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REVIEW

The role of endocytosis in activating and regulating signal transduction

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Abstract Endocytosis is increasingly understood to play crucial roles in most signaling pathways, from determining which signaling components are activated, to how the signal is subsequently transduced and/or terminated. Whether a receptor-ligand complex is internalized via a clathrin-dependent or clathrin-independent endocytic route, and the complexes' subsequent trafficking through specific endocytic compartments, to then be recycled or degraded, has profound effects on signaling output. This review discusses the roles of endocytosis in three markedly different signaling pathways: the Wnt, Notch, and Eph/Ephrin pathways. These offer fundamentally different signaling systems: (1) diffusible ligands inducing signaling in one cell, (2) membrane-tethered ligands inducing signaling in a contacting receptor cell, and (3) bi-directional receptor-ligand signaling in two contacting cells. In each of these systems, endocytosis controls signaling in fascinating ways, and comparison of their similarities and dissimilarities will help to expand our understanding of endocytic control of signal transduction across multiple signaling pathways.

Keywords Endocytosis · Clathrin · Dynamin · Caveolin · Primary cilium · Signaling · Wnt · Notch · Eph · Ephrin · EGF

Abbreviations

ADAM	A disintegrin and metallo-protease
AP1/2	Adaptor protein one or two
Arf	ADP-ribosylation factor
Arp2/3	Actin-related protein 2/3
Cav1/2	Caveolin one or two
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CDR	Circular dorsal ruffles (also known as waves)
CE	Convergent extension
CLASP	Clathrin-associated sorting proteins
CLIC-GEEC	Clathrin-independent carrier/GPI-anchored protein-enriched early endosomal compartment
CME	Clathrin-mediated endocytosis
CSL	CBF1/Suppressor of Hairless/LAG-1
Dll1/3/4	Delta-like one, three or four (Notch ligands)
Dsh/Dvl	Disheveled
EEA1	Early endosomal antigen one
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Fc	Fragment crystallizable region (tail region of antibody)
Flot1/2	Flotillin one or two
GPI	Glycosylphosphatidylinositol
GTPase	Guanosine triphosphate hydrolase enzyme
Hek cells	Human embryonic kidney cells
HeLa cells	Cervical cancer cells from Henrietta Lacks
HGF	Hepatocyte growth factor
Jag1/2	Jagged one or two (Notch ligands)
LacZ	Beta-D-galactosidase
LDL	Low-denstity lipoprotein
Lef1	Lymphoid enhancer-binding factor 1
Lqf	Liquid facets (*Drosophila* epsin homolog)
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain

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N-WASP	Neural Wiskott-Aldrich syndrome protein (aka WASL, Wiskott-Aldrich syndrome-like)
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PM	Plasma membrane
Ptc	Patched (Shh receptor)
Rab11	Rab-protein 11
Rac1	RAS-related C3 botulinum substrate 1
Rin1	Ras and Rab interactor one
Ror1/2	Receptor tyrosine kinase-like orphan receptor one or two
Ryk	Receptor-like tyrosine kinase
Shh	Sonic hedgehog
TCF	T-cell factor
TGF-β	Transforming growth factor beta
Vav2	Vav 2 guanine nucleotide exchange factor

Introduction

In order for multi-cellular organisms to thrive, communication between cells is necessary to co-ordinate such disparate processes as proliferation, patterning, migration, cell-cycle exit, and differentiation. As such, communication between cells is precisely regulated by a number of different signaling mechanisms. Endocytosis, a process by which eukaryotic cells internalize plasma membrane (PM), along with cell-surface receptors and diverse soluble molecules, is used by the cell for a number of different purposes. Within the realm of cellular signaling, it plays critical roles in initiating and spreading signals, determining which specific sub-pathway to activate, and in terminating signaling. In some systems, the very outcome of cell signaling depends on which endocytic routes are used, and how the various components of the signaling machinery are trafficked.

The cell has a multitude of mechanisms it can employ to endocytose signaling components, including clathrin-dependent and clathrin-independent endocytic mechanisms such as caveolin-mediated endocytosis, arf6-dependent endocytosis, the clathrin-independent carrier/glycosylphosphatidylinositol (GPI)-anchored protein-enriched early endosomal compartment (CLIC-GEEC) endocytic pathway, and flotillin-dependent endocytosis (for review, see [1]). Clathrin-independent endocytic pathways used for internalization of large-sized particles, such as phagocytosis and macropinocytosis, are outside the scope of this review and have been reviewed elsewhere [1].

Originally, endocytosis was thought to merely down-regulate signaling. Some of the earliest indications that endocytosis plays a role outside of signal down-regulation came from studies on epidermal growth factor (EGF) signaling, in which dynamin-mediated endocytosis was shown to be necessary for the EGF-induced phosphorylation of the EGF receptor (EGFR) [2]. Later studies also revealed that while lower doses of EGF are internalized through clathrin-mediated endocytosis, higher doses of EGF are associated with ubiquitination of EGFR, endocytosis by lipid-raft- and, possibly, caveolin-mediated endocytosis and degradation [3]. Therefore, endocytosis appears to be crucial in the regulation of EGF signaling. However, caveolin knock-out mice are viable and fertile [4–8], and caveolin-deficient brown adipocytes do not show perturbations in EGF-induced ERK/MAPK signaling at high or low doses of EGF [9], so the requirement for caveolin in EGF signaling is not perfectly clear. Similar to EGFR, the transforming growth factor beta (TGF-β) receptor is endocytosed both by clathrin-mediated endocytosis into EEA1-positive early endosomes, and by clathrin-independent endocytosis into caveosomes, endosomes enriched in caveolin [10]. Signaling induced by TGF-β also depends on the endocytic route utilized, wherein uptake by clathrin-mediated endocytosis promotes TGF-β signaling, but incorporation into caveosomes leads to receptor turnover and signal downregulation [10]. These examples demonstrate that ligands can induce different endocytic pathways, depending for example on concentration, and thus activate different signaling pathways within the cell.

It is becoming increasingly evident that specific endocytic pathways are essential for the appropriate cellular responses to different signaling cues. This review aims to examine the diverse ways in which the cell utilizes endocytosis to achieve signaling. Specifically, the role of endocytosis in assembling "signalosomes" in a single "signal-receiving" cell (*Wnt signaling*), the role of endocytosis in cell pairs to activate signaling in one signal-receiving cell (*Notch signaling*), and the role of endocytosis in cell pairs during bi-directional signaling (*Eph/ephrin signaling*) will be addressed.

