Published in final edited form as:
Physiol Rev. 2012 January ; 92(1): 273–366. doi:10.1152/physrev.00005.2011.

Endocytosis and Signaling: Cell Logistics Shape the Eukaryotic Cell Plan

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Abstract

Our understanding of endocytosis has evolved remarkably in little more than a decade. This is the result not only of advances in our knowledge of its molecular and biological workings, but also of a true paradigm shift in our understanding of what really constitutes endocytosis and of its role in homeostasis. Although endocytosis was initially discovered and studied as a relatively simple process to transport molecules across the plasma membrane, it was subsequently found to be inextricably linked with almost all aspects of cellular signaling. This led to the notion that endocytosis is actually the master organizer of cellular signaling, providing the cell with understandable messages that have been resolved in space and time. In essence, endocytosis provides the communications and supply routes (the logistics) of the cell. Although this may seem revolutionary, it is still likely to be only a small part of the entire story. A wealth of new evidence is uncovering the surprisingly pervasive nature of endocytosis in essentially all aspects of cellular regulation. In addition, many newly discovered functions of endocytic proteins are not immediately interpretable within the classical view of endocytosis. A possible framework, to rationalize all this new knowledge, requires us to "upgrade" our vision of endocytosis. By combining the analysis of biochemical, biological, and evolutionary evidence, we propose herein that endocytosis constitutes one of the major enabling conditions that in the history of life permitted the development of a higher level of organization, leading to the actuation of the eukaryotic cell plan.

The line between disorder and order lies in logistics

Sun Tzu

I Introduction: The Vantage Point of Endocytosis on Signaling (and Vice Versa)

A search for the term *endocytosis* in any major cell biology textbook, in the *Encyclopedia Britannica*, or even in Wikipedia will result in a definition corresponding to a more or less

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Disclosures
No conflicts of interest, financial or otherwise, are declared by the authors.

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sophisticated version of the following (taken from Wiki): “Endocytosis is the process by which cells absorb molecules … that cannot pass through the plasma membrane.” This is indeed true; endocytosis serves to bring nutrients and/or other types of molecules into the cell and, at the same time, to regulate the composition of the plasma membrane (PM). Indeed, endocytosis most likely evolved for this purpose. However, this is only a fragment of the whole picture. Based on present knowledge, a more precise definition of endocytosis should read: “a vast program, deeply ingrained in the cellular master plan and inextricably intertwined with signaling, which constitutes the major communications infrastructure of the cell. As such, it governs almost all aspects of the relationships of the cell with the extracellular environment and of intracellular communication. Its evolution constitutes, arguably, the major driving force in the evolution of prokaryotic to eukaryotic organisms.” A shorter version might read as follows: “Endocytosis is the logistics of the cell,” where logistics (again as defined by Wiki) “involves the integration of information, transportation, inventory, warehousing, material handling and packaging, and security,” or in brief “having the right thing, in the right place, at the right time.” This latter definition of endocytosis underscores a shift from a limited perspective (what we might call a “traditional view”) to a much wider one, in which endocytosis is the cornerstone of the eukaryotic cell project. The declared intent of this review is to illustrate how we have moved, in the course of the past 15 years or so, from one outlook to the other.

The attentive reader will notice that this review ends where it should have started, i.e., with a discussion of the evolution of endocytosis and of the endomembrane system. This is because the past is paradoxically the best bridge between the present and the future, or, in other words, because the evolutionary perspective, though speculative, can help us to rationalize a number of heterogeneous observations and can indicate which experimental strategies might serve to achieve a coherent biological picture of endocytosis. For the sake of clarity, however, we would be wise to take things from the beginning. Early prokaryotic life forms used relative simple devices, such as pumps or channels, to transport essential molecules, such sugars, amino acids, and ions, through the PM. As prokaryotes evolved to eukaryotes, more complex “entry portals” began to appear. As Christian De Duve suggested (151), this might have happened as a consequence of the selective pressure provided by the transition from “the primordial soup to oceans,” i.e., from environments in which nutrients were present in a concentrated form to diluted environments. Such a transition might have provided selective pressure for the survival of life forms capable of actively searching for, and concentrating nutrients.

Regardless of how endocytosis came to be, this “simple” evolutionary perspective also pretty much encapsulates our traditional view of endocytosis that spanned a number of years. The problem is that we then started to discover things that were (and still are, to some extent) more and more difficult to reconcile with the traditional view, starting from the tight connection between endocytosis and signaling. From the signaling viewpoint, our outlook on endocytosis started to change with the pioneering studies of Brown and Goldstein (12), which established the concept of receptor-mediated endocytosis. By using surface receptors, the cell cleverly concentrates macromolecules at the PM and reduces the energy consumption necessary for their internalization. However, this mechanism of internalization

has other interesting implications that are not immediately connected with the uptake of nutrients.

Receptor-mediated endocytosis can be either constitutive or ligand induced. In the constitutive process, membrane receptors are continuously internalized and then recycled back to the cell surface, after sorting in the endosomal compartment. The internalized ligand is destined to different metabolic fates: either recycling to the surface or routing to lysosomes for degradation. In the ligand-regulated process, internalization is triggered by the interaction of a ligand with its surface receptor. The receptor-ligand complex is normally routed to the lysosomal compartment with ensuing degradation. However, the internalized receptors (and sometimes their ligands) can be redelivered to the PM, in a recycling process not dissimilar to that of constitutive endocytosis.

Ligand-mediated endocytosis is typical of signaling receptors, such as receptor tyrosine kinases (RTKs). In this case, one important role of endocytosis is to remove active, signaling receptors from the PM and to destine them for degradation, thus achieving signal extinction and long-term attenuation. An interesting side effect (so to speak) of this process is that endocytosis continuously remodels the composition of the PM, thus allowing plasticity in the cellular responses to the microenvironment, something that, as we will see, plays a determining role in many biological processes, first and foremost cell fate determination. Another relevant implication of these findings concerns recycling. While recycling was initially considered to be a device that simply replenished the PM with receptors, this process is now understood to be a major tool for redistributing receptors and other signaling membranes to specific regions of the PM where polarized signaling needs to occur.

The discovery of receptor-mediated endocytosis led to further signaling-relevant insight. In the endosomal compartments, signaling receptors are frequently still bound to their ligands, and are therefore active. Thus the possibility existed that signaling might not occur from the PM exclusively, but could persist throughout the endosomal route. More interestingly, signaling receptors in the endosomal compartment could potentially be exposed to substrates that were inaccessible at the PM. Under this scenario, endocytosis would be a mechanism to sustain signaling and to achieve signal diversification and specificity.

The first compelling functional evidence in this direction came from a study published by the Schmid laboratory. These researchers exploited a dominant negative mutant of dynamin, which blocks most endocytic events, to show that while some signaling pathways were augmented (as would be expected if signaling were to occur exclusively from the PM and endocytosis were simply an attenuation device), other pathways were dampened (807). This study established the concept that endocytosis is required for at least some forms of signaling. In the decade that followed this seminal discovery, the field witnessed a tumultuous expansion in knowledge that firmly established the fact that the signaling and endocytic programs are profoundly interconnected. This bi-univocal correspondence is witnessed by *1*) the regulation of signaling pathways by endocytosis, *2*) the reciprocal regulation of endocytosis by signaling, and *3*) a shareware situation in which several molecules have a dual role in endocytosis and signaling. Over recent years, progress in the deconvolution of the biochemical wirings that connect endocytosis and signaling has been

mirrored by a spectacular series of studies that have highlighted how these circuitries actuate and coordinate the execution of several biological programs. Not surprisingly, efforts directed at discovering how things can go wrong have also highlighted an important contribution of endocytosis to several pathological human conditions.

And yet, this might still represent only part of the entire story. In recent years, numerous studies have reported a role for endocytic proteins in cellular programs that cannot be directly linked to biomembranes, such as the control of gene transcription, cell cycle progression, and mitosis. Many of these connections cannot intuitively be rationalized within a canonical view of endocytosis. They appear, therefore, to identify "noncanonical" functions of the process. Regardless of nomenclature, the real question to resolve is the relationship between the molecular machinery of endocytosis and that of apparently very distant cellular processes. One possibility is that endocytic proteins participate in these events as "freelancers," so to speak: their functions in these processes would be unrelated to the roles they play in endocytosis. There is, however, an alternative and much more appealing hypothesis, i.e., that endocytosis is integrated with, and necessary for, the execution of these cellular programs, however distant they may appear to be from membrane dynamics.

The declared intent of this review is to try and provide a unitary framework that can accommodate all these apparently heterogeneous functions of endocytosis. We will try to support the hypothesis, originally put forward by evolutionary biologists, that the acquisition of a system of endomembranes constitutes a "quantum leap" in evolution that allowed the actuation of the eukaryotic cell plan, thus justifying the involvement of endocytosis in so many cellular functions. To build our case, we will start by providing a necessarily succinct account of the mechanisms of endocytosis. This section (sect. II) is essentially for the benefit of the nonexpert reader and makes no pretense of exhaustiveness (interested readers will be directed to several in-depth reviews). Then, we will explore the wealth of evidence that established the "inseparable partnership" between signal transduction and endocytosis, from the biochemical perspective (sects. III–V). Next, we will review a number of examples that, at the systems biology level and at the biological and cellular biochemical levels, show how endocytosis is an integral part of most cellular programs in eukaryotic cells (sects. VI and VII), including those identifying apparently "noncanonical" functions of endocytosis. The pervasiveness of endocytosis within the cell is mirrored by the frequent subversion of endocytosis and/or trafficking in pathological conditions (sect. VIII). In the last part of the review, we will analyze how an understanding of the evolution of endocytosis (sect. IX) might provide reasonable explanations or, at minimum, a viable working hypothesis for a unitary view of endocytosis. By building on an evolutionary perspective, we will finally discuss a concept that we have recently proposed, that of the "endocytic matrix" (sect. X), which views endocytosis as the major enabling condition that allowed the emergence of many other features and programs of the eukaryotic cell.

II At the Cell's Gates: Entry Portals and Endocytic Routes

The vastness of the impact of endocytosis on cellular homeostasis is revealed through the intricacy of entry portals and through the subsequent modalities of cargo sorting in

intracellular membranous compartments. An explosion of knowledge, in the past few years, has forced the field to abandon the idea of a simple and universal mechanism of internalization/trafficking in favor of the notion of multiple and interconnected entry routes, which coexist in the same cell type and are more or less selective for different types of cargoes. Many attributes of the system, including molecular determinants, cargo specificity, and lipid requirements, are also frequently cell context dependent, thereby increasing its convolution. Here, we will cover some basic aspects of the endocytic routes, and refer the reader to a number of recent reviews for a more comprehensive account of their complexity and of the present level of knowledge and/or uncertainty about their nature (for comprehensive reviews, see Refs. 173, 509, 517, 586, 623, 631, 781, 889). A note about nomenclature: throughout this review, the names of proteins are given in capital letters, regardless of the species of origin, except for cases in which descriptive names, such as dynamin, clathrin, or integrin, are used.

The complexity of the system kicks off at the PM where multiple entry portals have been described (Table 1). A rough classification is based on the size of the initial membrane invagination. Large particles (>500 nm) are taken up by phagocytosis (reviewed in Ref. 763), as in general is the case for bacteria or for apoptotic cells, whereas fluid uptake occurs by macropinocytosis (reviewed in Refs. 391, 516). Both processes involve large rearrangements of the PM guided by actin cytoskeleton remodeling, and coordinated by the stepwise involvement of RHO-GTPases (reviewed in Ref. 345). Micropinocytic events are instead characterized by smaller invaginations (<200 nm) and include clathrin-mediated endocytosis (CME) and non-clathrin endocytosis (NCE) (reviewed in Ref. 173).

CME is the best-characterized endocytic route. This pathway involves the recruitment of PM-resident cargo into clathrin-coated pits (CCPs), in a process in which adaptor molecules, such as AP-2, bridge the internalizing cargo with clathrin (reviewed in Ref. 781). Clathrin polymerization then drives the progressive invagination of the pit, which is eventually released into the cytoplasm as an endocytic vesicle, through the action of the GTPase dynamin (reviewed in Ref. 521). The apparent simplicity of this process is belied by the fact that more than 50 different proteins are associated with CCPs, where they assist coat formation and vesicle release (Table 1 and Refs. 173, 781). Some of these accessory proteins (such as epsins and β-arrestins) have been demonstrated, or proposed, to work as substitute adaptors for AP-2, since they can bind both cargo and clathrin. Furthermore, the existence of such a wealth of proteins involved in CME, together with the large variety of sorting signals, has raised the possibility that they might be required for the formation of distinct types of CCPs, specialized in terms of cargo selection and possibly of specific intracellular fate, a concept that is, however, still debated (393, 423, 520, 543, 627, 779; a stimulating discussion of this issue can be found in Ref. 781). The exact mechanism of formation and release of CCPs and the role of the several participating proteins are currently the focus of intense investigation based on biochemical, structural, and live imaging approaches (see, for instance, and limitedly to recently published studies, Refs. 118, 180, 225, 226, 247, 309, 354, 387, 475, 524, 620, 767), and we refer the reader to the cited reviews for an in-depth analysis of this aspect of endocytosis.

Compared with CME, the current picture of NCE is at a lower level of resolution. The term *NCE* is used to refer to a heterogeneous group of pathways that share the common property of being insensitive to clathrin depletion, but that frequently depend on cholesterol-rich PM microdomains, thus being sensitive to pharmacological cholesterol depletion. While knowledge of the molecular machinery of NCE(s) is still somewhat limited, attempts to classify these pathways rely on three major criteria (Table 1, see also Refs. 173, 516): *1*) dependency on dynamin for vesicle release; *2*) presence of "coatlike" proteins involved in membrane curvature and stabilization, such as caveolins or flotillins, in the case of caveolae-mediated or flotillin-mediated internalization, respectively; and *3*) dependency on small GTPases, which control the entry of specific cargoes such as interleukin (IL)-2Rβ (dependent on RHO-A), major histo-compatibility complex (MHC)-I (dependent on ARF6), or fluid-phase markers, which enter through the so-called CLIC/GEEC pathway (CDC42 dependent, GRAF-1 dependent). A recent study has provided a precise quantitative assessment of the CLIC/GEEC pathway in fibroblasts, which surprisingly revealed that it is the major endocytic pathway for fluid-phase and bulk membrane endocytosis, being three times more active than CME (327). In addition, the biochemical purification of vesicles internalized through this pathway has highlighted unexpected links with the actin-based cell migration machinery (327). While a detailed description and analysis of NCE pathways would exceed the scope of this review, caveolae deserve a particular mention, as they not only represent one of the best-known example of non-clathrin internalization portals, but their function intersects with several other aspects of control of signaling, possibly also in a manner unrelated to endocytosis. The role of caveolae in internalization and signaling will be discussed in detail in section VII*A*.

Cargoes, internalized either through CME or NCE, are routed to early endosomes, where they are subjected to distinct trafficking paths that ultimately determine their fates: degradation in the lysosome, recycling to the PM, or retrotransport to the Golgi (reviewed in Refs. 330, 370, 751). Early endosomes represent in all likelihood a common sorting station for all internalization pathways. In the case of caveolar trafficking, the existence of a separate intermediate station, the caveosome, was proposed (598), although more recently this concept has been revised and reincorporated into a standard endosome-lysosome perspective (195, 299; discussed in Ref. 585).

The mechanisms governing the specificity of sorting at the endosomal station reveal a surprisingly dynamic picture. Growing evidence indicates that early endosomes are a morphologically and functionally heterogeneous population, whose complexity is enhanced by the presence of biochemically distinct membrane subdomains, which ultimately influence the signaling capacity and fate of receptors within individual organelles (423, 523, 740, 885). Furthermore, it has been shown that newly formed endocytic vesicles can convert directly into early endosomes (617), and early endosomes into late endosomes (652). Cargo selection is, therefore, predicted to specifically and dynamically control the continuous remodeling of the downstream endocytic pathway, a concept that will be elaborated in detail in section IV*A*.

At the molecular level, small GTPases, mostly, but not exclusively, belonging to the RAB subfamily, play a paramount role in orchestrating the different sorting fates of cargoes in

endosomal stations (reviewed in Ref. 757). For instance, once delivered to early endosomes, cargoes can be recycled to the PM through either a fast or a slow recycling route, depending on RAB4 and RAB8/RAB11, respectively (757). An additional recycling pathway relies on ARF6 activity. This latter route is mainly followed by receptors internalized through NCE, such as MHC-I (reviewed in Ref. 693), although CME-internalized cargo can also be recycled through this pathway (580).

In other instances, cargoes are committed to degradation in lysosomes via sorting through late endosomes and multivesicular bodies (MVB). This route depends on another RAB family member, RAB7 (757). Cargo ubiquitination provides the crucial signal for entering this pathway. Indeed, several protein complexes harboring ubiquitin (UB)-binding domains recognize ubiquitinated cargoes and escort them along the degradative route to the lysosome (reviewed in Ref. 169). These complexes called ESCRT (endosomal sorting complex required for transport) act sequentially at various stations of the degradative route. ESCRT-0, composed of two interacting proteins HRS and STAM, works at the level of the endosome, by sequestering ubiquitinated cargo. Three additional complexes, ESCRT-I, ESCRTII, and ESCRT-III, act at the MVB membrane. ESCRT-I and ESCRT-II mediate the invagination of the membrane into which cargo is sorted, and ESCRT-III is involved in the pinching off and release of the invaginations that constitute the intraluminal vesicles (ILVs) of the MVB (852, 853); deubiquitinating enzymes, recruited by ESCRT-III, remove UB from the cargo so that it enters the ILVs in a deubiquitinated state. This ensures that the free UB pool in the cell is continuously replenished. Finally, ESCRT-III oligomers are dissociated through the activity of the ATPase VPS4 (for reviews, see Refs. 338, 631). The impact of cargo ubiquitination, and of the UB signal in general, on the endocytic pathway will be discussed in detail in section V.

III The Partnership: Endocytosis Controls Signaling

In the following three sections, we will take a close look at the biochemical foundations of the partnership between endocytosis and signaling by reviewing evidence of *1*) how endocytosis controls signaling, *2*) how signaling controls endocytosis, and *3*) how the UB system plays a special and pivotal role in this partnership.

As highlighted in section I, endocytosis is a major mechanism of long-term signal attenuation. However, the liaison between endocytosis and signaling runs much deeper than signal extinction: endocytosis enables cells to adopt several strategies for the regulation of signal propagation and duration. The emerging picture is that the biological outcome of signaling pathways depends on physical constraints resulting from the association of signaling molecules with biomembranes, in turn regulated by endocytosis, and by cycles of endocytosis and recycling (endo/exocytosis cycles, EECs) to the PM. This not only allows cells to resolve signals according to precise time kinetics and spatially defined sites of action, but also to fine-tune a diverse array of biological outputs in response to extracellular stimuli, thereby determining signal specificity. In this section, we review current knowledge of how endocytic circuits control signaling. We will focus on general principles, while referring the reader to recent reviews for more extensive coverage of the molecular details (694, 744).

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A Endocytosis Regulates Signaling at the Plasma Membrane

Receptors at the PM are the first line sensors of extracellular signals. Not surprisingly, therefore, the regulation of their surface levels has an immediate impact on how a cell responds to environmental stimuli. There are several mechanisms through which endocytosis controls receptor signaling specifically at the cell surface.

1 Regulation of receptor availability at the cell surface—Soluble and cell-associated ligands have long been known to promote ligand-induced internalization of their cognate receptors. As a consequence, signaling receptors are rapidly removed from PM, providing a mechanism that directly limits the magnitude and duration of signaling from this site. At steady state, continuous stimulation with soluble ligands, such as those activating RTKs, frequently causes a permanent reduction of the number of cell surface receptors, which are routed to degradative pathways (reviewed in Ref. 744) (Figure 1*A*). This negative-feedback loop is essential to circumvent excessive signaling and to maintain homeostasis. The risks of losing this safety net can be seen in pathological conditions such as cancer, where the frequently seen overexpression of both ligands and receptors contributes to malignant transformation (reviewed in Ref. 538).

Removal of surface receptors does not necessarily lead to a reduction of maximal signal stimulation. Rather, it causes a dose-response shift so that higher ligand concentrations are required to elicit the same magnitude of signal response. This regulatory mechanism is operational, for instance, during chemotaxis in response to soluble ligands. A number of PM receptors, including RTKs and G protein-coupled receptors (GPCRs), function as motogenic sensors, able to respond to gradients of chemotactic factors that guide the migration of cells to their final destination (reviewed in Ref. 177). In addition to moving directionally, migrating cells must also be able to stop at their target sites, where the concentration of chemotactic factors is highest. It was shown, for example, that in the case of epidermal growth factor (EGF)-dependent chemotaxis of mammary carcinoma cell lines, the EGF receptor (EGFR) is uniformly distributed over the cell surface. Ligand binding at the fore end of the cell causes ligand sequestration. This prevents lateral diffusion of the ligand and reduces the gradient. The subsequent internalization and degradation of the ligand-receptor complex, at the fore end, renders cells progressively less sensitive to the chemotactic stimulus, until they stop at their target sites where the concentration of the chemotactic factors is the highest (37). Thus a ligand-dependent internalization/sorting mechanism, driving a motogenic receptor toward a degradative pathway, is critical for both sensing and switching off a migratory signal.

Mechanisms of this kind have been shown to regulate the migration of primordial germ cells (PGCs) toward the gonads during zebrafish development. PGCs express the chemokine receptor CXCR4b and migrate directionally toward sites in the embryo at which the ligand SDF-1a is expressed (174, 644). An internalization defective receptor led to aberrantly elevated receptor signaling levels, increased nondirectional cell migration, and increased ectopic cell migration (527). Similar mechanisms have also been described, during *Drosophila melanogaster* oogenesis, in border cells that migrate directionally in response to platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)

gradients (362). Mutants in critical endocytic factors, such as the E3 ligase CBL or the RAB5 activator SPRINT, showed impaired endocytosis with uniformly localized surface signaling, leading to loss of the spatial information necessary for directed cell migration. In this context, a balance between pro- and antiendocytic activities is also required to control the localization and amount of guidance receptors at the cell surface. Genetic ablation of the endocytic inhibitor MIM (missing in metastasis), for example, leads to increased receptor internalization, resulting in a lack of directional movement by the border cell cluster (629). Thus the antiendocytic function of MIM might be required to maintain the level of guidance signaling within a functional range that regulates the ability of cells to select the direction to follow and prevent their migratory arrest (629).

2 Regulation of ligand accessibility to the receptor—Signaling can also be modulated by the regulation of ligand accessibility through endocytosis. NOTCH receptor signaling provides a specialized example of this kind. We will briefly review here the complex role played by endocytosis in NOTCH activation, which also involves the regulation of the accessibility of NOTCH ligands to the receptor.

Notch signaling is induced through the direct engagement of the receptor by ligands of the DSL (DELTA, SERRATE, LAG-2) family that are present, in a membrane-anchored form, on an adjacent signal-sending cell. Following binding, a series of events ensues in the NOTCH-containing (signal-receiving) cell leading to two proteolytic cuts in NOTCH (the so-called S2 and S3 cuts). The S3 cut, executed by γ-secretases, occurs in the transmembrane region of NOTCH, leading to the release of a soluble cytoplasmic fragment of NOTCH, which translocates to the nucleus and activates the expression of target genes. While the exact mechanism through which DSL ligands activate NOTCH is still the object of intense scrutiny and debate (reviewed in Refs. 224, 237, 406, 592), endocytosis is involved in every proposed model, also in light of genetic evidence that the signaling function of NOTCH requires dynamin, and therefore presumably endocytosis (700).

Endocytosis seems to be required both in the signal-receiving and in the signal-sending cell. In the signal-receiving cell, endocytosis of NOTCH is required for its activation, since the final activating S3 cut probably takes place in endosomes (although it may also occur at the PM), as supported by evidence that *1*) γ-secretase is present at the PM and on endosomes; *2*) γ-secretase displays peak activity at low pH (587), suggesting that endosomal transit is necessary for Notch activation; *3*) DELTA:NOTCH interaction is favored at low pH (595); and *4*) endocytosis is necessary for the cleavage of NOTCH (788). The endocytic regulation of NOTCH activation in the signal-receiving cell is likely to include additional levels of complexity, as ligand-independent NOTCH internalization pathways controlled by the UB ligase DELTEX also exist (847).

Surprisingly, NOTCH activation in the signal-receiving cell requires endocytosis to occur in the signal-sending cell as well (Figure 1*A*). One model contemplates a mechano-transduction mechanism in which pulling forces exerted by the internalizing DSL ligand "strip" the extracellular domain of NOTCH from the intracellular membrane-anchored moiety (NOTCH is a heterodimeric receptor), thereby allowing proteolytic cleavage of NOTCH (possibly including an internalization step in the signal-receiving cell, see above),

as recently demonstrated in *Drosophila* germ lines (849). However, for DELTA at least, endocytosis also appears to be necessary for ligand "activation." Endocytosis and recycling of DELTA to restricted regions of the PM are probably required to maintain sufficiently high local levels of ligand to cause robust NOTCH activation, as also supported by recent findings in *Drosophila* (632; see also section VII*C*). Consistent with this idea, clustering of DSL ligands can potentiate their signaling effects in mammalian cell culture assays (311, 718). Within this context, specific posttranslational modifications of DSL ligands in the sorting or recycling compartments, such as monoubiquitination of their intracellular domains, might also activate the ligands (reviewed in Ref. 223), through mechanisms that remain to be clarified (Figure 1*A*).

Like NOTCH, EPH receptors also recognize membrane-bound ligands, called ephrins, present on neighboring cells. This subfamily of RTKs is involved in development of the nervous system and is important in establishing cell-cell contacts. Ligand-receptor interactions trigger bidirectional endocytosis, during which the ephrin-containing cell internalizes EPH receptor, and vice versa. Ultimately each cell contributes to controlling its neighbor's ligand and surface receptor availability. This regulatory mechanism is important for the activation of RHO GTPases and results in changes in the actin cytoskeleton that mediate the repulsion or attraction of neighboring cells (reviewed in Ref. 187).

3 Regulation of the assembly of PM-specific platforms—The differential distribution of signaling effectors between the PM and the endosomal compartment contributes to the spatial and temporal regulation of signals. Removal of receptors from the PM, through endocytosis, additionally extinguishes certain signals that depend on PM-specific molecules. For example, GPCR signaling via PM potassium channels requires that both receptors and K^+ channels are present in the same membrane in order for the trimeric G proteins to initiate signaling (502). Furthermore, GPCR-mediated signaling via phospholipase C (PLC) requires the prevalent, if not exclusive, localization of the PLC substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), at the PM (511). Similarly, PLC and phosphoinositide 3-kinase (PI3K) signaling by the EGFR is virtually abrogated by receptor internalization due to the lack of PIP_2 in endosomes (295) (Figure 1*A*).

The interplay between PM-enriched inositol-containing phospholipids, such as PIP_2 and phosphatidylinositol 3,4,5-triphosphate (PIP_3), endocytosis and signaling is rather complex, and extends well beyond the mere extinction of PIP_2-requiring signals at the PM. It is known that both PIP_2 and PIP_3 regulate CCP dynamics (142, 369, 550, 886). Indeed PIP_2 can bind, and concentrate at the PM, a variety of endocytic proteins including clathrin adaptors (reviewed in Ref. 168) and dynamin (633). PIP_3 can also bind to endocytic adaptors (243, 351, 638). The requirement for tight regulation of phosphoinositide turnover in the formation of CCPs is underscored by the presence in CCPs of enzymes that synthesize PIP_2 and PIP_3 [type I PIP kinases, (38, 410, 550, 769)], as well as of enzymes responsible for their catabolism [the inositol-5-phosphatases synaptojanin-1, OCRL, and SHIP2, (126, 196, 493, 550, 604)]. The various phosphatases are recruited to CCPs with time kinetics that are partly overlapping and partly sequential, suggesting that *1*) a coordinated action of multiple phosphatases is required in CCP formation and *2*) a certain level of redundancy and robustness is built in the system. A recent study elucidates how the action of one of these

phosphatases, synaptojanin 1, might be regulated by a geometry-based mechanism to contribute to the spatial restriction of PIP_2 elimination in certain membrane domains (115). Synaptojanin 1 is recruited to sites of internalization predominantly through interaction with the BAR-containing protein endophilin (242, 651). Through the action of its BAR domain, endophilin participates in and senses membrane curvature. It was shown that, by acting in concert with endophilin, synaptojanin 1 preferentially removes PIP_2 from curved membranes, with respect to flat ones (115). Interestingly, these data are in line with recently developed systems models, developed for yeast endocytosis, highlighting the importance of the connection between chemical reactions and mechanical deformation of the PM (465, 466; see also sect. VI*B1*).

Finally, it is worth mentioning here that GPCR-mediated signaling is regulated by β-arrestin (ARR)-mediated internalization (reviewed in Refs. 292, 495) (Figure 1*A*), a process that is intimately linked with endocytosis and signaling. The complexity of this liaison represents an almost paradigmatic case of the partnership between these two cellular programs, something we will cover briefly in section VII*F2*, while referring the reader to a recent excellent review for an in-depth analysis (485).

B The Integration of Different Endocytic Routes Controls the Net Biological Output

The biological output of a specific signal can be controlled not only by internalization of receptors and/or ligands into endosomal organelles, but also by the routes through which receptors reach the different compartments (Figure 1*B*).

Many signaling receptors, including RTKs, GPCRs, tumor growth factor β receptor (TGFβR), WNT, and NOTCH, undergo both CME and NCE, and the relative partitioning of receptors between the two entry routes determines the final net signaling/extinction output. TGFβR, for example, can be internalized by both CME and NCE (through the caveolar pathway). Binding of the FYVE-domain containing adaptor protein SARA (smad anchor for receptor activation) to the TGFβR at the PM causes receptor routing to signaling-competent endosomal vesicles. Conversely, recruitment of SMAD7 to the receptor is associated with additional binding of the E3 UB ligase SMURF, leading to receptor ubiquitination, internalization through an NCE route, and subsequent degradation (167). A similar scenario might also be operational during signaling and internalization of the EGFR. In this case, EGFR is committed to a signaling-competent or a degradative pathway as a function of ligand dose (722). At low doses of EGF, the EGFR is almost exclusively internalized through CME; at higher doses, the receptor is internalized through both CME and NCE (721, 722). CME leads to recycling of the receptor and sustained signaling, with limited degradation (721). The NCE route [which in the case of EGFR is still poorly characterized, although it has been shown to be clathrin- and caveolin-independent (721)] leads instead to an MVB-dependent degradative pathway, limiting both the duration and the intensity of EGFR-dependent signaling pathways (721) (see also sect. V*C1*). In other studies, however, the internalization of EGFR was reported to be exclusively through CME at all concentrations of EGF (386, 639). The discrepancy between these two scenarios may lie in cell-specific differences between the EGFR internalization models studied by the different groups.

Whereas TGFβR and EGFR (at least in some cases) utilize CME for signaling/recycling, and NCE for degradation (Figure 1*B*), other cargoes exploit the two types of internalization routes in the opposite manner, as is the case for the WNT3a-activated low-density receptor-related protein 6 (LRP6). Signaling by LRP6 is associated with internalization by NCE, while degradation is clathrin dependent. In the presence of WNT3a, LRP6 is phosphorylated and internalized into a caveolin-positive vesicular compartment, where it can stabilize β-catenin and transduce the signal via the CK1γ kinase. LRP6 can also bind the WNT3a antagonist DKK, which diverts it from the caveolin to the clathrin pathway. This prevents the encounter of LRP6 with CK1γ (that is restricted to caveolin-positive compartments), ultimately resulting in enhanced β-catenin degradation (865).

Notably, the regulation of the WNT-LRP6 canonical signaling can also take place at the endosomal levels. A well-known target of this signaling axis is represented by glycogen synthase kinase 3 (GSK3), which is normally active and free in the cytoplasm to phosphorylate β-catenin, thereby promoting its degradation (14). Upon internalization, however, activated LRP6 is directed to endosomes where it can sequester GSK3, preventing its encounter with β-catenin (143). Remarkably, the sequestration of GSK3 can also occur in late endosome/MVB, and this appears to be critical for long-lasting β-catenin activity (764). Collectively, these results illustrate how internalization portals and endocytic routes contribute to finely tune signaling outcome.

C Endosomes Are Signaling Stations

A large body of evidence supports the notion that signaling is not restricted to the PM. As internalization proceeds, activated transmembrane molecules, with their tails exposed toward the cell cytoplasm, are confined and enriched within endomembrane organelles, from where they continue to signal. Such structures are bona fide signaling platforms that influence the duration, amplitude, and specificity of the downstream signals (reviewed in Refs. 265, 744). Consistent with this view, a growing number of signal transduction pathways are reported to require active endocytic machinery, or to originate exclusively from various types of endosomes. The term *signaling endosome* embodies this concept (Figure 1*C*). The concept was first proposed by the Bergeron group showing that EGFR internalization leads to the assembly of a variety of signaling complexes and their compartmentalization into endosomal vesicles (166). The actual term *signaling endosome* (Figure 1*C*) was later introduced as a hypothesis to account for the long-distance retrograde transmission of neurotrophic signals along axons (54, 272). In the last decade, this concept has received overwhelming experimental support, and it has now entered in the common language of signaling to indicate that endosomes (and MVBs) are signaling compartments, which confer time- and space-resolution to signals that would otherwise be only partially informative, and which add specificity to signaling through a variety of molecular mechanisms.

A number of specific features of endosomes make them ideal for both signal propagation and specificity. Endosomes are characterized by *1*) a limited volume that may favor the interaction between receptor and ligand when the two are internalized in the same vesicle, further sustaining receptor activity; *2*) a relatively longer resident time of activated receptors, with respect to the PM from which active receptors are rapidly removed as a consequence of

internalization; *3*) a scaffold-promoting microenvironment, thanks to their enrichment in particular lipids or proteins [such as the lipid phosphatidylinositol 3-phosphate (PI3P), or the protein P18] that are able to assemble specific signaling complexes; *4*) a chemically defined microenvironment characterized by low pH, which favors specific reactions, such as proteolysis of signaling molecules. Put simply, endosomes can influence signaling either by sustaining signals originating from the PM or by contributing to signal specificity through their provision of a platform for the assembly of specific signaling complexes that are prohibited at the PM. These concepts have been discussed in detail in a series of recent reviews (265, 694, 744). Here we provide a few examples of variations on these themes.

