Intracellular trafficking and signaling in development

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Abstract

Tissue patterning during development relies on cell communication by secreted proteins and receptors that engage in complex signaling crosstalk to induce distinct cell behaviors in a context-dependent fashion. Here I summarize recent insights into basic mechanisms that control the distribution and activities of transforming growth factor beta, Wnt, Hedgehog, and Notch proteins, by regulating trafficking decisions during secretion and endocytosis.

Introduction and context

How signaling molecules enable cells of multicellular organisms to communicate and assemble tissues and organs is a central question in biology. Embryo manipulation and molecular genetics established that a surprisingly small number of secreted proteins of only a handful of conserved gene families govern a multitude of cell-fate decisions. Much of the diversity in signaling outputs at the level of target gene regulation is attributed to tissue-specific signal integration in elaborate networks. But while the number of known interactions between signaling pathways increases daily, our conventional charts of this crosstalk give little account of how the molecules involved reach their correct localization. In part, this oversimplification is due to the fact that the role of trafficking and its regulation are not well understood. However, advances in cell biology and improved imaging technologies now allow us to follow protein trafficking at high resolution and in real time. Imaging, combined with sensitive genetic screens and sophisticated manipulation of protein and membrane trafficking, established that localization is one of the key determinants regulating signal outputs.

The present article will review the most recent important findings on how trafficking controls signaling at the level of polarized secretion, protein processing, and endocytosis. As each of these fields are limitless, they are only represented here by illustrative examples to emphasize that they are intertwined, and that they need to be integrated to appreciate how intimately signal regulation is coupled to trafficking.

Major recent advances

Regulated apical-basal sorting directs signal deployment

The most basic decision in trafficking is whether a protein is sorted apically or basolaterally in polarized epithelial cells – for example, to position ion channels and proton gradients. The diffusion barrier of tight junctions, which insulate apical from basolateral membranes, can also limit access of ligands to receptors, as shown for transforming growth factor beta (TGF-ß) [1]. Protein sorting into distinct exocytic carriers occurs in the trans-Golgi network (TGN) or in endosomes [2]. Determinants of apical sorting include glycosylphosphatidylinositol (GPI) anchors, which mediate membrane attachment and oligomerization in lipid rafts [3,4] (reviewed in [5]), as well as crosslinking of carbohydrate side chains by apical sorting receptors such as the mannose-binding lectin VIP36 (vesicular integral membrane protein of 36 kDa), or the raft-independent transporter, galectin-3 [6-8]. By contrast, basolateral sorting involves cytosolic tails of transmembrane proteins that bind endosomal AP-1B (adaptor protein complex 1B) [9] or other cytoplasmic adapter proteins interacting with clathrin. Depletion of clathrin in MDCK

(Madin Darby canine kidney) cells thus disrupts basolateral sorting without diminishing polarized secretion of apical proteins [10].

But how do proteins reach lateral membranes if they lack a cytosolic domain? Studies on the morphogens Hedgehog (Hh) and Wingless (Wg) in *Drosophila* wing imaginal discs suggest that specific carrier proteins mediate transcytosis from the apical to the lateral plasma membrane. In this system, production of Hh in the posterior compartment gives rise to a signaling gradient in anterior cells, whereas Wg secreted at the dorso-ventral boundary moves dorsally and ventrally. Secretion of Wg and Hh is regulated by palmitoylation and, in the case of Hh, a cholesterol adduct [11,12]. Both Wg and Hh are released basolaterally in association with lipoprotein particles [13-15], but how lipoproteins extract their cargo from the plasma membrane is poorly understood. Recent work in mammalian cells confirmed that binding of the Wg-related protein Wnt3a to lipoprotein and basolateral secretion depend on Wnt3a palmitoylation [16]. Wnt proteins are palmitoylated in the endoplasmatic reticulum by Porcupine and exit the TGN upon association with Wntless (Wls), a transporter that is recycled from endosomes to the Golgi by the retromer complex [17-20]. If endocytosis in *Drosophila* imaginal discs is blocked by dominant negative dynamin [21], or by deleting the receptors DFz2 (*Drosophila* Frizzled 2) and Arrow [22-24], extracellular Wg is trapped on the apical plasma membrane, suggesting that Wls targets Wnt proteins apically. So how is Wg forwarded from apical to lateral membranes?