Types of endocytic pathways

There is a growing roster of recognized endocytic pathways (Fig. 1). Moreover, there are a number of ways in which to classify these endocytic pathways, including distinctions based on the proteins required for a certain pathway (for example dynamin, clathrin, caveolin, or actin), the type of cargo being internalized (large versus small, specific receptors or extracellular fluid sampling), the morphological appearance of the endocytic process (vesicular or tubular appearance, or a ruffled appearance

as seen in circular dorsal ruffles), or the sub-cellular compartment/organelle it is associated with (such as the primary cilium). As such, there is a degree of overlap between different definitions and it is important to accurately define each endocytic mechanism as completely as possible. Therefore, this review begins with a description of some of the different endocytic pathways available to the cell, with a focus on the two most thoroughly characterized pathways, the clathrin- and caveolin-mediated endocytic pathways.

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is considered the best characterized endocytic pathway. Clathrin, which was

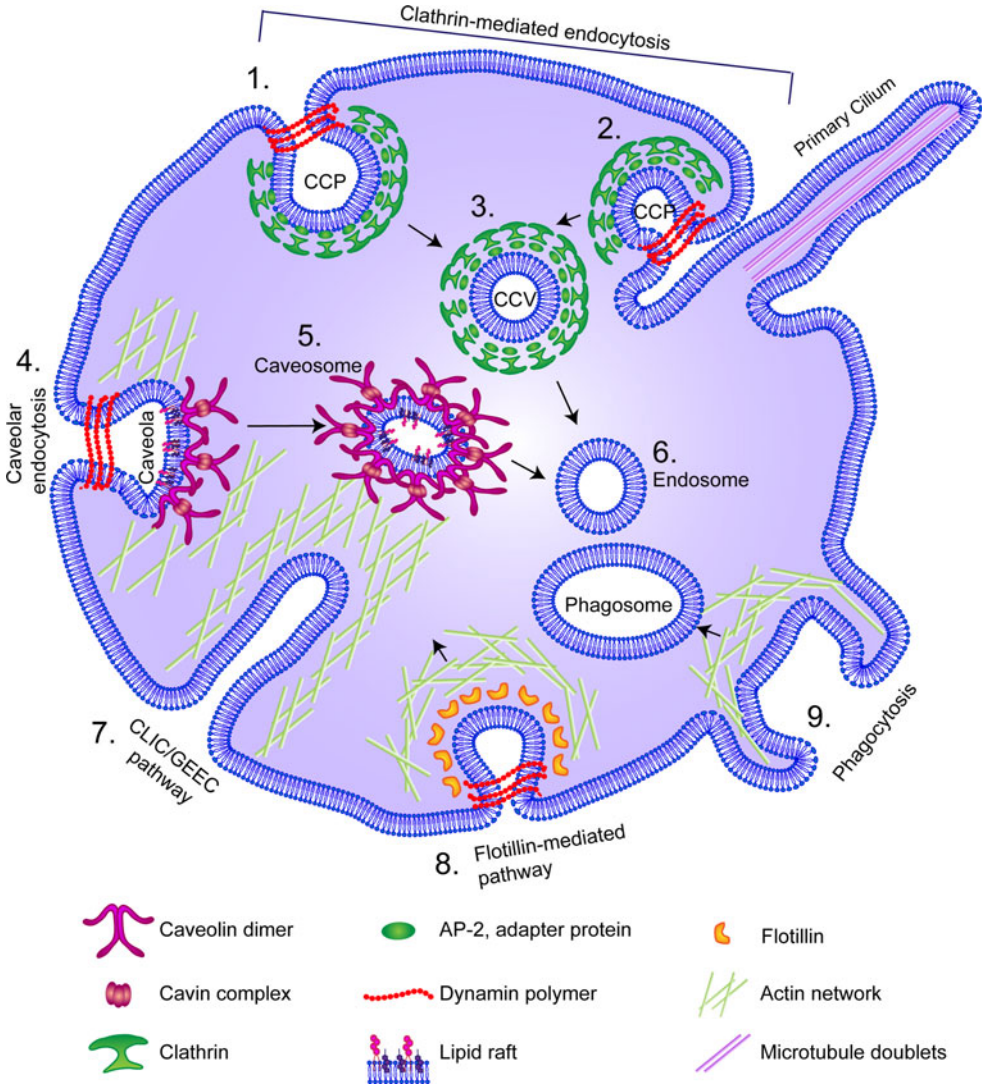

Fig. 1 Endocytic mechanisms employed by the cell. Mammalian cells can internalize plasma membrane and receptors through a number of different routes, which nevertheless may utilize some of the same cellular machinery. Clathrin-mediated (*1*, *2*) and caveolin-mediated (*4*) routes both require dynamin. Clathrin-mediated endocytosis begins with the coating of clathrin-coated pits (CCP) from the cell membrane (*1*) or from clathrin-coated pits at the base of the primary cilium (*2*), which are pinched off by dynamin to yield clathrin-coated vesicles (CCVs) (*3*). Caveolae (*4*) are flask-shaped invaginations in the plasma membrane formed by caveolins and cavins, which are enriched in lipid rafts, and which require actin. Endosomes enriched in caveolin are termed caveosomes or cavicles (*5*). In some cases, caveosomes and CCVs can converge (*6*). The clathrin-independent carrier (CLIC)/GPI-anchored protein-enriched early endosomal compartment (*GEEK*) pathway is dependent on actin, but is clathrin and caveolin-independent (*7*). The *flotillin-dependant* pathway (*8*) has been suggested as an alternative route to caveolin-mediated pathways, since cells devoid of caveolin still manifest flask-shaped invaginations, in which flotillins can be found. Phagocytosis (*9*), which can endocytose large cargo such as apoptotic cells, also relies on actin and forms large endosomes termed phagosomes, but is primarily used by specialized cells such as neutrophils and macrophages (for further details, please see the text)

first described in 1976 [11], is recruited from the cytoplasm by adapter proteins to form a polygonal lattice coating the plasma membrane (PM) intracellularly, resulting in the formation of clathrin-coated pits (CCPs) [12]. These subsequently pinch off in a dynamin-dependent fashion and form clathrin-coated vesicles (CCVs) of 100–200 nm in diameter. CCVs are uncoated after endocytosis and can then fuse with early endosomes for further sorting. Clathrin is used for endocytosis from several sub-cellular compartments, including the PM and the ciliary pocket. It is important to note that dynamin is used by several endocytic pathways in addition to CME, including caveolin-mediated endocytosis, the flotillin pathway, and circular dorsal ruffles [1].

