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# Endocytosis Conducts the Cell Signaling Orchestra

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**Endocytosis is used by eukaryotic cells to regulate nutrient internalization, signal transduction, and the composition of the plasma membrane. However, a more complex picture is emerging, in which endocytic pathways integrate diverse signals, thereby contributing to a higher level of cellular and organismal organization. In this way, endocytosis and cell signaling are intertwined in many biological processes, such as cell motility and cell fate determination.**

Although the existence of different endocytic routes is well known, their exact biological impacts are only beginning to be understood. Clathrin-mediated endocytosis (CME) has traditionally attracted the most experimental attention; however, it is now clear that clathrin-independent endocytosis also plays important roles. One form of clathrin-independent endocytosis relies on cholesterol-rich membrane domains, such as lipid rafts and caveolae. Herein, we refer to this form of endocytosis as raft/caveolar endocytosis (RCE). In this review, we provide snapshots of complex situations, in which different endocytic routes orchestrate biochemical pathways and biological behavior. This work indicates that endocytosis is a fundamental organizer of the cell, which coordinates the core variables in cell signaling—duration, intensity, integration, and spatial distribution—to control such processes as cell fate determination and cell migration.

## Endocytosis Integrates and Attenuates Signaling

Some plasma-membrane receptors are internalized through CME, some through RCE, and some through both. The question is why and under what circumstances. The answer may be that the two routes serve different purposes. As an example, when the TGF-β receptor is internalized through CME, it is routed to "canonical" endosomes, where it signals through the Smad-dependent pathway. Conversely, the fraction of receptors internalized through RCE is delivered instead to a degradative compartment (Di Guglielmo et al., 2003).

Work on the epidermal growth factor receptor (EGFR) suggests a mechanism by which the choice between CME and RCE is made. When stimulated with low doses of ligand, EGFRs are almost exclusively internalized through CME. However, at higher doses of EGF, RCE is also used. This shift at higher doses of EGF correlates with the monoubiquitination of EGFR. Moreover, chimeric proteins, harboring ubiquitin (Ub) as the sole intracellular signal, are internalized through RCE (Chen and De Camilli, 2005; Sigismund et al., 2005). One might speculate that CME is preferred under conditions of scarce ligand because it sustains prolonged endosomal signaling, whereas, when abundant ligand is present, excess stimulation might be avoided by routing part of the receptor population to a degradative (RCE) pathway. This scenario finds support in mathematical simulations showing that receptor internalization and endosomal signaling are critical for signal output only at low doses of EGF (Liu et al., 2005). Cumulatively, this work suggests that the net outcome for signaling events is dependent on the ratio of CME to RCE.

However critical questions remain. For instance, how does RCE direct receptors to degradation? Is signaling by receptors other than EGFR and TGF-β receptor also integrated by the decision between CME versus RCE? Also, in those cases in which CME functions as a "signaling route," is the cargo receptor eventually targeted for degradation? At least in the case of the TGF-β receptor, evidence suggests that CME sustains continuous shuttling of the receptor between the plasma membrane and the signaling endosome rather than leading to degradation (Di Guglielmo et al., 2003).

## "Endocytomics" and Cellular Organization

A recent paper inaugurated the era of functional genomics of endocytosis in mammalian cells (Pelkmans et al., 2005). By RNA interference of the human kinome, an unexpectedly high number of kinases were implicated in endocytosis. Two different viruses (VSV and SV40, which are internalized via CME and RCE, respectively) were used to probe distinct endocytic routes. Surprisingly, the majority of kinases regulate only one of the two endocytic pathways, and, of the 36 kinases that affect both, 23 have opposing effects, enhancing one pathway while suppressing the other. Also, many of the "endocytic kinases" connect endocytosis to other aspects of cellular activity, such as the cell cycle, adhesion, and metabolism. For example, a significant negative correlation is scored between cell proliferation and

the RCE pathway. Moreover, the RCE pathway requires kinases of the integrin pathway, such as FAK, underscoring a connection between cell adhesion and RCE. Conversely, a number of kinases that control the CME pathway, such as Mylk and PKCζ, are involved in cytoskeleton-dependent transport and cell polarity.