A first hint was that Wg fails to move across mutant clones of cells lacking the glypicans Dally (Dly) and Dally-like protein (Dlp) [22]. Glypicans are GPI-anchored heparan sulfate proteoglycans that are internalized by the lipid raft scaffold protein flotillin/reggie via a clathrin- and caveolin-independent route [25]. Immunostaining and antibody uptake by a green fluorescent protein-Dlp fusion protein showed that Dlp initially localizes to the apical membrane, but that it is efficiently recycled basolaterally within less than 90 minutes after endocytosis [21]. Moreover, in mutant cells lacking dynamin function or Dlp, apical-basal transcytosis of Dlp or the basolateral targeting of Wg, respectively, were blocked, indicating that Wg is targeted laterally by Dlp via a dynamin-dependent endocytic route [21].

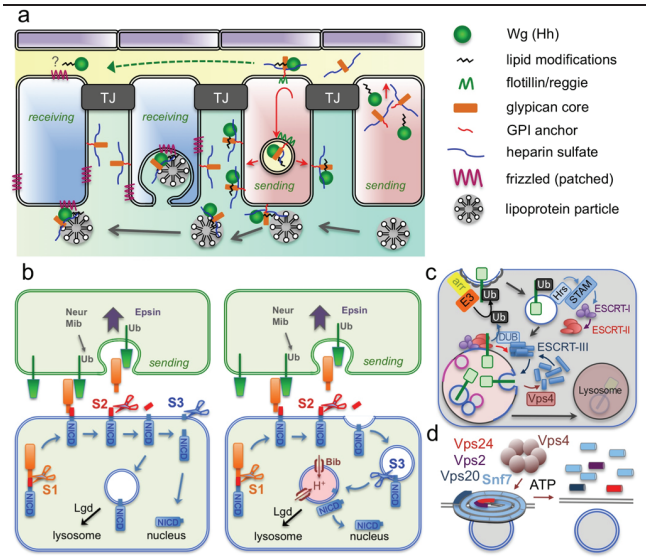
Dly and Dlp also enhance the activity and spreading of Hh [26], bind lipoprotein particles, and colocalize with Hh and its receptor Ptc (Patched) in vesicles of signal receiving cells [27,28]. Colocalization and stimulation of Hh signaling were lost if the GPI anchor of Dlp was substituted by a transmembrane domain [21]. Does this mean that both Wg and Hh rely on glypicans for apical-to-basal transcytosis as a gate to board the basolateral 'lipoprotein shuttle' that controls gradient formation? In support of this model, secretion of Wg and Hh in the wing imaginal disc also depends on reggie-1/flotillin-2 [29]. Furthermore, mutant forms of Hh that cannot be lipid-modified fail to sort basolaterally and instead diffuse apically, giving rise to an abnormally shallow signaling gradient [30]. Taken together, these studies suggest that Wg and Hh are transcytosed from the apical to the lateral plasma membrane and transferred to lipoprotein particles by glypicans (Figure 1a).