Although CME is an umbrella-term for all endocytic pathways utilizing clathrin, there are a remarkable number of different adapter proteins coupling clathrin to the cargo being endocytosed [13], which help to provide signaling specificity. Endocytic adapters comprise two groups based on their multimerization capacity: multimeric adapter proteins such as adaptor protein 2 (AP-2) [14], and monomeric or dimeric adapter proteins, such as the clathrin-associated sorting proteins (CLASPs), which include proteins such as epsin [15–17], β-arrestin [18], and Numb [19, 20]. AP2 binds to lipids, cargo, accessory proteins and clathrin, while CLASPs may bind to all or only some of the above. For example, internalization can be elicited by ubiquitination of transmembrane receptors, which is recognized by epsin, a protein that contains an ubiquitin-interacting motif, a phospholipid-binding motif, a clathrin-binding motif, an accessory-protein-binding motif, and an AP-2 binding motif [15, 16]. Thanks to this vast array of clathrin-binding adapters, CME is a very adaptable endocytic pathway, which can target endosomes to different compartments in order to achieve specific signaling aims.

Clathrin-mediated endocytosis, the primary cilium

The primary (non-motile) cilium was first identified in 1898, but its signaling role in signaling has not been appreciated until more recently. This structure consists of a cellular protrusion containing a cytoskeleton of nine microtubule doublets, and is found on most differentiated cells in vertebrates (for review, see [21]). At its base is a ciliary pocket, an invagination in which the cilium is deeply rooted, which is rich in clathrin-coated pits [22]. As such, the ciliary pocket has been proposed to be a specialized endocytic membrane domain. Interestingly, in Trypanosomatids, the flagellar pocket (which shares many characteristics with the vertebrate ciliary pocket) is the obligate site of endocytosis and exocytosis [23]. While the same is not true for vertebrate cells, which can endocytose from the rest of the plasma membrane as well, it is interesting to note that ciliary proteins undergo very slow exchange with the rest of the cell due to the presence of a diffusion barrier [24], which may complicate analysis of endocytosis and trafficking in this region. The importance of primary cilia to signaling is well demonstrated by its requirement for activation of Sonic hedgehog (Shh) signaling [25]. However, while the Shh receptor patched (Ptc) can endocytose Shh, in a dynamin-dependant manner [25], it is not yet clear whether endocytosis from the primary cilium/ciliary pocket itself is required for Shh signaling. Thus, it is important to note that some clathrin-dependent signaling pathways may be specifically initiated at or near the primary cilium.

Raft-dependent endocytosis/caveolin-mediated endocytosis

One of the most widely studied endocytic pathways requiring dynamin, but not clathrin, is the caveolin-mediated endocytic pathway. This pathway is named for the appearance of flask-shaped PM invaginations termed caveolae, from the Latin for "little caves". Found in most cells, they are especially abundant in adipocytes and endothelial cells [1]. Caveolae are enriched in lipid rafts, membrane microdomains containing cholesterol, glycosphingolipids, sphingomyelin, phospholipids, and glycosylphosphatidylinositol (GPI)-linked proteins. The formation of caveolae requires caveolin (Cav), of which there are three in mammals (Cav1-3). Cav1 appears to be required for caveolae formation in most cells, as Cav2 is not required, and Cav3 is found predominantly in muscle cells [26]. Cav1 binds to cholesterol and fatty acids and forms oligomers to induce the caveosome structure. In addition to caveolin, a second family of proteins, named cavins, (four subtypes in mammals) is required for caveolae formation [27, 28]. Cavin1 (or PTRF, polymerase I and transcript release factor), cavin2 (or SDPR, serum deprivation protein response) [29], cavin3 (or SRBC, sdr-related gene product that binds to-c-kinase) [30] and cavin4 (or MURC, muscle restricted coiled–coiled protein) are found in caveolae and contain putative leucine zipper-like domains and PEST (proline, glutamic acid, serine, and threonine-rich) domains, which target proteins towards proteolytic degradation. Intriguingly, cavin1 contains nuclear localization signals (NLSs) while cavin2 and cavin3 have been associated with protein kinase C (PKC) α and δ. Cavins may thus contribute to directing signaling in a specific direction from caveolae depending on cavin composition.

Caveolae themselves are highly reliant on the presence of cholesterol-rich lipid rafts and can be disrupted by depleting cholesterol from the cell membrane. While

caveolin is required for the formation of caveolae, it is less clear what its role is in endocytosis per se. It was previously thought that caveolin was required for endocytosis of caveosomes, but this view is more recently being challenged. Budding off of caveolin positive vesicles, termed caveosomes or cavicles, requires dynamin [31], but the rate of caveolar endocytosis is negatively regulated by cav1 [32, 33] and positively regulated by increased raft-lipid levels. Similarly, increasing the levels of cav1 increases the number of caveolae but not endocytosis of these. Therefore, it has been suggested that caveolins stabilize caveolae at the plasma membrane but inhibit their endocytosis. Also, some caveosomal structures, which appear to be distant from the plasma membrane may, in fact, still be connected to the plasma membrane [34]. In addition, non-clathrin/cavaeolar and clathrin-derived endosomes can converge [35, 36], adding yet another layer of complexity to deciphering endocytic routes.

It is important to note that much of the information we have regarding trafficking and signaling by different pathways is derived from studies utilizing over-expression in mammalian cell culture or from studies performed in *Drosophila*. Unfortunately, many of the central players in clathrin-mediated endocytosis are so crucial to life itself that murine knockouts fail to thrive, and die during embryonic development, as in the case of AP1 [37] and AP2 [38], making analyses of these mammalian counterparts more difficult. In contrast to clathrin-related proteins, cav1, cav2, and cav3 appear to be dispensable for embryonic development [6–8], arguing that caveolin cannot be required for mediating signaling pathways during development.

Other endocytic pathways, circular dorsal ruffles, flotillin-mediated endocytosis, the CLIC-GEEC pathway

Besides clathrin and caveolin-mediated endocytosis, additional internalization pathways are being unveiled, which display quite different characteristics, from different morphologies to different requirements for dynamin or small GTPases (for review see [1]). While these are, as yet, less well characterized, it is important to bear these alternative routes in mind when considering the different endocytic conduits employed by signaling pathways.

Circular dorsal ruffles

Circular dorsal ruffles (CDRs), or waves, are transient structures that form and propagate across the dorsal PM of cells grown in vitro in response to growth factors such as EGF [39], hepatocyte growth factor (HGF) [40], and platelet-derived growth factor (PDGF) [41, 42], which causes integrins to re-localize to CDRs to be endocytosed by micropinocytosis [43]. CDR-associated endocytosis is independent of clathrin and caveolin [44] but requires dynamin and cortactin, an actin-binding protein that is important for actin remodeling [45]. CDRs are thought to be regulated by phosphoinositide metabolism [46] and activation of actin polymerization and curved membrane-bound proteins [43]. PDGF-induced CDRs require N-WASP and Arp2/3 [47], as well as small GTPases, as Vav2 and Rac drive CDR formation in response to PDGF [48].