Future "omics" studies will likely define different subgroups within CME and RCE. A recent study analyzed the impact of six regulatory kinases on the caveolar pathway (Pelkmans and Zerial, 2005). This revealed two modalities of caveolar dynamics, whereby individual caveolae undergo rapid cycles of fusion and internalization whereas multicaveolar assemblies are static and connected to the extracellular space. Interestingly, the two categories may be regulated by different kinases, and stimulation of RCE by SV40 infection could induce rapid exchange between these two pools of structures.

From a wider viewpoint, these studies unveil an unexpectedly vast regulation of endocytosis by signaling and argue that the two programs might be so deeply intertwined that they in fact constitute a single system. Theoretical modeling has already paved the way for such thinking. For instance, the association of signaling molecules with biological membranes is predicted to increase the number (and/or the average lifetime) of signaling complexes (Kholodenko, 2003). In addition, trafficking membranes could represent an efficient way to deliver messages to biologically relevant locations, such as the nucleus (Kholodenko, 2003). In this framework, the kinases that control both cytoskeleton-dependent traffic and CME (Pelkmans et al., 2005) could deliver signals to appropriate subcellular locations. Thus, theoretical modeling and experimental evidence (Miaczynska et al., 2004) strongly suggest that endocytosis provides necessary spatial and temporal dimensions to signaling. How this idea relates to the "real" biological world is illustrated by recent discoveries in the fields of cell motility and cell fate determination.

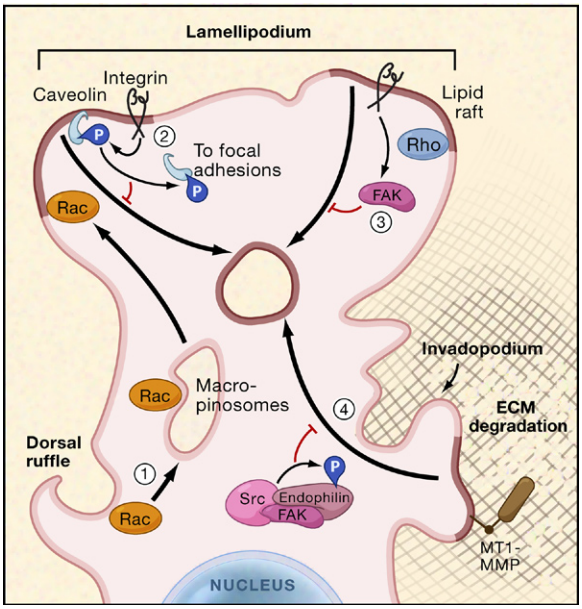


**Figure 1. Endocytosis Regulates Cell Motility and Invasiveness**
Rac is internalized via macropinosomes and recycled to rafts (1). The internalization of Rac at rafts is inhibited by integrin signaling (2) through the relocalization of phosphocaveolin to focal adhesions (del Pozo et al., 2005). A similar mechanism (3), but requiring FAK (Palazzo et al., 2004), targets Rho to rafts. In Src-transformed cells (4), FAK acts as a scaffold to promote the phosphorylation of endophilin-A2 by Src (Wu et al., 2005). Phosphorylation of endophilin disrupts its association with dynamin. This event may inhibit endocytosis of the matrix metalloproteinase MT1-MMP and thereby contribute to tumor invasiveness.