1 Endosomes sustain signals—RTK endosomal signaling serves as a canonical example of continuous signaling. The first compelling evidence in support of this concept was provided by the selective inhibition of EGF-induced activation of PI3K and extracellular signal-regulated kinases (ERKs) by a dominant-interfering mutant of dynamin that arrests EGF internalization (807). Indeed, several signaling receptors, including RTKs and the TGFβR, remain bound to their ligand and active once internalized within endosomes, thus being capable of sustained signaling (93, 166, 272, 294, 300, 325, 420, 603, 813, 824, 829) (for a notable exception in the case of the TGFα:EGFR interaction, see sect. III*D*).

An additional level of endosomal regulation of signals is provided by the observation that all components of the ERK activation cascade can be detected in endosomes (613, 666, 859). Endosomal-specific proteins that serve to sustain signaling have been identified, such as P18 that serves as an anchor for an ERK-activating scaffold and is required for the maximal amplitude of ERK1/2 phosphorylation (547) (Figure 1*C*). This further reinforces the notion of endosomes as specific signal-sustaining stations. A similar situation also occurs in the case of GPCR signaling where ARR, similarly to P18, acts as a specific scaffold stably anchoring ERK1/2 to the endosome. This seems to prolong signaling, and it has also been proposed to bias signaling towards cytosolic rather than nuclear ERK substrates (163).

Two recent studies have also highlighted the requirement of GPCR/G protein internalization for persistent Gα_s signaling (leading to cAMP production) from an endosomal compartment (98, 218) (Figure 1*C*). In the case of the parathyroid hormone receptor (PTHR), the stability of the receptor-hormone complex is critical. The PTHR responds to two native ligands; the endocrine parathyroid hormone (PTH) and paracrine acting PTH-related protein (PTHrP) with divergent downstream effects. Treatment with PTH, but not PTHrP, causes prolonged GPCR activation and G protein signal response (218). PTH also stimulates cotrafficking of PTHR with Gα_s into early endosomes. A dynamin dominant negative mutant prevents sustained cAMP production in response to PTH. Conversely, PTHrP causes the dissociation of Gα_s from its receptor prior to PTHR internalization (218). Thus PTH-induced internalization of the receptor into the endosomal compartment is required for sustained signaling, possibly protecting the receptor from ARR-mediated desensitization.

2 Endosomes transmit signals over long distances—Neurotrophin receptors at nerve terminals must send their signals to the neuronal body and the nucleus that may be up to a meter away. The speed with which signals travel cannot be accounted for by simple diffusion. Internalized nerve growth factor (NGF) bound to its cognate TRKA receptor is

sequestered into early endosomes, where it remains fully competent to signal to the ERK1/2 and PI3K/AKT pathways. The NGF-containing endomembranes can be transported by retrograde microtubule- and dynein-based transport to their targets where signal is delivered, promoting survival (reviewed in Refs. 324, 343). Recently, alternative routes of NGF-TRKA internalization have been unveiled, showing that the activated receptor can also undergo clathrin-independent macropinocytosis, which requires the activity of the small GTPase RAC and the trafficking protein PINCHER (790, 791). NGF-TRKA-positive macropinocytic endosomes are resistant to degradation and are long-lived structures, suggesting, by analogy to early endosomes, that they may be involved in promoting sustained or long-range ERK1/2 signaling.

3 Endosomes as intermediate stations between PM and nucleus—In confirmation of the signaling endosome hypothesis, it has been demonstrated in a few cases that endocytic structures can function as obligatory intermediate signaling stations between the PM and the nucleus. For instance, in response to EGFR stimulation, APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) translocates from these endomembranes to the nucleus, where it interacts with the nucleosome remodeling and histone deacetylase multiprotein complex NURD/MECP1, an established regulator of chromatin structure and gene expression (42, 523).

A somewhat similar mechanism exploiting the endosomal machinery appears to be involved in the propagation of signaling from the RTK MET to the activation of the multifunctional transcription factor STAT3 (signal transducer and activator of transcription). Stimulation of MET by its ligand (the hepatocyte growth factor, HGF) results in recruitment of STAT3, which is believed to be activated by the receptor at the PM. STAT3 then travels, via endocytic organelles, to the nucleus where it controls gene transcription. Recent observations have demonstrated that the strength of the signaling response is related to trafficking of the receptor and downstream signaling components (390) (Figure 1*C*). Endosomal trafficking may thus not only serve as an efficient directional transport mechanism of STAT3 into the nucleus, but it may also protect weakly activated STAT3 from cytosolic phosphatases (390).

4 Endosomes specify signaling—An increasing number of reports have demonstrated the existence of endosome-specific signaling platforms. This is the case for so-called SARA-endosomes and the APPL-endosomes, involved in signaling by the TGFβR and by RTKs, respectively.

Endosomal recruitment of SMAD2 by the FYVE domain adaptor SARA allows efficient phosphorylation of SMAD2 by internalized TGFR. Once phosphorylated, SMAD2 dissociates from the complex and interacts with SMAD4. The SMAD2-SMAD4 complex then translocates to the nucleus, where it regulates gene transcription (122, 300, 783) (Figure 1*C*).

APPL1-containing endosomes are a population of precursors of early endosomes that contain RAB5 but lack the RAB5-binding protein EEA1. The interaction of APPL1 with RAB5 appears to be part of a control mechanism that couples the release of APPL1 from

these very early endosomes to growth factor signaling and trafficking (458, 523). It was recently proposed that APPLs and EEA1 compete for binding to GTP-bound RAB5, and their recruitment to early endosomes is regulated by phosphoinositide content (885). Thus the acquisition of PI3P through the recruitment and activation of the class 3 phosphatidylinositol 3-kinase, PI3KC3 (also called Vps34), which can directly phosphorylate phosphoinositol, results in the accumulation of EEA1 and the concomitant dissociation of APPLs in maturing early endosomes. This phosphoinositide-dependent switch mechanism has important consequences on signal duration since APPL-1-positive earlier-stage endosomes are competent for AKT signaling, which is terminated following the acquisition of EEA1 and maturation of the vesicular compartment (885).

Several other receptor systems, such as GPCRs, NOTCH, tumor necrosis factor-1 receptor (TNF1R), and TOLL-like receptors, also exploit signal-specific endosomes and have been reviewed elsewhere (671, 744).

Differential subcellular compartmentalization of the three main RAS isoforms (H-RAS, N-RAS, and K-RAS) is believed to underlie their biological differences. For example, endosomal localization of certain RAS isoforms has been shown to attenuate RAS-ERK signaling in mammalian cells (372). It was shown that shuttling of RAS between the PM and the endosome is mediated by RAS mono- and di-ubiquitination catalyzed by RABEX-5, an E3 ligase and RAB5 exchange factor (862). This process is also conserved in *Drosophila,* where RABEX-5-mediated ubiquitination of RAS leads to endosomal relocalization of RAS, thus restricting its signaling during the establishment of organism size, wing vein pattern, and eye versus antennal fate (866). In this case, the canonical function of endosomes in signal attenuation is therefore coupled to signal specification. This latter mechanism seems also to operate in specifying the signaling of different Ras isoforms. Using live imaging with probes that allow the monitoring of activated Ras and binding to its effectors, it was shown that not only can K-RAS (but not H-RAS or N-RAS) enter endocytic CME pathways, but it is also transported along early endosomes, late endosomes/MVB, and finally into lysosomes. K-RAS is active on late endosomes/MVB, where, together with the p14-MP1 scaffolding complex, it provides an active intracellular signaling platform (480). Thus, although the late endocytic compartment is thought mainly to downregulate signaling by destining receptors and transducers to degradation (see below), it can also selectively regulate specific signaling events.

D Regulation of Signaling by Endosome Sorting

Endosome sorting plays an essential role in the spatial restriction of signals, which prevents signals from becoming uniformly distributed throughout the cell, and consequently uninformative. EECs are necessary for the execution of a number of polarized cellular functions, including directed cell migration, cell-fate decisions, epithelial-cell polarization, growth cone movement, tissue morphogenesis during development, and cell invasion into the surrounding tissues of metastatic cells (see sect. VII, *B* and *C*, for an in-depth discussion).

Endosome sorting also controls signaling fate. Routing of cargo to degradative pathways effectively terminates signaling, while recycling pathways that lead cargo back to PM replenish the supply of substrates for a further round of activation. Both these mechanisms

are exploited by cells and therefore affect temporal and spatial limitation of signaling (reviewed in Refs. 495, 744). Transfer of activated receptors to late endosomes/MVB terminates signaling, either by sequestering receptors in ILVs, thus preventing their interaction with effectors, or by promoting their lysosomal degradation. Receptor ubiquitination plays a critical role in this process (see sect. V for details). On the other hand, recycling of internalized receptors to the PM replenishes the cell surface with ligand-free receptors and also restores receptor sensitivity to extracellular ligands, as is the case for GPCRs. β2 Adrenergic receptor (β2AR) coupling to trimeric G proteins is inhibited by receptor phosphorylation events at the PM (see, for instance, Refs. 56, 57, 610; also reviewed in Ref. 389), which cause functional desensitization of signaling in the absence of endocytosis. However, sorting of internalized ARR-bound receptor into a rapid recycling pathway promotes receptor dephosphorylation by an endosome-associated PP2A protein phosphatase, thus ensuring the return of intact receptor for successive rounds of signaling (resensitization) (611, 801, 868) (Figure 1*A*). The mechanism is signaling-regulated, as it was shown that PI3K hampers βAR resensitization and that it does so by inhibiting PP2A activity (801).

A functionally similar process also occurs during EGFR signaling. Both TGF-α and EGF elicit rapid internalization of EGFR. EGF binding to EGFR is relatively stable at the pH of endosomes, so EGFR remains active in these organelles, before being ubiquitinated (see sect. V) and transported to lysosomes for degradation. In contrast, TGF-α rapidly dissociates from the receptor when exposed to the acidic endosomal environment. As a consequence, the receptor becomes deactivated and is recycled back to the PM (153, 183, 229, 476). This differential trafficking fate is crucial to the duration of the EGFR signal. Following receptor degradation, the EGFR signal will be diminished until the number of EGFRs at the PM has been reestablished by protein synthesis. In contrast, following receptor recycling, the cell is immediately able to undergo an additional round of EGFR activation. In accordance with this, TGF-α is a more potent mitogen than EGF (832). Thus the endosome regulates signaling output for the EGFR, as a function of ligand type bound to the receptor.

IV The Partnership: Signaling Controls Endocytosis

As previously mentioned, endocytosis and signaling are biunivocally related, meaning that the regulation of signaling by endocytosis is mirrored by a reciprocal control of endocytosis by signaling. Section III described the first half of this relationship. This section will instead provide an overview of the latter half of these events.

A Plasticity of the Endocytic Compartment in Response to Signaling

The regulation of endocytosis by signaling events can be directly inferred from the remodeling of endocytic compartments upon growth factor stimulation. Such rearrangements involve the entire endocytic pathway, from vesicles internalizing at the PM all the way down to the endosomal and lysosomal compartments.

1 Plasticity at the plasma membrane—At the PM, it has been reported that that activation of EGFR or TRKA is able to induce newly formed clathrin nucleation sites in epithelial and neuronal cells, respectively (53, 136, 366, 625, 844). Mechanistically, this

correlates with the fact that EGFR activates SRC kinases, which in turn phosphorylate the clathrin heavy chain in the hub domain, causing redistribution of clathrin at the cell periphery and increasing EGFR endocytosis, possibly through the regulation of clathrin assembly (844). It is noteworthy that, even in the case of nonsignaling receptors, such as the transferrin receptor (TFR), it has been recently shown (463) that clustering of receptors can promote initiation of CCP formation, an event that correlates with increased TF uptake induced by TFR clustering. CCPs containing clustered TRF mature more efficiently and exhibit longer lifetime than other CCPs in the same cell (463). The relative contribution of receptor clustering (it should be remembered that signaling receptors cluster as a consequence of ligand binding) and/or of true signaling to the initiation of CCPs remains, therefore, an open question. Regardless, the sum of the results indicates a major impact of cargo composition in the dynamics of CCPs.

Signaling-regulated plasticity of entry portals might not be limited to CME. During mild stimulation of neurons, synaptic vesicle recycling, regulated by clathrin endocytosis, is the main mode of vesicle recovery after neurotransmitter release (269, 883). Such vesicle retrieval occurs in response to exocytosis at the synapse as a result of calcium activation of calcineurin, which causes a rapid dephosphorylation of numerous endocytic proteins, including dynamin and amphiphysin (51, 497). However, during stronger neuronal stimulation, clathrin-independent bulk endocytosis (also called ADBE, activity-dependent bulk endocytosis) steps in to become the dominant mode of internalization (129, 130). The switch to ADBE is regulated by GSK3 activity, possibly through the phosphorylation of dynamin I (130). In the case of EGFR, EGF has been reported to increase the number of caveolae and caveolae-like structures at the PM of epithelial cells (573). Moreover, caveolin and flotillins, two major regulators of non-clathrin pathways of endocytosis (Table 1), are phosphorylated in response to EGF or PDGF, and this has been proposed to be important for the assembly of caveolin- or flotillin-containing pits (33, 436, 563, 573). It should be noted, however, that the relevance of caveolae to the internalization of the EGFR has been questioned (386) and that the emerging signaling role of caveolin-1 imposes caution in extrapolating results obtained on this protein to the physiology of caveolae tout court (an issue that will be dealt with in detail in sect. VII*A*). Thus it remains to be established whether the reported effects of signaling pathways on caveolin (and perhaps flotillin) result in regulation of NCE routes or are part of signaling circuitries not directly pertinent to endocytosis.

2 Plasticity at the endosomal and multivesicular body level—Early endosomes are a critical sorting station in the endocytic pathway, as a number of fate decisions are made in this compartment according to which cargoes can be recycled to the PM, retrotransported to the Golgi, or further routed to late endosomes for eventual degradation in the lysosome. We will concentrate here on the transition from early to late endosomes, in which recent findings highlight an important impact of signaling, while we refer the reader to reviews for other aspects of sorting at the early endosomal station (330, 370, 751).

The debate on the mechanisms of transition from early to late endosomes is almost as old as the concept itself of endosomes (305). In one “classical” model (the vesicular transport model), vesicles budding from early endosomes fuse with late endosomes; according to this

hypothesis, endosomal compartments are more or less static and connected by vesicular transport. In another, more dynamic, model (the maturation model), a series of events progressively occurring on the surface of early endosomes convert them into late endosomes. At the biochemical level, RAB GTPases play an essential role in the control of sorting events. These proteins determine the functional organization of different endosomal compartments, by generating biochemically distinct RAB-containing membrane domains both in early and late endosomes (excellently reviewed in Ref. 757). With reference to the early-to-late endosome switch, two RABs are critical, RAB5 and RAB7, which label, by and large, early and late endosomes, respectively (119). However, RAB5 and RAB7 are also simultaneously present on a subpopulation of early endosomes, in two distinct membrane domains (652, 810). The question is, therefore, how the two domains become separated, to give rise to RAB7-containing endosomes. In support of a classical vesicle transport model, budding and fission of RAB7 domains from RAB5-positive endosomes have been observed (810). However, the Zerial group has observed that endosomal membranes switch from being RAB5-positive to being RAB7-positive as they proceed from early to late endosomes, or, in other words, that the RAB5 domain decreases while the RAB7 one increases, on individual endosomes (652). This mechanism, dubbed “RAB conversion,” directly supports a maturation model. Additional support for maturation mechanisms in the endocytic pathway has come from recent studies of the De Camilli group that showed PI3P-dependent maturation of APPL endosomes (see sect. III*C4*) (885), and of the Donaldson group that showed that newly formed endocytic vesicles can convert directly into early endosomes (617).

From the signaling perspective, it is relevant that the RAB conversion is controlled by the progressive concentration of specific cargoes at the endosomal station. For instance, ligands destined for degradation, such as low-density lipoproteins (LDL), progressively concentrate in fewer and larger endosomes that migrate from the cell periphery to the center, where RAB5 is replaced by RAB7 (618, 652). Importantly, growth factor receptors influence the kinetics of this kind of transport, as shown by the fact that treatment with EGF slows down LDL transport possibly by activating RAB5 and delaying the RAB5-to-7 conversion (618, 652) (for systems biology approaches to the phenomenon of RAB conversion, see section VI*B1*).

Another station at which signaling exerts control over endocytic dynamics is the MVB. It was shown that EGF stimulation controls *1*) MVB biogenesis, by increasing the number of MVBs per unit of cytoplasm, and *2*) inward vesiculation, by increasing the number of ILVs per MVB (840).

The effect of EGF on MVB biogenesis was found to depend on TSG101, a component of the ESCRT-I machinery (643). Although the molecular mechanisms are unknown, the involvement of TSG101 must rely on an EGFR-mediated signaling component since the effects of TSG101 depletion on MVB formation were much greater in EGF-stimulated versus unstimulated cells (643). A more recent study, in which all four ESCRT complexes were simultaneously silenced, shed additional light. It was found that, in pan-ESCRT-depleted cells, the formation of EGF-induced MVBs was inhibited; however, the formation of EGF-independent MVBs was still allowed, albeit with considerable alterations in

morphology (759). Thus at least two major mechanisms of MVB formation seem to exist: an ESCRT-dependent one, which is regulated by EGFR signaling, and an ESCRT-independent one, which is EGFR independent. It remains to be seen whether this latter mode is constitutive or whether it depends on other types of, as yet unknown, signals.

Similarly, the stimulation of inward vesiculation was specific to the subset of EGFR-containing MVBs and required the phosphorylation of annexin I (840), which is known to be a major substrate of the EGFR in the MVB (241). Importantly, annexin I was neither required for the formation of ILVs in unstimulated cells, nor for EGF-induced MVB biogenesis (840), arguing that there are at least two mechanistically distinct pathways through which EGFR regulates MVB function. In ILV formation, the EGFR-mediated signal, through annexin I, operates downstream of ESCRT proteins (840), raising the possibility that this mechanism, as well, is ESCRT independent. In this latter contention, it is to be noted that at least two ESCRT-independent mechanisms of ILV formation have been reported (768, 780) (see also sect. VII*E*).

At the functional level, the dual control by EGF on MVB function probably results in different mechanisms of signal attenuation. The effect on MVB biogenesis is most likely functional to EGFR degradation, since all ESCRT proteins are equally required for the degradation of the EGFR (630). On the other hand, loss of annexin I (which impacts on EGF-induced ILV formation) has little effect on EGF degradation (840). In this case, increased sequestration of EGFR into ILVs might represent a mechanism to remove the EGFR catalytic kinase domain from the cytosol, thereby terminating its signaling from the endosomal compartment. It was recently shown that this process is regulated by the tyrosine phosphatase PTP-1B, which dephosphorylates the EGFR at the limiting membrane of the MVB (186). Interestingly, PTP-1B localizes at the cytoplasmic face of the endoplasmic reticulum (ER) (228), and it was shown that the interaction between EGFR and PTP-1B is made possible by membrane contacts between the MVB and the ER (186, 285).

3 Genome-wide approaches to plasticity—System-wide analysis of the endocytic pathway (132, 484, 597, 735) has helped the understanding of some of the regulatory networks at the basis of the endocytic system (see also sect. VI). In one recent study, RNAi-based high-throughput screening on a genome-wide scale was combined with multiparametric image analysis to profile EGF and transferrin (TF) endocytosis (132). This analysis further reinforced the idea that the endocytic pathway does not involve simple transport from one static compartment to another, but, rather, it is dynamically regulated and rearranged depending on the type and intensity of signaling and the cargoes engaged. Indeed, various signaling pathways, such as integrin, TGFβR, NOTCH, and WNT, were found to affect both EGF and TF endocytosis, possibly by exerting feedback mechanisms on the endocytic machinery itself. In addition, cells seem to specifically regulate the number of EGF-positive endosomes, tightly coupling their number, size, and intracellular location to cargo concentration (132). This might have a significant impact on endosomal signaling, controlling both quality and strength of signaling outputs, ultimately influencing cell fate.

A similar systems biology approach was applied to investigate population context-dependent phenotypes in endocytosis, using viruses as tools to follow endocytic pathways (735). This

analysis revealed that CME (determined as a function of TF internalization and MHV, mouse hepatitis virus, infection) is mostly active in densely populated cell cultures, while SV40 and cholera toxin entry, which depend on lipid microdomains, are more efficient in sparsely populated cell cultures. Local cell density regulates endocytosis through the modulation of surface levels of the sphingolipid GM1 and activation of focal adhesion kinase (FAK). Therefore, a specific signaling pathway, controlled by population context, regulates virus infection and endocytosis. Follow-up of these "high-throughput" studies is needed to gain a deeper understanding of the molecular mechanisms responsible for these signaling circuitries and their biological implications (see also sect. VI).

B Endocytic Regulation by Actin Signaling

Endocytosis and actin dynamics are intimately connected. On the one hand, endocytosis and recycling can exert spatial and temporal control over a number of critical regulators of actin dynamics, thus influencing the biological outcome of a variety of actin-based processes, as will be discussed in section VII*B*. On the other hand, there is a clear requirement for the actin cytoskeleton along the various steps of the endocytic process, both to provide structural support for membrane trafficking intermediates, such as tubules emanating from endocytic vesicles, and to generate forces that aid either the deformation of membranes into invaginations or the scission of vesicles and their motility. In addition, actin assembly and polymerization are spatially and dynamically controlled by signaling, suggesting that pathways regulating this process may therefore also influence membrane trafficking. However, evidence in support of this latter contention has only recently started to emerge (reviewed in Refs. 431, 440, 614). In this section, we briefly illustrate established and evolutionarily conserved mechanisms through which actin dynamics modulate and control the different steps of the endocytic process; more extensive descriptions can be found in recent reviews (135, 245, 375).

A tight regulation of the actin cytoskeleton is crucial for various modes of cellular uptake, including phagocytosis, macropinocytosis, and some forms of NCE (see sect. II). However, the role of actin dynamics in CME is still matter of debate. Much of the discussion stems from the fact that while CME in yeast is entirely dependent on actin dynamics for curvature of the membrane, stabilization of vesicular intermediates and pinching-off, a similar requirement in mammals, has not yet been established. In mammals, for instance, proteins such as dynamin appear to have a greater role both in deforming the membrane and pinching off vesicles, thus making the role of actin dynamics less critical than in yeast (465). Consistently, drugs that disrupt the actin cytoskeleton and its dynamics have been reported to affect CCP dynamics and lateral mobility within the PM, although their effects appear to be cell-type dependent (519, 672, 871). In keeping with this notion and further complicating the matter, recent observations revealed the existence of two classes of clathrin-containing structures at the PM with distinct kinetic properties, namely, coated pits, the classical short-lived endocytic invaginations, and coated plaques, longer-lived structures present on the basal membrane of some adherent cell types. While the former are internalized in an actin-independent manner, the latter rely entirely on a functional actin cytoskeleton for their dynamics and internalization (672).

This notwithstanding, the evidence implicating the actin cytoskeleton in aiding or facilitating CME is mounting also in mammals. For example, a plethora of mammalian proteins directly link the endocytic pathway with the actin cytoskeleton, potentially regulating actin assembly at endocytic sites (Figure 2A). Just to name a few: HIP1R binds clathrin, actin, and cortactin and may locally slow down actin assembly rates (432); intersectin binds to N-WASP, a well-established nucleator promoting factor that is recruited to CCP and facilitates CME (340); dynamin directly binds to cortactin and ABP1 (both inducers of actin polymerization), and these interactions may support endocytosis by stimulating cytoskeletal functions as well as regulating cell shape and movement (392, 513). In addition, live imaging studies have shown that actin, together with actin-regulatory proteins, is invariably recruited to CCPs before their detachment from the PM, in a fashion and timing not dissimilar to what has been demonstrated in yeast (374, 519, 871) (Figure 2*A*). Finally, biochemical reconstitution experiments, using purified proteins and lipid vesicles to mimic the initial endocytic steps, have demonstrated that a stabilized actin network is involved in the generation of pulling forces necessary to bend the membrane for invagination and subsequent scission (665) (Figure 2*A*). Remarkably, these latter findings are entirely consistent with the role attributed to actin in yeast, where the presence of the cell wall and an elevated turgor pressure requires actin-based forces to allow early endocytic events to occur (3). In support of this model, increasing turgor pressure has been shown to hinder endocytosis, whereas reducing the pressure suppresses the internalization defects of actin-bundling mutants and of cells treated with actin depolymerizing drugs (3).

The actin cytoskeleton, in addition to generating pulling inward forces that aid the bending of the PM, may also contribute to local changes in the lipid organization that correlate with the formation of membrane microdomains on model membranes, suggesting that domain boundary forces are driving tubule membrane constriction. It was recently shown that an actin shell that nucleates along the sides of invaginating membrane tubules causes local membrane reorganization, lipid domain repartition, and line tension (659) (Figure 2*A*). These areas of membrane discontinuity and accumulated tension may facilitate scission by a physical mechanism that can function independently from or in synergy with pinchase activities, such as the one provided by dynamin. It must be pointed out that this mechanism has been shown to operate in clathrin-independent endocytic processes, but its relevance in CME remains to be established.

Compared with yeast, mammals have a relatively lower internal counter pressure, and thus a lower force to oppose invagination. This may also account for early findings suggesting that dynamin may be sufficient to promote constriction and generate tension for vesicle pinching-off (665), although at the neck of budding vesicles other factors leading to tension have been proposed to cooperate with the activity of dynamin (665). Indeed, work in dynamin 1 and 2 double-knockout (KO) mice showed that while vesicle scission was hindered, membrane invagination and tubule formation still occurred through the coordinated action of membrane bending (mediated by BAR-containing proteins) and actin dynamics. BAR protein and actin likely act upstream of dynamin (through clathrin-dependent recruitment mechanisms), generating tubular intermediates of size and composition that are remarkably similar to the endocytic tubular invaginations in yeast (215, 350, 857) (Figure 2*A*). All these studies contribute to reconcile divergent views of

endocytosis in different organisms and suggest that the underlying processes are fundamentally conserved. Thus actin dynamics may modulate local membrane tension to facilitate invagination, scission, and vesicle movement of CCPs both in yeast and mammals. Yet, a number of unresolved discrepancies still remain. These differences likely reflect the plasticity and increasing complexity of uptake mechanisms in various organisms and cell types, as witnessed by the observations that a single mammalian cell can employ a large spectrum of endocytic mechanisms to internalize different cargoes, each characterized by different requirements for clathrin, dynamin, actin regulators, and lipids (as discussed in sect. II).

Actin is further involved, in both yeast and mammals, in moving vesicles away from the PM, for their subsequent trafficking along the endocytic pathway (reviewed in Refs. 262, 615, 739) (Figure 2*A*). Within this context, actin dynamics provide a default basic engine to propel vesicle motility inside the cells, in close cooperation with the microtubule-dependent transport system (reviewed in Ref. 660). However, recent evidence indicates that actin polymerization can also be regulated in a cargo-dependent manner at specific endocytic sites to control cargo sorting. At the endosomal station of mammalian cells, the actin machinery can generate specialized subsets of tubular microdomains involved in sequence-dependent recycling of the β2AR family of signaling and endocytic receptors (626). An actin shell is specifically recruited along β2AR-positive tubules, emanating from early endosomes, and this shell is significantly more stable compared with the highly dynamic tubules involved in bulk endocytosis. This stability allows slow-diffusing, sequence-dependent cargoes enough time to enter into recycling pathways, and thus actin dynamics can ultimately control the fate of internalized, recycled receptors. Notably, the actin cytoskeleton at these sites also promotes the concentration of cargo, which is specifically recruited to these microdomains through interactions between PDZ domains, contained in actin regulatory proteins, and PDZ-binding motifs in β2AR (626). Therefore, efficient sorting at the endosomal station is exerted through a combination of kinetic and affinity mechanisms, both of which are mediated by actin. These results have established a connection between endosomal and actin dynamics that is clearly distinct from the function of actin in endosome motility. Finally, it is worth noting here that the connection between actin and endocytosis is bidirectional, and membrane dynamics control, in turn, the actin cytoskeleton (Figure 2*B and* sect. VII*B*)

C Transcriptional Programs Controlling Endocytosis

Signaling also controls the endocytic pathway through the activation/repression of specific transcriptional programs. Several stress-induced signaling pathways directly modulate the transcription of endocytic proteins to induce specific cellular responses, as illustrated here using a few specific examples (see also Figure 3).

Under hypoxia conditions, the hypoxia-inducible factor HIF1α was shown to inhibit the transcription of RABAP-TIN-5 gene, a critical RAB5 effector (825). This impairs RAB5-mediated early endosome fusion and delays the endocytic pathway. As a consequence, the resident time of activated EGFR in endosomes is prolonged, and signaling is sustained leading to cell proliferation and survival (825). In agreement with this mechanism, tumor

hypoxia and HIF1α overexpression correlate with an aggressive phenotype and poor patient prognosis (825, 878) (Figure 3*A*).

Lysosomal stress can activate a transcriptional circuitry controlling endocytosis. In lysosomal storage disorders (LSDs), the transcription factor TFEB translocates from the cytoplasm to the nucleus and mediates the coordinated transcriptional activation of lysosomal genes to stimulate lysosome biogenesis and function, which facilitates the clearance of nondegraded molecules (684). These observations illustrate how lysosomal function is tightly and specifically modulated in response to cellular needs (Figure 3*B*).

Stress signals activating TP53 transcriptional programs have been also shown to affect the endocytic compartment. Indeed, TP53 regulates genes, such as TSAP6 (also known as STEAP3) and CHMP4C (which encodes a subunit of the ESCRT-III complex localized to the MVB), that are involved in exosome production (see also sect. VII*E*), as well as DRAM that encodes a lysosomal membrane protein required for the induction of the autophagy pathway (872). In addition, TP53 transcriptionally regulates caveolin-1, a regulator of caveolar function (see sect. VII*A*). As a consequence of TP53 activation, caveolin-1 and EGFR are reported to be simultaneously internalized from the PM and to be directed to the MVB compartment (872). Therefore, one of the mechanisms through which the TP53 program suppresses cell growth and division in response to stress might be the regulation of endocytosis (Figure 3, *C* and *D*).

Indeed, it appears that the oncogenic potential of TP53 point mutants found in human cancer may partly lie in their regulation of the endocytic pathway (542). It has recently been demonstrated that these mutants not only lose tumor suppression activity, but that they also promote invasion and metastasis by inducing RCP (RAB-coupling protein)-mediated recycling of integrins and EGFR, thus sustaining AKT signaling and inducing cell migration (542). The mechanism involved is not completely understood, but it possibly relies on the inhibition of P63 (a TP53 family member) transcriptional activity (Figure 3*E*).

TP53 may also regulate the endocytic machinery through mechanisms that are independent from its role as a transcription factor. In fact, TP53 has been found to localize in the cytoplasm, mitochondria, and centrosomes (reviewed in Ref. 270), where it has been proposed to prevent tumorigenesis through nontranscriptional mechanisms (see also sect. VII). It was recently shown that TP53 binds to the clathrin heavy chain not only in the nucleus (as we will discuss in sect. VII*FI*), but also in the cytoplasm, and it was found to colocalize with clathrin heavy chain and EGFR at the PM upon EGF stimulation. Thus a possible role of TP53 in the regulation of EGFR endocytosis has been suggested (194). TP53 ablation delays receptor internalization and increases EGFR signaling, suggesting that TP53 might regulate EGFR endocytosis to control specific signaling outcomes (194). While the transcription-independent connection between TP53 and endocytosis needs further validation and independent confirmation, it might add to the nonnuclear functions of TP53.

V Multiple Roles of Ubiquitin in the Connection Between Endocytosis and Signaling

Posttranslational modification of signaling receptors by the covalent attachment of one, or often more, UB moieties has emerged as the major regulatory mechanism responsible for receptor "downregulation." Ubiquitination is a complex process executed by a cascade of enzymes, whose final effectors, the UB ligases, or E3 enzymes, catalyze the addition of a UB moiety or of a UB chain to their substrates. E3 substrates can therefore be monoubiquitinated (when a single UB is appended), multiple monoubiquitinated (when single UBs are appended to multiple sites), or polyubiquitinated (when substrates are conjugated to a UB chain). In addition, UB chains display different topologies, according to the linkages joining the various UB moieties in the chain. The reader interested in understanding the enormous impact of ubiquitination on cell physiology and pathology has only to start picking among the ~4,000 reviews retrievable from PubMed using the search word *ubiquitin*. Here we will limit ourselves to the connections between UB and endocytosis, exclusively from the signaling perspective.

Pioneering work in yeast has demonstrated that UB is required for the first step in cargo internalization, as well as for targeting cargoes to vacuoles (the yeast equivalent of lysosomes) (310, 404). Following these initial observations, there are now numerous reports of ubiquitination of a vast array of mammalian signaling receptors, such as RTKs, GPCRs, growth hormone receptor (GHR), MHC-I, NOTCH, various channels and transporters, cytokine, and interferon receptors. The molecular basis of UB-dependent regulation of endocytosis is being clarified. UB-mediated internalization/sorting of membrane receptors requires accurate recognition of the ubiquitinated cargo by endocytic UB receptors, proteins containing one or more UB-binding domain (UBD). Such UB-binding "route-controllers" inexorably ferry the internalized receptor towards a degradative fate in lysosomes and away from a recycling pathway. We will describe two reciprocal aspects of the process, namely, how signaling controls ubiquitination (ligand-induced ubiquitination of receptors and of endocytic adaptors) and how ubiquitination controls endocytosis, fate, and consequently signaling.

A Ligand-Induced Ubiquitination of Cargo

Activation of signaling receptors can transmit signals to the ubiquitination machinery that then modifies the receptors themselves. The best-characterized circuitry involves the E3 ligase CBL, which is responsible for the ubiquitination of several RTKs. In the case of EGFR and MET, the molecular mechanism of receptor ubiquitination has been investigated in detail. In both cases, CBL binds directly to phosphotyrosine (pY)-sites on the activated receptor through its NH_2-terminal tyrosine kinase binding (TKB) domain (450, 606), as well as indirectly through its constitutive partner GRB2, which is recruited to receptors via other pY sites (336, 364, 831). Once bound, the ligase is phosphorylated and consequently activated (383). Both direct and indirect interactions of CBL with EGFR or MET are required for the full ubiquitination of these receptors (Figure 4*A*).

Another class of E3 ligases, the HECT NEDD4 family (663), whose regulation has been extensively studied, also regulates endocytosis and sorting of numerous signaling receptors. Of these ligases, AIP4 can interact directly with CXCR4 via a noncanonical WW domain-mediated interaction involving serine residues within the COOH-terminal tail of CXCR4. These serine residues are phosphorylated upon agonist activation and are critical for mediating agonist-promoted binding of AIP4 and the subsequent ubiquitination and degradation of CXCR4 (62) (Figure 4*B*). Once again, the ligase appears to be regulated by its phosphorylation. AIP4 phosphorylation is activated by JNK1 (244), which presumably leads to conformational changes that disrupt the inhibitory intramolecular interactions between its WW and the HECT domains. A similar regulatory mechanism has been suggested for SMURF2, NEDD4, and WWP2, for which an intramolecular interaction between the C2 and HECT domains has been shown (843). For SMURF2, this in *cis*-autoinhibition can be relieved by binding of SMAD7 to the E3 HECT domain (565).