Flotillin-mediated endocytosis

Another endocytic pathway independent of clathrin and caveolin is instead dependent on flotillin (also known as reggie in zebrafish), a protein that shares homology with caveolin [49] and that requires actin for its raft/membrane association and lateral mobility [50, 51]. Despite this homology, over-expression of flotillin-1 (Flot1) and -2 (Flot2) cannot rescue caveolae production in cav-null cells [52]. These results may be due to the fact that reggies appear to hetero-oligomerize, and the stoichiometry of this hetero-oligomerization regulates endocytosis [53]. In any case, flotillin/reggie appears to share some functions with caveolin, as it is responsible for some of the internalization of cholera-toxin B [49, 54], which was previously described to be endocytosed through caveolin-dependent mechanisms [55, 56]. In addition, flotillins are required for a number of different cellular processes, including PKC-mediated endocytosis of the dopamine transporter (DAT) and excitatory amino acid transporter 2 (EAAT2) [57], axon regeneration in zebrafish (reviewed in [58]), and matrix degradation by macrophage podosomes [59]. Interestingly, it has been shown that flotillin requires cholesterol to cluster amyloid precursor protein (APP) at the cell membrane, which is required for its subsequent endocytosis through clathrin-mediated routes [60]. EGF, which has been shown to traffic through caveolin and clathrin-dependent routes, as mentioned above, also lead to the phosphorylation of reggie and endocytosis of reggie-positive endosomes [61]. Finally, it is important to note that reggie has also been implicated in exocytosis, as it is required for secretion and spreading of Wnt and hedgehog in *Drosophila* [62].

The CLIC/GEEC pathway

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are internalized via a route not requiring caveolin, clathrin or dynamin, but requiring cdc42 [63–65]. This route has

been termed the CLIC/GEEC pathway since it is mediated by clathrin-independent carriers (CLIC), and the endocytosed GPI-APS can be found in GPI-AP enriched early endosomal compartments (GEECs). The CLIC/GEEC pathway depends on actin instead [63] to form long tubular structures that are budded off by myosin motors [66]. It is unclear at present how, or whether, the CLIC/GEEC pathway overlaps with the flotillin pathway, which is also responsible for uptake of GPI-anchored proteins.

Endocytosis in signaling

The functions of endocytosis in signaling are numerous. In its simplest form, endocytosis can act to down-regulate the levels of a receptor at the cell-surface to inhibit further signaling. However, it is becoming increasingly clear that endocytosis can modulate signaling in a number of other manners. Compartmentalization of the plasma membrane, for example in cholesterol-rich lipid microdomains, serves to cluster receptors and signaling components in specific constellations to accomplish particular signaling aims. Endocytosis can direct signaling-active endosomes to different intracellular compartments for degradation, modification of the signal, or recycling of the signaling components. It can act as a sheer mechanical force to move or "pull" on membrane-bound proteins. In this review, three examples of signaling pathways will be discussed in light of the specific properties of each signaling pathway and the role that endocytosis plays in mediating signaling. In the first example, the Wnt pathway, a diffusible ligand activates signaling by binding to a membrane-bound receptor and co-receptors. Endocytosis is required to activate signaling, even though (at first glance) this would appear to be an unnecessary constraint. In the second example, the Notch pathway, a membrane-bound ligand, binds to a membrane-bound receptor on an adjacent cell. Here, endocytosis is required in both the receptor- and the ligand-expressing cell to activate signaling in the receptor-expressing cell. In the last example, the Eph/ephrin pathway, a membrane-bound ligand binds a membrane-bound receptor. Signaling by Eph/ephrins requires endocytosis and can occur both in a forwards and in a reverse manner, into the receptor or the ligand expressing cell, or both. These three examples offer different perspectives of how endocytosis may be utilized during signaling to accomplish specific objectives.

Ligand-induced endocytosis in order to signal: Wnt signaling

Wnts comprise a family of 19 secreted ligands that can activate multiple intracellular signaling pathways through stabilization and activation of β-catenin (Wnt/β-catenin), activation of small GTPases (Wnt/planar cell polarity [PCP]), or calcium signaling, among others [67] and with a degree of cross-talk between different Wnt pathways [68]. In Wnt/β-catenin signaling, a Wnt ligand binds to a frizzled (Fzd) receptor and the low-density lipoprotein 5 or 6 (Lrp5/6) co-receptor to destabilize the β-catenin destruction complex, which is comprised of axin, adenomatous polyposis coli (APC), casein kinase 1 alpha (CK1α) and glycogen synthase 3 kinase beta (GSK3β) [67, 68]. β-catenin thus accumulates and translocates to the nucleus where it associates with the transcription factors T cell factor (Tcf) and lymphoid enhancer-binding factor 1 (Lef1) to induce transcription of Wnt target genes. In Wnt/β-catenin-independent pathways, Wnt ligands can also utilize frizzled receptors, but other co-receptors have been implicated, including atypical receptor-related tyrosine kinase (Ryk) [69, 70], receptor tyrosine kinase-like orphan receptor 2 (Ror 2) [71, 72], van gogh/vang gogh-like (vang in *Drosophila*/vangl in mammals) and starry night (Stan), previously known as flamingo (Fmi) in *Drosophila* (cadherin, EGF LAG seven-pass G-type receptor [Celsr] in mammals) [73]. This leads to the activation of small GTPases, which are required for the actin cytoskeleton rearrangements necessary for the organization of PCP or convergent extension (CE) movements. Wnt pathways act through the scaffolding protein disheveled (Dsh in *Drosophila*, Dvl in vertebrates), which also plays a role in receptor endocytosis [74].

Both clathrin and caveolin-mediated endocytosis have been implicated in Wnt/β-catenin signaling, although most studies point to a predominant role for clathrin. Early studies in *Drosophila* showed that clathrin was required to degrade wingless (*Drosophila* Wnt) in a gradient [75] over the ventral cuticle, which supported the classical view of endocytosis as contributing to down-regulating signaling. Later studies revealed the positive role of endocytosis in activation of signaling itself; dynamin and Rab5 are required for activation of a Tcf/Lef luciferase reporter (TopFlash) in *Drosophila* S2R + cells [76], and clathrin and dynamin were found to be required for Wnt/β-catenin signaling in mouse L cells (L cells stably harboring the TOPFLASH and LacZ constructs, "LSL cells") [77]. One hint as to the function of clathrin-mediated endocytosis is offered by studies on β-arrestin, a CLASP that binds to clathrin, AP-2, and G-protein coupled receptors, in Wnt/β-catenin signaling. Co-expression of β-arrestin-1 and Dvl is sufficient to induce LEF-mediated transcription [78], and β-arrestin can bind both Dvl and axin [79], thus it is possible that β-arrestin couples frizzled receptors to phosphorylated Dvl, forming the complexes required for active signaling while disrupting the destruction complex. Importantly, β-arrestin is required for Wnt/β-catenin

signaling since β-arrestin morpholinos reduce the activation of endogenous β-catenin and block the axis duplication otherwise induced by X-Wnt-8 [79].