## Endocytosis and Cell Motility

Cell motility has traditionally been regarded as a plasma-membrane-based signaling process in which the engagement of cell-surface receptors leads to actin rearrangements and the formation of motile structures such as lamellipodia and dorsal ruffles. However, there is emerging evidence that endocytosis is an essential component of cell motility. For instance, in migrating border cells of the fruit fly *Drosophila*, endocytosis is used to shuttle the receptors that interact with guidance cues to specific regions of the plasma membrane (Jekely et al., 2005). In this way, endocytosis redirects molecules to regions of "high signaling." Similarly, studies of the GTPases Rac and Rho, which regulate cell motility, suggest that the recruitment and retention of signaling molecules at specific locations of the plasma membrane may be facilitated by the differential regulation of endocytic pathways (Figure 1). Upon integrin-mediated cell adhesion, high-affinity binding sites for Rac become available at the plasma membrane. Signaling downstream of active Rac promotes the formation of lamellipodia, which are characteristic of the leading edge of migrating cells. Integrins act locally to prevent the internalization of lipid rafts by RCE, which serve as anchorage points for Rac. This process maintains active Rac near sites of integrin-mediated signaling (del Pozo et al., 2004, 2005). A similar mechanism, but involving different molecular effectors, might also be responsible for localizing Rho (Palazzo et al., 2004).

In addition to RCE, macropinocytosis is another form of non-clathrin-mediated endocytosis, in which membrane protrusions fuse back with the plasma membrane to produce large vesicles. Membrane bound Rac is also internalized by macropinocytosis. If this process is inhibited, lamellipodia are lost and active Rac accumulates in aberrant membrane ruffles, which could be aborted macropinosomes (Schlunck et al., 2004). Taken together, a complex trafficking pattern emerges: Rac is internalized through macropinosomes and then recycled to specific regions of the plasma membrane where integrin-mediated inhibition of RCE makes rafts (and their components) available for binding to Rac (Figure 1). This circuitry requires that opposing effects on two endocytic pathways be coordinated, a concept that also emerged from the recent genomic studies (Pelkmans et al., 2005).

Endocytosis also contributes to the migratory and invasive behavior characteristic of transformed cells. Invasive