Separate entry pathways mediate distinct signal outputs

Another critical aspect of protein trafficking concerns the mechanisms of signal regulation in endocytic compartments. It is well established that endocytosis is important for ligand-induced receptor degradation during signal attenuation and reduction of the extracellular concentration of morphogens in the extracellular space. However, endocytosis is equally important for signaling molecules to access specific endosomal signaling platforms and sorting stations (for a survey of existing endocytic pathways, see [31,32]). The best known uptake routes initiate from clathrin-coated pits or caveolae, which are severed from the plasma membrane by dynamin. TGF-ß receptors enter both compartments, but only coated pits enable access to the Smad anchor for receptor activation (SARA) on early endosomes, and hence activation and nuclear translocation of cytoplasmic Smad transcription factors. By contrast, uptake via caveolae leads to recruitment of the ubiquitin ligase Smurf2 and receptor degradation [33]. Degradation of the related bone morphogenetic protein (BMP) receptors via a caveolar route increases upon dephosphorylation by Dullard, which attenuates the anti-neuralizing function of BMP signaling during *Xenopus* gastrulation [34]. Surprisingly, a caveolar uptake has now been found to also mediate ligand-degradation of the epidermal growth factor receptor (EGFR), whereas clathrin-mediated uptake allows recycling and sustained EGFR signaling [35]. This is unexpected in light of the conclusion from earlier studies that EGFR is internalized mainly through clathrin-coated pits (reviewed in [36]).

Caveolar uptake can also promote signaling, for example, of Wnt proteins. To signal via the canonical pathway, Wnt ligands bind complexes of Frizzled (Fz) receptors with the co-receptor lipoprotein-related protein (LRP)5 or LRP6. In response to Wnt3a, LRP6 binds caveolin and together with Dvl (Dishevelled) forms intracellular aggregates that inhibit the kinase GSK3ß (glycogen

Figure 1. Signal regulation by glypicans, endosomal proteolysis and lysosomal sorting hinges on intracellular trafficking decisions



(a) The glypicans Dally (Dly) and Dally-like protein associate with lipid rafts and mediate basal transport of the morphogens Wingless (Wg) and Hedgehog (Hh) in *Drosophila* wing imaginal disc epithelial cells to access lipoprotein particles in the hemolymph. Cleavage of the glycosylphosphatidylinositol (GPI) anchor facilitates endocytosis of glypican and associated cargo in signal-receiving cells. Some shedding of Dly also occurs apically [27], but whether any Hh protein moves through the apical lumen is controversial (stippled green arrow) [26,30,86]. **(b)** Two models for the localized activation of the signaling receptor Notch. Left panel: after S1 cleavage by intracellular Furin, the Notch extracellular domain (orange) is engaged by membrane-bound ligands, which are ubiquitinated in their cytosolic tails by neuralized (Neur) and Mind bomb (Mib). Endocytosis of Notch ligand by Epsin in signal-sending cells (purple arrow) enables activation of the S2 site by ADAM-10 or -17, followed by γ-secretase cleavage of the intramembraneous S3 site and nuclear translocation of the Notch intracellular domain (NICD) [43]. Right panel: new data suggest that S3 cleavage mainly occurs after endocytosis [47,48]. Nuclear translocation of NICD in addition requires acidification of the endosome by the aquaporin Big Brain (Bib) [49]. Loss of Bib suppresses Notch hyperactivation in *lgd* mutants, suggesting that NICD matures on endosomes, rather than at the plasma membrane. **(c)** Lysosomal targeting of transmembrane proteins. Ubiquitination of transmembrane proteins (green) by E3 ligases during endocytosis is guided by arrestins (arr) and mediates binding to endosomal Hrs (hepatocyte growth factor receptor-regulated tyrosine kinase substrate), a subunit of endosomal sorting complex required for transport (ESCRT)-0. Sequential assembly of ESCRT-I, -II and -III complexes culminates in the recycling of ubiquitin (Ub) by a deubiquitylating enzyme (DUB), followed by invagination of the limiting membrane and associated cargo into the endosome lumen. The endosome membrane is a mosaic of different subdomains, which sort cargo for delivery to lysosomes or other destinations (see text for details). **(d)** A protein lattice consisting of the ESCRT-III components vacuolar protein-sorting (Vps)20, Vps24 and sucrose non-fermenting 7 (Snf7) is sufficient to induce intralumenal budding of the limiting membrane of giant unilamellar vesicles. Recruitment of the ATPase Vps4 by Vps2 disassembles ESCRT-III components for recycling (after [64]). STAM, signal transducing adapter molecule; TJ, tight junction.