With regards to caveolin-mediated endocytosis in Wnt/β-catenin signaling, both positive and negative regulation has been observed. Wnt3a treatment of HEK 293 or HeLa S3 cells induces trafficking of LRP6 from lipid rafts at the PM to caveosomes, and caveolin is required for this trafficking and the stabilization of β-catenin [80]. However, in the absence of co-expressed LRP6, Wnt3a-induced internalized FZD5 co-localizes with clathrin, but not caveolin, and is inhibited by clathrin siRNA, suggesting that multiple endocytic routes may be activated by Wnt3a, depending on which receptors are present. When both LRP6 and FZD5 are expressed, these co-localize with caveolin upon Wnt3a treatment. These results were extended by the finding that Wnt3a-stimulation of HeLa cells induces DVL oligomerization at the PM and the formation of LRP6 aggregates, which then induce CK1γ phosphorylation of LRP6 and the recruitment of AXIN to lipid rafts. These "signalosomes", which include FZD, GSK3-β, DVL, and AXIN, co-localize with caveolin but not clathrin and are thought to promote the stabilization of β-catenin by AXIN sequestration and inhibition of GSK3 activity [81].

Pharmacological disruption of lipid rafts and caveolae by filipin has revealed a role for caveolin-mediated endocytosis in LRP6 turnover, while inhibition of clathrin using monodansylcadaverine had no effect [82]. These findings highlight the importance of studying not only how signal transduction is affected by different endocytic routes but also how steady-state levels of signaling components are affected by increased or decreased endocytic capacity, as these will affect signal transduction indirectly.

Endocytosis is also important for β-catenin-independent Wnt signaling, as seen during *Xenopus laevis* gastrulation. Both Wnt5a and Wnt11 are well characterized as non-canonical Wnt ligands that act through β-catenin-independent pathways. During gastrulation, Wnt11 signals through the receptor Ryk to induce β-arrestin2-mediated endocytosis of Fz7 and Dsh. Accordingly, depletion of Ryk and Wnt11 prevents Dsh endocytosis in dorsal marginal zone tissues [83] and the endocytic function of β-arrestin2 is required for convergent extension movements [84]. Fz4 internalization, which can be induced by Wnt5a, is regulated in a clathrin-dependent manner together with AP-2, β-arrestin-2 and Dsh during convergent extension in *Xenopus* [85, 86]. Wnt5a can also activate the small GTPase Rac1 in several cell types [87, 88], and this too, is endocytosis-dependent, acting via clathrin-dependent mechanisms through the receptors Fzd2 and Ror1/2 [88].

In sum, endocytosis is required for Wnts to signal through both β-catenin-dependent and -independent pathways. Further studies would be of great interest to elucidate the degree to which endocytosis contributes to Wnt pathway specificity; for example whether all Fzd receptors are targeted through the same endocytic pathways, regardless of the Wnt activating them, or whether some of the pathway specificity arises from specific endocytic adapters and endocytic targeting. It would be interesting to determine whether specific Wnts are preferentially endocytosed through various endocytic routes. Unfortunately, the current paucity of commercially available Wnts, and the difficulty associated with purifying Wnts, makes such analyses difficult. An additional consideration is that Wnts, for example Wnt3a and Wnt5a, appear to have relatively different activation concentrations (from 10 ng to 300 ng/ml) and thus it is difficult to assess whether the pathway activated and the endocytic route employed is a high- or low-dose pathway, making comparisons between Wnts difficult. Nevertheless, further investigation into this and other questions surrounding Wnt endocytosis and pathway activation will certainly help to improve our understanding of pathway specificity.

Endocytosis in two cells to elicit signaling in one signal-receiving cell: Notch signaling

The Notch pathway represents an especially interesting example of endocytic regulation of signaling in that both the receptor and the ligand are membrane-bound on contacting cells and both require endocytosis for functional signaling to occur. Notch signaling is initiated when a Notch receptor on one cell is bound by a Notch ligand on a contacting cell. The Notch receptor is then sequentially cleaved to generate the Notch extracellular truncation (NEXT) and then release the Notch intracellular domain (NICD), which translocates to the nucleus to initiate transcription of Notch target genes together with the transcription factor CSL (CBF1/suppressor of hairless/LAG-1). Vertebrates have four Notch receptors (Notch 1–4) and five canonical Notch ligands (jagged 1,2 [Jag1, Jag2], and delta-like-1,3,4 [Dll1, Dll3, Dll4]), while *Drosophila* has one Notch receptor and two ligands, delta and serrate (for review, see [89]).

The first indication that endocytosis was required for functional Notch signaling came from the identification of a temperature-sensitive *Drosophila* dynamin mutant (*shibire*, shi^{ts}), which displayed a Notch loss-of-function phenotype [90] with an excess of neuroblasts and neurons [91, 92]. Later studies revealed that dynamin-mediated endocytosis was required in both the ligand-expressing, "signal-sending", and the receptor-expressing, "signal-receiving", cells [93].

On the signal-sending side, mutational analyses of delta [94] and serrate [95] revealed the requirement of

intracellular lysine residues in these ligands for proper Notch signaling. Lysine residues on transmembrane proteins are targets for monoubiquitination and thus serve as a signal for endocytosis [96]. Neuralized (Neur) [97–100] and mind bomb (Mib) [101, 102] have been identified as two E3 ligases required for ubiquitination of Notch ligands and thus activation of Notch signaling. Epsin, a CLASP mentioned previously, which is also known as liquid facets (Lqf) in *Drosophila*, recognizes the ligand monoubiquitination and is additionally required for ligand endocytosis and initiation of Notch signaling [17, 103–105], and loss of epsin results in Notch loss of function phenotypes in mouse [106], *Drosophila* [17], and *C. elegans* [104]. Epsin was previously regarded as an adapter protein mainly involved in clathrin-mediated endocytosis, and thus it is tempting to assume that Notch ligands are internalized via clathrin-dependent routes. However, more recent studies have shown that, at least in *Drosophila* oogenesis, ligand endocytosis does not require clathrin, but does require dynamin [107].