In some cases, such as for the epithelial Na^+ channel (ENaC), receptor:ligase interaction, and consequent receptor ubiquitination, is the default pathway, with phosphorylation negatively regulating ligase activity. NEDD4–2 binds constitutively to ENaC PPxY-containing motifs and catalyzes its ubiquitination, internalization, and lysosomal targeting. This prevents Na^+ overload in epithelial cells and is necessary for the maintenance of salt and fluid balance in the body. To increase ENaC abundance at the surface and enhance epithelial Na^+ absorption, NEDD4–2 is phosphorylated by various kinases, including PKA, SGK, and IKKβ (reviewed in Ref. 736) (Figure 4*C*). Phosphorylation induces binding of 14–3-3, which prevents NEDD4–2 from binding to ENaC (61, 344).

Finally, specific binding proteins can regulate the process of ubiquitination by acting as adaptors to recruit the E3 to receptors that lack a direct binding motif for the ligase (reviewed in Ref. 446). Recently, the yeast family of ARR-related proteins (ARTs) was shown to direct the yeast HECT RSP5 activity towards PM receptors (457, 556) (Figure 4*D*).

B Ligand-Induced Ubiquitination of Adaptors

Similar to direct receptor ubiquitination, the ubiquitination of endocytic adaptors plays a critical role in endocytosis. The ARR family of proteins is able to direct internalization of the GPCR cargo. Signaling from activated GPCRs is terminated when GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs), leading to the recruitment of ARR that binds to AP-2 and clathrin, causing the whole complex to be internalized. Agonist-stimulated ubiquitination of ARR mediated by the E3 UB ligase MDM2 is critical for rapid receptor internalization (710). MDM2-ARR binding occurs constitutively and does not persist after receptor activation, suggesting that UB modification might cause a conformational change on ARR required to promote internalization. GPCRs themselves can also be ubiquitinated, most likely by NEDD4, an event required for cargo degradation but not internalization (711). Thus after the “phosphorylation code” on the receptor carboxyl tail, UB modifications on both adaptors and receptors result in a “ubiquitination code” that fine-tunes signal strength, localization, and cellular functions of GPCR (Figure 4*E*).

ARR is not the sole example of endocytic adaptor subjected to UB modification. Several components of the downstream endocytic machinery are modified by monoubiquitination

upon RTK activation (284, 384, 616, 716). In most cases, these adaptors are UB receptors that are ubiquitinated by the E3 ligase NEDD4. The presence of a UBD is required for monoubiquitination of the UBD-harboring adaptor, in a process termed "coupled monoubiquitination," whose molecular workings have been elucidated using the endocytic proteins EPS15 and epsin-1 as model systems (206, 851) (Figure 4*F*). On the contrary, the mechanism by which the upstream signal induced by the activated EGFR causes NEDD4 recruitment remains to be clarified.

What is the role of adaptor ubiquitination? Monoubiquitination might permit the formation of several tiers of ubiquitination-dependent interactions in the endosome, by allowing binding of ubiquitinated cargo (through UBDs) and recruiting another layer of UB receptors through a monoUb signal. The result would be signal amplification and progression of ubiquitinated cargoes along the endocytic pathway. Alternatively, it has been proposed that ubiquitination (with particular reference to coupled monoubiquitination) could represent a signal to "switch off" the binding activity of the adaptor (or of other endocytic proteins that undergo the same process), by allowing intramolecular interactions between the UBD and the UB moiety present in *cis* (319). This mechanism might in turn harbor a series of consequences, for instance, the release of ubiquitinated cargo that would thus become available for the next tier of interactions along the endocytic route. In favor of this possibility, it was shown that EPS15-UB fails to localize properly on endocytic vesicles containing internalized EGFR, thereby preventing the interaction between the UBDs contained in EPS15 and EGFR-UB, an event associated to delayed internalization and degradation of the receptor (206, 319). A similar mode of regulation was proposed for RABEX-5, which despite not being an endocytic adaptor is also subject to coupled monoubiquitination (439, 602). In this case, it was shown that monoubiquitination of RABEX-5 was sufficient to prevent its recruitment to endosomes (505). It is to be noted, however, that in many cases endocytic proteins are ubiquitinated at a rather low stoichiometry: an occurrence not immediately compatible with a "switch off" function of ubiquitination, unless the process is tightly regulated locally (i.e., it occurs and it is relevant only on a minor fraction of the endocytic protein in a particular location). In addition, it was recently reported that monoubiquitination of Vps27 (vacuolar protein sorting 27, the yeast homologue of HRS), a component of ESCRT-0, is not required for cargo sorting along the degradative endocytic route (758).

In conclusion, while the relevance of the ubiquitination of endocytic proteins is clear in some cases, it remains obscure in others. One possibility is that the simple idea of a general mechanism should be abandoned and that the role of ubiquitination in endocytosis be established on a per-case basis. This would not be inconceivable, given the extreme versatility and plasticity of ubiquitination as a regulator of protein function. For instance, a relatively unexplored aspect concerns the signaling properties of UB as a tool for the propagation of effector signals, something that might involve also endocytic proteins. In this contention it is of note (as also reviewed in sect. VI*A*) that recent studies highlight how the UB modification induced by activation of the EGFR involves a network of proteins as vast (or vaster) than that based on the more "canonical" modality of signal transmission through pY (22).

C Impact of Ubiquitination on Internalization and Fate

In this section, we will concentrate on the relevance of ubiquitination on *1*) the internalization step of endocytosis and *2*) the determination of fate at the endosomal level. While at the endosomal level the picture is reasonably well defined and most likely identifies a stereotyped and general mechanism to commit cargo to degradation in the lysosome, at the internalization step the situation is much more heterogeneous and, in some instances, still controversial.

1 Cargo ubiquitination and internalization—In yeast it is well established that monoubiquitination of several PM receptors (α-factor receptor, permeases, and transporters) is sufficient to trigger their internalization, although modification with Lys63-linked UB chains speeds up this process (reviewed in Ref. 428). In mammalian cells, the situation is far more complex, and the regulation by UB often varies depending on the receptor system. In the case of several membrane transporters (ENaC and DAT being the best studied examples, reviewed in Refs. 339, 528), ubiquitination is required both for internalization and trafficking of the cargo to the lysosomes. In many other cases, however, receptor ubiquitination does not seem to be essential for the internalization step (while still being essential for sorting at the endosomal level). This has been shown by mutational studies on different receptors, such as EGFR (332, 335, 365, 722), FGFR (296), GHR (266, 796), and GPCRs (e.g., β2-adrenergic receptor and CXCR4, Refs. 494, 709).

The fact that receptor ubiquitination is not indispensable for the internalization step, in the mentioned cases, does not imply that it has no role at all. Indeed, at least three different sets of observations should be taken into account to fully understand the liaison between ubiquitination and internalization in mammalian cells.

1) UB is just one of the multiple internalization signals that might be present in various receptors. This is the case of RTKs where multiple docking sites for the internalization machinery have been identified. EGFR provides the best understood model: through the combination of biochemical, proteomic, and mutational studies, multiple endocytic signals have been identified in the intracytoplasmic moiety of the receptor (259). These include linear recognition motifs [e.g., dileucine and tyrosine-based motifs (333, 743)], pY-based motifs (306, 321, 365, 745), phosphoSer/Thr-based motifs (43, 140, 212, 304, 570, 775), UB sites (332, 335), and acetylation (259) and neddylation sites (577), although the relevance of these two latter modifications to EGFR endocytosis is not established. Such a plethora of signals defines a probable scenario in which *1*) optimal internalization requires cooperation of different signals and of their recruited pathways and *2*) multiple layers of redundancy might be built in the system to ensure robustness. Thus individual signals, such as UB, might not be indispensable, but still participate in the process under physiological conditions.

2) At least in some cases, the UB modification might selectively couple the same receptor with different entry portals. In the case of the EGFR, molecular genetic evidence obtained with receptors mutated in the E3 ligase-binding sites or in the UB-acceptor lysines showed that direct EGFR ubiquitination is not essential to

promote CME (332, 335, 365, 722), while it is essential for NCE (722). Also in the case of TGFβR, receptor ubiquitination is associated with the caveolar endocytic pathways and not with CME, although in this case it is not clear whether receptor ubiquitination is the signal that triggers caveolar endocytosis (167). In addition, different types of ubiquitination might direct the cargo to distinct endocytic routes. In the case of the IGF-1R, the E3 ligase Mdm2 catalyzes the formation of Lys63-linked UB chains and targets receptors to CME, while CBL preferentially utilizes Lys48 and, under these conditions, the internalization of IGF-1R seems to proceed via caveolae (697).

3) Ubiquitination of the endocytic machinery, and not the cargo itself, is often required for internalization, as exemplified by the case of GPCRs or of GHR. Ubiquitination of ARR, but not of the cargo itself, is required for GPCR recruitment to CCPs (708, 709). Recruitment of the E3 ligase TrCP and an intact UB machinery, but not receptor ubiquitination, are required for GHR internalization through CME, possibly through ubiquitination of endocytic adaptors (266, 796). In these cases, therefore, it is the cargo-associated adaptor that provides the signal for ubiquitination.

In conclusion, it is becoming clear that ubiquitination regulates internalization via multiple mechanisms, which are frequently cargo-specific, and in some instances coupled to different entry portals. In addition, cells may have learned how to exploit cargo ubiquitination to add redundancy and robustness to their internalization.

2 Cargo ubiquitination and endosomal sorting—Following internalization, ligand-induced ubiquitination plays a key role in the lysosomal targeting and downregulation of signaling receptors. UB-directed sorting into MVBs is mediated by the ESCRT multiprotein complexes (31, 32, 34, 385, 481, 852, 853; see also sect. II). This conserved machinery performs three distinct but connected functions: *1*) it recognizes ubiquitinated cargoes and prevents their recycling and retrograde trafficking; *2*) it deforms the endosomal membrane, allowing cargo to be sorted into endosomal invaginations; and *3*) it catalyzes the final abscission (breaking off) of the endosomal invaginations, forming intraluminal vesicles that contain the sorted cargo (for exhaustive reviews, see Refs. 338, 631).

Since the rate of receptor downregulation and MVB targeting typically correlates with the extent of receptor ubiquitination in endosomes, interference with this posttranslational processing enhances signaling, such as for mutants in EGFR ubiquitination sites (335). Similarly, RNA or genetic interference with the UB adaptor HRS in mammalian cells or in *Drosophila* results in enhanced signaling by various RTKs, including EGFR and VEGFR (286, 471, 860). The opposite effect (signal impairment of various RTKs) is observed in conditional mouse knockouts of the deubiquitinating enzyme UBPY/USP8 (555). Furthermore, genetic disruption of members of the ESCRT complexes, which are required for membrane fission events, including those that lead to endosomal intraluminal vesicle formation, leads to sustained EGFR signaling in mice (35, 491), and, in *Drosophila*, to NOTCH hyperactivation and neoplastic transformation (788). This latter observation

underscores the emerging involvement of endosomal sorting, and endocytosis in general, in tumorigenesis (for recent reviews, see Refs. 425, 538 and also sect. VIII*B*).

VI Integrating the Partnership: Systems Biology of the Endocytic Network

Even when considering "isolated" signaling circuitries, i.e., under conditions in which reactions and cascades are modeled in a "well-stirred space," there is a consensus that intuition alone will often fall short of providing realistic accounts and interpretations of the results. When space constraints and spatiotemporal dynamics are factored in, systems approaches become indispensable. A true understanding of the integration between endocytosis and signaling circuitries therefore requires systems biology.

In this section, we review some of the most recent advances in the field of endocytosis from a systems perspective. We will cover two rather different, though complementary, approaches to the analysis of molecular networks. The first, the top-down approach, addresses the property of large networks, generally obtained with high-throughput data. Such data are generally static and are analyzed with the tools of network biology (44). Following a general trend, we will also include, in our discussion, papers reporting high-throughput data per se without network analysis. The second brand of systems biology, the bottom-up approach, deals with the analysis of much smaller networks generated by molecular biology and genetics techniques. In this case, the approach involves the formulation of mathematical models and their numerical simulations. Endocytosis has been analyzed both via top-down and bottom-up approaches. In recent years, reviews have discussed the bottom-up approaches of systems biology (in particular, see Refs. 29, 64), while top-down approaches have not been systematically compared. Here, we will present an overview of both approaches, with particular emphasis on results produced in recent years.

A Top-Down and High-Throughput Studies

The analysis of the CME interactome was one of the first examples of network analysis applied to an endocytic pathway (690; see also Ref. 889 for a further network-based analysis). The CME network analysis draws on the extensive amount of biochemical, structural, and proteomic data relating to CME and collected over the last 20 years or so (reviewed in Refs. 173, 185, 371, 781). These studies were integrated with RNA interference screenings that provided a functional characterization of this pathway (132, 315, 334, 539, 597). In addition, the advent of mass spectrometry coupled to organelle purification has recently produced a large amount of quantitative data (see, for instance, Refs. 69, 75, 94, 256), which not only gives information on the identity of the associated proteins, but also on stoichiometry of interaction and extent of contamination by other compartments, particularly in the case of comparative mass spectrometry techniques (for exhaustive reviews, see Refs. 26, 514). RNAi-based screening and mass spectrometry studies were also recently applied to identify components of various NCE pathways (203, 327, 597).

The CME interactome revealed that clathrin and AP-2 are two important hubs of the pathway (i.e., highly connected nodes that regulate the organization of the network) and provided a first framework to understand the properties of the CME network in terms of

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robustness, adaptability, and evolution of the pathway (690). One characteristic of the network is its modularity: small modules can be plugged-in and accommodated at the level of hubs still using the same overall network. This is the case for alternative cargo adaptors that are added to the network by binding to the AP-2 and clathrin hubs for the internalization of specific cargoes (see below and sect. II). Importantly, the interactions are frequently of low affinity, and multiple interactions ensure avidity, thus stabilizing the network (322, 620). This gives rise to a dynamic instability of the network, and a certain number of interactions are required to allow network assembly and pathway progression. Importantly, many of the accessory factors interact with AP-2 and clathrin in a mutually exclusive manner. Indeed, biochemical experiments revealed that the clathrin hub displaces the AP-2 hub, ensuring the timing and directionality of the process (689). It is therefore crucial that network analysis takes into account the dynamic nature of the pathway, where at each step there are significant changes in the interactome picture (690). Modularity of the pathway also emerges by looking at the conservation of the CME network: hubs are conserved across species, while other nodes are sometimes lost in species distant to mammals. In addition, clathrin and AP-2 have maintained their specialized functions across evolution: a non-self-polymerizing cargo recognition module (AP-2), and a cage-forming module (clathrin). This allows flexibility in cargo repertoire and ensures in-built fidelity.

Dynamin forms another important hub. In this case, the connectivity relying on this protein might have even more far-reaching implications for cell physiology. Dynamins intersect a variety of pathways. For instance, dynamin is required both for CME and for some forms of NCE (see sect. II and Table 1). However, it is also crucial for actin dynamics (reviewed in Ref. 71; see also sect. VII*B*), directed cell migration (sect. VII*B*), centrosome cohesion at mitosis (sect. VIID), cytokinesis (sect. VII*D*), and apoptosis (not reviewed here, but see Refs. 219, 747). Thus the interconnectivity exerted by the dynamin hub might reflect a higher level of integration among different (or perhaps only apparently different, see sect. X) territories of cellular regulation.

Another interesting example of network analysis applied to a proteomic screening is provided by the recently obtained EGF-regulated UB proteome (22). This study revealed that in addition to well-established liaisons with endocytosis-related pathways, the EGF-Ubiproteome intersects many circuitries of intracellular signaling involved in DNA damage checkpoint regulation, cell-to-cell adhesion mechanisms, and actin remodeling. Moreover, the EGF-Ubiproteome was enriched in hubs, and a significant overlap was observed between the EGF-Ubiproteome and published EGF-induced pY-proteomes (66, 287, 578). Pathway analysis of UB/pY-containing proteins revealed a significant enrichment in endocytic and signal transduction pathways, while “hub analysis” revealed that UB/pY-containing proteins are enriched in highly connected proteins to an even greater extent than UB-containing proteins alone. These data point to a complex interplay between the UB- and pY-networks and suggest that the flow of information from the receptor to downstream signaling molecules is driven by two complementary and interlinked enzymatic cascades: kinases/phosphatases and E3 ligases/DUBs.

One very important implication of network analysis that is potentially relevant to the design of new therapies for human diseases is that, in addition to acting as critical interconnections

between signaling pathways, hubs are also points of fragility of signaling networks (11). As such, they represent ideal targets for pharmacological intervention. Identifying a protein in the network as a "hub" or as a "regular node" has important implications in term of predictions of biological outcomes. Depletion of a hub is predicted to eliminate the pathway [this is the case, for instance, of clathrin hub depletion (334, 539)], while depletion of regular nodes is predicted to have a lesser impact, as a consequence of pathway redundancy [as in the case of accessory factors such as epsins, EPS15/R, or intersectins (309, 334, 722)]. An interesting example in this sense is the depletion of the AP-2 hub, which affects CME to different extents, depending on the type of cargo (315, 334, 423, 539). In this case, alternative clathrin adaptors ("regular nodes") confer robustness to the network, allowing specific cargoes to be internalized in an alternative AP-2-independent manner (discussed in sect. II).

Detailed molecular knowledge of the mechanisms of interconnectivity of hubs is indispensable to predict the results of "hub interference." Recent systems analysis studies aimed to achieve this goal, focusing on the identification of new components of the endocytic network from a functional perspective, employing large RNAi screening and high content analysis. A siRNA screening study for genes involved in endocytosis in *C. elegans*, led to the identification of genes coding for the PAR-complex, required for asymmetric cell division, and thus for the establishment and maintenance of polarity in embryos and epithelial tissues (40). A similar approach was taken by Zerial and colleagues (132) who developed a siRNA screening for identifying genes involved in endocytosis, and also their contribution to 10 specific phenotypes of endocytosis which were rigorously quantified (number of endosomes, distance from the nucleus, size of endosomes, etc.). This group used a quantitative multiparametric image analysis approach to assess the variation of the 10 selected phenotypes in cells treated with both EGF and TF for 10 min. Their study showed that cells can adjust endosome size, number, and location (distance from the nucleus). The study also demonstrated that genes regulating EGF endocytosis, and thus cargo uptake, were different from those involved in TF endocytosis, and thus in cargo recycling. Finally, they confirmed a strong feedback between endocytosis and signal transduction pathways.

To be understood at the mechanistic level, these high-throughput data need to be integrated into mathematical models of signaling, which we will briefly review in the next section. We shall see that the gap between these two approaches has been narrowed, as high-throughput data have become more quantitative and as models have started to keep track of both spatial and time resolved signaling cues.

B Bottom-Up Approaches

Endocytosis has been described in mathematical models of two specific subjects: *1*) signal transduction pathways and *2*) the formation of polarized structures during asymmetric cell division. While the role of endocytosis in setting the timing of the different events has always been thoroughly investigated, the spatial dimension, that is more difficult to address mathematically, has received less attention.

1 Signal transduction pathways—Models of signal transduction pathways were produced well before the term *systems biology* was coined. These were among the first of only a handful of successful models coupled to experimental results that led to new ideas and experimental tools, such as the endocytic rate constant, a measure that is still widely used by experimental biologists to quantify internalization (845, 846). Over the years, new molecular details have been introduced into signal transduction pathway models, particularly for EGF signaling, to produce some of the most detailed models developed by systems biologists so far, involving a large number of reactions and molecular players, and generally described by ordinary differential equations (73, 548, 692). These models primarily focus on the timing of signal transduction pathways. Their broad view of signaling necessarily omits a precise and accurate description of endocytosis (described simply as the first element of the transduction pathway) and endomembranes such as endosomes and lysosomes [included, if at all, as intermediate steps for receptor recycling at the PM and/or degradation (647)].

Both the dynamics of endomembranes and endocytosis, though, are well described by specific models devoted to their particular analysis. The original model of Heinrich and Rapoport that describes the vesicular transport system (303) was updated by more recent models that have addressed the transition from early to late endosomes. This process involves the so-called RAB conversion, whereby early endosomes, carrying a high density of the small GTPase RAB5, are irreversibly transformed into late endosomes, with RAB7 being the prevailing species (see sect. IV*A2*). The presence of a positive-feedback loop in the interaction between RAB5 and its guanine nucleotide exchange factor (GEF) RABEX-5 has been identified as an important source of nonlinearity that underlies the switch. The positive-feedback loop guarantees the enrichment of RAB5 in the early endosomes and needs to be inactivated during the conversion to late, RAB7-enriched, endosomes. To this aim, a negative-feedback loop whereby RAB5 activates RAB7 which inactivates RAB5 has been invoked (155). More recently, SAND-1 (the *C. elegans* ortholog of mammalian MON1A and MON1B) has been identified as the molecular switch underlying the conversion (Figure 5*A*) (618, 800).

As for endocytosis itself, a recent biophysical model of endocytosis in yeast has stressed the importance of the interplay between chemical reactions and mechanical deformations of the PM (465, 466). The model is based on data describing the recruitment of different molecular players to the PM before and during endocytosis. Here again, positive-feedback loops have been proposed to be required for the process (Figure 5*B*). This time, the loops are based on the interaction between enzymes that control pulling forces and pinching of the membranes, and the resulting membrane curvature that enhances the activity of the enzymes. Along these lines, a recent study in mammalian cells supports the model with respect to the generation of PIP_2-depleted domains on the PM, created through the coupling of specific phosphatases with molecular machinery capable of sensing membrane curvature (115; see also sect. III*A3*).

The dependency of signaling on space and time has been modeled by the group of Boris Kholodenko who pioneered the study of the role of spatial gradients in signaling (87). Their results showed how in the presence of constant inactivating signals distributed all over the cytoplasm, a very steep gradient of signals would form if signal transduction pathways were

to deliver their signal simply by free diffusion. Using sensible parameters for diffusion coefficients, they demonstrated that in some instances it is the signaling endosomal compartments that permit the signal to pass through this cytoplasmic inactivating barrier for delivery to the nucleus (395).

A further step towards the integration of time, space, and signaling (see Refs. 396, 402 for a general discussion) which includes also endocytosis, exocytosis, and endomembranes has recently been developed by the group of Philippe Bastiaens, using both mathematical models and experimental measurements. The most thorough analysis produced so far addresses the activation of H- and N-RAS (which we will refer to globally as RAS) following growth factor treatment (478), whose presence activates a signal differentiated both in time and in space: while RAS activation at the PM is fast and quickly disappears, signaling continues for a longer time from the Golgi. With a combination of models and single-cell live cell measurements, it was shown that two overlapping dynamics contribute to guarantee the spatial-temporal dynamics of RAS activity after growth factor treatment in MDCK cells. The first is the so-called acylation cycle, which controls RAS localization. Growth factors induce the recruitment of activated RAS to the PM, from where it is internalized. Ubiquitous depalmitoylation decreases the affinity of RAS for endomembranes, thus increasing its diffusion rate, while repalmitoylation, operated at the Golgi, stabilizes RAS in this compartment. In this way, RAS localizes preferentially to the Golgi, from which it is sent back to the PM via the secretory pathway. Besides localization of RAS, the localization of GTPase-activating proteins (GAPs) and GEFs contributes to the particular spatially and temporally resolved activation of RAS, its regulators being absent from the Golgi but present in the ER, the intermediate “stop” between the PM and the Golgi.

Arguably, systems biologists working from a bottom-up perspective on endocytosis will have to introduce space as a fundamental component of their models to further understand the intricate connection between endocytosis and signaling. Along this line, it will be possible to make use of the data obtained by the high-throughput studies of endocytosis described above (132).

2 Polarization—Cell polarization is a field that requires the contribution of endocytosis to be necessarily described both in space and time. *Saccharomyces cerevisiae* has successfully been used for the development of models to analyze the establishment and maintenance of polarity in cells. In particular, the process of bud formation, typical of yeast, has been analyzed with systems biology approaches that have underlined the role of endocytosis in creating one single focus of budding precursors on the PM. A proposed model suggests that the symmetry breaking, taking place during bud development, is triggered by a positive-feedback loop whereby CDC42, a small RHO GTPase required for budding, favors the positioning of actin cables which in turn contribute to cluster CDC42 on the membrane (496). The removal of CDC42 from the membrane via endocytosis plays a key role in this mechanism, as it guarantees that the protein is not equally redistributed all over the PM (Figure 5*C*). In this sense, the interplay between endocytosis and delivery to the PM via actin cables, to generate a spatially uneven distribution of CDC42, resembles the above-mentioned acylation cycle of RAS. This result has been challenged by stochastic models that explicitly include vesicle fluxes by endocytosis and exocytosis at the PM.

Results of stochastic stimulations suggest that integral membrane proteins, which have slow diffusion rates, do become polarized as a result of actin-dependent endo/exocytic traffic provided they are actively concentrated in both endocytic and exocytic vesicles. Conversely, loosely associated membrane transducers, including CDC42, have much faster diffusion rates that, coupled with actin-directed vesicle traffic, are predicted to hinder, rather than to reinforce, polarization in yeast (429). Thus a CDC42/actin positive-feedback loop is probably not solely responsible for polarization. Both experimentalists and theoreticians have suggested an alternative and not exclusive mechanism based on the fast diffusion of BEM1, a scaffold protein that recruits CDC24, a GEF for CDC42, to the PM, thus leading to CDC42 activation and anchoring on the membrane (263, 326, 408). In this latter scenario, endocytosis does not play a role in the establishment of cell polarity (Figure 5*D*). However, the actin/CDC42 positive-feedback loop could still apply in the presence of other factors limiting CDC42 rapid diffusion rate. One of these components may be represented by molecules, such as septin, that set a diffusion barrier on the plasma membrane, thereby limiting lateral diffusion of CDC42 along the cell cortex. Consistently, septins, which are small GTPases enriched at the bud site of a dividing yeast (reviewed in Ref. 113), have recently been shown to provide a barrier function that is required to counteract the dispersal of CDC42 (572). In the daughter cell, this event is necessary to maintain CDC42 polarized localization, which may be initiated by directed exocytic vesicle delivery. Thus a synergic action between membrane trafficking and septins may operate to maintain the dynamic polarization of CDC42 during asymmetric growth in yeast.

VII Integrating the Partnership: Biological Programs Controlled by Endocytosis

In this section, we tackle the issue of how endocytosis and signaling are integrated during the execution of complex biological programs. From what we have reviewed so far, it should be evident that all proliferative, differentiative, apoptotic, metabolic, and developmental cellular programs controlled by membrane receptors are also governed by endocytosis. We do not dwell, therefore, on biological aspects that are evident consequences of endocytic control over signaling at the circuitry level; rather, we try to provide an account of biological programs in which the impact of endocytosis is (or was) less obvious and more complex. We will start by reviewing knowledge on an endocytic organelle, the caveola, whose study is unveiling surprising overlapping levels of complexity in the interconnection between endocytosis and signaling. We then move to the description of cellular programs in which the impact of endocytosis is paramount, i.e., in the control of *1*) polarized motility functions, *2*) cell fate determination, *3*) mitosis, *4*) cellular reprogramming and biogenesis of miRNAs, and *5*) transcription. The examples we provide are organized according to a “gradient” of functions that are progressively more and more distant from membrane dynamics, up to the point of representing apparently “noncanonical” functions of endocytosis.

A Caveolae: An Example of a Multifunctional Integrator of Endocytosis and Signaling

Caveolae are small (60–80 nm in diameter), flask-shaped, invaginations of the PM. Despite having been first observed more than half a century ago (579), their function is still the object of intense investigation and debate. They have been implicated in NCE, cell adhesion,

signal transduction, redox signaling, lipid and cholesterol regulation, mechanosensing, and possibly even in the regulation of transcription. Thus, despite the incompleteness of available knowledge, we feel that they represent an almost paradigmatic example of how a single "endocytic" organelle might integrate diverse biological functions (for recent reviews, see Refs. 50, 173, 290, 517).

1 Structure of caveolae—Caveolae are enriched in certain sphingolipids, cholesterol, and PIP_2 (235, 236, 574, 612). They represent therefore a subset of membrane (lipid) rafts. For this reason, the caveolar pathway of endocytosis is frequently referred to as "caveolar/raft endocytosis." This nomenclature is perhaps misleading, since caveolae-independent endocytosis of rafts can also occur (for reviews on membrane rafts and on the relationships between caveolae and rafts, see Refs. 724 and 422, respectively). Caveolae are associated with microtubules (302, 649) and with the actin cytoskeleton, this latter connection possibly being mediated by filamin (755). Two families of protein components are crucial structural and regulatory components of caveolae: caveolins (caveolin-1 through -3) and cavins (cavin-1 through -4).

The relevance of caveolins to the biogenesis of caveolae was established through the genetic disruption of caveolin-1 gene, which resulted in mice lacking caveolae (181), and by overexpression of caveolin-1 in caveolae-deficient cells, which resulted in caveolae formation (462). The interaction between caveolin-1 and cholesterol is critical for the oligomerization of the former (544), and this is probably important for the ability of caveolin to influence membrane curvature by inducing or stabilizing it (358, 596, 662). The exact structural role of caveolins in the formation of caveolae is still the object of investigation and debate (see Refs. 50, 173, 290, 517), as is its functional role in caveolar endocytosis (see below). In addition to caveolins, four cavins are also critical for the formation of caveolae at the PM (49, 289, 314, 468, 512). Cavins form a multiprotein complex (298) that is recruited by caveolin-1 to the PM, in a cavin-1-dependent manner (49), where it stabilizes caveolae. Interestingly, cavin-1 does not associate with other pools of caveolin-1 (for instance, that present in the Golgi, Refs. 298, 314), indicating that it recognizes a PM-specific form of caveolin-1, possibly in association with other components of the caveolin-1 enriched surface domain. In this contention, it is of note that cavins bind to phosphatidylserine in vitro and that caveolins might generate phosphatidylserine-enriched domains at the PM (815). While the interested reader will find a wealth of additional information on cavins in recent reviews (50, 290), it is of interest that cavin-1 was originally identified as a transcription termination factor, named PTRF (polymerase I and transcript release factor, Ref. 357) (see below).

2 Functions of caveolae—Caveolae have been implicated in the endocytosis of several ligands, including integrins, glycosphingolipids, and certain viruses, such as polyoma and SV40 (reviewed in Refs. 125, 517). Caveolar endocytosis might be tightly linked to the process of cell adhesion, as supported by findings that, in the case of caveolae-mediated SV40 internalization (598, 599), several kinases regulating the process are also involved in cell adhesion (597). In addition, integrin activation might regulate caveolar endocytosis, and in turn, caveolar internalization might remove integrins from the cell surface, suggesting bidirectional communication between the two processes (704). True enough, evidence

supporting opposite contentions, stimulation versus inhibition of caveolar endocytosis by integrins, has been provided (156, 728, reviewed in Ref. 50; see also sect. VII*B2* for a specific example); however, while differences need to be resolved, the concept of connection between caveolae and adhesion seems established. It should also be said that the exact endocytic function of caveolae remains the object of debate in the field. First, many proteins that enter the cell through caveolae might also be internalized through different portals. Second, caveolae are by-and-large relatively immobile and stable structures at the PM (596, 600, 774; but see also below for a recent revisitation of this concept), and also caveolins and cavins are remarkably long-lived proteins undergoing very slow turn over (298, 299). Indeed, caveolin-1 has even been proposed to function as a negative regulator of caveolae endocytosis, by slowing down their turnover and stabilizing them at the PM (435). Finally, even SV40, a traditional caveolar cargo, was recently also found in noncaveolar vesicles (201), and it was shown to be internalized with faster kinetics in caveolin-1-null cells (148). A stimulating account of the debate on the endocytic function of caveolae can be found in Reference 173.

While the above evidence does not deny the endocytic nature of caveolae, it draws attention to the facts that *1*) probably not all internalization events thought to be executed through caveolae are really as such, and *2*) even bona fide caveolar internalization events must be stringently regulated to account for the rather nondynamic nature of these organelles. A recent study unveils the regulation at the basis of caveolae and caveolin-1 assembly, disassembly, and degradation (299). Indeed, by altering the balance of core caveolae components (caveolin-1, cavins, and cholesterol), it is possible to accelerate caveolin turnover, by inducing caveolae disassembly, and caveolin ubiquitination and degradation into the lysosome (299). It was proposed that this process might be involved in the normal life cycle of caveolae: trafficking to early endosomes following internalization might cause the disassembly of the caveolar scaffold due to cavin loss, followed by caveolin-1 degradation (299). In this contention, a recent paper unveils a more dynamic nature of caveolae than previously thought (77). Caveolae have been reported to exist in two pools at the PM, a static predominant one and a minor highly mobile one which undergoes continuous rapid release and transient fusion with the PM without full collapse of the vesicle ("kiss-and-run" behavior) (596, 600, 774). By monitoring caveolae for long periods of time, it was found, however, that the vast majority of caveolae are dynamic with lifetimes ranging from a few seconds to several minutes. Thus probably two pools of caveolae exist: a short-lived and a long-lived one (77). Interestingly, the dynamics of caveolae are affected during mitosis, when the arrival and departure of caveolae, at the PM, becomes skewed towards the latter, causing a redistribution of caveolin-1 from the PM to intracellular compartments: an observation that adds to the involvement of endocytic dynamics in mitosis (see sect. VII*D*), although its exact role remains to be determined (77).

Caveolae have also traditionally been regarded as assembly platforms for signal transduction machinery. This property has been largely ascribed to the protein-protein interaction abilities of caveolin-1, which can act as a scaffold for a surprisingly large number of signaling proteins, such as growth factor receptors and their downstream transducers, SRC-like tyrosine kinases, G proteins, GTPases, GPCRs, steroid hormone receptors, and the endothelial nitric oxide synthase (eNOS) (reviewed in Ref. 590). While not all of these

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interactions are validated at a high level of resolution and functional certainty, together they define the idea that caveolin-1, and, by association, caveolae, function as a platform to regulate signaling. New developments in the field, however, compel some reevaluation of these findings. It is clear now that caveolins are expressed in cells that do not show caveolae, such as neurons, in which they control signaling by neurotrophins and synaptogenesis, or leukocytes, where they exert control over inflammatory responses and T-cell activation (reviewed in Ref. 301). In addition, a wealth of evidence (reviewed in Ref. 301) shows that caveolins can act as scaffolds to organize signaling (and other) proteins in both caveolar and noncaveolar regions on the PM, even in cells that possess caveolae. Thus the scaffolding and signaling properties of caveolin-1 cannot be automatically extended to caveolae, and case-by-case validation is needed. One such case is eNOS, whose association with caveolae is well established (250, 748). The interaction of eNOS with caveolin-1 inhibits the function of eNOS (90, 250), as also supported by findings in caveolin-1-deficient mice that display increased nitric oxide production (642). Importantly, the connection between eNOS and caveolae is part of a larger emerging role of these organelles in the compartmentalization of redox signaling machinery in cells, which includes binding and regulation also of NADPH oxygenase, heme oxygenase, and other redox systems (reviewed in Ref. 589).