synthase kinase 3 beta) in order to stabilize ß-catenin and induce target genes [37,38]. Depletion of caveolin in cultured cells inhibits both Wnt3a-induced endocytosis and signaling of LRP6, indicating that canonical Wnt signaling depends on caveolar uptake [37]. By contrast, in cells depleted of clathrin, canonical Wnt signaling was not impaired. However, clathrin and the adapter molecule ß-arrestin internalize complexes of Fz with Wnt5a and Wnt11, which activate distinct, non-canonical signal transduction pathways to specify planar cell polarity and induce convergence extension movements during gastrulation [39-41]. Interestingly, binding to the secreted Wnt antagonist Dkk1 (dickkopf-1) redistributes LRP6 from caveolae to a clathrin-dependent uptake route [42]. Together, these studies show that receptor endocytosis is essential for Wnt signaling, and that uptake by distinct entry pathways is tightly regulated and mediates the activation of different intracellular signaling cascades.

Coupling of endocytosis and protein processing to regulate signal activation

During endocytosis, signaling molecules are often processed by proteases to remove an inhibitory propeptide, or to shed a trans-membrane domain or release a cytoplasmic tail. Sequential cleavage of Notch by furin, ADAM10 (a disintegrin and metallopeptidase domain 10), and γ-secretase leads to the release of the Notch intracellular domain (NICD), which enters the nucleus to regulate target genes [43]. It is well established that Notch is hyperactivated in *lethal (2) giant discs* (*lgd*) mutant flies, where transport from early to late endosomes is inhibited [44-46]. However, whether γ-secretase cleavage occurs in endosomes has been controversial [43]. A recent study in *Drosophila* wing and eye imaginal discs now has shown that null mutations in dynamin, Rab5 or the endocytic syntaxin Avl (Avalanche) all diminish the amount of NICD, suggesting that cleavage occurs after endocytosis [47]. Consistent with this view, immunostaining of Notch and NICD in dividing *Drosophila* sensory organ precursor cells revealed the presence of cleaved Notch on SARA-positive early endosomes of only one of the asymmetric daughter cells [48]. In addition, nuclear translocation, but not the production of NICD, requires acidification of endosomes by the aquaporin Big Brain [49]. Together, these observations suggest that the production and nuclear translocation of NICD are coupled to endosome maturation, but limited by rapid sequestration of Notch in lysosomes (Figure 1b).

Proteolytic processing also regulates the function of secreted proteins derived from soluble precursors. Propeptides contain sorting signals, mediate interactions

with transporter proteins or sterically hinder precocious binding to receptors. It is important, therefore, that propeptides are removed in the appropriate subcellular compartments. Proprotein processing also balances the activities of precursor and mature forms of their substrates, as shown for Nodal [50] or the neurotrophic growth factor precursor, respectively [51]. Nodal is essential in the implanted blastocyst to inhibit precocious neural differentiation of pluripotent progenitor cells and to pattern the surrounding primitive endoderm, whereas later it induces mesoderm and definitive endoderm formation [52]. Endoderm and mesoderm are specified during gastrulation by distinct signaling thresholds of Smad2 and Smad3 transcription factors, which are phosphorylated by endosomal signaling complexes of activin receptors, processed Nodal and a GPI-anchored co-receptor of the EGF-like Cripto/FRL-1/Cryptic (CFC) family [53]. But how is this complex activated?

Cripto already binds Nodal in the endoplasmatic reticulum, in part via the prosegment of the uncleaved precursor. Cripto also recruits the subtilisin-like proprotein convertases Furin or PACE4 (paired amino acid cleaving enzyme 4), which cleave Nodal [54]. However, Furin and PACE4 are not coexpressed with Cripto *in vivo*, but secreted by cells in the microenvrionment [55,56]. In addition, Cripto and Nodal access the plasma membrane by an unconventional route bypassing proprotein convertases in the TGN [54]. Nodal thus is not processed until arrival at the cell surface. Upon maturation, Nodal is rapidly endocytosed, whereas in the absence of cleavage it is only slowly internalized and secreted via the TGN. The advantage of maturing in a complex with Cripto is that the GPI-anchor of Cripto localizes Nodal processing to membrane microdomains that access endosomal signaling platforms, whereas Cripto-independent Nodal processing favors uptake in degradative compartments.