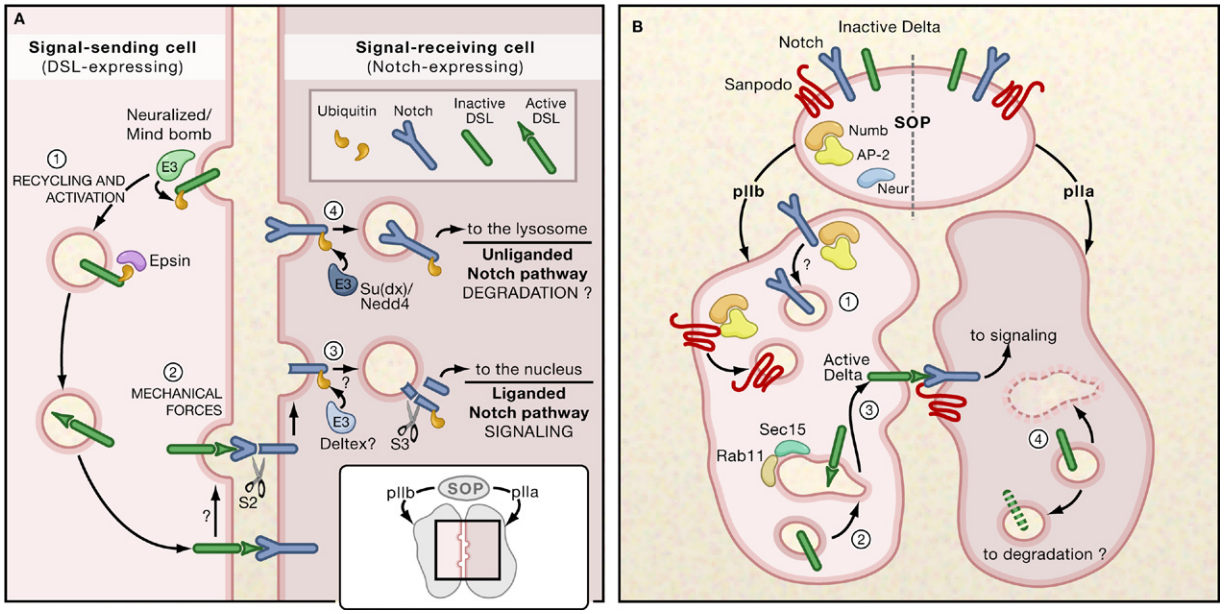


**Figure 2. Endocytosis and Cell Fate**

(A) The endocytosis of Notch and its ligands, Delta, Serrate, and Lag2 (DSL), is required for Notch activity. Internalization of DSL (in *Drosophila* and zebrafish) depends on ubiquitination by the E3 ligases Neuralized and Mind bomb. Downstream events require the Ub binding protein epsin/*lqf* (Le Borgne et al., 2005). Models to explain why DSL endocytosis is required for Notch activation are shown. (1) Inactive DSLs are endocytosed, "activated" in endosomes, and recycled to the surface. (2) Endocytosis of DSLs generates conditions (by mechanical "pulling" forces) that unmask the Notch S2 site. (3) Ligand-engaged Notch requires endocytosis (possibly dependent on ubiquitination by Deltex) for its activation. (4) Unliganded Notch is continuously endocytosed, through ubiquitination by the E3 ligases, Su(dx)/AIP4 and Nedd4, to prevent sporadic activation. This might route Notch to an endosomal compartment where it can interact with presenilin, an effector of the S3 cut (Le Borgne et al., 2005).

(B) Creation of asymmetry in pIIa and pIIb cells in *Drosophila* is regulated by endocytosis. The SOP cell is shown with relevant molecules (dashed line, mitosis). (1) Notch is nonfunctional in pIIb cells because it is internalized/degraded or because Sanpodo is internalized. A working model (Hutterer and Knoblich, 2005) attempts to reconcile these two possibilities. Sanpodo itself regulates Notch endocytosis. In the absence of Numb (pIIa), Sanpodo might route Notch to an "activating" endosomal compartment (the "liganded" pathway in [A]). In the presence of Numb (pIIb), Sanpodo might participate in Notch downregulation (the "unliganded" pathway in [A]). The E3 ligase Neuralized is asymmetrically partitioned in pIIb, allowing endocytosis of Delta. (2) Following endocytosis, Delta is routed to a Rab11 endosome, and then to the plasma membrane (3). (4) In pIIa, this pathway is blocked, possibly because a critical Rab11 partner (Nuclear fallout/Arfophilin 1) is inactivated (Emery et al., 2005). How Delta is internalized in pIIa in the absence of Neuralized is not clear, although there may also be Neuralized-independent mechanisms of Delta internalization (Wang and Struhl, 2005). Delta might also be internalized before mitosis of the SOP cell; in pIIb, it could be recycled to the plasma membrane, whereas in pIIa cells, it might be destined to a degradative pathway (Emery et al., 2005). The events shown need not be all or none, but still occur in both cells but be biased in favor of one.

tumor cells degrade the extracellular matrix through membrane-anchored metalloproteinases, such as MT1-MMP. In Src-transformed cells, a FAK-dependent mechanism is activated, which attenuates endocytosis of MT1-MMP. This results in increased degradation of the extracellular matrix, which could contribute to tumor invasion and metastasis (Wu et al., 2005) (Figure 1).

## Endocytosis and Cell Fate Determination

Upon engagement by membrane bound ligands of the DSL (Delta/Serrate/Lag2) family, the plasma-membrane receptor Notch is cleaved in the extracellular region (the S2 cut), followed by a cleavage in the transmembrane region (the S3 cut). The liberated intracellular domain translocates to the nucleus, where it acts as a transcriptional regulator (Figure 2A). Genetic evidence in *Drosophila* shows that endocytosis of both DSL and Notch is required for Notch activation (Figure 2A; Le Borgne et al., 2005). Endocytosis of DSL follows at least two routes, one dependent on ubiquitination (Le Borgne et al., 2005) and the other relying on canonical endocytic motifs (Wang and Struhl, 2005) (Figure 2A). Whether the two pathways represent RCE and CME, and how they are integrated, is uncertain. Notch also undergoes at least two different kinds of internalization, both dependent on ubiquitination (Figure 2A). Unliganded Notch is continuously endocytosed, probably to prevent its sporadic activation. Conversely, ligand-engaged Notch requires endocytosis for its activation. How ubiquitination couples Notch to both a degradation/recycling route (when not bound by ligand) and activation (when ligand bound) is presently debated (Le Borgne et al., 2005).