Caveolae are also involved in lipid homeostasis. While we will not cover this aspect of caveolar function in detail (for reviews, see Refs. 433, 545, 608), available evidence supports an important role for caveolae in fatty acid uptake and storage in lipid droplets, in adipocytes, as well as a role in regulating the levels of free cholesterol and cholesterol export. Studies of caveolin-1 knockout mice and cells further support a role in lipid and cholesterol regulation, as these mice show reduced body fat, reduced cholesterol in adipocytes, and resistance to diet-induced obesity (434, 641). Of note, these findings correlate with the presence of mutations in the caveolin-1 gene in human lipodystrophies (100, 398).

Finally, recent developments implicate caveolae in cellular mechanosensing. It was previously known that caveolin-1 (and henceforth probably, but not necessarily, caveolae) was required for cellular responses to hyposmotic shock (784) and for the mechanosensitive activation of PI3K and AKT (695). In addition, based on theoretical modeling, it was proposed that changes in the mechanical tension of composite lipid membranes are buffered by the invagination of membrane domains (698). Support for the relevance of this concept to caveolae was brought by findings that caveolae act as membrane reserves that attenuate swelling in hyposmotic conditions, thereby limiting the mechanosensitive activation of some ion channels (407). A recent study provides a mechanistic framework for the idea that the membrane reservoir represented by caveolae allows the cell to readily respond to mechanical stress (729). It was found that cells react to cell stretching or osmotic swelling through the rapid flattening of caveolae into the PM and their disassembly. This ability is intrinsic to the caveolar structure, being independent of ATP and actin. On the contrary, the reassembly of caveolae after stress is assisted by ATP and actin remodeling. Caveolae indeed promptly reassemble when the mechanical stress is relaxed, suggesting the existence of a mechanosensitive signaling pathway that mediates this response (729). It has been proposed that caveolar components, such as caveolins and cavins, which are released upon caveolae flattening/disassembly, may act as signal transducers to mediate long-term cell response to

mechanical stress (508, 729). One intriguing possibility is that cavin-1/PTRF after release is translocated into the nucleus where it might activate a transcriptional program necessary for caveolae neosynthesis (508).

In conclusion, while caveolae still hold onto a number of their secrets after almost 60 years of research, their function is emerging as that of a critical signal integrator regulated by membrane dynamics and endocytosis.

B Endocytosis and Motility

Cells of unicellular and multicellular organisms must recognize and process spatial information. This is achieved by adapting cytoskeletal and membrane components and signaling molecules, so as to acquire and maintain an asymmetric architectural organization and a polarized distribution of signaling molecules whose output, thus, becomes spatially restricted. One way to confer spatial and temporal dimensions to signaling is through EECs. Endocytic internalization of membrane and membrane-associated proteins is, indeed, frequently accompanied by recycling of these factors back to the PM. While EECs serve to replenish ligand-free receptor for the next round of signaling and transport, they can also redirect and confine signaling molecules to specialized and distinct areas of the PM, such as the apical and basal membranes of polarized epithelial monolayers (reviewed in Ref. 88). This function can also act as a positive-feedback mechanism to maintain the polarization state of critical signaling molecules (see sect. VI*B2* and Refs. 496, 792) as long as they feature slow diffusion rates, such as integral membrane cargo proteins (see sect. VI*B2* and Ref. 429). Spatial restriction of signaling has, thus, emerged as a critical device for the execution of a number of polarized cellular functions, including directed cell migration, cell-fate decisions, epithelial-cell polarization, growth cone movement, tissue morphogenesis during development, and cell invasion by metastatic cells into their surrounding tissues (reviewed in Ref. 170). At the molecular level, a complex network of different pathways orchestrates the transmission of signals in both space and time, enabling cells to initiate movement in response to specific extracellular cues, and also to arrest precisely at target sites. Not surprisingly, there are multiple mechanisms through which trafficking of membrane and membrane-associated motogenic transducers directly impinge on cell migration (see also Figure 2*B*).

1 EEC and membrane flow—A default mechanism linked to EEC that potentially has direct consequences on polarity phenotypes is the generation of membrane flow. By analogy with actin tread-milling, the flow of internalized and recycled membrane was proposed more than a decade ago either to generate forces for the extension of migratory protrusions (84), or to promote the rearward movement of molecules bound to the surface of these protrusions during cell motility. Results consistent with membrane flow have been obtained in various cell types (83, 323, 691, 699), although, for other motile cell types, a number of experiments failed to detect any significant rearward membrane flow (415, 437). Thus membrane flow may not be a universal property of moving cells, although it may be important for some of them. This notwithstanding, the requirement for a continuous flow of membranes propelled by endocytic molecules is essential for the highly dynamic changes of cell shape that occur during directional, chemotactic migration of the amoeba *Dyctiostelium discoideum*, a

professional mover (782, 836). Clathrin-null *Dyctiostelium* mutants, in addition to displaying dysfunctional cytokinesis, are characterized by increased roundness, defective polarity, reduced cell velocity, and inefficient chemotaxis (836). This was originally proposed to be due to an impaired ability to extend polarized cell protrusions at the front of the cell. More recent evidence suggests, instead, that an intact clathrin-dependent EEC of membrane is necessary for a moving cell to adjust its cell surface area to match changes in cell shape. Lack of this adaptation system thus severely impairs cell locomotion (782).

2 EEC spatially restricts signals for directional migration—One instance where the processing of spatial information through EEC becomes critical is during chemotactic cell migration. Under these conditions, cells must reorient directionally by polarizing PM sensors according to the direction of travel. One obvious way to achieve signal polarization and directional motility is through localized redistribution, via EEC, of signaling molecules in response to extracellular cues. The first genetic evidence in support of this concept was produced in *Drosophila melanogaster*. Disruption of typical endocytic regulators, such as the E3 UB ligase CBL, or the RAB5 activator SPRINT (the homolog of mammalian RIN1) resulted in aberrant cell migration in response to stimulation (362; see also sect. III*A1*), by affecting the EEC of the motogenic RTKs of the EGFR and PVR (PDGF/VEGF receptor) families. Thus endocytic pathways, particularly those impinging on RAB5, are required to ensure the spatial resolution of chemotactic signaling emanating from different RTKs, to regulate actin-based, polarized protrusive activity and motility.

There is evidence that a similar circuitry also operates in mammalian cells to modulate polarized cellular function (111). Endocytic trafficking of RAC and its recycling to the PM is required for the transduction and spatial resolution of information emanating from motogenic stimuli (580). As occurs in *Drosophila*, an endocytic RAB5-based circuitry is pivotal. By activating endocytosis, RAB5 causes internalization of RAC, its activation in recycling endosomes, and its subsequent delivery through ARF6-dependent routes to specific regions of the PM. Once redelivered to the membrane, polarized RAC-dependent functions take place, leading to the formation of migratory protrusions that promote a mesenchymal mode of cell motility (580) (Figure 2*B*).

The importance of endosomal recycling routes for directional migration is highlighted by various studies in different mammalian cells. There is evidence, for example, that inhibition of the slow recycling pathway by expression of dominant negative RAB11 or truncated myosin Vb or RAB11-FIP, an effector of RAB11, impairs cell migration (492) and chemotaxis of basophilic leukemia cells (207). These latter results have been recently confirmed in epithelial PtK1 cells, where, however, interference with the RAB11 recycling pathway increased random motility and impaired directional and persistent migration, possibly as a consequence of the delocalized formation of protrusive lamellipodia (621). Thus polarized endosomal recycling is not required for cell locomotion per se, but rather, it appears to be critical for the maintenance of the polarity of cell migration, which when disrupted leads to disorganized motility.

A similar endo/exocytic cycle appears to control the cellular trafficking of integrins. These major cell surface adhesion receptors play a critical role in cell migration. Several different

mechanisms control their activity, including expression and subunit heterodimerization patterns, clustering and lateral diffusion in the plane of the PM, and interaction with the actin cytoskeleton and the inside of cells (reviewed in Ref. 97). In addition to this, many integrins are continually internalized from the PM into endosomal compartments and are subsequently recycled, prompting the proposal that spatially polarized EEC of these adhesion receptors is essential to control various aspects of cell locomotion (reviewed in Refs. 112, 841). Consistent with this view, for instance, the blockade of integrin $\alpha 5\beta 1$ recycling by functional interference with the integrin-associated RAB25, a member of the RAB11 family of proteins that control endosomal recycling, impaired the formation of "pseudopodal protrusions" (mesenchymal motility) and directional motility during three-dimensional cell migration (111) (Figure 2*B*).

Mechanistically, one important question that these findings raise is how signaling molecules are recycled to specific regions of the PM (as opposed to the bulk PM) to execute spatially restricted signaling. In the case of RAC and integrins, one possible answer came from recent studies connecting localized RAC activation with integrin-mediated adhesion and lipid raft internalization (Figure 2*B*). These studies suggested that RAC positioning at, and trafficking from and to specific locations of the PM may be regulated through raft-dependent endocytosis. This process is needed, in turn, to specify the localization of RAC activity for the execution of relevant biological processes. Thus, upon activation of integrins, sites of high RAC affinity become available on the PM, preventing RAC internalization, which only occurs following cell detachment in a dynamin- and caveolin-1-dependent manner (157). Indeed, caveolin-1-deficient cells show increased RAC activation, which however is not spatially confined, leading to loss of directional migration (268). The RAC/integrin EEC and targeting circuitry appears to require the coordinated action of two different routes of endocytosis [clathrin dependent (580) and raft mediated (156)]. Within this context, ARF6-dependent recycling appears to be the critical factor controlling not only the redelivery of RAC (580) and integrins (156–158), but also of lipid rafts, back to the PM, ultimately coordinating RAC signaling and directional migration with adhesion-dependent cell growth (39) (Figure 2*B*).

One additional attractive hypothesis to account for how various endocytic routes may promote directional migration in a coordinated fashion is based on observations that caveolar endocytosis frequently occurs only at the trailing edges of migrating cells (581), while CME, coupled to fast recycling, is restricted to the advancing leading edges (for a review, see Ref. 220). These findings suggest that polarized locomotion may be facilitated by a front-rear distribution of diverse endocytic routes. This notion has recently been extended to include clathrin- and raft-independent endocytic routes as well as macropinocytosis. The clathrin-independent carrier (CLIC) internalization pathway has been shown to be responsible for the vast majority of bulk endocytosis in lamellipodia and to be required for directional cell migration by promoting rapid non-clathrin-mediated EEC of focal adhesion cargoes (327). Conversely, macropinocytosis induced by PDGF was shown to promote the rapid redistribution of both $\beta 1$ and $\beta 3$ integrins to circular dorsal ruffles, their subsequent internalization through macropinosomes, and redelivery to nascent focal adhesions at the leading edge of migratory fibroblasts (275), ultimately promoting cell locomotion.

In the case of integrin trafficking, questions that still remain to be addressed are as follows: *1*) whether integrins that undergo EEC are the active ones bound to their ECM ligand, and *2*) whether their activation status affects their endocytic routes and intracellular fate. One recent report shed lights on these issues, further providing evidence in support of the notion that proper targeting of activated integrins to lysosomal degradation is required for cell motility (474) (Figure 2*B*). A sizable fraction of internalized fibronectin (FN)-bound $\alpha 5\beta 1$ integrin dimers are specifically directed to lysosomes for degradation through a mechanism involving integrin ubiquitination and recognition by the ESCRT machinery. Cells expressing an $\alpha 5\beta 1$ integrin mutant, that could no longer be ubiquitinated, were severely impaired in cell migration, suggesting that FN-integrin complex turnover is essential for locomotion. Since FN degradation is also required for cell migration (318), it is possible that the FN-integrin complex must be degraded, instead of being continuously recycled, to avoid the formation of dysfunctional adhesion sites that would result in increased adhesion and buildup of extracellular matrix, both of which would hinder cell migration. Alternatively, degradation, as opposed to or in equilibrium with recycling, may be required for the proper attenuation of integrin signaling to have an impact on migration.

3 Endocytosis acts locally—A key aspect of directional migration of well-adherent cells is the establishment of transient attachments to the extra-cellular matrix (ECM) through integrin clusters that form plaques known as focal adhesions. Focal adhesions establish a connection between the ECM and the actin cytoskeleton and serve as points of traction for the cell. The contraction of focal adhesion-associated actin stress fibers is thought to propel the cell body forward. During migration, there is a constant turnover of focal adhesions that form at the leading edge, often as focal complexes that mature into focal adhesions as tension builds up, and that are then disassembled, allowing for tail retraction, and integrin detachment from the ECM (reviewed in Ref. 650). While the mechanisms of adhesion assembly have been largely defined, focal adhesion disassembly still remains unclear. Given the importance of integrins in adhesion and the role of integrin trafficking in migration, a prevailing idea is that the formation and disassembly of focal adhesions during cell migration are coupled to the endo/exocytic cycles of integrins (111, 558, 601). In keeping with this notion, focal adhesion disassembly was shown to be dependent on the activity of dynamin, which can form a complex with the kinase FAK and the adaptor GRB2, and is essential for microtubule-dependent focal adhesion disassembly (205, 823). Additionally, clathrin and various clathrin accessory proteins can accumulate at focal adhesion sites where, following targeting by microtubules, they promote the localized internalization of integrin and focal adhesion disassembly (116, 117, 204) (Figure 2*B*). Thus, while integrin EEC may globally serve as a device to maintain a spatially confined front-to-back gradient of adhesion receptors, focal adhesion-restricted CME may terminate mechanosignaling, suggesting that membrane trafficking is a versatile system for the temporal and spatial control of motogenic inputs.

Endocytosis is also emerging as a critical factor for the spatial control of biological processes in the morphogenesis of polarized epithelial tissues. To form functional and organized tissues, cells need to control their morphology, especially during certain development stages. Epithelial cells can, for example, lose attachments to each other,

depolarize and undergo a process called epithelial-mesenchymal transition (EMT) (377, 770). At other stages during development, epithelial tissues must change their shape as the result of a coordinated rearrangement and movement of individual cells. Under these conditions, epithelial cells maintain their polarized features, but dynamically remodel their contacts with neighboring cells. A wealth of recent evidence has demonstrated a crucial role for endocytosis and recycling of cell adhesion molecules, and in particular of E-cadherin, during each of these developmental processes. We cannot cover here all these findings, so we refer the reader to a recent review (271). We note, however, a recent study that exemplifies the importance of coordinating spatially restricted junctional E-cadherin endocytosis with intracellular actomyosin-based tension to ensure proper epithelial morphogenesis. In the early *Drosophila* embryo, myosin II controls the planar polarized remodeling of cell junctions to enable convergent tissue extension, which is a process whereby the epithelial layer that forms the thorax and abdomen of the embryo narrows along the vertical axis and lengthens in the perpendicular, horizontal axis (405, 499). The distribution of E-cadherin is also planarly polarized along "vertical" junctions (that are oriented along the dorsoventral axis of the developing embryo), but complementary to that of the myosin II, which is restricted primarily to horizontal junctions (oriented along the anteroposterior axis of the embryo). Remarkably, such a precise spatial organization depends on the restricted and polarized distribution of endocytic factors, such as dynamin, clathrin, and AP2 (447). Blocking CME of E-cadherin results in an alteration of the epithelial morphogenetic programs in *Drosophila* embryos, causing intercalation (a process during which mediolateral cells converge along the dorsoventral axis intercalating with neighboring cells) defects. Endocytic molecules are kept planarly polarized by the concerted action of actin and myosin regulatory factors, including the formin mDia and myosin II, which generate actomyosin filaments along the ventrolateral region and in "vertical" junctions during cell intercalation of a developing *Drosophila* embryo. These contractile structures favor the clustering of E-cadherins, the recruitment of endocytic components, and ultimately promote laterally localized CME that is, in turn, essential to establish and maintain the planar distribution of E cadherins.

4 Cross-talk between PM receptors within the endocytic network—An additional emerging level through which PM motogenic receptors, including mechanosensors, such as integrins, and canonical signal transducers, such as RTKs, influence cell migration is by exerting a reciprocal control over their trafficking routes.

It is well established that RTK and integrin signaling are inextricably linked in such a way that full activation of various RTK pathways can be achieved only if cell adhesion is engaged, while inside-out integrin activation is frequently promoted by growth factors in a variety of cellular processes ranging from cell spreading, epithelial cell morphogenesis, and cell migration (reviewed in Ref. 483).

One mechanism to initiate inside-out signaling is through the mobilization of the endosomal pool of integrin heterodimers for rapid redelivery to the PM (reviewed in Ref. 112). PDGF selectively promotes the recycling of integrin $\alpha v\beta 3$, but not of integrin $\alpha 5\beta 1$, through RAB4-dependent endosomal routes, enhancing cell adhesion and spreading (653, 854). Endocytic and signaling pathways are deeply integrated as indicated by the observation that

integrin αvβ3 primarily activates RAC, which is essential for the formation of lamellipodia and focal complexes, and which drives directional cell migration, while integrin α5β1 controls RHOA-dependent stress fiber formation and cell contraction (149). The selective activation of integrin heterodimers, therefore, influences the balance of their signaling to RHO-GTPases, ultimately controlling the mode of cell motility. Fibroblast growth factor receptor 1 (FGFR1) and cell-cell adhesion molecules display a similar mode of interaction. For example, neural cell adhesion molecule (NCAM) associates with FGFR1 (227). FGF induces endocytosis and degradation of FGFR1, while NCAM instead promotes stabilization of the receptor, which is recycled to the cell surface in a RAB11- and SRC-dependent manner, resulting in sustained signaling. This promotes NCAM-induced cell migration, and presumably, also accounts for the NCAM proinvasive role during tumor progression (reviewed in Ref. 875).

There is accumulating molecular evidence of interactions between different integrin heterodimers along the endocytic routes and of integrin regulation of RTK trafficking (reviewed in Refs. 112, 841). A specific case in point is provided by studies linking integrins αvβ3 and α5β1 with EGFR trafficking networks. Upon αvβ3 ECM ligand engagement, the heterodimer is actively routed to RAB11 recycling compartments through its interaction with RAB-coupling protein, RCP (839). Disruption of αvβ3 function causes RCP to dissociate from αvβ3 integrin and to bind α5β1 instead. This mechanism results in efficient rerouting of integrin α5β1 back to the PM, effectively accomplishing an integrin heterodimer switch that enhances RHOA signaling with concomitant enhanced turnover of lamellipodial extension and increased random cell migration. Importantly, disruption of αvβ3 by cyclic peptidomimetic drugs not only drives the recruitment of RCP to the cytoplasmic tail of β1 integrin, but also enables RCP to associate with EGFR (839). α5β1 Integrin and EGFR thus coordinately recycle to the PM, with a striking effect on EGFR and integrin signaling that enhances the invasiveness of various epithelial tumor cells (839). The “endocytic interaction” of integrins with RTKs is not limited to this specific example. In endothelial cells, pharmacological inactivation of αvβ3 results in enhanced VEGFR2 recycling through a RAB4-dependent pathway, diverting this receptor away from degradation, while boosting VEGFR2 cell surface levels, endothelial sprouting, and tubulation, ultimately resulting in neoangiogenesis (648). This is the opposite effect to that originally predicted for αvβ3 interfering drugs, such as Ciliengitide, highlighting the importance of understanding endocytic networks in regulating complex pathophysiological processes.

C Endocytosis and the Determination of Cell Fate

While the majority of cell divisions generate two identical daughter cells, in a number of cases the two progenies assume different fates; in these instances, one of the two daughters might retain the mother cell fate, or both daughters can assume fates that are different from the mother and from each other. These events are crucial in development and in the maintenance of stem cell (SC) compartments in adult life. Furthermore, their subversion is thought to play a central role in cancer (reviewed in Refs. 19, 239, 261, 553, 787).

The phenomenon defined as asymmetric cell division (ACD) sits at the heart of the process of cell fate determination. ACD can be influenced by intrinsic and extrinsic mechanisms. In the former case, the unequal partitioning of molecular machinery at mitosis gives rise to daughters that are intrinsically different. In the latter, the influence of external stimuli, for instance, a SC niche, imparts different cues to the two progenies, helping shaping their fates. Endocytosis plays a paramount role in both intrinsic and extrinsic mechanisms, and it is one of the major programs (arguably, the major) through which ACD, and ensuing cell specification, is achieved.

1 Lessons from genetics: the SOP system in the fruit fly—The most advanced mechanistic knowledge of how endocytosis impacts on ACD derives from studies in the fruit fly, and in particular on the bristle sensory organ. This organ is formed by four cells that originate from a mother cell (the sensory organ precursor, SOP, cell) through a pattern of ACDs. The SOP cell (Figure 6*A*) divides asymmetrically along the anteroposterior axis to originate an anterior pIIb cell and a posterior pIIa cell. The pIIb and pIIa cells give rise, through further ACDs, to the final four cells of the organ, the sheath cell and the neuron (from the pIIb), and the socket and shaft (hair) cells (from pIIa) (for details, see Refs. 261, 553). Although the SOP cells do not constitute a bona fide SC compartment, being devoid of self-renewal ability, the study of the divisions it undergoes to form the pIIa and pIIb cells has enormously advanced our knowledge of the intrinsic mechanism of ACD.

It has been known for many years that a protein called NUMB partitions asymmetrically at one of the poles of the dividing SOP, thereby being inherited almost exclusively by the pIIb cell and imparting cell specification (for extensive reviews of the mechanism of the asymmetric partitioning of NUMB, and of several other cell fate determinants identified in *Drosophila*, see Ref. 553). Genetically, NUMB counteracts the action of the signaling receptor NOTCH (277, 749, 750, 879). It has also been known for several years that, in *Drosophila* neurogenesis, the signaling function of NOTCH requires dynamin, and therefore presumably endocytosis (700). These two observations, however, were not rationalized together until the discovery that NUMB was an endocytic protein (679, 683). It was then discovered that NUMB binds to the major endocytic adaptor AP-2 (58, 683) and determines its asymmetrical segregation in the pIIb cell (Figure 6*A*). A flurry of papers subsequently defined how a series of differential endocytic/recycling events, taking place in the pIIb and pIIa cell, creates sufficient asymmetry in the repertoire of signaling molecules, at the PM and in intracellular signaling compartment, to allow directional signaling from the pIIb cell (which behaves as a signal-sending cell) to the pIIa cell (the signaling-receiving cell). At least four endocytic-based circuitries concur to create this asymmetry (Figure 6*B*):

1) Numb (and/or AP-2)-dependent endocytosis in the pIIb cell of NOTCH or of SANPODO, a positive regulator of NOTCH signaling during ACDs in *Drosophila* (58, 341, 658). It is of note that recent evidence in mammalian cells argues for a role of NUMB as an inhibitor of NOTCH recycling to the PM (510), rather than as a positive modulator of internalization, suggesting that the function of NUMB in the pIIb cell might be that of preventing NOTCH recycling to the PM, favoring its commitment to degradation. Whatever the case,

the presence of NUMB determines a functional NOTCH-null (or attenuated) situation in the pIIb cell (Figure 6*B*).

2) DELTA activation by neuralized-dependent endocytosis in the pIIb cell. The UB-ligase neuralized is also asymmetrically segregated during SOP cell mitosis, being preferentially partitioned into the pIIb cell, where it ubiquitinates the NOTCH ligand DELTA, thereby promoting its internalization (419, 430). In this case, internalization functions as an "activation" strategy, as it is known that the internalization of DELTA and its recycling to the PM (through as yet unknown molecular mechanisms) are necessary for the ability of DELTA to engage NOTCH on neighboring cells (349, 582, 822). Neuralized might actually promote endocytosis of DELTA through a particular pIIb-specific pathway. Indeed, DELTA is trafficked differently in pIIa and pIIb cells. In the latter, it is routed through RAB11-positive recycling endosomes, and probably recycled to the PM. In pIIa cells, conversely, RAB11 endosomes do not form, and DELTA cannot be recycled and is presumably destined to degradation (191). This mechanism would ensure that the expression of DELTA (and possibly of "activated" DELTA) at the PM is skewed towards the pIIb (signal-sending) cell. These results reinforce the notion of recycling as an important aspect of the mechanisms of cell fate specification, as also supported by the involvement of SEC15, a component of the exocyst (a complex involved in tethering and spatially targeting exocytic and recycling vesicles to the PM), in the ACD of SOP cells (355) (Figure 6*B*).

3) Recycling- and actin-dependent topological segregation of signaling molecules. In *NUMB* or *α-ADAPTIN* SOP mutants, SANPODO is enriched at the pIIa-pIIb cell interface (777). In addition, it has been recently reported that the apical surface of pIIa and pIIb cells display actin-rich microvillar structures, to which DELTA is preferentially recycled. The formation of these structures depends of the presence of the actin nucleator complex ARP2/3, and the presence of ARP3 is required in the signal-sending pIIb cell for fate specification (632). In a system in which both ligands and receptors are membrane-tethered, the PM region within the area of cell-to-cell contact is clearly the most relevant for directional signaling. This suggests that the overall PM level of effector molecules (SANPODO, NOTCH, or DELTA) might not matter as much as their levels within defined signaling domains, which in the case of pIIa and pIIb might be represented by microvillar structures that would greatly amplify the surface area available for cell-cell contacts (Figure 6*B*).

4) Asymmetric partitioning of SARA-endosomes. The asymmetric partitioning of entire endocytic compartments also plays an important role in cell fate specification. Unequal distribution of endosomes between daughter cells is observed frequently during ACD, for instance, at the first cleavage of the *Caenorhabditis elegans* embryo (13), or during ACD of mammalian hematopoietic SCs (55). In SOP cells, both NOTCH and DELTA are trafficked to SARA endosomes before ACD (138) (Figure 6*B*). These endosomes are then directionally transported to the nascent pIIa cell (138). This is functionally

important, since mistargeting of SARA endosomes to the pIIb cell causes ectopic activation of NOTCH in that cell (138). These findings define a mechanism, operating in the pIIa cell that acts synergistically with the other described mechanisms towards the generation of asymmetry.

2 Endocytosis and stem cells—ACD is crucial in the maintenance of adult SC compartments, in which SCs divide asymmetrically to give rise to a daughter cell that retains the mother fate (i.e., becomes a SC and withdraws into quiescence) and to a daughter, the progenitor, which undergoes multiple rounds of cell division to generate a vast progeny that eventually differentiates. Most of what we know about the role of endocytosis and SC compartments revolves, not surprisingly, around the role of NUMB as an intrinsic determinant of ACD in SCs. Such a role has been demonstrated, both in lower organisms and in mammals, in neuroblasts (451, 607, 640, 707, 750, 879), retinal neuroepithelial cells (114), muscle satellite cells (104, 134, 670), hematopoietic SCs (848, 858), and mammary SCs (127, 594). We have recently reviewed this topic in depth, and therefore refer the reader to that review (592). At the mechanistic level, one issue deserves additional comments, since it has been shown that in neuroblasts, NUMB might couple with different signaling pathways, in a context-dependent manner. One such pathway involves ACBD3, a NUMB-interacting Golgi protein, which undergoes changes in its subcellular distribution during the cell cycle (881). When ACBD3 is redistributed in the cytosol after Golgi fragmentation at mitosis, it acts synergistically with NUMB in specifying a SC fate, whereas, when it is associated with the Golgi during interphase, it can promote neuronal differentiation in postmitotic neurons (881).

The evidence reviewed so far concerns a role for endocytosis in the intrinsic mechanism of cell fate determination. There are also, however, indications of an impact of endocytosis on extrinsic mechanisms, in particular in determining cues imparted by osteoblasts of the hematopoietic niche to hematopoietic SCs. In this case, a specialized membrane domain of the hematopoietic SC is *trans*-endocytosed by the osteoblast and trafficked to SARA endosomes where it remains (without being degraded) and triggers signaling pathways leading to attenuation of the SMAD2/3 pathway and to expression of chemokines promoting hematopoietic SCs homing (255). Mechanistically, this aspect of non-cell autonomous endocytic control in cell fate specification can be viewed as a variation on the theme of the wider impact of endocytosis on the reprogramming of adjacent cells, reviewed in section VII*E*.

D Endocytosis and Cell Division

Emerging evidence points to a critical role for the endocytic machinery in cell division (reviewed in Refs. 238, 694). Two levels of regulation have been described and will be reviewed in this section: *1*) endocytosis is required for the dramatic reshaping of the PM that occurs during mitosis, and *2*) several components of the endocytic machinery seem to play a direct role in chromosome segregation and cytokinesis.

During mitosis, cells need to change their surface area rapidly (79, 280, 522). First, they reduce their PM, which permits cell shrinkage and detachment from the substrate. Later,

cells increase their surface at the level of the cytokinetic furrow, to allow newly formed daughter cells to spread out. Plant biologists have long known that internalization and recycling of PM at mitosis play a crucial role in these remodeling events, with membrane trafficking representing a critical event for the completion of cell division. Now, however, it is becoming apparent that plant and animal cytokinesis hold many similarities in this respect (reviewed in Refs. 41, 238). Indeed, in contrast to the previous idea that animal cells shut down endocytosis during mitosis (59, 60, 674), it was recently found that CME remains active throughout the cell cycle (78). However, the recycling pathway is regulated at two levels during mitosis. *1*) Entry into metaphase is associated with a dramatic decrease in recycling events (78, 830). The net outcome of continuous internalization in the absence of recycling is a reduction of PM area and a concomitant accumulation of an intracellular pool of vesicles. One of the consequences of this phenomenon is PM depletion of TFR and an apparent decrease in its internalization: events that were erroneously interpreted in the past as a general shut down of internalization (674). *2*) At telophase, the recycling pathway recovers: endosomes and late endocytic compartments are recycled to the midbody and fused to the cytokinetic furrow, allowing the two daughter cells to divide. This polarized recycling towards the midbody is regulated by different components of recycling endosomes (RAB11, ARF6, RAB35) and of the secretory machinery (SNARES), and is directed by plus-end-oriented microtubular motor proteins (Figure 7*B*).

At the midbody, fusion events of endosomes to the PM mediated by SNARE protein complexes (such as syntaxins, Refs. 80, 273) generate distinct membrane subdomains, with specific lipid compositions, that work as signaling platforms to allow the completion of the mitotic process. For instance, PI3P localization during cytokinesis is spatially restricted at the midbody (675), where it helps to recruit a centrosomal protein, FYVE-CENT, required for progression through cytokinesis. Indeed, through FYVE-CENT and its partner TTC19, CHMP4B, a component of the ESCRT-III complex, is able to engage at the midbody (675) (Figure 7*A*). This complex, which participates in the inward membrane budding events involved in MVB sorting, has been recently shown to be involved in virus budding and midbody abscission; all three events involve cleavage of membrane necks with similar unconventional membrane topologies (for a review, see Ref. 338). Several studies have shown that cytokinesis requires both ESCRT-III components and VPS4 as well as ESCRT-I and ALIX in differing degrees, to mediate constriction of midbody membranes (103, 537, 753). This process has been conserved across species, and indeed is found even in a subset of *Archaea* (103, 460). A recent study (190) employed imaging techniques to characterize the organization and dynamics of ESCRTs during cytokinesis. Remarkably, ESCRTs are recruited at the midbody center following the same scheme of sequential recruitment thought to operate at the endosome/MVB. In particular, the levels of ESCRT-III peak closely to abscission, followed by VPS4, when ESCRT-III becomes concentrated in the narrow constriction site where abscission takes place (190).

It is also of note that dynamin is a midbody protein, required for cytokinesis (213, 733, 773, 842), and that a number of functional RNAi screens have identified it as a player in cell division, suggesting that this GTPase coordinates the membrane remodeling events that occur during cytokinesis (184, 188, 378, 397; reviewed in Ref. 71). Although the mechanistic details of how all these endocytic events are regulated and coordinated with

mitotic progression remain elusive, there is no doubt that this evolutionarily conserved mechanism is crucial to orchestrate cell division in space and time.

Finally, the master endocytic regulator RAB5 is involved in nuclear membrane breakdown at mitosis, as depletion of this GTPase in *C. elegans* leads to multiple defects in nuclear envelope disassembly (27). In this case, the role of RAB5 is part of a wider function in maintaining the structure of the ER, of which the nuclear membrane represents a functional district (27). Although the molecular mechanisms are unclear, it has been proposed that RAB5 might act in *trans*, while localized on endosomes, by interacting with effectors on the ER membrane to induce their homotypic fusion. Evidence has also been provided that such a mechanism might be conserved in mammals (27).

Beyond cytokinesis, several endocytic factors (e.g., clathrin, dynamin, ARH, RAB6A, ARR) have been found to colocalize with and regulate different components of the chromosome segregation machinery, namely, centrosomes and the mitotic spindle, as briefly summarized below (Figure 7*B*).

1) Clathrin heavy chain has been found at the mitotic spindle (498, 569). Depletion of clathrin heavy chain causes destabilization of kinetochore fibers, leading to chromosome misalignment, persistent activation of the spindle checkpoint, and a consequent delay in mitotic progression (667). Structure-function reconstitution experiments revealed that stabilization of the kinetochore fibers was dependent on the capability of clathrin to trimerize, indicating that this function of clathrin depends on its unique structural features (667, 668). In addition, the clathrin heavy chain was recently found to mediate recruitment to mitotic spindles of phosphorylated TACC3 (transforming acidic coiled-coil-containing protein 3), a substrate of the kinase AURORA-A required for mitotic spindle stability (232, 456).

2) RAB6A is recruited to the kinetochores during metaphase, where it cooperates with the MAD2-dependent spindle-checkpoint pathway to ensure the attachment of the spindle microtubules to the kinetochores (529).

3) The GTPase dynamin was shown to bind to γ-tubulin and to be required for centrosome cohesion, as its depletion causes centrosome separation (368, 772).

4) The endocytic adaptor ARH has been proposed to be involved in dynein-mediated transport to the centrosomes, since it can bind to dynein and since it is found to localize first at centrosomes and then at kinetochores, as cells progress from interphase to metaphase. In agreement with this, ARH-null fibroblasts have smaller centrosomes than their wild-type counterparts (444).

5) The membrane bending activity of epsin-1 is required for mitotic membrane organization and proper spindle morphogenesis in *Xenopus laevis* egg extracts. Interestingly, this function seems to be independent of the molecular mechanism of action of epsin during endocytosis that occurs during interphase (470).