While it is clear that endocytosis is required in the signal-sending cell, its actual function in this cell is still debated. Two main models have been proposed to explain the role of endocytosis in signal-sending cells (Fig. 2): the ligand-maturation or recycling model, and the pulling-force model [108]. In the ligand-maturation model, endocytosis and recycling of the ligand is required for its final maturation into a Notch-activating ligand, either through modification(s) of the ligand itself, or through appropriate sub-cellular re-distribution, thus being presented either at the cell surface in specific microdomains, or packaged into exosomes, which are capable of signaling [109]. In the pulling-force model, the binding of Notch receptor by the ligand, and subsequent pulling away of the extracellular domain through endocytic force, is required for the successive cleavage events of the Notch receptor. There is some evidence to support each of these models, which are, in fact, not mutually exclusive.

In the case of ligand maturation, there is some evidence to support this model, but also some evidence that speaks against it. For example, Dll4 has been found in exosomes from endothelial cells and Dll4-overexpressing tumors, but these exosomes do not activate Notch signaling. Instead, they are incorporated into the Notch-expressing cells and

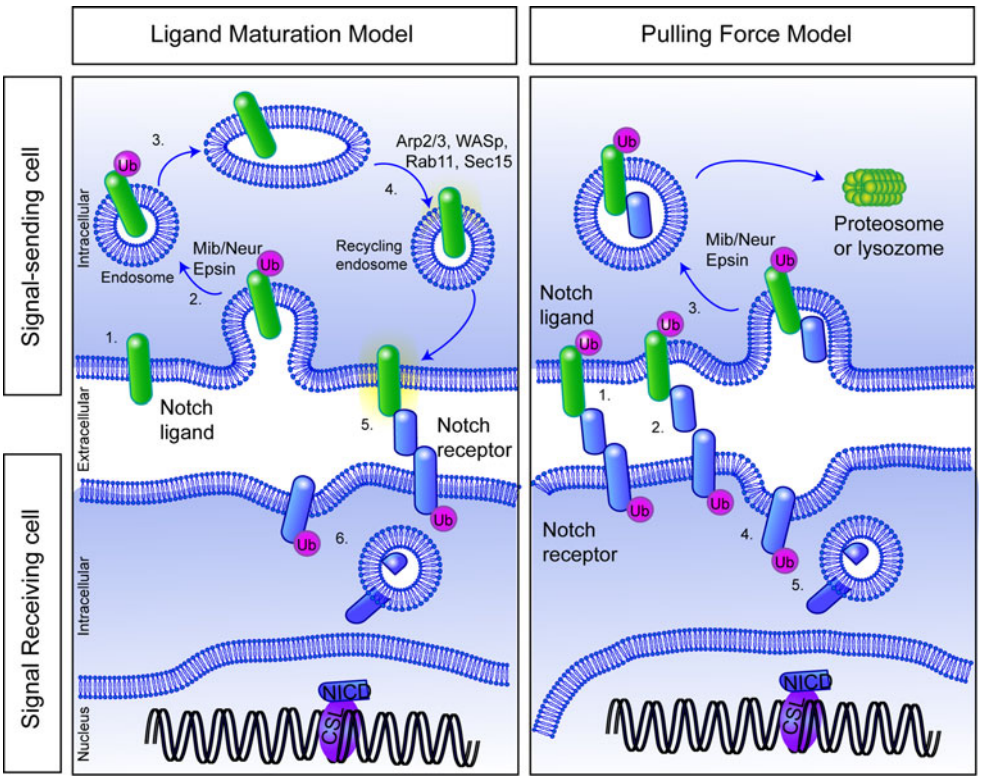

Fig. 2 Alternative roles for endocytosis in the ligand-expressing cell during Notch signaling. In the ligand maturation model, **a** Notch ligand (*1*), undergoes ubiquitination, for example by mind bomb or neuralized (*2*), is endocytosed and is sent to an as-yet undefined compartment (*3*) to be modified to be activation-competent. The ligand is then recycled back (*4*) to the membrane where it can now activate Notch on an adjacent cell (*6*). **b** In the pulling-force model, the Notch ligand binds to the Notch receptor (*1*) and when both are subject to endocytic forces, the ligand "pulls" on the receptor (*2*), revealing cleavage sites for ADAM secretase. The ligand is endocytosed into the ligand-expressing cell, carrying the Notch extracellular domain with it (*3*), while the Notch receptor is now further cleaved and activated, either at the plasma membrane (*4*) or in endosomes (*5*) (for further information, please see the text)

cis-inhibit Notch signaling there [110]. Epsin, as mentioned previously, is necessary for ligand endocytosis and activation of Notch signaling, but not for bulk endocytosis of ligand [105], and replacing the intracellular domain of delta with an LDL internalization signal bypasses the requirement for epsin. This led to the suggestion that monoubiquitination of the ligand is required for its endocytosis and activation of Notch signaling. Furthermore, delta is proteolytically cleaved in wild-type cells, which contain a full-length (~105 kDa) and a cleaved fragment (~50 kDa), while *lqf*- cells only contain the full-length version [105]. However, studies of ligand cleavage are complicated by the fact that different groups report different sized fragments, and a more recent investigation of the role of the actin-related protein 3 (Arp3) and Wiskott–Aldrich syndrome protein (WASp) found protein bands for delta of multiple sizes, including a doublet at 98 kDa, one band at 68 kDa, and one at ~55 kDa. There were no dramatic differences in the levels of each of these in wild-type or *arp3*- cells, although this is not overly surprising considering Arp3 was proposed to regulate a step in the post-endocytic sorting and recycling of delta, and thus delta may have been cleaved prior to its interaction with the Arp3+ compartment [111]. Therefore, Arp3 is required for the appropriate sub-cellular distribution of delta [111], as are the recycling proteins Rab11 [112] and Sec15 [113]. Sucrose-gradient fractionation has shown that wild-type Dll1 is enriched in detergent-insoluble lipid raft microdomains, rich in caveolin, while mutated versions of Dll1, which are not found in lipid raft fractions, are incapable of eliciting Notch signaling [114]. Jagged1, however, is not enriched in lipid rafts [115]. In sum, Notch ligands can be recycled from the plasma membrane and can be found in specific sub-cellular domains, which includes apical versus. basal redistribution and membrane microdomain targeting, which may be especially important in polarized cells.

In support of the pulling-force model, Notch ligands are ubiquitinated in response to interaction with the receptor [116], a signal which induces endocytosis [96], and the Notch extracellular domain is trans-endocytosed into the ligand-expressing cell [117, 118], dissociating the Notch receptor and unfolding the negative regulator region (NRR) of Notch to reveal cleavage sites for a disintegrin and metallo-protease (ADAM) secretases [119, 120]. Thus, both models are supported by a number of observations and it is possible that both mechanisms are used in different contexts.