How all of these endocytic pathways converge to execute a multifaceted biological program is exemplified by studies of cell fate determination. In asymmetric cell division, fate determinants are differentially partitioned between daughter cells. In the genesis of the sensory organ of *Drosophila*, the precursor cell (SOP) divides asymmetrically, generating a pIIa and a pIIb cell (Figure 2B), which have distinct fates because Notch signaling is activated only in pIIa. This is due to asymmetric partitioning of Numb (an endocytic

protein and an antagonist of Notch) in the pIIb cell. Mutants of α-adaptin, a component of the endocytic adaptor AP-2, mimic Numb loss of function (Le Borgne et al., 2005). Because Numb binds to α-adaptin and Notch, it might induce Notch endocytosis in pIIb. An alternative possibility is suggested by studies of Sanpodo, a protein required for Notch signaling. Although Sanpodo is not asymmetrically partitioned, its subcellular localization is different in pIIa and pIIb cells. In pIIa, Sanpodo is at the plasma membrane, whereas in pIIb, Sanpodo is internalized through Numb/α-adaptin-dependent endocytosis (Hutterer and Knoblich, 2005). Thus, Numb might regulate endocytosis of Sanpodo, making it inaccessible to Notch, thereby suppressing Notch signaling (Figure 2B).

The endocytosis of Delta is also involved in pIIa/pIIb specification (Figure 2B). Once internalized, Delta passes through Rab11-positive recycling endosomes (Emery et al., 2005). The Rab11 endosome should recycle Delta to the plasma membrane of pIIb, allowing engagement of Notch. This possibility is corroborated by findings that Sec15, a putative effector of Rab11, is critical in this pathway (Jafar-Nejad et al., 2005). Sec15 is a component of the exocyst in the secretory pathway, which mediates tethering of vesicles to the plasma membrane. In pIIa, the Rab11-based mechanism is suppressed (Emery et al., 2005), thereby hindering recycling of Delta and generating asymmetry.

## Outlook

As our understanding comes into focus, endocytosis and signaling appear as two sides of the same coin. This raises interesting questions to be tackled in the future. For instance, can the same biochemical pathways achieve different biological outcomes simply by being constrained by different configurations of membrane organelles and having different patterns of endocytosis? Similarly, given the high degree of overlap between the pathways that are activated by diverse signaling receptors, is "coincidence detection" on endomembranes a mechanism to resolve these many inputs into specific signals? There is already strong evidence that this happens in phosphoinositide (PI) signaling—PIs and PI binding proteins adopt a restricted configuration on cellular membranes through a series of signaling-mediated events (Carlton and Cullen, 2005).

Also, the need for increased signaling complexity in the course of evolution might have been met, at least in part, by increasing the complexity of the endocytic membrane system. An opportunity to test this possibility is offered by studies of the GTPase dynamin (Elde et al., 2005). The primordial function of dynamins is related to mitochondrial inheritance. During evolution, some of the dynamins were "recruited" to the endocytic pathway, where they execute the fission of vesicles. The study by Elde et al., (2005) shows that the acquisition of an endocytic role by dynamin occurred independently during the ciliate and metazoan radiations. If convergent evolution of this "endocytic" event is related to the independent acquisition of the same signaling-related properties or phenotypes, this would constitute a spectacular demonstration of the concept.

Finally, the increasing understanding of the link between endocytosis and signaling raises the possibility that targeted interference of endocytosis might alter disease-linked phenotypes, especially those that are associated with aberrant cell specification. This might lead to new ways of manipulating stem cells and could increase our understanding of pathological conditions such as cancer.

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