6) Intersectin 2, which regulates CDC42 activation during epithelial morphogenesis, has been observed to be centrosomally localized. Both

intersectin 2 and CDC42 are required for normal positioning of the mitotic spindle (656).

7) Finally, ARRs have been found associated with centrosomes (534, 703), and their knockdown triggers multinucleation, centrosome amplification, and mitotic defects (703).

Despite the accumulating evidence, more work is needed to clarify whether these endocytic players conserve their "endocytic" mechanisms of action during cell division, or whether they display a "split personality," acquiring additional modes of action during mitosis, as will be further discussed later.

E Endocytosis, Genetic Reprogramming of the Microenvironment, and Regulation of miRNAs

Recent developments have highlighted another complex mechanism through which endocytosis can control signaling, i.e., through genetic reprogramming of the cellular microenvironment. Quite surprisingly, in this case, the cellular target of the process is not the cell in which endocytosis occurs, but its neighbors. This is achieved by endocytic-controlled cell-cell communication via exosomes. Exosomes are small vesicles (40–100 nm in diameter), essentially constituted from the intraluminal vesicles of MVBs. MVBs typically fuse with lysosomes, delivering their cargo for degradation. However, they can also fuse with the PM, in an exocytic fashion, to release their vesicular content, the exosomes, extracellularly. These are recognized by surface proteins on the surrounding cells (531, 561), thus allowing their endocytosis. Studies of exosomes have traditionally concentrated on their protein content, with implications for several physiological functions, such as the shedding of the TFR during the maturation of reticulocytes into red blood cells, the release of decoy receptors, or the process of antigen presentation during the immune response (reviewed in Ref. 725). Recently, a DSL ligand has been shown to be secreted via exosomes, thus activating NOTCH from a distance, in the absence of cell-cell contacts (706). However, the relevance of exosomes is not limited to their protein-carrying ability, as it has been shown recently that they can also contain and deliver genetic material, thus functioning as a potential vehicle for genetic reprogramming of the recipient cell. Mast cells, for instance, secrete exosomes that contain more than a 1,000 mRNA species and more than 100 types of microRNAs (miRNAs), both of which can genetically reprogram a cell when taken up (789). Genetic reprogramming by exosomes can be exploited by cells for highly selective targeting functions, as recently shown by the unidirectional transfer of miRNA-containing exosomes by T cells to antigen-presenting cells at the level of the immunological synapse (530). The process becomes extremely relevant if one considers that exosomes can deliver miRNAs to the recipient cell. miRNAs are endowed with extensive regulatory capacity, since a single miRNA species can control the expression of scores of target mRNAs and proteins (see, for instance, Refs. 352, 412, 730 and references therein). Thus there is potential for extensive non-cell-autonomous regulation, through exosome-mediated transfer of miRNAs.

It was found that subunits of the ESCRT-II complex can selectively bind to mRNAs (346). The role of ESCRT-II in mRNA binding, however, seems to be independent of its role in endosomal sorting (346, 780). Indeed, it was shown that at least one population of ILVs/

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exosomes buds into MVBs by an ESCRT-independent mechanism, i.e., through the action of ceramide, produced by neutral sphingomyelinase at the cytosolic face of late endosomes (780). In this case, therefore, the endocytic (ESCRT) machinery in the donor cell does not constitute the "hardware" of exosome production, but possibly a tool for the selection of exosomal cargo for secretion. Whether this is a general principle that applies to many mRNA and/or miRNA species remains to be established. Similarly, it is unknown how (and if) the process of ESCRT-dependent recognition of mRNAs is coordinated with the ESCRT-independent mechanism of ILV/exosome budding. An alternative possibility is that the ESCRT-mediated localization of mRNAs can be part of the assembly of miRNA processing complexes, as reviewed below.

The liaison between endocytosis and miRNAs is even more articulated and might extend beyond exosomes, as it has been recently shown that endosomes and MVBs might actually represent platforms for the assembly of miRNA processing complexes (254, 442). Small regulatory RNAs (which include miRNAs and small interfering RNAs, siRNAs) are associated with Argonaute (AGO) proteins in the so-called RISC (RNA-induced silencing complexes), which directs the degradation or translational repression of target mRNAs. Components of RISC, including AGO proteins and GW182, are enriched in endosomes and MVBs (254, 442). The association has functional significance since blocking the formation of MVBs from early endosomes decreases RISC activity. Conversely, by inhibiting the fusion of MVBs with the lysosome, and thereby reducing the disposal of RISC through lysosomal degradation, RISC activity is increased. These results are compatible with a model in which the MVB membrane is a platform for the assembly of miRNA processing complexes (254, 442, 731) (Figure 3*D*).

The connection between endocytosis and microenvironment reprogramming through exosomes has important implications not only for physiology, but also for human diseases, such as neurodegenerative disease (see Ref. 805 and references therein). Indeed, recent findings have demonstrated that α-synuclein, a protein central in the pathogenesis of Parkinson's disease, is secreted via exosomes and probably serves to amplify and propagate the disease (192). Even more significantly, cancer cells secrete exosomes that can deliver RNAs, angiogenic proteins, and even mutated cancer proteins to the surrounding normal cells, thus promoting tumor growth (7, 394, 518, 568, 732, 834). Furthermore, cancer cells apparently secrete more exosomes than their normal counterparts, suggesting the idea that the exosome cycle can be hijacked by mutated cancer proteins to obtain genetic reprogramming of adjacent cells, much in the same way as exogenous pathogens, such as HIV1 and prions, ensure their release (Figure 3*D*).

F Endocytosis and Transcription

An intriguing facet of endocytosis is the frequent detection of endocytic proteins in the nucleus. This inevitably leads to questions of how and why endocytosis, an all-cytoplasmic event, might be connected to the regulation of nuclear events. Above, we have reviewed evidence that endocytic proteins are involved in mitosis and cytokinesis. This connection, albeit unexpected, is still rationalizable, at least in part, within the context of the dynamics of biomembranes, since some mitotic events, such as spindle formation, are deeply

interconnected with biomembranes, which probably provide an elastic module to support microtubules during spindle assembly (10, 30, 470, 833). In other cases, such as those reviewed below and dealing with the control of transcriptional events, the involvement of endocytic proteins appears to constitute a true noncanonical function of these proteins, not immediately linked to membrane dynamics. In the following paragraphs, we will provide some general principles of how endocytic proteins control transcription and then we will analyze in more detail two cases, those of ARRs and of the multilayered involvement of TP53 with the endocytic machinery. A word of caution: in this section a number of connections between the endocytic and transcriptional machineries are described. The evidence for the functional relevance of these connections is not always firmly established. Whenever such evidence is present in the literature, we report a brief description of it. In the other cases, it is implicit that the relevance remains to be ascertained.

1 Modalities through which endocytic proteins influence transcription—

Various clathrin adaptors and endosomal proteins undergo nuclear translocation, and operate in the nucleus to regulate transcription (342, 619, 803, 821; also reviewed in Refs. 74, 628). Transcriptional regulation by endocytic proteins occurs at multiple levels (Figure 8):

1) Control of RNA polymerase, exerted by subunits of the ESCRT-II complex (Figure 8*A*). Indeed, in mammals, the ESCRT-II complex was initially identified as a group of proteins that increases the catalytic rate of transcriptional elongation by RNA polymerase II in vitro (379, 717).

2) Chromatin remodeling, regulated by APPL1/2 (42, 523; see sect. III*C3*) and ESCRT-III proteins (756), which bind to chromatin remodeling complexes (Figure 8*B*). The involvement of both ESCRT-II (see above) and ESCRT-III components in the regulation of transcription is particularly interesting and also supported by recent network analysis studies which evidenced tight high-confidence functional interactions between nuclear and vesicular proteins, especially those related to the UB modification, such as ESCRTs (806).

3) Regulation of transcription initiation, by endocytic proteins that act as transcriptional coregulators by binding known transcription factors (Figure 8*C*). TSG101, a subunit of the endosomal ESCRT-I complex, interacts with the androgen receptor and the glucocorticoid receptor (both representing transcription factors) (92, 316, 347, 761). Binding to the androgen receptor has been reported to elicit either stabilization of the receptor with ensuing enhanced transcriptional activity (92), or further association with the transcriptional cofactor P300 with attenuation of the transcriptional activity of the androgen receptor (761). Thus, while the transcriptional activity of TSG101 is established, the details of its function remain unclear, and possibly context dependent. Another case is represented by HIP1, which works in endocytosis at the interface between CME and actin dynamics. HIP1 also interacts and increases the transcriptional activity of the androgen receptor (359, 525). These latter findings are particularly relevant in light of the demonstrated involvement of HIP1 in cancer, and in particular in prostate cancer in which the role of androgen receptor is paramount (636, 637). Finally, the clathrin heavy chain also can be

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found in the nucleus, where it specifically enhances TP53-dependent transactivation by binding to the TP53-responsive promoter and stabilizing the interaction between TP53 and P300 histone acetyltransferase (193, 567).

4) Retrograde delivery of transcriptionally relevant cargo (Figure 8, *E–G*). A number of membrane-anchored growth factor receptors and growth factors display nuclear localization, and their presence in that location has been linked to control of transcription (reviewed in Ref. 106, 827). Evidence is particularly compelling for members of the EGFR family and for their cognate ligands. The major question, in this case, is how membrane-bound protein can be delivered to the nucleus. Evidence has been provided that two membrane-anchored growth factors of the EGF family, proAR and pro-HB-EGF, are delivered, in a signaling- and endocytosis-dependent manner, to the inner nuclear membrane (INM), through a retrograde transport pathway, whose molecular workings have however not been clarified (Figure 8, *E* and *F*). Once on the INM, they can act as chromatin remodeling agents or sequesterers of transcriptional repressors, respectively (312, 348) (Figure 8, *E* and *F*). The situation is more complex for the EGFR itself, which also exhibits nuclear localization and possibly acts as a transcription factor (459) (Figure 8*G*). This property, which is also shared by other members of the EGFR family of receptors, relies both on the ability of the EGFR to bind directly to a number of promoters and to transactivate them, or to bind to well-known transcription factors (e.g., STAT3, E2F1, STAT5) (reviewed in Ref. 827). The surprising fact is that EGFR appears to localize to the nucleoplasm, i.e., in a non-membrane-anchored state. A number of pieces of evidence are compatible with a model in which EGFR traffics to the nuclear pore complex in a membrane-bound environment, through some form of retrograde transport. However, it does so in association with importin β, which interacts with putative nuclear localization sequences in the EGFR (and in the related receptor ERBB-2) (257, 472). Importin β is responsible for nuclear translocation, by directly associating with components of the nuclear pore, and might therefore aid in the translocation of the EGFR through the pore onto the INM. Interestingly, the translocon SEC61β also resides on the INM, where it associates with EGFR, and might be responsible for the extraction of the receptor from the membrane and its release in the nucleoplasm (828) (Figure 8*G*).

One general question is whether the regulation of nuclear events represents a "moonlighting" function of some endocytic proteins or betrays a deeper level of integration between these cellular functions, enabling, for instance, the efficient transfer of extracellular information to the nucleus. In some instances, the endocytic and nuclear functions appear to be mutually exclusive, such as in the case of HIP1 (525). Furthermore, the transcriptional activity of the clathrin heavy chain does not require its trimerization domain, which is instead indispensable for its endocytic coat protein function (567), again arguing in favor of distinct endocytic and nuclear functions. On the other hand, there are instances in which the endocytic and the nuclear transcriptional functions are linked, as it is the case for ARRs (see below, and Figure 8*D*) or for APPL, which, upon ligand stimulation, travel through the

endocytic routes as bonafide trafficking molecules, and eventually translocate to the nucleus to regulate transcription (523), or for membrane-anchored growth factor and receptors for which retrograde transport directly implicates endocytosis as the vehicle of nuclear delivery of transcriptionally relevant cargo. As we will discuss at the end of the review, scenarios can also be envisioned in which the apparent heterogeneity of the functions of some endocytic proteins can be reconciled.

2 ARRs and the control of transcriptional programs—The ARRs were originally discovered for their role in the desensitization of GPCRs. GPCR, once activated by their ligands, essentially work as GEFs for heterotrimeric G proteins (Figure 1*A*); this activity constitutes one of their major modality of signal transmission. Following activation, however, these receptors also become serine and/or threonine phosphorylated, which allows high-affinity binding of ARRs. The binding of ARRs precludes the receptor from further coupling with G proteins (desensitization). Furthermore, ARRs bind to clathrin and clathrin adaptors, thereby removing the receptor from the cell surface. Thus ARR-mediated desensitization and endocytosis represent short- and long-term mechanisms of GPCR attenuation, respectively (reviewed in Ref. 485; see also Figure 1*A*).

Recent findings have considerably changed this relatively simple outlook. It was found that ARRs have a dual role, as attenuator and propagators of signaling. This “signaling” role is in part connected to the endocytic role (along the lines and the principles reviewed in sections III, *A* and *D*, and V*B*), but largely constitutes an effector role in itself. The current view, therefore, is that GPCRs switch between two modalities of signaling: G protein-dependent signaling and ARR-dependent signaling, with the latter modality also being capable of attenuating the former. ARR-mediated signaling impinges on a number of relevant circuitries, including regulation of non-receptor tyrosine kinases, of ERKs and of E3 ligases (not reviewed in detail here, but see Ref. 485 and references therein).

A sizable part of the effector function of ARRs is connected to their ability to modulate transcription. In some cases, this is the consequence of their regulation of transcriptionally relevant signaling pathways. For example, ARRs negatively regulate transcription activated by the ERK (252, 438, 835), NFκB (208, 246, 482, 826, 850), and STAT1 (532) pathways, by titering out critical components of these pathways. In other cases, the transcriptional role seems more directly connected with the modulation of real transcription factors. ARR binding to MDM2, the major E3 ligase responsible for TP53 degradation, inhibits the ubiquitination of TP53, therefore stabilizing it, with ensuing enhancement of TP53 signaling (820).

Even more importantly, perhaps, it has been shown that ARRs undergo nucleocytoplasmic shuttling, exhibiting a distinct nuclear phase (Figure 8*D*). The shuttling is directly controlled by signaling (it follows activation of GPCRs) and leads to the formation of ARR-based complexes that contain the P300 histone acetyltransferase. Since ARRs can also directly bind to promoters (such as the P27, the FOS, or the BCL2 promoter), they might work as chaperones for P300, allowing increased local histone H4 acetylation and stimulating transcription (380, 552, 714) (Figure 8*D*). In addition, in zebrafish, it has been shown that ARRs bind and sequester the polycomb group recruiter YY1, in turn leading to a release of

the polycomb-mediated repression of the CDX4-HOX pathway, involved in the specification of the hematopoietic lineage (873). These studies have probably uncovered only the tip of the iceberg, as a recent proteomic study revealed that around one-third of the ARR interactome consists of nucleic acid-binding proteins (861). Thus ARR-controlled transcriptional regulation is an area in which we should witness important and surprising progress in the near future.

The signaling role of ARRs (including its transcriptional role) is very relevant not only to biology, but also to medicine. Currently, perhaps half of all drugs in clinical use are directed to modulate GPCR function. In addition, there is evidence that the two major signaling modalities of GPCRs (G proteins and ARRs) can be pharmacologically uncoupled, leading to the possibility that "biased GPCR drugs" might be developed for clinical use in many diseases.

3 Multiple points of contact between TP53 and endocytosis—We have reviewed evidence that clathrin and ARRs can control the transcriptional activity of TP53. Recently, one additional connection emerged, as it was shown that NUMB (an endocytic protein, reviewed in sect. VII*C1*) controls the cellular levels of TP53 (131). This action of NUMB is determined by its ability to bind to and inhibit the E3-ligase MDM2, in a circuitry reminiscent of the ARR/TP53 one (820). The regulation of MDM2 by NUMB occurs in the context of a NUMB/TP53/MDM2 tricomplex (Figure 8*H*) (131). The functional ablation of NUMB, in a model of normal human mammary epithelial cells, results in reduced TP53 levels and activity, with impaired apoptosis, DNA-damage, and cell cycle checkpoint activation response (131).

The potential relevance of these findings is that they project a role for the NUMB:TP53 axis in the maintenance of the SC compartment, a cellular territory in which the impact of endocytosis and recycling is paramount, as discussed. Investigations of the role of TP53 in SCs has so far focused on the induction of cellular senescence by TP53, which in turn can be linked to the depletion of SCs and to organism aging (reviewed in Ref. 884). However, a role for TP53 as a cell-autonomous asymmetric kinetics control gene has been proposed (712), which might be due to its involvement in regulating immortal DNA strand cosegregation, a phenomenon that is closely linked to ACD (634). This function might be directly connected to the asymmetric inheritance of NUMB, as supported by findings that: *1*) NUMB is a critical determinant of ACD, *2*) NUMB directly controls the level of TP53 (131), and *3*) in mammary SCs, the genetic removal of TP53 skews the cell division from an asymmetric to a symmetric mode, with both daughter cells acquiring a proliferative destiny (Figure 8*I*) (127).

One major question remains to be resolved: Does the control of NUMB over TP53 occur in the nucleus, since NUMB also shuttles in and out of the nucleus (373), or in the cytosol, possibly in association with biomembranes. This latter occurrence is not implausible, since a number of non-nuclear functions of TP53 are known, mostly connected with autophagy and apoptosis (reviewed in Ref. 270). In addition, in section IV*B*, we have reviewed evidence arguing for transcriptional and nontranscriptional functions of TP53 in endocytosis and traffic; thus, based on available knowledge, the existence of a feedback loop linking endocytosis → TP53 → endocytosis is not inconceivable. In such a case, the analysis of the

connections between endocytic pathways and TP53 will be an important area of future developments.

VIII Endocytosis and Diseases

The pervasiveness of endocytosis in virtually every program of cell regulation predicts that alterations of the endocytic machinery, or of intracellular sorting mechanisms at large, should play an important role in several human pathological conditions. This is indeed the case, and the pathogenesis of many diseases can be traced back to subversion of intracellular traffic. Here, we will briefly highlight the impact of endocytosis on human diseases, with particular emphasis on genetic diseases and cancer.

A Endocytic Trafficking and Human Genetic Diseases

Alterations of intracellular traffic have been described in a vast array of human diseases. They include defects at the level of the membrane-associated protein sorting and lipid trafficking machineries. Essentially all stations of the endocytic pathway are affected, from the internalization step to endosomal sorting, to lysosomal biogenesis and function. The endocytic proteins involved can be mutated or altered in their level of expression (overexpressed or underexpressed) or become the target of autoimmune responses. This, in turn, results in a role for endocytic proteins in several inherited, neurological, metabolic, autoimmune, infectious, and hyperproliferative diseases, among which there are many pathologies of high social impact such as Alzheimer's disease, diabetes, or cancer (reviewed in Refs. 16, 425, 856, respectively). A comprehensive discussion of endocytic alterations and human diseases is impossible here; however, the interested reader is referred to reviews on this specific subject in which a systematic classification of endocytosis (or traffic)-related pathologies has been described (23, 24, 624); in addition, the journal *Traffic* maintains a collection of published papers on this topic in a virtual issue "Diseases of membrane traffic" (http://www.traffic.dk/virtual_issues.asp).

In Table 2, we report an updated list of alterations of endocytic genes in Mendelian disorders, obtained by searching the OMIM (Online Mendelian Inheritance in Man) and the GENE databases at NCBI with a manually curated list of 339 genes encoding "endocytic/trafficking and actin regulator/dynamics proteins" [277 "endocytic/trafficking proteins, including structural/accessory endocytic proteins, ARF GTPases and their effectors, endocytic and non-endocytic RABs, proteins belonging to the ESCRT complexes, SNARE proteins, sorting nexins and synaptotagmins, proteins associated with lysosomes or endosomes and important for their biogenesis or function, and 62 "actin regulator/dynamics proteins" (see legend to Table 2 for details)]. Not surprisingly, a sizable number of alterations affect RAB proteins and RAB regulators/effectors, underscoring the pivotal master regulator role of these GTPases in the maintenance of endocytic and trafficking homeostasis. What is remarkable, however, is the frequency of alteration of endocytic genes in monogenic (Mendelian) disease. Of the 339 genes of our list, 289 are present in OMIM, as of May 2011. Of these 289 genes, 72 are responsible for monogenic diseases, corresponding to a frequency of ~25% (Figure 9*A*).

We sought to compare this frequency to that of the alteration of all human genes in Mendelian disorders. This is not a straightforward task, since public databases do not contain a downloadable list of all human genes responsible for monogenic diseases. Several efforts have been published, however, to produce such a manually curated list. In the most recent one, published in May 2009 (95), genes were retrieved from OMIM if labeled as disease-causing mutations and then ”contaminants,“ such as non-disease genes and genes not annotated as ”susceptibility genes,“ were filtered out. This yielded a list of 2011 genes (95). A more thorough effort of manual curation of a list of human monogenic disease genes (always using the OMIM database as a starting point) was reported a year earlier in June 2008 (68): this list contained 1,039 distinct genes. While differences in the two lists likely reflect those in the selection of the curation criteria, one can reasonably assume that ~1,000 genes and ~2,000 genes represent the lower and the upper limit of human Mendelian disease genes, respectively, at the present state of knowledge. These genes can be compared with the total number of human genes (~20,000), or more conservatively to the number of genes listed in the OMIM database (~14,000 as of May 2011). This creates a number of scenarios, depicted in Figure 9*A*, in which the fraction of all human genes responsible for monogenic diseases ranges from 5 to 14%. In all cases however, the frequency of alteration of endocytic genes is vastly (and very significantly, Figure 9*A*) superior to that of all human genes: from approximately two- to fivefold more.

The question arises therefore as to what is the meaning of the enrichment in monogenic disease genes of the class of ”endocytic“ genes. Several characteristics, which distinguish disease genes from non-disease genes, have been reported. First, Mendelian disease genes are under strong functional constraints, as it has been shown that they evolve more slowly than complex disease genes and non-disease genes as the result of stronger purifying selection (68, 95). In addition, disease genes are expressed more heterogeneously across tissues than non-disease genes (95). It was proposed (95) that disease genes, on the whole, are assigned to more essential functions than non-disease genes, thus explaining the strong purifying selection. At the same time, restrictions in the expression patterns allow their mutant alleles to go through germ-line without causing embryonic lethality. What is perhaps even more interesting is that disease genes tend to be older than non-disease genes (95, 175). Once again, a hierarchy might exist with Mendelian disease genes being older than complex disease genes, which are in turn older than non-disease genes (95). The fact that endocytic genes are a class strongly enriched in Mendelian disease genes, therefore, might mean that they are, on the average, older and more essential than other genes, a possibility that would fit well with the major thesis on this review, i.e., that they shape the eukaryotic cell plan, as will be discussed in section X. We directly tested this hypothesis by analyzing the ”age“ distribution of endocytic genes, with respect to all other human genes, using the gene classes identified by Cai et al. (95). As shown in Figure 9*B*, endocytic genes were remarkably, and very significantly, enriched in old genes and depleted in the classes of middle-aged and young genes.

B Alterations of the Endocytic Machinery in Cancer

Several lines of evidence support a role for endocytosis in cancer, and these are mostly connected to its role as a regulator of signaling events. Indeed, a wealth of studies have

shown how alteration of the endocytic machinery can induce transformation in several model systems, including mammalian cells in vitro (reviewed in Refs. 425, 538) and developmental model systems (especially *Drosophila*) (reviewed in Refs. 139, 787). With reference to naturally occurring tumors in humans, a synthetic list of the connections between endocytosis and cancer would include (due to space limitations, we cannot be as comprehensive as this topic would require, and we refer to more exhaustive reviews, cited along with each item below, on specific issues).

1) Endocytosis is an important regulator of RTK signaling, which is frequently subverted in cancer (see below and Refs. 538, 742).

2) Endocytosis is involved in the activation of oncogenic receptors, such as NOTCH, by regulating the accessibility of both receptors and ligands (237).

3) Endocytosis is a major regulator of cell fate determination, and of the maintenance of SC compartments (see sect. VII*C*). This may be highly relevant to cellular transformation, in light of increasing support for the SC theory of cancer (128, 139, 238, 278).

4) As we extensively described in section VII*B*, endocytosis is involved in the spatial restriction of signals needed for directed cell movement, and for the switch between motility strategies (amoeboid vs. mesenchymal) adopted by metastatic cells, thus implicating endocytosis in tumor progression.

5) Related to this, endocytosis and trafficking of adhesion molecules (cadherins and integrins) is often misregulated during cancer progression (110). This represents a crucial mechanism that cooperates with transcriptional programs leading to the acquisition by cultured epithelial cells of a mesenchymal-like and SC-like motile phenotype, a transition required for metastatic dissemination and possibly for reversion of progenitor cells into SCs during cancer development (377, 727, 785).

6) Autophagy, a degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome, is linked to tumor suppression and tumor promotion (82, 448). The relationship between autophagy and endocytosis is still largely undefined, although some connections are starting to emerge (144, 281), and this area might witness important developments in the future.

7) As we discussed in section VII, endocytic proteins are involved in the regulation of diverse cellular processes such cell cycle, mitosis, apoptosis, and genetic reprogramming that are known to be involved in cancer.

8) Finally, there is growing direct evidence for genetic alterations, or for subversion of their regulation, of endocytic/trafficking proteins in human tumors (425, 538).

In the remainder of this section, we focus on two aspects of the connection between endocytosis and cancer: *1*) a systematic analysis of alterations of endocytic/trafficking proteins and of actin regulators in human cancers, as obtained by extensive mining of public databases and of published literature, and *2*) a survey of the alterations of the endocytic determinants in signaling cargoes in cancer.

1 An atlas of the alterations of ”endocytic“ proteins in human cancers—To investigate the impact of deregulation of the endocytic and trafficking machinery in cancer, we used the same list of 339 ”endocytic and actin regulator proteins“ employed in Table 2, to screen the OMIM and GENE databases and published literature for alterations in cancer. In Table 3, we show all the identified alterations for which high-resolution studies are available. In addition, high-throughput studies are identifying a wealth of somatic mutations of endocytic proteins, whose impact remains, however, to be established. This latter series of potential alterations is reported in Table 4, as obtained by searching the COSMIC (Catalogue of Somatic Mutations in Cancer) database, using the list of 339 ”endocytic and actin regulator proteins.“ In this latter case, we found that 160 genes (47%) harbor at least one mutation in one tumor type (excluding silent mutations, which are also reported in the COSMIC database).

A close analysis of the high-throughput approach gene list of Table 4 revealed several interesting features, especially in light of the fact that high-resolution studies (Table 3) concentrated mostly on alterations in the expression of endocytic genes and comparatively less on their mutations. We focused on those genes displaying more than five total mutations (henceforth ”frequently mutated,“ see Table 4 and Figure 9*C*). In all these cases, the number of tumors screened is large enough (from 75 to 3,475 tumors) to allow for some tentative conclusions.

In the case of CBL, for instance, COSMIC data confirm, on a much wider scale, conclusions present in the literature about mutations of this gene in neoplastic diseases of the myeloid lineage. The vast number of cases permits the establishment of a frequency of ~6% for CBL alterations in myeloid malignancies. Interestingly, CBL was also mutated in ~3% of lung cancers (non-small-cell lung carcinoma, NSCLC; note that the mutations of CBL in NSCLC in the COSMIC database are those reported by Ref. 765). In myeloid malignancies, mutations are clustered in (or very close to) the Ring Finger region of CBL, which is essential for binding to E2 conjugating enzymes and, therefore, for the E3 ligase activity of CBL (680, 685) (Figure 9*D*). Interestingly, mutations in NSCLCs display a more widespread distribution, being present also in the TKB region (which is responsible for CBL binding to pY residues in RTKs, see sect. V*A*) and in the COOH-terminal region of the protein. Thus, although the real impact of its mutations in lung tumorigenesis remains to be established, CBL might participate in neoplastic transformation with different cell-specific molecular mechanisms.

Another eight endocytic and actin regulator genes were frequently mutated in the COSMIC database. Of these, only CYFIP1 was previously implicated in cancer through high-resolution studies (723). For the other seven (VPS13B, CUBN, LYST, TSC2, FLNB, RIMS1, FLNC), the involvement in cancer was previously unsuspected (see Table 3). We caution that in several tumors, the number of analyzed cases is too low to draw meaningful conclusions. In addition, the high frequency of mutations in ovarian cancers is suspect because too many genes were mutated at high frequency. Despite these limitations, in the case of breast cancer, the number of analyzed cases (30–50) and the frequency of mutation of the ”frequently mutated genes“ (in some cases as high as 10 –12%) suggest a significant impact of subversion of endocytosis in this type of neoplasm (Table 4).

There is one additional reason to suspect that mutations of the ”frequently mutated genes“ have a causal role in cancer. We noticed a singular overlap between endocytic genes that are mutated in Mendelian diseases (as from Table 2) and those that are frequently mutated in cancer. Table 4 shows that, already by visual inspection, a clustering of Mendelian genes is evident towards the top of the Table, where the frequently mutated genes are. In particular, eight of nine genes frequently mutated in cancer (>5 total mutations) are also monogenic disease genes. The calculated *P* for this event is highly significant (*P*: 0.0005); in addition, this is an exclusive property of the ”frequently mutated genes,“ since the overlap between the entire two sets of genes (all the genes of Table 2 and Table 4) is not significant (*P*: 0.5). This argues for the fact that a subset of endocytic genes are highly sensitive to mutations (meaning that they are sufficiently important for mutations to cause disease phenotypes), possibly for the reasons already discussed at the end of section VII*A*. Under this scenario, mutated alleles of these genes would give rise to Mendelian diseases or to cancer when mutated in the germ line or in somatic cells, respectively.

This hypothesis is supported by the analysis of the CBL mutations in the Noonan syndrome-like disorder (the monogenic disease in which CBL is implicated) and in cancer. The mutations in the genetic syndrome (501, 605) cluster in the Ring Finger region of CBL, and in several cases they affect exactly the same residue as in myeloid diseases (Figure 9*D*). Indeed, in the case of CBL, the tight relationship between cancer and monogenic diseases is established at the human genetics level by the fact that individuals with juvenile myelomonocytic leukemia harboring CBL mutations also show phenotypic traits of a Noonan syndrome-like disorder (605). In the case of the other ”frequently mutated in cancer“ endocytic genes, the connection between mutations causing cancer and Mendelian diseases is more elusive. This can be due to the low number of cancer mutations presently available and to the fact that, in many cases, the genetics of the paired conditions (cancer:Mendelian disease for each gene) might be different (dominant or recessive), suggesting different molecular pathogenesis. Whatever the case, the highly significant overlap between the two sets and the CBL paradigm suggest that the comparative analysis of alterations in cancer and in genetic disease might help to identify driver mutations in cancer, a possibility that we suspect might extend beyond the subset of endocytic genes herein analyzed.

2 Alterations of the endocytic determinants in signaling cargoes in cancer— Not only endocytic proteins, but also PM cargoes are frequently mutated in human cancers, in specific determinants that alter their vesicular traffic. In this instance, alterations usually affect the ability of the receptor to be properly ubiquitinated and downregulated, therefore causing sustained signaling. This is the case for several RTKs, like EGFR (reviewed in Ref. 609), MET (reviewed in Ref. 418), and KIT (876). The most frequent genetic alterations, in these occurrences, consist of deletions that affect the region encoding portions of the intracellular domains of RTKs, usually encompassing the binding region for CBL, the major E3 ligase involved in RTK ubiquitination.

In addition to this, other mechanisms are exploited by cancer cells to evade endocytosis-mediated desensitization. For instance, somatic mutations in the kinase domain of the EGFR have been reported in non-small-cell lung cancers, and they have been shown in vitro to

cause reduced receptor phosphorylation at Y1045, the major CBL binding site and, consequently, defective receptor downregulation (240, 719). Similarly, EGFRvIII, an oncogenic deletion mutant of the EGFR, frequently observed in glioblastoma, shows hypophosphorylation of Y1045 and reduced degradation (267, 288; see also Ref. 538 for more detailed explanations).

An endocytic-dependent mechanism has been proposed to contribute to the transforming effects of ERBB-2 over-expression in breast cancer. ERBB-2 belongs to the EGFR family of RTKs; at variance with EGFR, however, ERBB-2 is internalization impaired (52, 741). Heterodimerization of ERBB-2 with ligand-occupied EGFRs seems to influence the endocytic trafficking of both ERBB-2 and EGFR. Indeed, it has been shown that EGFR-ERBB-2 heterodimers display delayed endocytosis, are not efficiently sorted to lysosomes, and are preferentially recycled back to the cell surface, causing aberrant signaling (28, 307, 445, 855). One possibility is that EGFR and ERBB-2 are not fully ubiquitinated in the heterodimers. Indeed, while activated ERBB-2 can recruit CBL, this recruitment is less efficient compared with EGFR (449). An alternative possibility is that heterodimers display reduced affinity for EGF and dissociate from the ligand in endosomes, due to the release of the ligand in the acidic environment of endosomes, being recycled back to the surface (445). However, computational modeling of the trafficking of EGFR-ERBB-2 heterodimers predicted that elevated dissociation of ligand in endosomes could not explain the observed trafficking patterns of the heterodimers (308). Rather, the reduced degradation of EGFR might be explained by a mechanism through which ERBB-2 directly competes with EGFR for a stoichiometrically limited quantity of endosomal retention components, thereby reducing endosomal retention and degradation of EGFR (308). Whatever the case, it appears that altered trafficking of EGFR might be one mechanism through which ERBB-2 exerts its oncogenic potential.

In addition to RTKs, many GPCRs are overexpressed in human cancers and contribute to tumor progression (reviewed in Ref. 178). Recent work has revealed that deregulated trafficking of CXCR4 and PAR1 through the endosomal-lysosomal station leads to increased surface expression of these cargoes in breast cancer cells, contributing to cancer progression (72, 199, 200, 453). Interplay with ERBB-2 seems to have a role in breast cancers that display elevated CXCR4 surface levels. Indeed, ERBB-2 overexpression seems to enhance CXCR4 levels both by increasing protein synthesis and by impairing CXCR4 ubiquitination and lysosomal degradation mediated by AIP4, the E3 ligase involved in this process (453). It has been proposed that in this case the mechanism may involve CISK, a Ser/Thr kinase downstream of PI3K signaling, which phosphorylates and inactivates AIP4, thereby contributing to the increased CXCR4 levels (734).

In conclusion, while we have had to necessarily limit ourselves to the description of a few paradigmatic cases, it is evident that subversion of endocytosis might be involved in cancer in multiple ways. Given this, a deeper analysis of the endocytic process is predicted not only to advance our understanding of cell regulation and how it connects to the pathogenetic mechanisms of cancer, but should also help to identify novel targets for molecular therapies and clinically relevant biomarkers for prognostic, diagnostic, and therapeutic purposes.