On the Notch receptor-expressing, signal-receiving side, endocytosis via dynamin, epsin and clathrin is required for functional Notch signaling. In response to ligand interaction, the Notch receptor can be monoubiquitinated at lysine 1749 [121], and then de-ubiquitinated by elF3f (a subunit of the elongation factor 3 [Elf3]) which is required for Notch to be processed by γ-secretase [122]. In fact, there are a growing number of E3 ligases associated with Notch ubiquitination and signaling. For example, Numb, a CLASP also mentioned previously, acts as a suppressor of Notch signaling (for review, see [123, 124]) by recruiting the E3 ubiquitin ligase itchy (Itch), the mammalian homolog of *Drosophila* suppressor of deltex (Su(Dx)), to regulate post-endocytic sorting events for Notch [125], and degradation of the Notch receptor [126]. There is also the putative E3 ubiquitin ligase deltex, which regulates Notch internalization and processing [127–131], and acts as a bridging protein between elF3f and Notch in early endosomes [122]. Deltex has been ascribed both positive [129, 130, 132, 133] and negative [134, 135] effects on Notch signaling, and perhaps some of the differences can be explained by the recent finding that canonical Notch signaling, activated by interaction with ligand, and noncanonical, deltex-activated, Notch signaling may be separate events activated in different endocytic compartments [131]. In sum, there appear to be a number of different E3 ligases that may regulate Notch receptor ubiquitination and thus its endocytosis, targeting, and activity.

Once the NECD has been trans-endocytosed into the ligand-expressing cell, the remaining membrane-tethered portion of Notch, the Notch extracellular truncation (NEXT), becomes a substrate for subsequent site 2 (S2) cleavage by ADAM protease and site 3 (S3) cleavage by a multi-subunit protease complex known as the γ-secretase complex [136].

Different groups have observed different localizations for Notch cleavage; either occurring predominantly at the cell surface [137 – 139] or mostly after internalization of the receptor by endocytosis [140]. In fact, S3 cleavage itself may be regulated by endocytosis: Notch ICD fragments generated from S3 cleavage will have an amino-terminal valine (Val) if cleaved at the plasma membrane, but have an amino-terminal serine/leucine (Ser/Leu) if cleaved in endosomes [141]. Ser/Leu-NICD fragments have a shorter half-life than Val-NICD fragments, which should therefore affect the duration of Notch signaling.

While endocytosis of NEXT requires clathrin and dynamin, its cleavage by γ-secretase does not, and in fact inhibition of endocytosis enhances S3 cleavage and Notch target gene transcription [138], which could be because of increased plasma-membrane cleavage rather than endosomal cleavage of NEXT. Meanwhile, loss of caveolin-1 expression results in an increase in γ-secretase-mediated cleavage of Notch, and over-expression of caveolin-1 attenuates γ-secretase-mediated proteolysis of Notch [142]. It is worth noting that γ-secretase is most active in lipid raft-like membrane domains [143].

All in all, clathrin-mediated endocytosis appears to be the predominant endocytic route required for Notch

signaling, a signaling pathway that requires active endocytosis by both the receptor- and the ligand-expressing cell. Notch endocytosis and signaling is fine-tuned by the action of different CLASPs, such as epsin and Numb, and signaling longevity may be determined by whether the Notch receptor is cleaved at the PM or in endosomes. It is therefore clear that endocytosis plays several important roles in Notch signaling, and it would seem that endocytic regulation of Notch signal initiation, and transduction, may be one way in which complex signaling is achieved from an otherwise relatively straightforward signaling pathway [89].

An additional point of interest is a recent finding describing a role for primary cilia in Notch signaling. Knockdown of intra-flagellar transport proteins during embryonic development resulted in the loss of cilia and causes differentiation defects that are Notch-dependent but Shh-independent [144], adding yet another dimension to endocytosis in Notch signaling.

Trans-endocytosis and bi-directional signaling: Eph receptors and ephrin ligands

Trans-endocytosis is the process by which one cell endocytoses material from another cell. In some cases, cells can trans-endocytose entire membrane-bound molecules from one cell to the other, sometimes bringing over portions of the plasma membrane and other associated proteins. This is not to be confused with the process of trogocytosis, implemented by immune cells, in which lymphocytes extract cell surface molecules from antigen-presenting cells to present them on their own cell surface (reviewed in [145]). Examples of membrane-bound receptor/ligand pairs that display trans-endocytosis include Eph receptors/ephrin ligands [146], the sevenless (sev) receptor/bride of sevenless (boss) ligand [147], and CD47/Src-homology-2-domain-containing protein tyrosine phosphatase substrate 1 (Shps-1) [148]. Of these, the most thoroughly studied group is the Eph/ephrin family.

Eph receptors are a family of receptor tyrosine kinases, activated by interaction with ephrin ligands, which are especially important in adhesion and repulsion during axon guidance (reviewed in [149]). Eph-ephrin interactions can result in forward-signaling in the Eph receptor cell, and reverse-signaling in the ephrin ligand cell, which is known as bi-directional signaling. Both forward and reverse signaling are of importance in vivo, for example EphB1/EphB2 forward-signaling and ephrin-B2 reverse signaling guide retinal ganglion cells axons to their proper termination zone in the superior colliculus [150]. Similarly to Notch signaling [151], co-expression of receptor and ligand in the same cell (in *cis*), inhibits trans-activation of the receptor by ligand on an adjacent cell [152, 153].

With regards to which endocytic pathway Eph/ephrins utilize, there appears to be some controversy as to whether clathrin- or caveolin-mediated endocytosis plays a more important role. Ephrin-B1 endocytosis of EphB1-Fc is dynamin-dependent, indicating that either caveolin or clathrin could be involved [154]. Supporting a role for caveolin-mediated endocytosis, ephrin-B1 can be found in lipid raft microdomains [155] and colocalizes with caveolin [154]. Also, EphB1 co-fractionates, co-immunoprecipitates and co-localizes with Cav1 upon ephrin-B2-Fc stimulation, and EphA2 similarly binds Cav1 in response to ephrin-A1-fc [156]. In support of a role for clathrin-mediated endocytosis, EphA8-mediated endocytosis of ephrin-A5-Fc co-localizes with transferrin, clathrin and EEA1 [157], and EphB2 co-localizes with transferrin, but not EEA1, upon ephrin-B2 treatment [158]. In vivo, EphA4 can be found in clathrin-coated vesicles [159], and functionally, ephrin-B1 endocytosis of EphB1-Fc is disrupted by potassium depletion, which inhibits clathrin-mediated endocytosis [154]. However, potassium depletion affects several other endocytic pathways [160, 161], and it will be important to further investigate the functional roles of caveolin-mediated, clathrin-mediated, and other endocytic pathways in Eph/ephrin internalization.