Endosomes as a sorting station to regulate signal duration

Endosomes also emerge as critical sorting platforms for degradation and recycling. Cell surface receptors are marked for degradation by ubiquitination, a covalent modification that targets the internalized protein to multivesicular bodies (MVBs) for delivery to lysosomes. Ubiquitin-independent MVB targeting has also been described [57]. Studies in yeast suggest that substrate specificity of the E3 ubiquitin ligases is conferred by adaptors of the arrestin family [58] (Figure 1c). Upon arrival in early endosomes, the ubiquitin moieties bind hepatocyte growth factor receptor-regulated tyrosine kinase substrate (Hrs), which associates with STAM (signal transducing adapter molecule) to form the endosomal sorting complex required for transport (ESCRT)-0 and thereby initiate the sequential assembly of the multiprotein complexes ESCRT-I, -II and -III [59,60]. The interactions among ESCRTs culminate in the recruitment of deubiquitylating enzymes and of the AAA+ ATPase vacuolar protein-sorting 4 (Vps4). Deubiquitylation by ubiquitin-specific protease Y (UBPY, Doa4 in yeast) and Ubp2 allows recycling of ubiquitin and advances cargo to intralumenal vesicles of endosomes that mature into MVBs [61-63] (Figure 1c).

How intralumenal vesicles are induced to bud from the limiting membrane into the endosome lumen has long remained elusive. A landmark study using a novel *in vitro* reconstitution assay has now shown that addition of only three yeast ESCRT-III subunits [Vps20, Vps24, and sucrose non-fermenting 7 (Snf7)] to synthetic giant unilamellar vesicles is sufficient to induce budding and scission of intralumenal vesicles from the limiting membrane (Figure 1d). The Vps2 subunit subsequently recruits Vps4 to disassemble and recycle the components of this complex for further rounds of budding [64]. Saksena *et al*. [65] independently demonstrated that the Vps4 ATPase is required to disassemble the protein lattice of Snf7 oligomers *in vitro*, although in their model Vps4-induced disassembly is linked to membrane scission. Accordingly, the primary role of ESCRT-0, -I and -II probably is to localize ESCRT-III to endosomal membrane microdomains loaded with cargo.

During development, TGF-ßs, receptor tyrosine kinases, and Notch proteins specify distinct cell fates at different signaling thresholds, which ultimately reflect the concentration and longevity of signaling complexes in endosomes. Since most if not all of these signaling molecules are degraded via the MVB pathway (reviewed in [36,66,67]), the new mechanistic insights provide a solid foundation to elucidate how the residence time of different receptors or combinations of receptors at the endosome-limiting membrane are regulated. The importance of ESCRT-mediated signal attenuation during development is further highlighted by the early embryonic lethality of mutant mice lacking Hrs or the ESCRT-III component CHMP5 (charged MVB protein 5) [68,69], and by the hyperactivation of Notch and excess proliferation of epithelial cells in *Drosophila* lacking the ESCRT-II subunits Vps25, Vps22 or Vps36 [70-73]. A future challenge will be to determine how the sorting of signaling molecules to intra-endosomal vesicles controls morphogenesis in specific contexts, and how this process is regulated *in vivo*.

Future directions

While aberrations in trafficking linked to cell polarization defects occur in a variety of disease syndromes and

contribute to tumorigenesis (reviewed in [2]), relatively little is known about the mechanisms regulating the polarized secretion of soluble ligands. The new role of glypicans discussed here during the secretion of Wnt and Hh proteins raises the question of whether these or similar transporters mediate basolateral delivery of other soluble proteins.