IX Evolution of Endocytosis

In this section, we review knowledge and hypotheses regarding the origin of endocytosis. We will do so, initially, with the underlying assumption that endocytosis evolved as a tool to increase fitness through the more efficient uptake of nutrients from the extracellular milieu (see sect. I and Ref. 151). An evolutionary perspective is, in our opinion, indispensable to understand how and why endocytosis has become what we know today. In the next section (sect. X), we will then try to put forward models that explain speculatively how a relatively simple device might have evolved to become a cornerstone of the eukaryotic cellular plan.

Even the simple ”will work for food“ outlook on endocytosis requires rather sophisticated tools, such as a functional system of endomembranes, the presence of accessory proteins that give plasticity to the membrane system (i.e., coat and transport/trafficking proteins), and an active cytoskeleton. Indeed, it is widely assumed that an internal and dynamic endomembrane system comprising a nuclear envelope, ER, Golgi system, endosomes, phagosomes, lysosomes, autophagosomes, peroxisomes, and mitochondria (chloroplast) must have been present in the last eukaryote common ancestor (LECA). In addition, until recently, the cytoskeleton, endomembranes, and endocytic accessory proteins were thought to be exclusive to eukaryotes. The picture has started to change, however, in the last few years with the realization that a number of elements of the system were already in place much earlier in evolution, in prokaryotic organisms. This bears important consequences on our understanding of how endocytosis came into being, and, as we will see, of how its ”simple“ beginnings already had embedded in them the prerequisites for it subsequent ”explosion“ in eukaryotic homeostasis.

A Actin in Prokaryotes

Actin filaments serve as a scaffold for motor proteins, e.g., in the distribution of mobile cellular elements such as transport vesicles and organelles. On the other hand, actin filament polymerization is the driving force in cellular shape changes such as the formation of pseudopods and amoeboid movement of cells. Actin homologs have been identified in prokaryotes (see Refs. 101 and 701 for a complete review). Both the actin homolog (Ta0583) of the archaeon *Thermoplasma acidophilum* and the eubacterial actin homolog (MREB) in *Bacillus subtilis* have been shown to possess biochemical and structural properties equivalent to those of eukaryotic actin (198, 293, 399, 657). The majority of the proteins involved in actin remodeling have no prokaryotic homologs or display only distant connections (such as a common structural fold) at the level of individual domains. In particular, no prokaryotic homologs were detected for the accessory subunits of the ARP2/3 complex that is highly conserved in all eukaryotes and serves as a nucleator of monomeric actin units to initiate polymerization (874). However, the presence of common structural features in ARP2/3 proteins and in the archaeal actins suggests that the common ancestors of the archaeal and eukaryotic actins were capable of forming branched filaments. In addition, the family of RHO GTPases, that are ubiquitous regulators of actin dynamics in eukaryotes, appears to be of bacterial origin (874).

Bacterial actin-like proteins have been shown to perform essential functions in several aspects of cellular physiology. They control cell growth, cell shape, chromosome

segregation, and polar localization of proteins and localize as helical filaments underneath the cell membrane. MREB forms dynamic helical structures and is required for the maintenance of a rod-shaped morphology. It has been shown to form spirals that traverse along the longitudinal axis of *Bacillus subtilis* and *Escherichia coli* cells. It has also been shown that the bacterial cytoskeleton and cell shape-determining proteins, such as MREB, function in concert to orchestrate the localization of cell wall synthetic complexes (171). In addition, MREB is involved in chromosome segregation. Eukaryotic cells use the tubulin-based cytoskeleton to segregate their chromosomes during mitosis. In bacteria, this task is accomplished by the actin homolog MREB, which specifically binds to and segregates the replication origin of the bacterial chromosome (414). *Bacillus subtilis* MREB and MBL (a second actin ortholog) have been shown to perform dynamic motor-like movements within cells (154). A proposed mechanism is that polymerization of MREB from the middle of the cells toward the cell poles pushes replicated regions on the chromosomes toward the poles (746). Thus a primordial actin-like cytoskeleton is present already in prokaryotes, ready to be harnessed (in evolution) by the future endomembrane system.

B Endomembranes and Coat Proteins in Prokaryotes

While the assumption that LECA possessed a well-developed endomembrane system is widely accepted, there is no established consensus regarding its origin and evolution. There are several models for the origin of endomembranes, which have been put forward mainly with the intent of explaining the origin of the eukaryotic nucleus and, the nuclear envelope itself (360, 500). Three major models are considered: *1*) a symbiontic scenario, which posits that the nucleus evolved from a symbiont (an archaeabacterium or enveloped virus); *2*) a de novo scenario, postulating that membrane genesis was gained by spontaneous lipid vesicle assembly; and *3*) an autogenous scenario, in which endomembranes evolved via the inward budding of a prokaryotic ancestor's PM.

A series of findings obtained with Planctomycetes (461) argues that an endomembrane system and compartmentalized cell organization, in an ancestral organism, could have developed without the need for contributions from cells of other domains of life. Indeed, a simple but functional system of endomembranes is present already in bacteria, in the phyla of Planctomycetes (reviewed in Ref. 234). Notably, the planctomycete *Gemmata obscuriglobus,* one of the few compartmentalized bacteria, seem to possess three distinct compartments; a "nucleoid" containing the DNA, a "riboplasm" a ribosome-containing cytoplasm, and a ribosomefree cytoplast, the "paryphoplasm" (461). In addition, in several members of this phylum, cytosolic membrane coat-like (MC) proteins were found, and for some of them a clear membrane-bound localization was observed (682). Finally, *Gemmata obscuriglobus* has the ability to uptake proteins present in the external environment in an energy-dependent process analogous to eukaryotic endocytosis, and the internalized proteins are associated with the membranes of internal vesicle (477). Thus an internal membrane system, responsible for endocytosis, has evolved within a simple prokaryotic cell and without the involvement of a symbiont.

The existence of Planctomycetes MCs can be viewed in light of another interesting concept that emerged from eukaryotic studies, that of the "protocoatomer." In eukaryotes, the

biogenesis of transport containers, which shuttle cargo between endomembranes and/or to and from the PM, is mediated primarily by coat protein complexes. These include the coat protein complex II (COPII) that mediates, ER-to-Golgi vesicular trafficking, coat protein complex I (COPI) that mediates intra-Golgi and Golgi-to-ER trafficking, and the clathrin-based protein complexes that are involved in endocytosis and trafficking between the Golgi, lysosomes, and endosomes. The core coat protein machineries are not only highly conserved throughout eukaryotic evolution, but also all evolutionarily related. An evolutionary link between the components of the COPI and clathrin adaptor complexes (AP-1, AP-2, and AP-3) has been demonstrated (688), as also supported by structural and biochemical comparisons of COPI and AP-2 or AP-1/AP-3 subunits (320). These findings support the idea that all eukaryotic coat proteins share some common ancestor, which is operationally defined as a "protocoatomer."

The question now is as to relationships between the Planctomycetes MCs and the hypothetical eukaryotic protocoatomer, and more in general between endocytosis in Planctomycetes and in eukaryotes. No significant sequence similarity can be detected between the bacterial and eukaryotic coat proteins. Although this seems to indicate that the two sets of proteins are unrelated, it is noteworthy that their core architecture is conserved and that sequence similarity is often lost during long periods of evolution (e.g., FtsZ and tubulin or MreB and actin). Indeed, low or no sequence similarity can be detected between the eukaryotic coat proteins themselves, despite a common origin and significant structural similarity. This leaves us with a number of possibilities. On the one hand, endocytosis, as found in Planctomycetes, may be an example of a parallel evolutionary development of an analog of the eukaryotic process. In other words, it is possible that both eukaryotic and bacterial membrane-coat proteins appeared separately, i.e., by convergent evolution. The alternative is that the two processes are the result of divergent evolution, in which the process originated in prokaryotes and then was either selectively lost in some branches of bacteria and in Archaea, or laterally transferred to Eukarya (see Ref. 222 for a more detailed discussion). The possibility also exists that bacteria of the superphylum Planctinomycetes-Verrucomicrobia-Chlamydiae (PVC) represent an intermediate evolutionary step between bacteria and a common eukaryotic and archaeal ancestor (161). Regardless, there is one fundamental lesson from the Planctomycetes studies, i.e., that generating an endomembrane system might not be that difficult after all. In the PVC superphylum, MC-like proteins are found only in those bacteria that possess a compartmentalized cell plan, i.e., with intracytoplasmic membranes (682). This might mean that the simple presence of a membrane-bending protein is enough to lead to the generation of an endomembrane system.

C From Prokaryotes to Eukaryotes

A system of endomembranes, an actin-like cytoskeleton and a repertoire of coat proteins mostly likely allowed, during evolution, the development of an internal trafficking system. As we have seen, all of these elements are already present at least in some prokaryotic cells. One could postulate that the loss of the cell wall in a prokaryote created the initial condition of membrane plasticity necessary for endocytosis. The development of protocoatomers allowed for membrane bending, and the harnessing of a primordial actin cytoskeleton provided the mechanical force to tubulate or vesiculate the PM. Ribosomes that were

initially attached to the PM then became internalized but stayed attached to a membrane, giving rise to a primitive endomembrane system, the rough ER, and finally to the nuclear envelope (152, 500). The acquisition of mitochondria then had a decisive impact on eukaryotic cell architecture. While such an outlook is obviously far from being proven (see Refs. 361 and 874, particularly in the Reviewers' Comments sections), it provides a plausible scenario under which an endocytic system might not only have evolved before eukaryote separation as a distinct lineage, but can also be considered a prerequisite for the formation of the endomembrane system, of the nuclear envelope, and mitochondrial acquisition, which are the fundamental features of all eukaryotes.

One important finding in favor of this model is the fact that membrane coat proteins and nuclear pore complex proteins are evolutionarily related at the structural level (160). Nuclear pores and vesicle-coating complexes may share these folds because both complex types originated from a common ancestor. In this scenario, a single protocoatomer would have been the progenitor for numerous vesicle coating complexes, as well as nuclear pore proteins. This model links vesicle coats and the nuclear pore protein complexes through a common ancestor, suggesting an evolutionary continuity of the corresponding membrane domains, i.e., the PM, the ER, the Golgi, and the nuclear envelope, and strongly argues that a secretory/endocytic compartment and its actively budding coated vesicles would predate the origin of the nucleus, and thus of eukaryotes (279, 360).

The sum of all data reviewed above, therefore, strongly support the autogenous scenario (see sect. IX*B*) for the origin of an endomembrane system and of the nuclear envelope via the inward budding of a prokaryotic ancestor's PM. While the driving evolutionary force might very well have been "competition for food," there is one major implication of this outlook (regardless of the driving force), i.e., that the starting point of any further molecular evolution in the endomembrane system must have been proteins originally associated with the PM, as supported by the relationship between coats and nuclear pores, an issue that will be further developed in section X.

X Outlook: Beyond the Partnership

It seems that endocytosis pops up at every stone that we turn in the cell. In this section we will speculate on why this might be so. Our *leitmotiv* is that endocytosis initially evolved as a simple stand-alone process in the competition for nutrients. However, the peculiar design of the system, even in its very primordial version, implicated a number of latent properties that created the enabling conditions for the explosion of a number of other features (Figure 10). These latent properties do not appear strictly related to the initial selective advantage provided by endocytosis and are thus true "emerging properties" of the system. They led to the development of a completely novel cellular plan, based on a novel system of cell logistics: the logistics provided by endocytosis and the eukaryotic cell plan.

While, for ease of understanding we will frequently use colloquial expressions such as "the cell learns," or "molecules learn," or the "purpose" of something, it goes without saying that these are not proper evolutionary terms. Our aim, though, is to present an intuitively understandable picture of how such a novel cellular plan may have come to be.

A A Logic for Logistics?

At the beginning of this review we provided a rather unusual definition for endocytosis: “Endocytosis is the logistics of the cell.” It is now time to explain, in light of all the facts that we reviewed, what we mean by that. From the evolutionary point of view, it does not take a large stretch of the imagination to see how the cell might have very rapidly learned how to exploit for other purposes, a system that originally developed under the simple pressure of competition for nutrients.

The appearance of receptor-mediated endocytosis is a first case in point. This is a process present in all eukaryotic cells and, therefore, must have been present in the LECA already. From the evolutionary viewpoint, it must have provided a considerable advantage, since it allows a switch in feeding habits from “sampling the milieu through bulk endocytosis” to “capturing and concentrating the nutrients.” Thus it might have evolved under the same selective pressure that allowed the development of endocytosis. And yet, the process came with an unexpected property: it modulated the composition of the PM, allowing for a higher level of molecular plasticity in the relationships between the intracellular and extracellular compartments.

Recycling is another example. One could easily envision a scenario under which recycling evolved as a simple tool to replenish the PM of components that were depleted during the internalization process. Similarly, when the cell learned how to employ receptor-mediated endocytosis, the ability to recycle receptors to the PM must have constituted a strong selective advantage in the competition for food. However, once the system was in place, it should not have taken much tinkering to develop a system of homing devices to obtain selective recycling to specific areas of the PM, as opposed to the bulk PM. The result of such a process is the ability to concentrate PM-resident molecules, such as receptors, in restricted areas of the PM, a prerequisite for the execution of polarized functions. In other words, the cell might have learned, by exploiting an emerging property of the system, that the quickest and most efficient way to move things around on the PM, was to move them away from the PM, and then back to it, through recycling. In this way the endocytic system might have been harnessed for the execution of a number of spatially restricted functions, such as directed cellular motility.

Another obvious emerging property of a vesicular system resides in the size of vesicles. The PM is a vast surface in which signaling molecules, which have been brought together to achieve effective concentrations required by the law of mass action, can rapidly diffuse away if not prevented from doing so by some energy-consuming mechanism. If signaling molecules, initially concentrated in a region of the PM, are internalized and sequestered in a vesicle, they simply have nowhere to go, and cannot diffuse away. This would create the conditions for sustained signaling and for “further improvements” such as the development (or the optimization) of “coincidence detectors,” i.e., molecular functions needing two or more simultaneous, relatively weak interactions to exert their function. Such a process, exemplified for instance by the simultaneous interaction of the endosomal protein EEA1 with RAB5 and PI3P (726), would obviously be favored on a small vesicle, with respect to the bulk PM. The physical separation of a primary and secondary membranous compartment (the PM and the endosomes) might additionally have allowed the cell to interpret time-

resolved signals, by converting an orderly temporal sequence into an orderly spatial sequence of compartments. This development would again represent an emerging property of the system that could readily be exploited to add complexity to signal deconvolution.

As we have already mentioned in the previous section, the likely origin of the cell nucleus from a PM-originated system of endomembranes carried the almost obligatory consequence of developing "nuclear" functions by tinkering with what was available, i.e., PM-originated proteins. In many cases (such as for coat proteins and nuclear pores), gene duplication and functional divergence might have been part of the evolutionary strategy. In others, one might imagine (an admittedly speculative scenario) that some proteins simply "learned" how to do additional things in the new environment, while retaining the original function. This in turn might help us rationalize why some endocytic proteins appear to perform moonlighting jobs in the nucleus (see sect. VII, *D* and *E*).

It should be also considered that some cellular processes were harnessed by endocytosis early in evolution, and therefore must have co-evolved with it from that point on. This is the case, as discussed already, for the actin cytoskeleton. Co-evolution would easily explain the numerous and bidirectional liaisons between endocytosis and actin dynamics. Another circuitry that must have been recruited to endocytosis in its early days is ubiquitination. Ubiquitination is certainly one of the distinctive, and highly conserved, features of eukaryotes; however, its ancestry can now be traced back to bacteria, both in terms of UB-like molecules and in terms of enzymatic molecular machinery (unfortunately, we cannot cover this fascinating story here, but see the beautiful review by M. Hochstrasser, Ref. 317). In addition, ubiquitination is firmly rooted in endocytic routes in all eukaryotic organisms, starting from yeast (see sect. V and Ref. 446 for a recent review). Therefore, we can postulate that there must have been very early mingling of endocytic and ubiquitination pathways leading to their subsequent co-evolution.

Signaling through phosphotyrosine (pY) might instead be a case of later convergence. This regulatory mechanism is relatively recent in evolution and can be traced back to ~600 million years ago just prior to the appearance of metazoans, during the transition from unicellular to pluricellular eukaryotes (reviewed in Ref. 455). In particular, while some elements of the system might be of rather old ancestry (455), tyrosine kinases (TKs) have been found only starting from choanoflaggelates, which probably are the closest unicellular relative of metazoans (400, 401, 696). In choanoflaggelates, TKs appear in rather explosive fashion, with ~120 TK domains in *Monosiga brevicollis* (401), and many of them already displaying the typical RTK configuration known in metazoans. This might mean that, from the very beginning, the evolution of pY signaling was constrained by the topology of an endomembrane system. In other words, what is considered the most distinctive signaling feature of metazoans (pY and TKs) might have been "forced" to evolve in a certain way because of its association ab initio with a preexisting system of spatial constraints. The vast interconnection between the UB and pY system, especially at the level of hubs (22), might very well have been directed and/or facilitated by the fact that the two signaling systems shared the same spatial platform (endomembranes).

In summary, a mixture of emerging properties, almost obligatory consequences, and coevolution of early and late convergent pathways might have transformed endocytosis from its primordial trade into something rather different: a powerful communication and compartmentalization infrastructure or, in essence, what we define as “the cell logistics of the cell.”

B A Picture Is Worth a Thousand Words

The evolution from prokaryotes to eukaryotes might have, therefore, been marked by a transition from an intracellular environment, in which communication was determined largely by free diffusion, to one in which a specialized infrastructure became available. The networking abilities of this infrastructure might have vastly transcended those required by the original “purpose” because of a number of emerging properties.

To illustrate how this might have been a turning point in evolution, so powerful as to become one of the founding blocks in the development of a completely new cell plan (the eukaryotic cell plan), we propose an analogy with the road system of the ancient Romans (694). There is little doubt that the might of Rome rested to a great extent on their road system. This system (some 50,000 miles) was built essentially for military purposes. Its emerging properties, however, were such that the system became central to the vitality of the Empire, as it fostered commerce, economy, the mail system, and prompted the development of new technology to maintain and develop the system itself for purposes different from the original ones. One property of the system that is of great relevance to our analogy is that it allowed the transfer not only of “hardware” (a legion, a payload, a letter), but also of “software” (laws, customs, religion). In the Internet era this might not appear to be a great accomplishment, but it is indeed the basis of civilization as we know it.

By analogy, the infrastructure that we call endocytosis (including all aspects of trafficking and of derivatives of endocytosis, such as the development of a cell nucleus) allows the intracellular movement of hardware (e.g., a nutrient) or of software (e.g., instructions on how to make a cell move directionally) and allow cell compartmentalization, regardless of how and why it came into being. As with all transport systems, there are structural components (all proteins needed for the actual functioning of the system, i.e., the majority of what we call endocytic proteins) and passengers (other molecules) that use the service. Passengers can be of different kinds: commuters would be the regular passengers (cargoes and associated machinery) for which the system was initially designed or that learned how to associate with it for the specific purpose of being carried around either to be delivered to a destination or to deliver the information that it is associated with them. Hitchhikers would be molecules that associate with the system (i.e., they hitch a free ride), for purposes unrelated to endocytosis, without altering the functioning of the system. One advantage that a hitchhiker might gain by doing so is, for instance, to remain physically segregated and blocked (or regulated) until the time is right for the execution of its function. The concept of hitchhiking is perhaps best visualized by considering its deviations, represented by hijackers. These are violent hitchhikers that sidetrack the system, causing its malfunction. Pathogens, such as viruses and bacteria, are examples of this situation (not reviewed here, but see Refs. 274, 517). In addition, increasing evidence (see sect. VII, *A* and *B*) indicates that cancer

proteins might usurp the endocytic system to confer a proliferative advantage to the transformed cell.

Of course, there is no sharp demarcation to distinguish commuters from hitchhikers. This would essentially depend on whether the association with the endocytic system is part of the "core" function of the molecule (as it would be the case of a receptor for a nutrient, e.g., the TFR, a true commuter) or an "accessory" one that helps the optimization of the function of the hitchhiker. Furthermore, hitchhikers (and to some extent also commuters) might very likely not maintain their status for long (in evolutionary time), as they might acquire, as a result of continuous evolutionary tinkering, some endocytic role, and thus start to contribute to the functioning of the endocytic system, while retaining their original occupation (we refer to this situation as that of ticket holders, i.e., of molecules that start to pay a price for the ride). Some structural components of the endocytic machinery might actually find themselves in a similar condition, in which they learn how to do things unrelated to their primary endocytic function, simply because they interact with hitchhikers on the endomembrane system. These "new jobs" might be so unrelated to the original ones, as to appear to be moonlighting jobs, thus explaining a number of instances in which endocytic proteins appear to execute completely unrelated functions.

The question is whether there is experimental support for this scenario. We believe so. For instance, the endocytic function of clathrin becomes increasingly important in evolution, from yeast to mammals (374, 778), suggesting increasing participation in endocytic events. A similar situation occurs in the case of clathrin adaptors, such as AP-2, that seems to have a limited function in endocytosis in yeast (108), but is pivotal in mammals (reviewed in Ref. 135). In the case of dynamin, it has been suggested that the primordial function of this GTPase is related to the regulation of mitochondrial inheritance. During evolution, some dynamins were "recruited" to the endocytic pathway to execute vesicle fission. Interestingly, this event seems to have happened through convergent evolution during the ciliate and metazoan radiation (189), thus indicating that the enrollment of dynamin to the endocytic machinery occurred more than once, and independently, during evolution. Recently, putative endocytic functions have been attributed to known tumor suppressor genes, such as MERLIN/NF2, VHL, and TP53 (194, 329, 490), which might further corroborate the idea of "recruitment" to the endocytic pathway of growth regulators.

The best example, however, is probably the protein NUMB (see sect. VII, *C* and *F3*). There appear to be three basic cellular functions intersected by Numb: *1*) endocytosis, and in particular recycling; *2*) the regulation of the UB network; and *3*) cell polarity, in connection with the PAR polarity complex (reviewed in Ref. 592). Numb appears in evolution roughly with bilateral animals (592). By this time, two of the functions to which Numb participates, endocytosis and ubiquitination, are already firmly planted, and interconnected, in the eukaryotic cell's make-up (see above). It is unlikely, therefore, that Numb might have evolved in direct conjunction with these processes. However, the appearance of Numb roughly coincides with the appearance of the PAR complex. While polarity (and the related event of differential inheritance) is perhaps as old as cellular life (for a review, see Ref. 488), a clear existence of proteins of the PAR complex can be traced back in animals only until roughly 500 million years ago, probably with the emergence of ancestors of bilateral animals

(e.g., nematodes, flies, and mammals) (reviewed in Ref. 260). It is possible, therefore, that Numb evolved (or co-evolved) together with the PAR complex, although it is impossible to say whether it would have been connected specifically to one of the multiple functions of the PAR complex in animal cell polarization. Regardless, Numb might represent (and might have evolved to be) a critical connector between polarization and endocytosis. Why this should be so is, obviously, a matter of speculation. However, many of the functions of the PAR complex, e.g., in the maintenance of epithelial cell polarity or in cell migration or in ACD, do require a tight coregulation with vesicular intracellular transport (discussed in Ref. 260) and, as discussed earlier in the context of SARA-containing endosomes, there is emerging evidence that unequal inheritance of endosomes might play a crucial role in ACD (see sect. VII*C1*). Thus Numb might have evolved with an original role in polarity, and because of its membrane location might subsequently have acquired additional roles in the connected endocytic/UB networks, liaising them with the hardware of polarity as well as participating with them in a polarity-independent fashion: in essence the characteristics that we expect of a ticket holder.

C Deconvoluting the "Matrix"

In the previous two paragraphs we have tried to depict a possible scenario to explain the fact that the present picture of endocytosis, as derived from a wealth of experimental evidence, is that of a very pervasive program that permeates basically every aspect of cell physiology and regulation. In our opinion, the simplest explanation for this is that endocytosis (in its wider meaning of cell's logistics) evolved not so much as a "stand alone" process that subsequently infiltrated other processes, but rather that it represented a quantum leap in cellular organization that allowed the development of a completely new cellular plan: the eukaryotic cell plan. In other words, what we call endocytosis is just one particular facet of a vaster code that supports the eukaryotic cell plan. "Endocytosis" in the classical sense is therefore one viewpoint of the code: the one that we initially discovered, and possibly the one that constituted the initial advantage for its selection. We have coined the term *endocytic matrix* to help visualize this concept (694) (Figure 10). The term *matrix* might be understood here roughly in the sense of computer sciences, to indicate the network of intersections between input and output functioning as a decoder. This might render justice to all the intersections between endocytosis and signaling, but is perhaps too limited. What we really had in mind was the science fiction movie "The Matrix" in which a hidden program (the Matrix) controls the life of an entire society. The program is paradoxically inconspicuous because society is so deeply built on it as to become unthinkable in the absence of the Matrix.

So, we have moved, in little more than a decade, from a view of endocytosis as a tool for transporting nutrients to a view in which endocytosis is so deeply interconnected with signaling that the two processes are impossible to distinguish, to the extent that they should be conceptualized as a single process. In previous work we defined this as "an inseparable partnership" (165). Perhaps it is time now to move to the next level of understanding, beyond the partnership, to the level of the endocytic matrix as the cornerstone (or one of the cornerstones) of the eukaryotic cell plan. In this new outlook, endocytosis and signaling are

no longer “simply” two deeply ingrained processes, but are instead two facets of an even wider program (Figure 10).

The major value of the concept of matrix is, in our opinion, heuristic, in that it provides guidance for what we need to do to unravel its workings. The properties of the matrix are, in what we propose, at the systems level; thus its deconvolution needs to be at this level. With this, we certainly do not mean to say that high-resolution mechanistic approaches are not essential. We will need, for instance, mechanistic-reductionistic knowledge to build a “reference” map of the endocytic matrix. This map can be obtained through the in vitro reconstitution of individual steps of the endocytic process coupled to single molecule resolution imaging, to add spatial and temporal aspects. Such a map will define the molecular workings of both core and accessory endocytic machinery. We will need to do this in a very quantitative way, to obtain parameters to feed into bottom-up mathematical modeling efforts. This will allow the incorporation of kinetics aspects and membrane constraints and dynamics into models of signal transduction. At the same time, systems approaches through probabilistic modeling will define the impact of single cell heterogeneity on various endocytic steps (see, for instance, Ref. 735).

Yet, the impact of endocytosis and traffic on cellular and organismal homeostasis might not be decoded solely through high-resolution studies (even if integrated by bottom-up modeling), and will probably require systematic strategies. This approach, pioneered by the group of Marino Zerial (132, 597), has been directed so far to the understanding of how the perturbation of genes affects endocytosis and traffic. We predict that an even higher level of knowledge might come from systematic studies of the impact of the endocytic machinery on nonendocytic phenotypes. This “functional map”, complemented by the various ongoing interactome studies, will provide us with a starting point to understand the full impact of the endocytic program and will be propedeutic to any attempt to reverse-engineer the eukaryotic cell plan. The vast involvement of subversion of endocytosis in human diseases forecasts that the eventual deconvolution of the endocytic matrix will be important not only for cell physiology, but also for our ability to fight diseases.

Acknowledgments

We apologize to all those colleagues whose primary references or important discoveries could not be properly acknowledged. We are in debt to Pascale R. Romano for critically editing the manuscript. We thank Prof. Dmitri A. Petrov for helping us with information relative to the classification of human genes on the basis of their age.

Grants

Work in the authors’ lab is supported by grants from the Associazione Italiana per la Ricerca sul Cancro (to P. P. Di Fiore, S. Polo, G. Scita, A. Ciliberto), the European Community (FP6 and FP7) (to P. P. Di Fiore, S. Polo), the European Research Council (to P. P. Di Fiore), the Italian Ministries of Education-University-Research (MIUR) and of Health (to P. P. Di Fiore, G. Scita), the Association for International Cancer Research (to G. Scita), the Ferrari Foundation (to P. P. Di Fiore), the Monzino Foundation (to P. P. Di Fiore), and the CARIPLO Foundation (to P. P. Di Fiore, S. Polo, A. Ciliberto).

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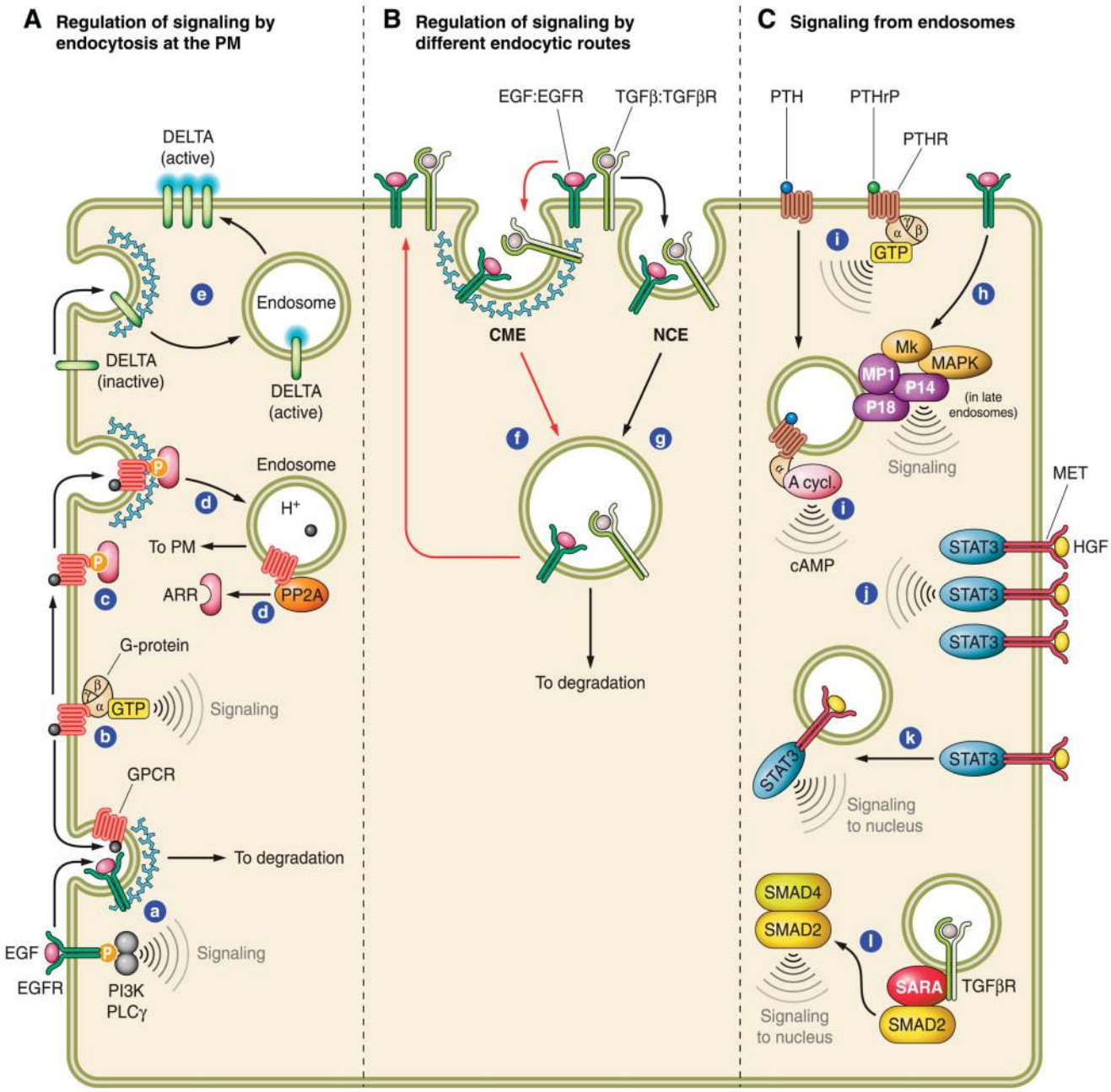

Figure 1.

Endocytosis controls signaling. Examples are provided of how endocytosis and recycling control signaling at different levels and cellular locations. References to the depicted circuitries (in this and all subsequent figures) are in the main text. *A*: endocytosis regulates signaling at the PM. Endocytosis extinguishes signals by routing PM receptors to degradation. In addition, even in the presence of continuous endosomal signaling (see below), endocytosis extinguishes signals dependent on the assembly and activation of molecular transducers exclusively localized at the PM, by removing receptors from the PM. Two examples are provided. In the case of RTKs (exemplified by EGFR, *a*), ligand binding

and receptor autophosphorylation allow binding to SH2-domain containing lipid kinases (e.g., PI3K) or lipases (e.g., PLC-γ), which mediate RTK-dependent signaling by using PM-enriched phospholipids as substrates. Endocytosis of the RTK, therefore, extinguishes this type of PM-restricted signaling. In the case of GPCR signaling (*b*), ligand binding permits the coupling with heterotrimeric G proteins (α and $\beta\gamma$). This allows GPCR to act as GEFs for Gα, a step necessary for the ability of Gα to activate adenylyl cyclase and signaling. Since heterotrimeric G proteins are PM-resident (with some notable exceptions, as depicted in panel *C*-*i*), the internalization of the GPCR [which can proceed through ARR-independent (*b*) or ARR-dependent (*c*) mechanisms] extinguishes the PM-based signal. Furthermore, upon ligand binding, GPCRs can become phosphorylated (*c*) and bind to ARR, which prevents the recruitment of stimulatory G proteins (desensitization) and promotes CME, thus terminating signaling (*c*). Internalized ligand-GPCR complexes are routed to early endosomes, where the reduced pH causes the dissociation of the ligands from their receptors, as well as receptor dephosphorylation by an endosomally localized PP2A phosphatase (*d*). The subsequent rapid recycling of receptors to the PM allows the reexposure of resensitized GPCRs at the cell surface (*d*). Ligand availability is also controlled by endocytosis. For example, in NOTCH signaling, endocytosis and recycling of DELTA to restricted regions of the PM may promote high local levels of ligand, thus causing robust NOTCH activation (*e*). Additionally, posttranslational modifications, such as monoubiquitination, of DSL ligands in the recycling compartments might also activate the ligands, via as yet ill-defined mechanisms (*e*). *B*: different endocytic routes modulate signal duration. Several receptors can be internalized through both CME and NCE, and the relative partitioning of receptors between the two entry routes determines the final biological output. For EGFR and TGF-βR, CME (red arrows) and NCE (black arrows), respectively, destine receptors preferentially to recycling to the PM (*f*) or degradation (*g*). Recycling leads to sustained signaling, while routing to lysosomes terminates signaling. Other cargoes exploit the two internalization pathways in the opposite manner (not shown). *C*: endosomes act as signaling platforms. The signaling endosome hypothesis was originally proposed in neurons where endosomes were postulated to serve as platforms for the assembly and transport of protein complexes for long-range signal transmission. Several neuronal and nonneuronal receptors exploit the unique physical-chemical properties of endosomal membranes to either prolong signals originating from the PM, or to specify and diversify signaling outcome. A paradigmatic example is provided by sustained endosomal activation of ERK kinases. Endosomes are enriched in specific adaptor proteins, such as P18 that serves as an anchor for an ERK-activating scaffold (MEK1/MP1/P14). This allows ERK signaling from the endosome upon activation of EGFR, in addition to PM-originated ERK signaling (*h*). A similar situation occurs for signaling along the GPCR-ARR-ERK axis (not shown). In this case, endosomes act as platforms that, in addition to prolonging ERK signaling, also bias it towards predominantly cytosolic rather than nuclear ERK substrates. In the case of the GPCR PHTR, different conformations of the receptor, associated with the binding to PTH or PTHrP, lead to different signals (*i*). The PTHrP:PTHR complex signals canonically from the PM. Conversely, PTH stimulates cotrafficking into early endosomes of PTHR with stimulatory Gα that promotes adenylyl cyclase (A cycl.) activation and production of cAMP from that location (*i*). Endosomes can also act as intermediate stations for the propagation of signals to the nucleus. Activation of EGFR stimulates the translocation of APPL1 from

endomembranes to the nucleus, where it controls the activity of chromatin remodeling enzymes (not shown). Similarly, HGF stimulation of MET receptor promotes STAT3 activation both at the PM (*j*) and on endosomes (*k).* STAT3 must translocate to the nucleus to promote transcription. When MET activation is weak, such as in the presence of limited amounts of ligand, endosomes are used to transport STAT3 to the nucleus, while protecting it from being deactivated by cytosolic phosphatases (*k*). Finally, there is increasing evidence of endosome-directed signal specificity. This is the case for SARA-endosomes mediating TGF-βR signaling. The recruitment of SMAD2 by SARA leads to phosphorylation of SMAD2 by internalized TGF-βR. Phosphorylated SMAD2 dissociates from the receptor and forms a complex with SMAD4 that translocates to the nucleus, where it regulates gene transcription (l).