While the relative roles of caveolin- and clathrin-mediated endocytosis have yet to be fully elucidated, a large number of small GTPases have been linked to trans-endocytosis of Eph/ephrin complexes. The Rho family of small GTPases, which includes the Ras, Ran, Rad, Rab, Arf, and Rho families, are mainly associated with regulation of the cytoskeleton, but also play important roles in endocytosis and trafficking [162]. EphB4+ fibroblasts trans-endocytose full-length ephrin-B2 in a Rac, Arp2/3 and dynamin-dependent manner [163], which is correlated with phosphorylation of EphB4 and activation of signaling in the receptor cell (forwards signaling). Similarly, AphA8+ 293 cells internalize ephrin-A5 into early endosomal antigen (EEA1)+, clathrin+ endosomes, in a process requiring Tiam1, a Rac-specific guanine nucleotide exchange factor [157]. Vav2, another Rho family GEF, physically interacts with both EphA and EphB receptors and is required for endocytosis of ephrin-A1-Fc by retinal ganglion cells [164], and EphA2-mediated activation of Rac1 during angiogenesis [165]. Rin1, a Rab5 GEF that targets phosphorylated receptors to Rab5 compartments during endocytosis, is required for EphA4 endocytosis upon ephrin-B3 stimulation [166]. EphA4 interacts directly with the Rho family guanosine exchange factor (GEF) ephexin upon ephrin-A1 stimulation, which upregulates RhoA activation [167] while EphA2 interacts with ephexin4, in a ligand-independent manner, to activate RhoG, but not Rac1 or RhoA [168]. Interestingly, ephexin1 constitutively activates RhoA, Rac1, and cdc42 in the absence of ephrin-A, but when ephrin-A is present, tyrosine phosphorylation of ephexin1 by EphA

receptors enhances ephexin1's exchange activity specifically toward RhoA [169].

It is of note that early studies found that Rac1 and RhoA both inhibit uptake of transferrin, implying that they inhibit clathrin-mediated endocytosis [170]. However, local rearrangements of the actin cytoskeleton, possibly mediated by RhoA or Rac, are required for clathrin-mediated endocytosis [171]. This reinforces the idea that further studies of Eph/ephrin endocytosis must aim to determine (1) which specific GTPases are involved in clathrin or caveolin-mediated endocytosis in forward and reverse-signaling, (2) whether they act on endocytosis and activation of signaling and/or (3) whether they act on the actin cytoskeleton to mediate, for example, axon guidance.

The directionality of trans-endocytosis is regulated by cytoplasmic determinants. An ephrin-B1 mutant lacking its cytoplasmic domain is preferentially trans-endocytosed into the EphB2 cell, while an EphB2 mutant lacking its cytoplasmic tail is preferentially trans-endocytosed into the ephrin-B1 cell [172]. Importantly, a kinase-dead version of EphB2 displays similar trafficking as the cytoplasmic truncation, demonstrating that phosphorylation is important for its endocytosis into the EphB2 cell. In this study, when both ligand and receptor are full-length, the majority of ephrin-B1 is internalized into the ephrin-B1 cell [172].

Many of these studies have been performed using Fc-fusion constructs, and as such, they frequently lack transmembrane or cytoplasmic domains and may not reflect true signaling states observable in vivo. Like the Notch receptor, Eph receptors and ligands can be cleaved by ADAM secretases and γ-secretase to release signaling fragments [173 –178]. Therefore, it will be of paramount importance to confirm the trans-endocytosis studies using full-length proteins and antibodies directed towards N- and C-termini of both Eph receptors and ephrin ligands to accurately map cleavage and/or trans-endocytosis of receptor and ligand. This would enable accurate assessment of the role of endocytosis and ligand/receptor cleavage in mediating such disparate events as cellular adhesion and repulsion.

In sum, Eph receptors and ephrin ligands appear to traffic through both clathrin- and caveolin-mediated endocytic routes, activating small GTPases, which are required for these endocytic events but also for actin remodeling. Determining which components control whether endocytosis occurs in a forwards or a reverse direction will doubtless increase our understanding of the fine-tuning of Eph/ephrin signaling, as well as other signaling pathways.

Concluding remarks

Although the three signaling pathways described here are very different mechanistically, they all require endocytosis for signaling to proceed in a correct fashion. In some cases, endocytosis may be required to assemble the correct signaling components into signalosomes, as described for Wnt signaling. In other cases, endocytosis may be required to exert a pulling force of the ligand on the receptor to reveal crucial cleavage sites required for activation, as suggested for Notch signaling. Additionally, correct targeting of the receptor, as in the case of Notch, may dictate at which amino acid and at what rate it is further processed/cleaved. In the case of Eph/ephrins, bi-directional signaling is finely regulated by endocytosis in each cell. Although these examples are quite dissimilar, certain questions and considerations may be applicable across several signaling pathways.

Eph/ephrins rely heavily on small GTPases for their endocytosis and for further signaling. Wnts can activate small GTPases, but the extent to which this is linked to endocytosis remains to be further explored. This is of particular interest since small GTPases link endocytosis with cytoskeletal rearrangements; in the case of Eph/ephrins to regulate axon guidance, and in the case of Wnts, to regulate convergent extension movements. Even Notch has been described to regulate axon guidance, in a non-canonical manner, through the activation of the small GTPase Rac1 [179]. Wnts activate a multitude of small GTPases [180], and therefore it would be of tremendous interest to determine which are required for endocytosis, which are required for transmitting the signal, and which are required for actin remodeling. Wnts also signal to the nucleus via small GTPases, which begs the question of whether Eph/ephrins or Notch can also signal to the nucleus via small GTPases, although it is thought that the main target of Eph/ephrin signaling is actin rearrangements, not transcriptional regulation.

Notch signaling shares several interesting features with Eph/ephrin signaling. Both of these pathways utilize membrane-bound receptors and ligands, and both pathways utilize ADAM and γ-secretase to cleave the receptors and ligands [175, 177, 181, 182]. It would be beneficial to determine whether the ligands and receptors are similarly targeted for their respective cleavage events. Additionally, it is still debated whether backwards-signaling occurs in the Notch ligand-expressing cell, similar to the ephrin-expressing cell. In support of bi-directional signaling in the Notch pathway, Notch ligands are cleaved and release signaling fragments, which have been described to activate an Ap-1 reporter [183], although this remains to be further investigated.

In sum, our knowledge of the vast array of possible endocytic routes is expanding rapidly, as is our knowledge of the complexity of the signaling networks activated by separate signaling pathways. The more we discover about each route and pathway, the more apparent it is that the cell reuses the same components to achieve slightly different

goals in different contexts. It is therefore fruitful to compare signaling pathways with respect to, not only which components are activated but also which endocytic pathways are utilized and how the endocytosed components are subsequently routed. Doing so will hopefully reveal cellular machineries linking initiation of signaling with signaling outputs, as well as divulge more universal paradigms applicable to signal specificity.

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