It is well established that Wnt, TGF-ß, Hh and Notch pathways, and other developmental signals are also regulated during endocytosis. That different entry pathways mediate degradation or recycling, or even activate distinct signaling branches, now adds an additional layer of complexity. However, irrespective of whether uptake involves clathrin or caveolin, EGFR is sorted to lysosomes via early endosomes [35]. So what is the advantage of a triage at the plasma membrane? Why tidy the house if the kids will make a mess again anyway? A possible answer is that endosomes are 'orderly kids' who do not randomly mix cargo, but instead discriminate between cargo from distinct endocytic origins based on differences in ubiquitination, clustering, or the composition of oligomeric receptor complexes at the plasma membrane. Consistent with this view, clathrin-dependent or -independent uptake of Wnts involves different co-receptors [74], and cluster size determines post-endocytic sorting of GPI-anchored proteins [75]. Moreover, the limiting membrane of early endosomes is a mosaic of discrete subcompartments [76] (Figure 1c). Likewise, Nodal molecules tethered to Cripto are not evenly distributed, indicating that early endosomes are unlikely to haphazardly mix cargo [77]. It is plausible, although not proven, that proteins accumulate in distinct subdomains of the endosome membrane for signaling or for deployment to other destinations. Based on their lipid content, endosomes also generate at least two different types of intralumenal vesicles for lysosomal targeting or recycling [78]. It will be exciting to learn how endosomes keep order among their cargo to correctly localize and integrate different signaling inputs.

Another question is how exocytic and endocytic sorting machineries respond to signaling. In the developing respiratory system of *Drosophila*, Wg modulates cell adhesion and intercalation by regulating the recycling of E-cadherin. This is achieved through upregulation of the transcription factor Spalt (Sal), which induces dRip11 (*Drosophila* Rab11 interacting protein) to stimulate the small GTPase Rab11 on recycling endosomes [79]. In vertebrates, fibroblast growth factor signaling induces the transcription factor Foxj1, a regulator of intraflagellar transport proteins, which drive the elongation of cilia [80-82]. Known functions of cilia in cell polarity and signaling, and their assembly by membrane trafficking, have been comprehensively reviewed elsewhere [83]. How these sensory organelles control, for example, the balance between canonical and non-canonical Wnt signaling [84], or the shape of Hh signaling gradients [85] is still not clear. A better understanding of the relationship between signaling and trafficking will surely help to resolve these questions.

Abbreviations

ADAM10, a disintegrin and metallopeptidase domain 10; AP-1B, adaptor protein complex 1B; Avl, Avalanche; BMP, bone morphogenetic protein; CHMP5, charged MVB protein 5; DFz2, *Drosophila* Frizzled 2; Dlp, Dally-like protein; Dly, Dally; Dkk1, dickkopf-1; dRip11, *Drosophila* Rab11 interacting protein; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complex required for transport; FRL-1, fibroblast growth factor receptor ligand 1; Fz, frizzled; GPI, glycosylphosphatidylinositol; GSK3ß, glycogen synthase kinase 3 beta; Hh, Hedgehog; Hrs, hepatocyte growth factor receptor-regulated tyrosine kinase substrate; lgd, lethal (2) giant discs; LRP, lipoprotein-related protein; MDCK, Madin Darby canine kidney; MVB, multivesicular body; NICD, Notch intracellular domain; PACE4, paired amino acid cleaving enzyme 4; Ptc, Patched; SARA, Smad anchor for receptor activation; Snf7, sucrose non-fermenting 7; STAM, signal transducing adapter molecule; TGF-β, transforming growth factor beta; TGN, trans-Golgi network; UBP, ubiquitin-specific protease; VIP36, vesicular integral membrane protein of 36 kDa; Vps, vacuolar protein-sorting; Wg, Wingless; Wls, Wntless.

Competing interests

The author declares that he has no competing interests.

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