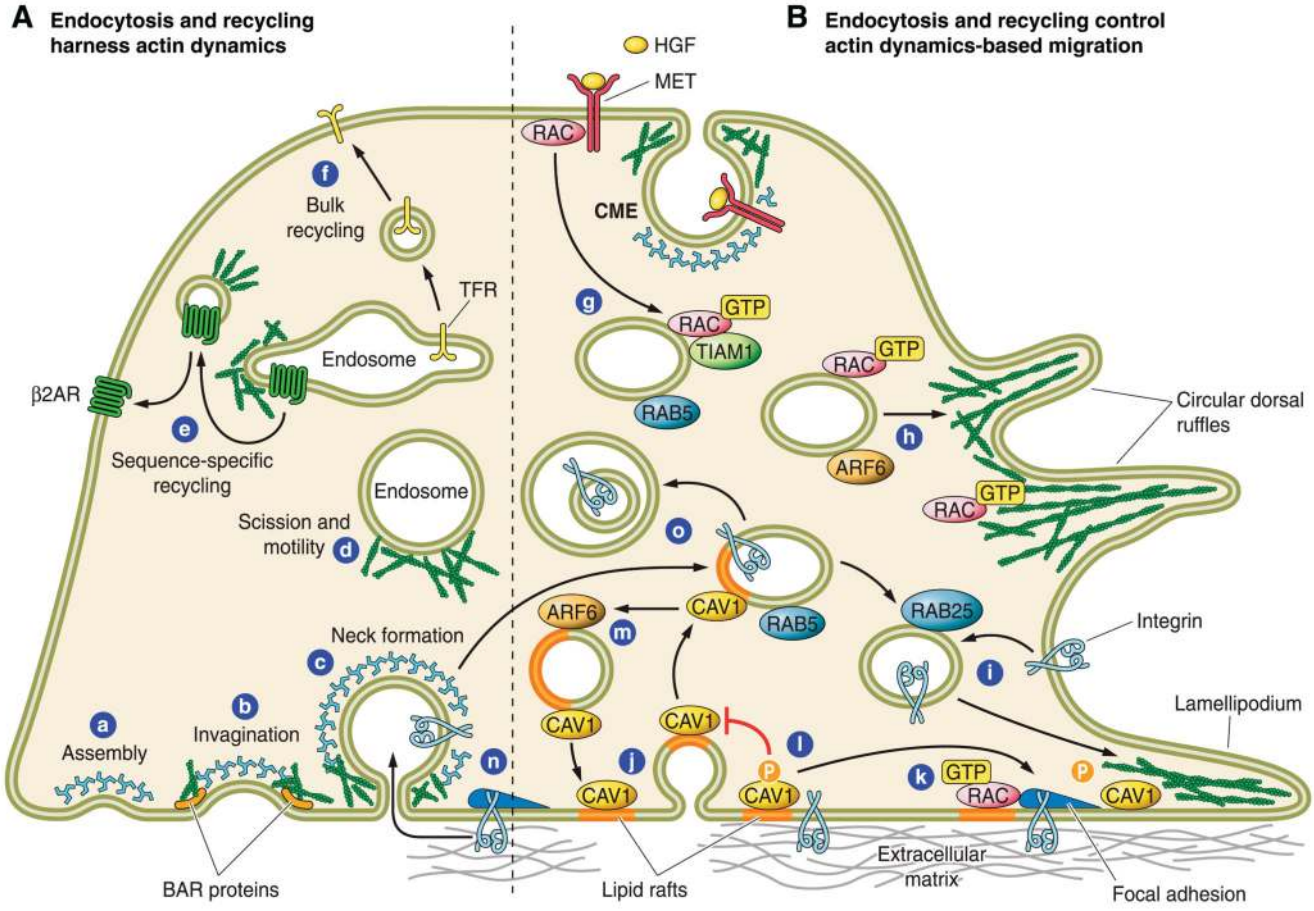

Figure 2.

Multiple and bidirectional connections between actin dynamics and endocytosis. *A*: endocytosis and recycling harness actin dynamics. Macropinocytosis, phagocytosis, and most forms of NCE are dependent on actin dynamics. The requirement for actin polymerization in CME in mammalian cells is less well established. However, recent evidence, mainly obtained using advanced live imaging, has shown that actin and actin regulatory proteins are invariably recruited along each of the various steps of CME and that they mediate key events in the transport and motility of endosomal vesicles. The initial curvature (*a*) of the PM is generated by the assembly of clathrin coats and additional endocytic proteins (not shown), such as F-BAR-containing membrane deforming proteins. As invagination proceeds (*b*), changes in curvature may be sensed by other BAR-domain-containing proteins that cooperate with the large GTPase dynamin (not shown) and actin polymerization regulatory factors to promote neck formation (*c*), which precedes vesicle scission (*d*). Proteins of the endocytic coat (not shown) may directly link the membrane to the actin network. Actin polymerization may generate the force necessary to promote pit invagination into the cell, until dynamin-mediated scission occurs. Alternatively, an actin shell that nucleates along the sides of invaginating membrane tubules may cause membrane reorganization, lipid domain repartition, and line tension, aiding dynamin-dependent scission. Actin is also involved in vesicle motility and trafficking inside the cells (*d*) and has

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recently been shown to influence cargo sorting (*e*). This is the case for β2ARs that generate endosomal, actin-coated, subdomains by recruiting actin regulatory factors through their PDZ-interacting motives. This provides a physical basis for sequence-dependent sorting of internalized membrane proteins from distinct domains within the same endosome, enabling separation and diversification between bulk [here, represented by TFR recycling (*f*)] and sequence-dependent (β2AR), actin-mediated recycling to the PM (*e*). Thus actin may be crucial not only for endosomal motility, but also for cargo-mediated regulation of receptor recycling. *B*: endocytosis and recycling control actin dynamics-based migration. Coordination between membrane traffic, cell substrate adhesion, and actin remodeling is required to generate and spatially confine the forces responsible for the formation of polarized cell protrusions, such as lamellipodia and circular dorsal ruffles (CDR). Two endocytic/signaling networks implicated in polarized migration are shown. In the first one (*g*–*h*), in response to stimulation of RTKs, such as HGF stimulation of the MET receptor, CME and RAB5 activation promote the internalization of RAC and its GEF, TIAM1, into early endosomes (*g*). Activated GTP-bound RAC is subsequently recycled through the ARF6 endosomal pathway (*h*) to confined PM regions, where actin polymerization supports the formation of CDR, a step that occurs prior to the extension of migratory protrusions. In the second network (*n*–*o*), the trafficking of integrins, through CME and raft-dependent NCE, enables sustained and polarized integrin signaling to lamellipodia as well as precise coordination of integrin activity with the changing dynamics of focal adhesions. Integrins, such as α5β1, are continuously internalized (*i*) and recycled to the PM, through RAB25-endosomes that are compartmentalized at the leading edge of cells for lamellipodial extension. Coordination of integrin adhesion and lipid raft endocytosis and recycling is also crucial to integrate RAC and integrin activation (*j*). Lipid rafts are endocytosed through caveolin-1 (CAV1)-containing caveolae (*j*). Lipid rafts are also binding sites for RAC (*k*). Integrin signaling blocks lipid raft internalization by promoting CAV1 phosphorylation and its retention in focal adhesions at the PM (*l*). Thus, when integrins are engaged by the ECM, RAC binding sites at the PM become available. On the contrary, cell detachment abrogates integrin activation and extinguishes RAC signaling at the PM, by enabling the relocalization and subsequent caveolae-mediated internalization of CAV1 and lipid rafts. Recycling of CAV1 (*m*), as well as of RAC (*h*) and integrins may be coordinated by ARF6. Mature, integrin-containing focal adhesions at the rear of the cells need to be disassembled to enable effective cell locomotion. This process involves dynamin and CME (*n*). Finally, internalized integrin, bound to ECM ligands such as fibronectin (FN), may be specifically directed to lysosomes for degradation, through a mechanism involving integrin ubiquitination and recognition by the ESCRT machinery (*o*). Cells expressing a ubiquitination-deficient α5β1-integrin mutant are impaired in cell migration, suggesting that FN-integrin complex turnover is essential for locomotion.

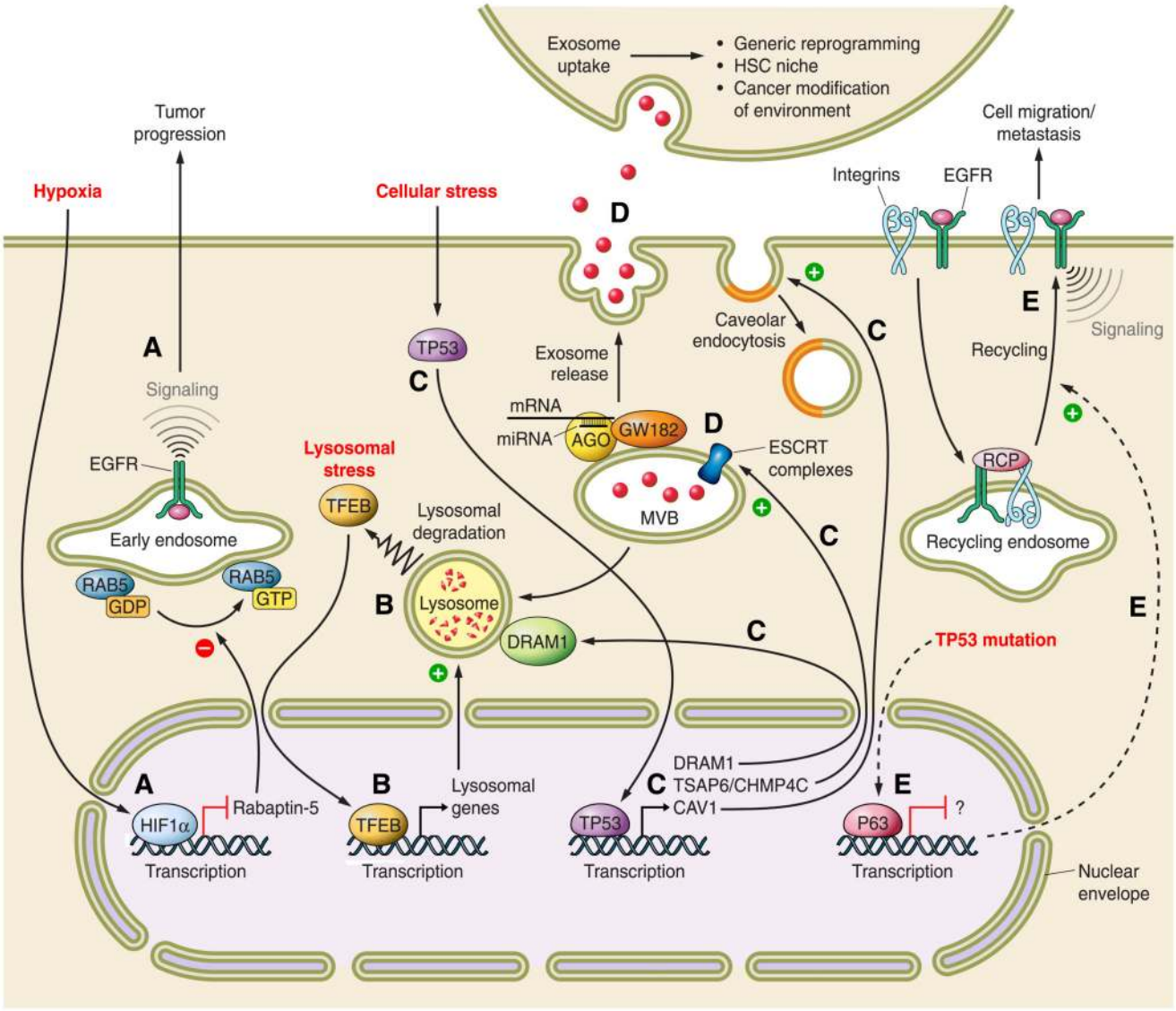

Figure 3.

Transcriptional programs controlling endocytosis and genetic reprogramming by endocytosis. *A*: activation of HIF1α during hypoxia causes the inhibition of the RABAPTIN-5 gene transcription and, consequently, endosomal retention of the EGFR, eventually leading to sustained EGFR signaling from the endosomal station and tumor progression. *B*: lysosomal stress causes nuclear translocation of the TFEB transcription factor, which induces transcription of a cluster of genes involved in lysosomal biogenesis. *C*: upon different types of cellular stresses, p53 translocates into the nucleus and activates the transcription of genes which play roles at different stations of the endocytic pathway: DRAM1, a lysosomal membrane protein; TSAP6 and CHMP4C, which are involved in exosome release from MVBs; CAV-1, which stimulates caveolar endocytosis. *D*: at the MVB, the ESCRT complex is assembled (CHMP4 being one of its components), which is involved in the recognition of ubiquitinated cargoes, and in invagination and scission of

intraluminal vesicles (see sect. V*C*). Subunits of the ESCRT-II complex can selectively bind to mRNAs. MVBs are also platforms for the assembly of the RISC complex, which directs the degradation or translational repression of target mRNAs. In particular, two components of the RISC are specifically enriched at this site, AGO and GW182. All these events participate in the regulation of exosome secretion, which in turn is a tool for genetic reprogramming of adjacent cells. *E*: TP53 mutations found in human cancers exert part of their oncogenic potential through the P63-dependent (transcriptional-dependent) stimulation of RCP-mediated recycling of integrin-EGFR complexes, leading to induced migration and metastasis. The molecular mechanism for this remains unclear.

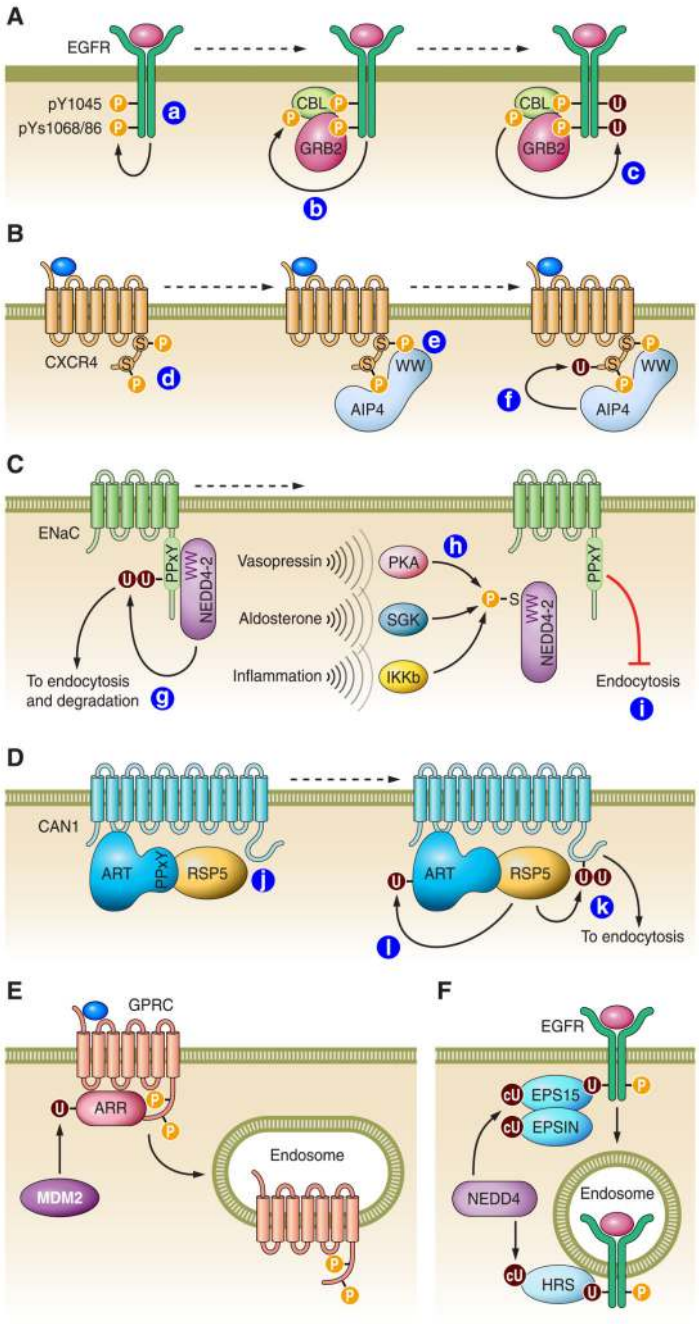

Figure 4.

The ubiquitin system and endocytosis. *A*: EGFR (and MET, not shown) ubiquitination by CBL. A GRB2-CBL complex binds to the receptor through interactions of *i*) the SH2 domain of GRB2 with pY1068 or pY1086 of EGFR, and *ii*) the tyrosine kinase binding (TKB) domain of CBL (either c-CBL or CBL-b) with pY1045. *a*: EGFR-bound CBL becomes phosphorylated and activated. *b*: Recruitment of E2 (not shown) to the RING domain of CBL results in covalent attachment of monoUb and polyUb chains to the kinase domain of the receptor (*c*). *B*: AIP4 mediates ubiquitination of CXCR4. Upon agonist-

mediated activation, CXCR4 becomes phosphorylated at Ser324 and Ser325 by an unknown kinase (*d*). This leads to the recruitment of the E3 ligase AIP4, through its WW domain (*e*), that ubiquitinates the receptor (*f*). *C*: ENaC ubiquitination by NEDD4–2. NEDD4–2 binds to ENaC PPxY motifs and catalyzes its ubiquitination (*g*). This induces ENaC endocytosis and lysosomal targeting, resulting in fewer channels at the cell surface (*g*). To increase Na^+ transport, NEDD4–2 is phosphorylated by kinases, including PKA, SGK, and IKKβ, in turn activated by various signaling pathways (*h*). Phosphorylation of NEDD4–2 induces binding of 14–3-3 dimers (not shown), which prevents NEDD4–2 from binding to ENaC. As a result, endocytosis of ENaC is inhibited (*i*), and increased ENaC presence at the surface enhances epithelial Na^+ absorption. *D*: RSP5 ubiquitinates permeases and transporters. In yeast, arrestin-related trafficking adaptors (ARTs) and the E3 UB ligase Rsp5 are recruited to the PM in response to environmental stimuli that trigger the endocytosis of proteins such as permeases and transporters (e.g., the arginine transporter Can1) (*j*). Through their PPxY motifs, ARTs bind to the WW domain of Rsp5 (*j*) and mediate ubiquitination of cargo (*k*). The ubiquitinated cargo is then internalized and degraded (*k*). ARTs are also ubiquitinated by Rsp5, an event required for endocytosis, though the mechanism remains unclear (*l*). *E*: ubiquitination of adaptors: ARR. Agonists induce rapid ubiquitination of GPCR-recruited ARR by MDM2, a process required for receptor internalization. *F*: ubiquitination of adaptors by EGFR. Activated EGFR is ubiquitinated at the PM by CBL (*A*) and recruits UBD-containing endocytic proteins such as EPS15, epsin, and HRS (at the endosome). These adaptors, in turn, are ubiquitinated by NEDD4 through a process known as coupled monoubiquitination (cU).

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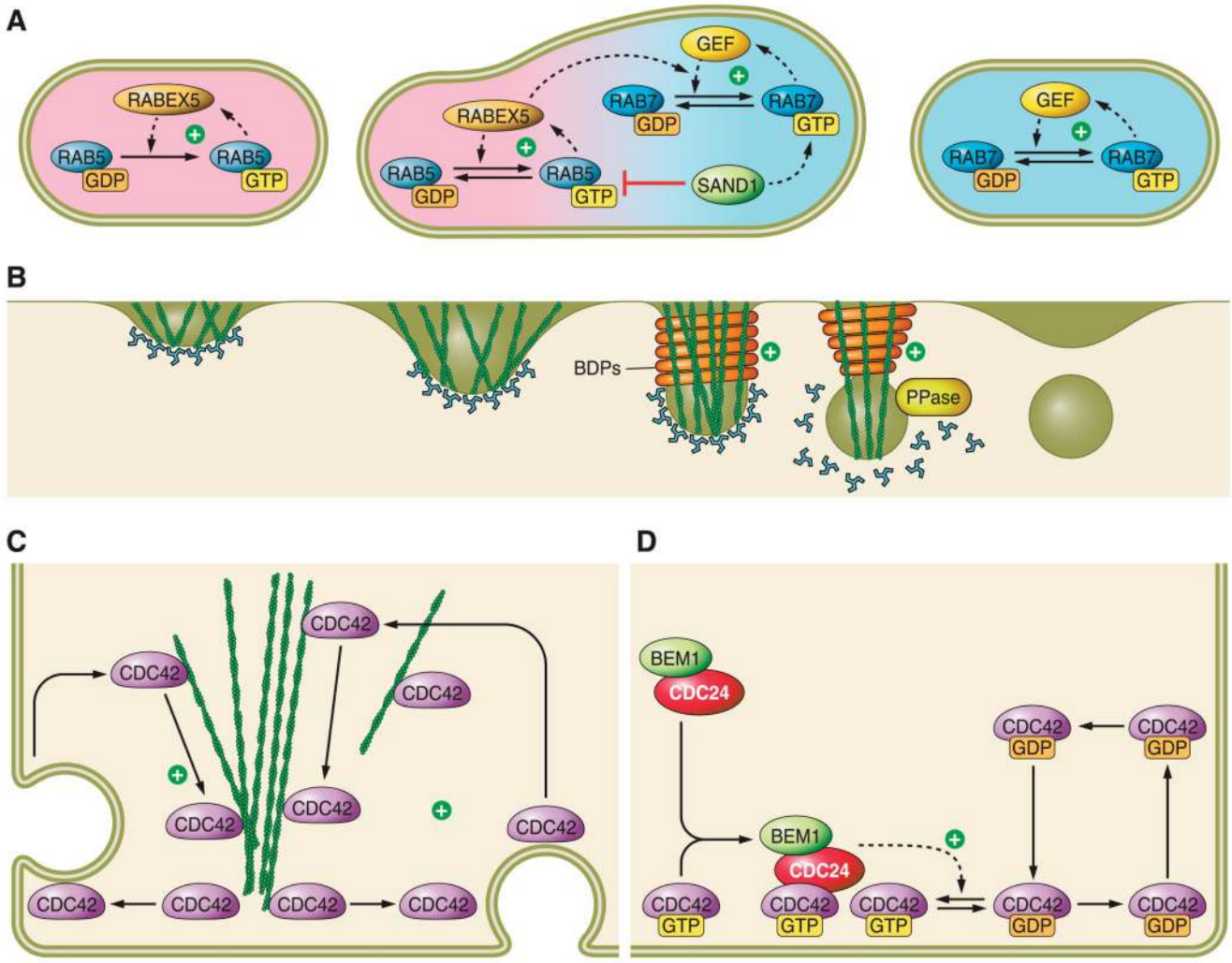

Figure 5.

Positive-feedback loops playing a role in systems level properties related to endocytosis. *A*: during the conversion from early (red) to late endosomes (blue), the GTPase RAB5 is replaced by RAB7. Two positive-feedback loops (shown as "+" in the figure) help the endosomes to maintain their enrichment in either of the two RABs. The first loop involves RAB5 and its GEF-complex (Rabaptin-5/RABEX-5), while the second involves RAB7 and the class C VPS/HOPS complex (GEF in the picture). To explain the switch from early to late endosomes, a negative-feedback loop has been hypothesized whereby RAB7 inhibits RAB5 (not shown) in the so-called "cut-out model." According to this model, after RAB5 reaches a critical level, it triggers the RAB7 feedback loop, which leads to both an enrichment in RAB7 and to the silencing of the RAB5 enrichment loop. More recently, it was reported that SAND-1 is involved both in the recruitment of RAB7 to endosomes, and in the inhibition of RAB5 activity (likely through the inhibition of RABEX-5). Thus SAND-1 might be the molecular switch driving endosomal RAB conversion. *B*: positive-feedback loops have also been invoked to explain the mechanical process of endocytosis. The model, originally developed for yeast, applies in general to eukaryotes. As actin

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remodeling leads to PM invagination, a first positive-feedback loop is created by BAR domain-containing proteins (RVS167 in yeast, shown as BDPs-BAR domain proteins, in the figure), which envelop the membrane, creating a curvature that further helps BDP binding to the tubular structure that has formed. The presence of BDPs protects part of the membrane from the activity of a PIP_2 phosphatase (PPase), which can act on the free part of the invagination (i.e., the bud). A second positive-feedback loop has been proposed whereby the effect of PIP_2 depletion from the bud increases the curvature at the interface between the bud and the tubule covered by BDPs, and PPase activity is further reinforced by this increase in curvature (not shown). As a result, the bud is eventually pinched off. *C*: during bud formation in budding yeast, CDC42 accumulates at the bud site. The asymmetric distribution of the protein has been proposed to be driven by two overlapping positive-feedback loops. In the first, slower, loop, the localization of CDC42 favors the accumulation of actin filaments, which in turn deliver more CDC42 to the site. Free diffusion on the membrane and endocytosis allow the redistribution of CDC42 away from the bud-site, while active transport along actin filament reverses this process. In this model, the transition between active CDC42 (CDC42-GTP) and inactive CDC42 (CDC42-GDP) is not affected by the distribution of CDC42; thus, in the figure, we do not specify the species to which CDC42 is bound. *D*: in the second, faster, positive-feedback loop, the activation/inactivation of CDC42 plays a key role. GTP-bound CDC42 is stably localized at the PM, whereas GDP-bound CDC42 shuttles freely between PM and cytoplasm. The presence of a pool of active CDC42 (CDC42-GTP) triggers a positive-feedback loop because it recruits the scaffold protein BEM1 and the GEF CDC24 to the PM. At the PM, CDC24 causes the activation of more CDC42, which in turn recruits more BEM1:CDC24 complexes, thereby producing a positive-feedback loop in the activation of CDC42.

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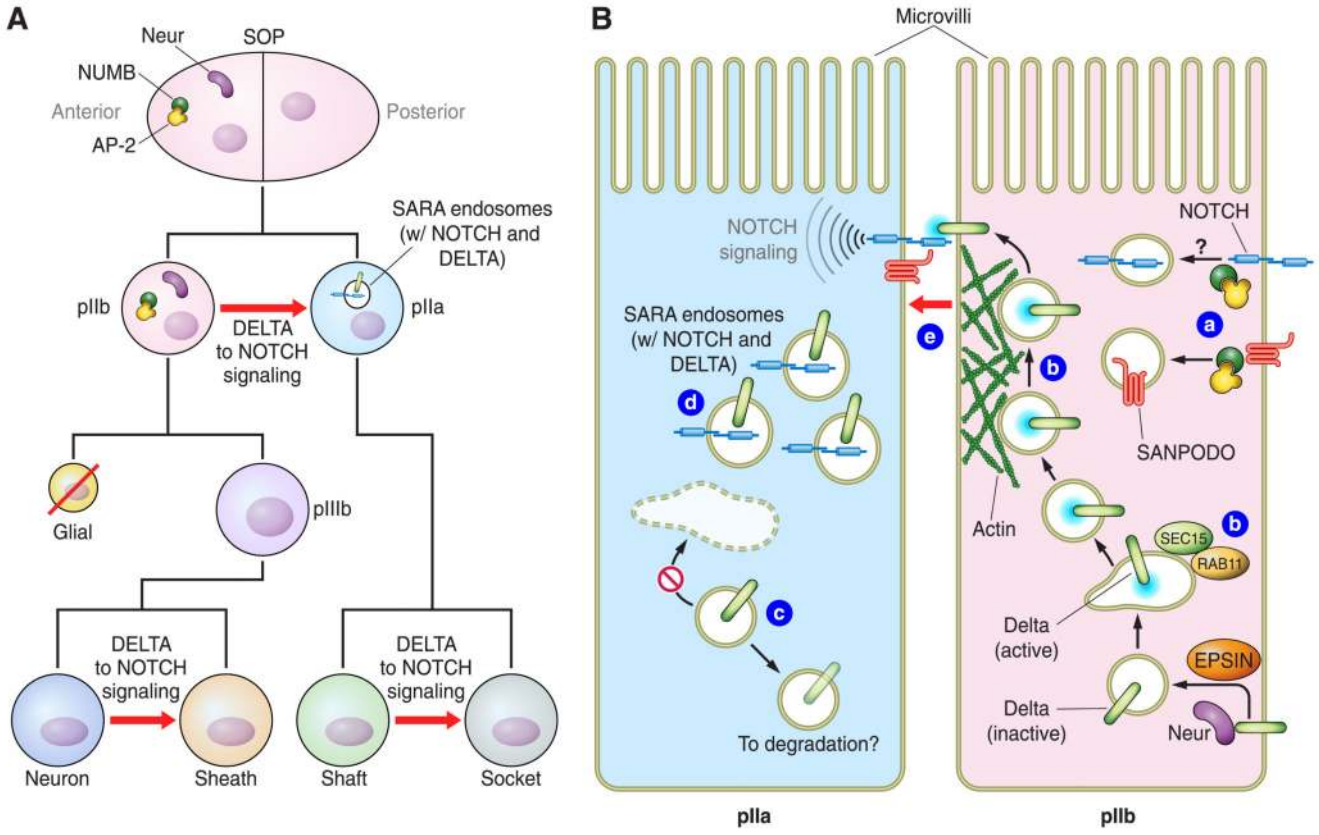

Figure 6.

Endocytic circuitries in asymmetric cell division. *A*: ACDs in the *Drosophila SOP lineage.* The SOP lineage is shown. All divisions are asymmetric and entail directional (DELTA to NOTCH) signaling between daughter cells (depicted by red arrows). The first division (from SOP to pIIa and pIIb) is shown in detail. The plane of division with orientations is indicated. Asymmetrically partitioned molecular machinery (NUMB, AP-2, and SARA endosomes) is also shown. *B*: endocytosis regulates the creation of asymmetry in pIIa and pIIb cells. *a*: NOTCH is nonfunctional in pIIb cells, because it is internalized/degraded or because SANPODO is internalized. While the internalization of SANPODO is established, it is not clear whether NOTCH is actually preferentially/internalized degraded in the pIIb cell (indicated by a "?"). However, recent evidence in mammals indicates that NUMB might be an inhibitor of NOTCH recycling, rather than a positive modulator of internalization. Thus, in the pIIb cell, the function of NUMB may be to prevent NOTCH recycling to the PM, so favoring its degradation. *b*: DELTA-related events in pIIb. The E3 ligase Neuralized is asymmetrically partitioned in pIIb, allowing endocytosis of DELTA. DELTA is trafficked by epsin to a RAB11/SEC15-positive endosome (this event might be preceded by a first pass onto the PM to "activate" DELTA, see Figure 1*A*). These endosomes are then directed, for cargo release, along a branched ARP2/3-dependent actin network to a microvillar-dense region of the apical membrane of the pIIb. This region has been shown to contract extensive interactions with a similar region of the pIIa cell. *c*: DELTA-related events in pIIa. DELTA is also internalized in the pIIa cell through a Neuralized and UB-independent mechanism. In

this cell, however, the recycling to the PM is blocked and DELTA is destined to degradation, because the RAB11-positive endosomal compartment cannot form, possibly because a critical RAB11 partner (Nuclear fallout/Arfophilin 1) is inactivated by as yet unclear mechanisms. DELTA might also be internalized before mitosis of the SOP cell; in pIIb, it could be recycled to the PM, whereas in pIIa it might be destined to a degradative pathway. *d*: Asymmetric partitioning of SARA-endosomes. In the SOP cell, both NOTCH and DELTA are trafficked to SARA endosomes before ACD. These endosomes are then directionally transported to the nascent pIIa cell, thereby contributing to asymmetry. The described events are not necessarily ”all or none“ situations. They might occur in both cells, with a cell-specific bias in favor of one of them that is further amplified through reinforcement/extinction events that lead from a quasi-symmetric situation to the final DELTA/NOTCH asymmetry needed for directional signaling (*e*).

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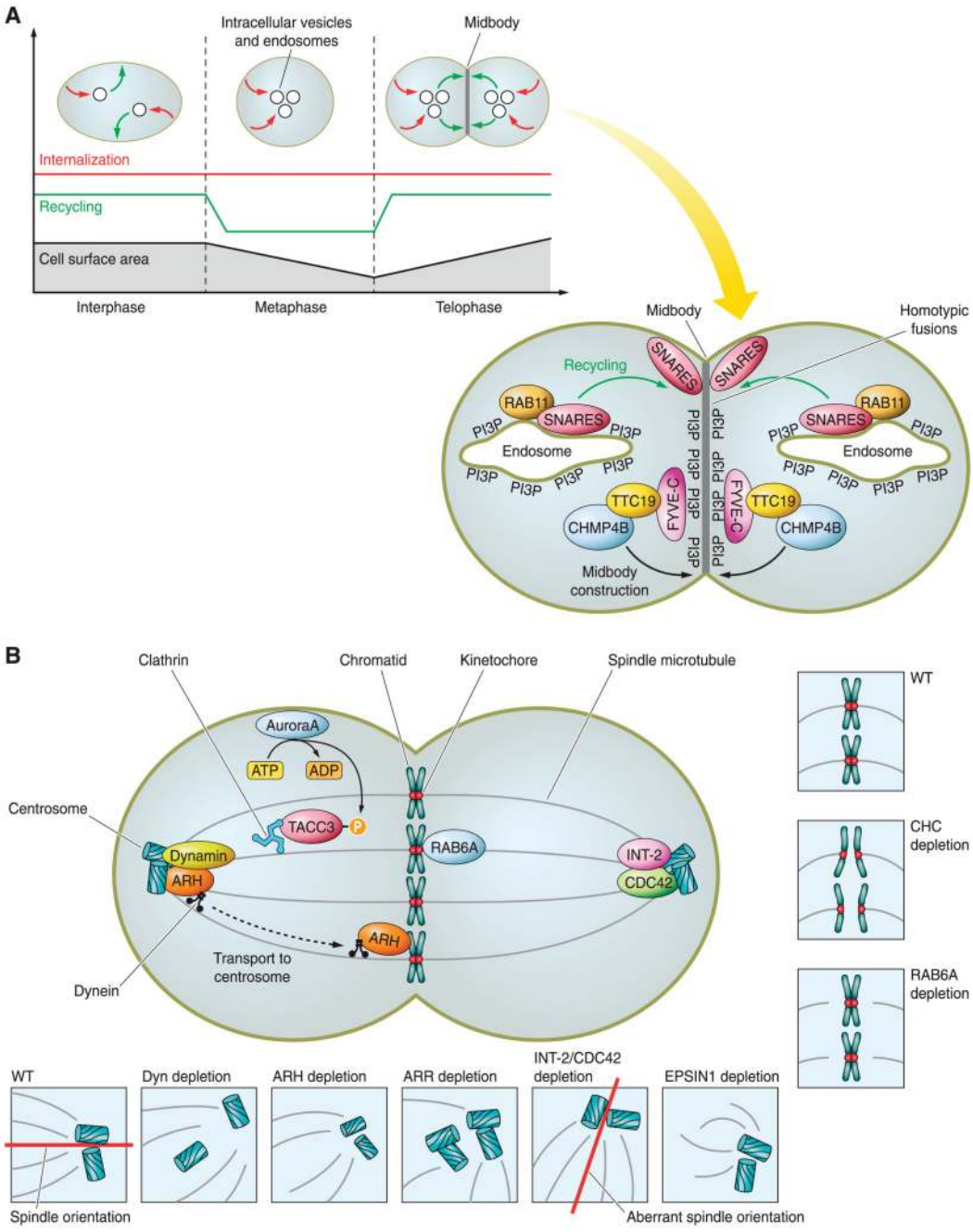

Figure 7.

Endocytic proteins in the control of cell division *A*: three phases of mitosis are represented in a temporal order (interphase, metaphase, and telophase). The internalization rate (red line) remains constant along the entire mitotic event. During interphase, internalization is balanced by high rate of recycling (green line). During metaphase, recycling decreases while internalization remains sustained. This leads to the accumulation of an intracellular pool of vesicles and endosomes, and to a reduction of the cell surface area. At telophase, recycling recovers and is polarized towards the midbody. In the blow up, the molecular details of

polarized recycling towards the midbody are depicted. PI3P-enriched endosomes are recycled towards the midbody in a microtubule-dependent manner. Recycling is mediated by RAB11 (as depicted in the picture, but also by RAB35 and ARF6, not depicted for simplicity). SNAREs mediate fusion events at the cytokinetic furrow. PI3P is enriched at this latter site, and this permits the recruitment of FYVE-CENT (FYVE-C). FYVE-C binds to TTC19 and CHMP4B, a component of the ESCRT-III complex, to allow midbody constriction. *B*: a mitotic cell in metaphase is depicted. Chromatids (green) are aligned on the metaphase plate and are connected to spindle microtubules by kinetochores (red). At the cell poles, centrosomes are depicted in blue. As discussed in the main text, different endocytic proteins bind to some of these mitotic structures: dynamin, intersectin 2 (INT-2), and CDC42 bind to centrosomes; ARH binds to dynein at kinetochores and is involved in transport to centrosomes; RAB6A is recruited to kinetochores; and clathrin heavy chain (CHC) binds to spindle poles where it recruits TACC3, a substrate of the AURORA A kinase. In the *bottom panels*, a depiction is shown of the effects of depletion of various endocytic proteins on centrosomes and mitotic spindle organization: dynamin depletion causes centrosome separation, ARH-null fibroblasts have smaller centrosomes, ARR depletion causes centrosome duplication, INT-2/CDC42 depletion causes aberrant spindle orientation, and epsin-1-depleted cells show aberrant organization of the mitotic spindle. In the *right panels*, the effects are depicted of the depletion of various endocytic proteins on chromosome attachment and alignment: depletion of CHC causes chromosome misalignment, while RAB6 depletion causes detachment of the spindle microtubules from the kinetochores.

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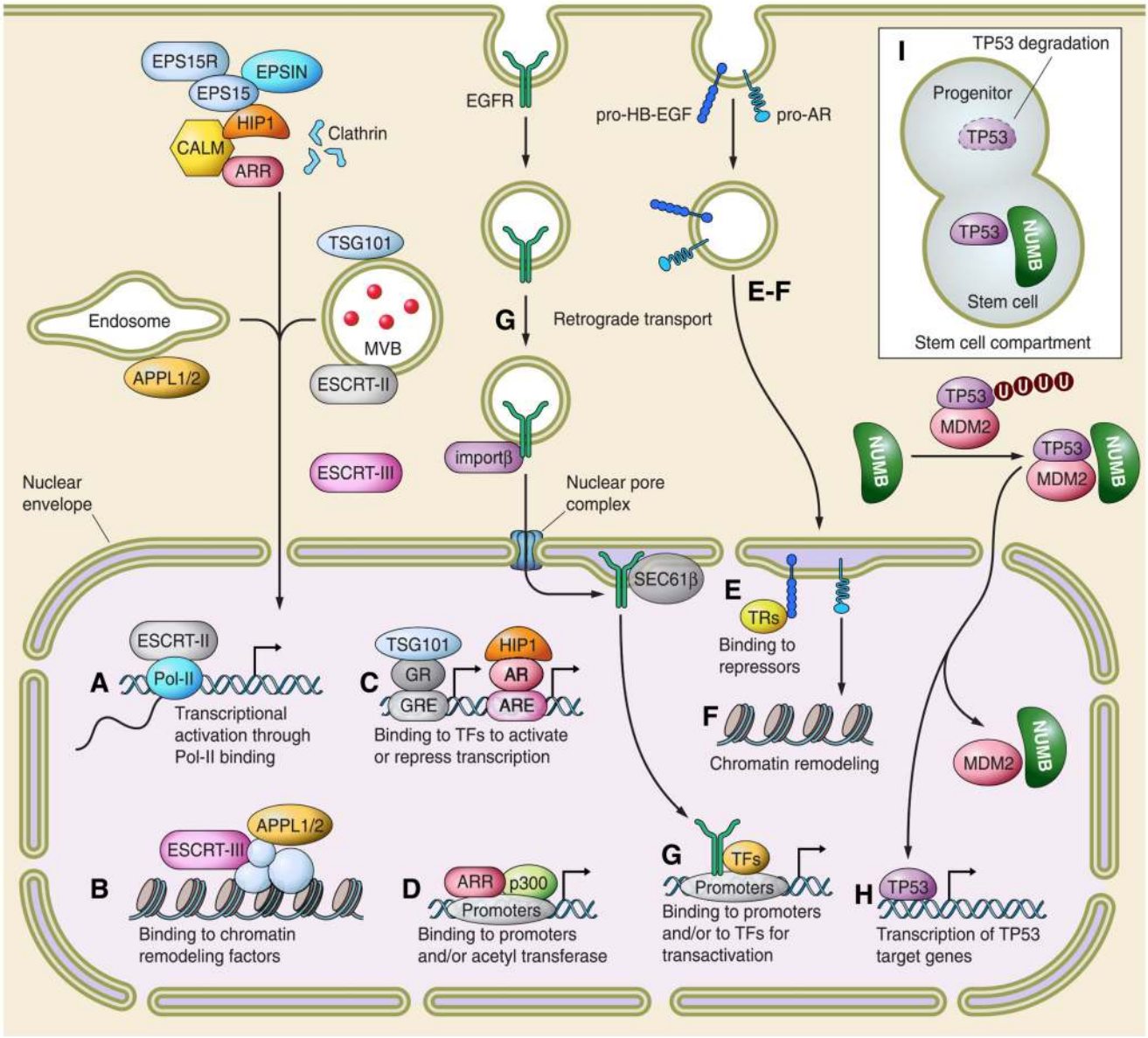

Figure 8.

The endocytic machinery controls transcription. Examples of endocytic proteins shuttling in and out of the nucleus, thereby affecting gene expression, are shown. *A*: subunits of the ESCRT-II complex activate RNA polymerase II (Pol-II)-dependent transcription. *B*: APPL1/2 and ESCRT-III components bind to chromatin remodeling complexes. *C*: HIP1 and the ESCRT-I component TSG101 bind to two known transcription factors (TFs), the androgen receptor (AR) and the glucocorticoid receptor (GR), respectively, to transactivate transcription at their sites (ARE, androgen responsive element; GRE, glucocorticoid responsive element). TSG101 can also either activate or inhibit AR transcription, through different mechanisms depending on the cellular context. *D*: ARR transactivates transcription either by binding to promoters directly or by binding to p300 histone deacetylase. Additional endocytic proteins depicted in the picture (EPS15, EPS15R, epsin, CALM, and clathrin)

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shuttle into the nucleus and affect transcription either by binding to TFs or to chromatin remodeling complexes (not depicted for simplicity). Endocytosis also delivers cargo to the inner nuclear membrane, by way of a retrograde transport mechanism. Two examples are shown. In the first, two membrane-anchored growth factors, pro-AR (precursor of amphiregulin) and pro-HB-EGF (precursor of the heparin-binding EGF-like factor), are delivered in a signaling-dependent and endocytosis-dependent manner to the inner nuclear membrane, where they sequester transcriptional repressors (*E*, in the case of pro-HB-EGF) or function as chromatin-remodeling agents (*F*, in the case of pro-AREG). In the second, the EGFR (*G*) is retro-transported via endocytosis in a complex with Importin β, which facilitates its translocation through the nuclear pore complex and its delivery to the inner nuclear membrane. Here, the receptor interacts with the translocon SEC61β, which catalyzes its membrane extraction and delivery to the nucleoplasm, where it activates transcription (*G*), either by direct binding to promoters or by binding to TFs. Finally, TP53 is controlled by the endocytic protein NUMB. NUMB inhibits the ubiquitination of TP53 by MDM2, thereby preventing its degradation, leading to increased TP53 levels and increased p53 transcriptional activity (*H*). Because the MDM2:NUMB complex shuttles in and out of the nucleus, it is not clear whether the regulation of TP53 by NUMB occurs in the cytosol or in the nucleus. In the mammary stem cell compartment (*I*), NUMB partitions into the daughter cell that adopts the stem-cell fate. One intriguing possibility is that this might drive high levels of TP53 in the daughter stem cell and its withdrawal into quiescence.

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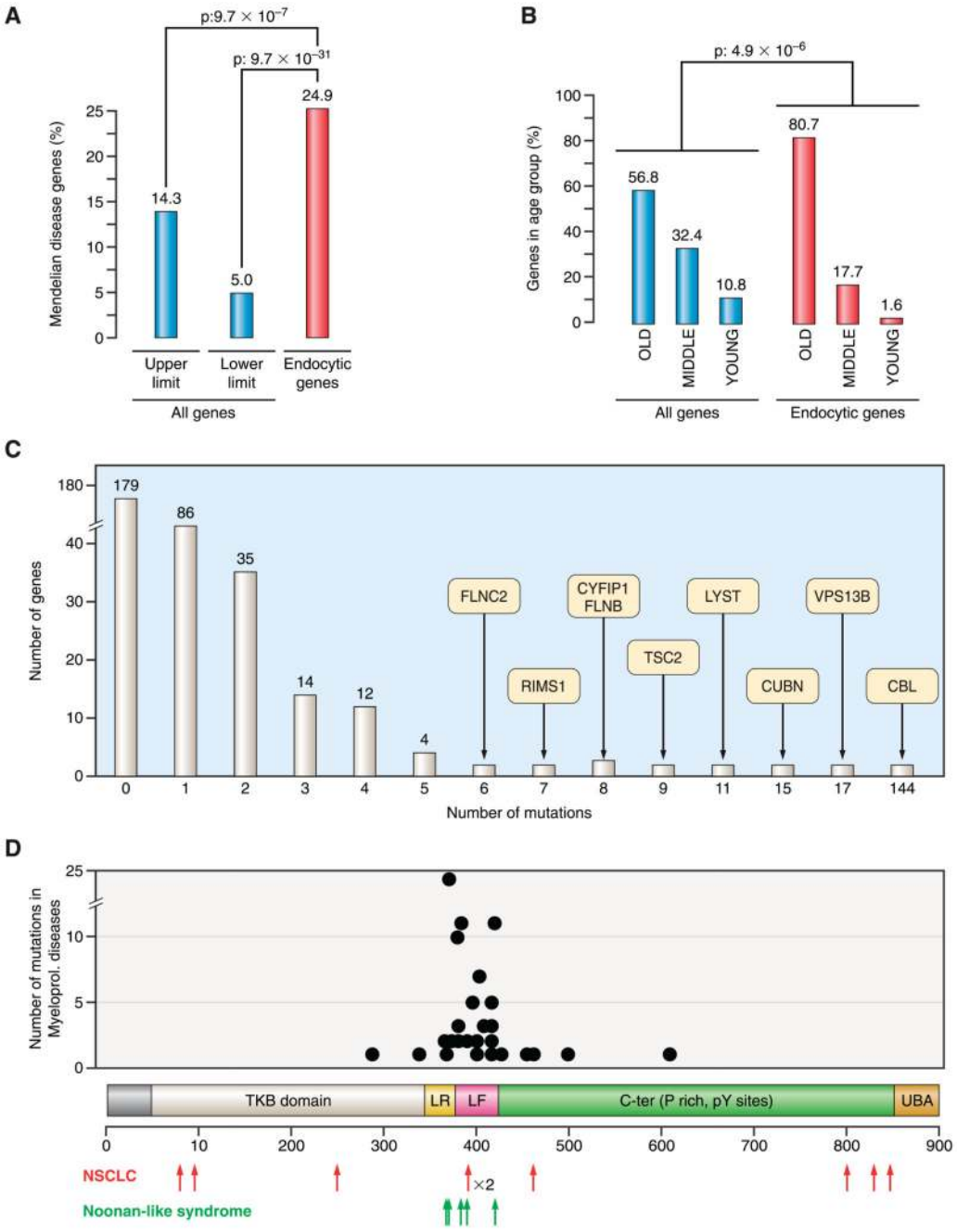

Figure 9.

Endocytic genes in Mendelian (monogenic) diseases and in cancer. *A*: endocytic genes and Mendelian diseases. A list of 339 genes, including 277 genes encoding proteins involved in endocytosis and traffic and 62 proteins involved in regulation of the actin cytoskeleton, was used to screen the OMIM and GENE databases (see Table 2 for details) for their mutations in Mendelian diseases. Of these genes, 289 were present in OMIM, and 72 were listed as the cause of at least one disease (the complete list is in Table 2), indicating a frequency of mutation of 24.9% (red bar). This value was compared with the frequency of Mendelian

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disease genes among all human genes. An upper and lower limit for this frequency is shown (blue bars), calculated as detailed in the main text. Significance of the enrichments was tested by hypergeometric tests. The *P* values were obtained using the *phyper* function from the R statistical language (http://www.R-project.org/). *B*: endocytic genes are enriched in ”old genes.“ Data relative to the phylogenetic age of all genes were downloaded from the Phylopat Database (www.cmbi.ru.nl/phylopat/). The three age groups (old, middle, young) were defined as from Cai et al. (95). The relative distribution in the three age groups of all human genes (blue bars) and of the endocytic genes (red bars) is shown. *P* values were calculated with chi-square test. *C*: mutations of endocytic genes in the COSMIC database. Of the 339 genes (described in *A*), 160 harbored at least one mutation in at least one type of cancer. On the top of each bar, the number of genes harboring the number of mutations indicated on the *x*-axis is shown. For the ”frequently mutated” genes (>5 total mutations), the gene symbol is also shown (details are in Table 4). *D*: mutations of CBL in cancer and Mendelian diseases. In the middle of the panel, a schematic of the CBL protein is shown with its functional domains (TKB, tyrosine kinase binding domain; LR, linker region; RF, ring-finger domain; UBA, UB-binding domain). The ruler underneath shows amino acid positions. On the *top*, the position and the frequency of the mutations detected in myeloproliferative diseases are shown by solid circles, aligned with the amino acid sequence. At the *bottom*, the position of the mutations detected in NSCLC and in the Noonan-like syndrome is shown by red and green arrows, respectively. In NSCLC, the mutation at position 391 was detected in two tumors (shown as x2). In the Mendelian syndrome, four of five mutations affect the same resides (371, 367, 382, 420) as in myeloproliferative diseases.

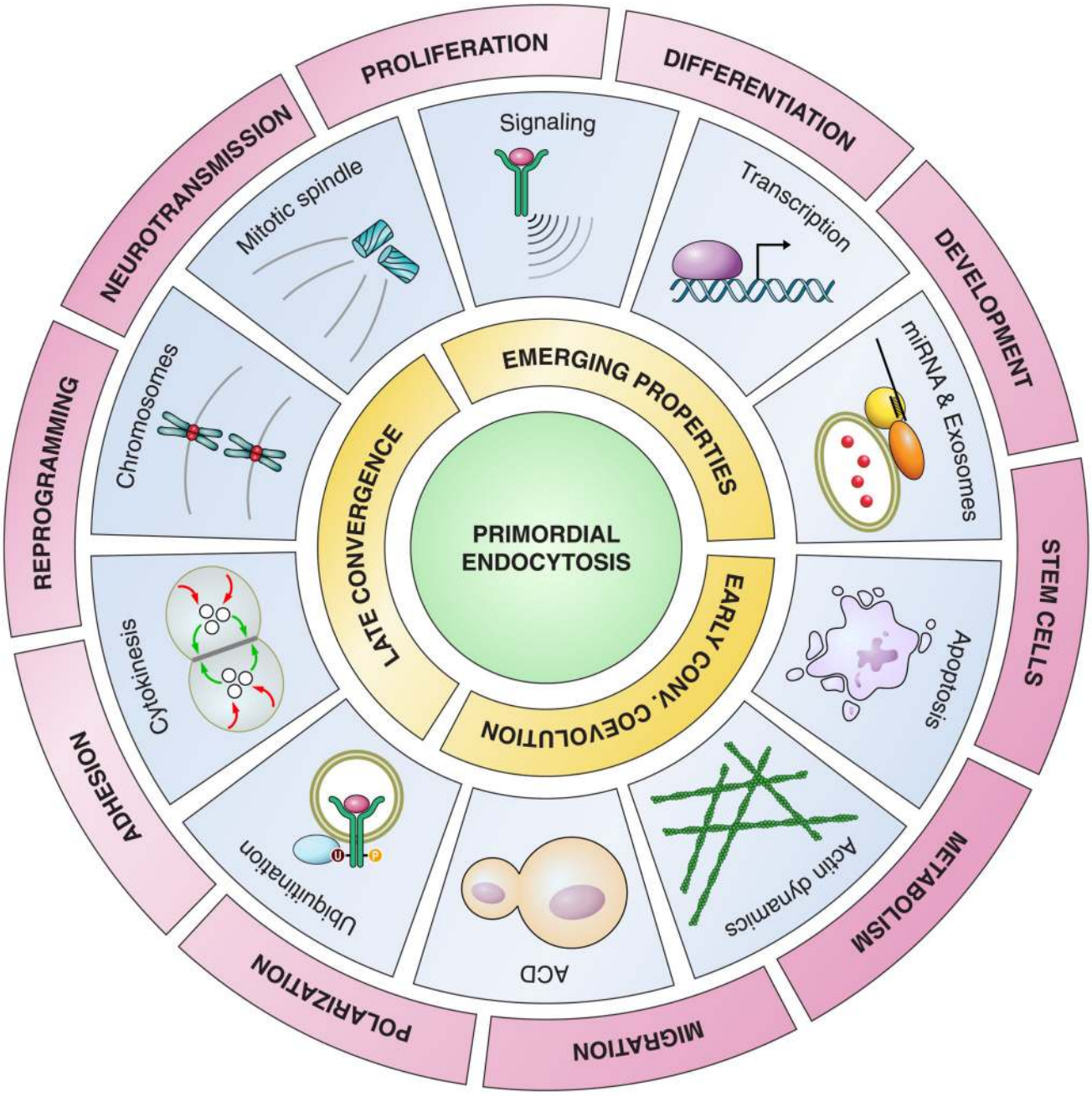

Figure 10.

The endocytic matrix. A conceptual drawing of the endocytic matrix is displayed. Starting from the primordial functions of endocytosis (green), connected with competition for food, a series of additional functions (yellow) became associated with the endomembrane system during evolution. These functions (yellow) were the consequence of *1*) emerging properties of the system, such as size of endosomes, physical separation of signaling compartments (PM and endosomes), and origin of the nuclear envelope from endomembranes (see sect. IX*B* and X*A*); *2*) early convergence of endocytosis with other cellular functions and

subsequent coevolution, as in the case of actin cytoskeleton and of the ubiquitination system (see sects. IX*A* and X*A*); and *3*) late convergence of endocytosis with other systems, such as pY-based signaling and the PAR complex (see sect. X, *A* and *B*). The consequence of these events is the pervasive presence of endocytosis and trafficking in virtually every cellular aspect of cell regulation (blue), and in the control of several cellular phenotypes (purple). The molecular (blue) and biological (purple) characteristics of this control are described in detail in the main text, with the exception of the role of endocytosis in neurotransmission, in particular at the synapse, which is not herein reviewed (for reviews on this issue, see Refs. 371, 720, 760).

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Table 1

Pathways of internalization

Pathway	Morphology and Size	Coat or Coat-like	Dynamin Dependence	Small GTPase Involved	Internalized Cargoes	Associated/Regulatory Proteins
Phagocytosis	Cargo-shaped >500 nm	None	No	RAC1/RHOA/CDC42 [depending on type (9, 105, 763)]	Pathogens, apoptotic cells, FcRs (738, 763)	Actin, ARP2/3 (507), Formins (133), PI3K (808), WASP (479), WAVE2 (2), amphiphysin (864), coronin (867), others (763)
Macro-pinocytosis	Ruffled 0.2–10 μm	None	In some cases (575)	RAC1, CDCD42 (560, 837), ARF6 (687), RAB5 (426, 427)	RTKs (575), fluids, bacteria (391, 763)	Actin, ARP2/3, cortactin (413), PI3K (17, 18), SRC (382, 391), PAK1 (164), RAS, CTBP1/BARS (454), others (763)
Clathrin-mediated	Vesicular 150–200 nm	Clathrin	Yes	RAB5 (89)	RTKs (742), GPCR (744), TFR (539), LDLR (539), toxins (681), bacteria (804), viruses (517)	AP-2, epsins, EPS15, intersectin, amphiphysin, ARRs, DAB2, ARH, others (>50) (388, 645, 781); FCHO1/2 (309), ARFGAP1(36)
Caveolae-mediated	Flask-shaped 50–120 nm	Caveolin 1 and 2	Yes	Not clear	GPI-linked proteins (291), CTxB (681), SV40 (517), TGF-βR (167), IGF-1R (678)	PTRF/cavin1 (290, 546), SDPR/cavin2 (289), SRBC/cavin3 (512), MURC/ Cavin4 (49), SRC (702)
CLIC/GEEC	Tubular	None	No	CDC42, ARF1 (416)	Fluids, bulk membrane, GPI-linked proteins (327)	Actin (327), GRAF1 (172), ARHGAP10 (416)
IL-2Rβ	Vesicular 50–100 nm	None	Yes	RHOA, RAC1 (424)	IL-2Rβ (424), γc-cytokine receptor (686)	PAK1 and 2, cortactin, N-WASP (424)
Arf6-dependent	Tubular	None	None as yet	ARF6 (147)	MHC I-II (67, 814), CD59 (551), CD55, GLUT1 (203), AchR (76)	None as yet
Flotillin-dependent	Vesicular	Flotillin 1 and 2	No	None	CTxB, CD59, proteoglycans (230, 258, 591), DAT, EAAT2 (141)	None as yet

The known pathways of internalization are shown together with the morphology and the size of the internalizing membrane structure, the coat involved, the dependence of the pathway on dynamin, the involvement of GTPases, the type of cargo internalized, and the known associated molecular machinery. Relevant references are in parentheses. CLIC, clathrin-independent carriers; GEEK, GPI-AP enriched early endosomal compartment; RTK, receptor tyrosine kinase; GPCR, G protein-coupled receptor; TFR, transferrin receptor; LDLR, low-density lipoprotein receptor; DAB2, disabled-2; ARH, autosomal recessive hypercholesterolemia; CTxB, cholera toxin B; SV40, simian virus 40; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; TGF-βR, transforming growth factor-beta receptor; IGF-IR, insulin-like growth factor I receptor; PTRF, polymerase I and transcript release factor; SDPR, serum deprivation response; SRBC, sdr-related gene product that binds to-c-kinase; MURC, muscle restricted coiled-coil protein; CtBP1, COOH-terminal binding protein 1; DAT, dopamine transporter; EAAT2, glial glutamate transporter; AchR, acetylcholine receptor.

Table 2

Alterations of endocytic/trafficking proteins and of actin regulators in human Mendelian disorders (source: OMIM database, Online Mendelian Inheritance in Man)

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
ACTA1 (*)	Actin, alpha 1, skeletal muscle	102610	Nemaline myopathy 3 (161800)	421, 564
	The ACTA1 gene encodes skeletal muscle alpha-actin, the principal actin isoform in adult skeletal muscle		Congenital fiber-type disproportion myopathy (255310)	
ACTA2 (*)	Actin, alpha 2, smooth muscle, aorta	102620	Aortic aneurysm, familial thoracic 6 (611788)	276
	This actin is an alpha actin that is found in smooth muscle			
ACTC1 (*)	Actin, alpha, cardiac muscle	102540	Dilated cardiomyopathy (613424)	504, 533, 571
	This actin is an alpha actin that is found in cardiac muscle		Familial hypertrophic cardiomyopathy (612098)	
			Atrial septal defect (612794)	
ACTB (*)	Actin, beta	102630	Dystonia, juvenile-onset (607371)	622
	Actin, beta is a cytoplasmic actin found in nonmuscle cells			
ACTG1 (*)	Actin, gamma-1	102560	Deafness, autosomal dominant 20 (604717)	646
	Actin, gamma 1 is a cytoplasmic actin found in nonmuscle cells			
ALS2	Amyotrophic lateral sclerosis 2 (juvenile)	606352	Juvenile amyotrophic lateral sclerosis-2 (205100)	202, 282, 869
	GEF for RAB5 (also contains a RHO-GEF domain)		Juvenile primary lateral sclerosis (606353)	
			Infantile-onset ascending hereditary spastic paralysis (607225)	
AP3B1	Adaptor-related protein complex 3, beta 1 subunit	603401	Hermansky-Pudlak syndrome type 2 (608233)	221
	Component of the AP-3 clathrin adaptor complex			
AP1S2	Adaptor-related protein complex 1, sigma 2 subunit	300629	*X*-linked recessive mental retardation (300630)	107, 766
	Component of the AP-1 adaptor complex			
ARL6	ADP-ribosylation factor-like 6	608845	Bardet-Biedl syndrome #3 (209900)	209
	Small GTPase of the ARF subfamily			
BIN1	Bridging integrator 1 (amphiphysin II)	601248	Autosomal recessive centronuclear myopathy (255200)	554
	MYC-interacting protein. Involved in synaptic vesicle endocytosis; and interacts with dynamin, synaptojanin, endophilin, and clathrin			
BLOC1S3	Biogenesis of lysosomal organelles complex-1, subunit 3	609762	Hermansky-Pudlak syndrome (203300)	535
	Component of the BLOC1 complex, required for normal biogenesis of			

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
	specialized organelles of the endosomal-lysosomal system			
CAV1	Caveolin 1	601047	Lipodystrophy, congenital generalized, type 3 also known as Berardinelli-Seip syndrome (612526)	398
	Caveolin-1, the major component of caveolae			
CAV3	Caveolin 3	601253	Long QT syndrome 9 (611818)	102, 526, 802, 811
	Caveolin-3, a muscle-specific isoform of caveolin		Rippling Muscle Disease (606072)	
			HyperCKemia (123320)	
			Muscular Dystrophy, limb-girdle, type 1C (607801)	
CBL	Cas-Br-M ecotropic retroviral transforming sequence	165360	Noonan syndrome-like disorder (613563)	501, 605
	E3 UB ligase			
CHM	Choroideremia (RAB escort protein 1)	300390	Choroideremia (300390)	793
(REP-1)	Component A of the RAB geranylgeranyl transferase holoenzyme. Binds unprenylated RAB GTPases and then presents them to the catalytic RAB GGTase subunit			
CHMP4B	Chromatin modifying protein 4B	610897	Autosomal dominant progressive childhood posterior subcapsular cataract, CTPP3 (605387)	715
	Component of ESCRT-III complex			
CUBN	Cubilin	602997	Megaloblastic anemia-1 (261100)	411
	Intestinal receptor for the endocytosis of intrinsic factor-vitamin B12			
DIAPH1 (*)	Diaphanous homolog 1 (*Drosophila*)	602121	Deafness, autosomal dominant 1 (124900)	486
	DIAPH1 have a role in the regulation of actin polymerization in hair cells of the inner ear.			
DNM2	Dynamin-2	602378	Dominant intermediate Charcot-Marie-Tooth disease (606482)	65, 182, 887
	GTPase involved in vesicle fission		Autosomal dominant centronuclear myopathy (160150)	
FLNA (*)	Filamin A, alpha	300017	Heterotopia, periventricular, *X*-linked dominant (300049)	47, 251, 417, 654, 655, 786
	FLNA is an actin-binding protein that regulates reorganization of the actin cytoskeleton by interacting with integrins and transmembrane receptor complexes		Otopalatodigital syndrome types I (311300) and II (304120)	
			X-linked cardiac valvular dysplasia (314400)	
			FG syndrome-2 (300321)	
			Frontometaphyseal dysplasia (305620	
			Melnick-Needles syndrome (309350)	
			Chronic idiopathic intestinal pseudoobstruction (300048)	

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
			Terminal osseous dysplasia (300244)	
FLNB (*)	Filamin B, beta	603381	Spondylocarpotarsal syndrome (272460)	63, 409
	FLNB is an actin binding protein that interacts with glycoprotein Ib alpha as part of the process to repair vascular injuries		Autosomal dominant Larsen syndrome (150250)	
			Type 1 atelosteogenesis (108720)	
			Type 3 atelosteogenesis (108721)	
			Boomerang dysplasia (112310)	
FLNC (*)	Filamin C, gamma	102565	Myopathy, myofibrillar, filamin c-related (609524).	812
	FLNC, as other filamin proteins, is an actin-binding protein that regulates reorganization of the actin cytoskeleton			
GDI1	GDP dissociation inhibitor 1	300104	Nonspecific, x-linked mental retardation (300104)	146
(RABGD1A)	Slows the rate of dissociation of GDP from RAB proteins and release GDP from membrane-bound RABs			
GSN (*)	Gelsolin	137350	Amyloidosis, Finnish type (105120)	506
	Gelsolin binds to the “plus” ends of actin monomers and filaments and functions in both assembly and disassembly of actin filaments			
HFE	Hemochromatosis	613609	Hereditary hemochromatosis (235200)	211
	Membrane protein that associates with β2-microglobulin and regulates the interaction of the TFR with TF			
HPS1	Hermansky-Pudlak syndrome 1	604982	Hermansky-Pudlak syndrome (203300)	566
	Component of the BLOC3, 4, and 5 complexes, required for normal biogenesis of specialized organelles of the endosomal-lysosomal system			
HPS3	Hermansky-Pudlak syndrome 3	606118	Hermansky-Pudlak syndrome (203300)	15
	Contains a potential clathrin-binding motif, consensus dileucine signals, and tyrosine-based sorting signals. May play a role in organelle biogenesis			
HPS4	Hermansky-Pudlak syndrome 4	606682	Hermansky-Pudlak syndrome (203300)	762
	This protein appears to be important in organelle biogenesis and is similar to the mouse “light ear” (“LE” or HSP4) protein			
HPS5	Hermansky-Pudlak syndrome 5	607521	Hermansky-Pudlak syndrome (203300)	877
	This protein may play a role in organelle. It interacts with Hermansky-Pudlak syndrome 6 protein and may interact with the cytoplasmic domain of integrin, α3			
HPS6	Hermansky-Pudlak syndrome 6	607522	Hermansky-Pudlak syndrome (203300)	877

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
	This protein may play a role in organelle biogenesis. It interacts with Hermansky-Pudlak syndrome 5 protein			
INF2 (*)	Inverted formin 2	610982	Focal segmental glomerulosclerosis 5 (613237)	86
	IFN2 functions in polymerization and depolymerization of actin filaments.			
LAMP2	Lysosomal-associated membrane protein 2	309060	Danon disease (300257)	559
	This glycoprotein provides selectins with carbohydrate ligands. It may also function in the protection, maintenance, and adhesion of the lysosome			
LDLR	Low-density lipoprotein receptor	606945	Familial hypercholesterolemia (143890)	150, 443
	Low-density lipoprotein receptor			
LDLRAP1	Low-density lipoprotein receptor adaptor protein 1	605747	Familial autosomal recessive hypercholesterolemia (603813)	20, 249
(ARH)	Clathrin adaptor			
LYST	Lysosomal trafficking regulator	606897	Chediak-Higashi syndrome (214500)	45
	Regulates intracellular protein trafficking to and from the lysosome			
MLPH	Melanophilin	606526	Griscelli syndrome type 3 (609227)	515
	RAB27A effector. Forms a ternary complex with GTP-RAB27A and myosin Va			
MCOLN1	Mucolipin 1	605248	Mucolipidosis IV (252650)	46
	Member of the transient receptor potential (TRP) cation channel family. It localizes to intracellular vesicular membranes, and functions in the late endocytic pathway and in lysosomal exocytosis			
MYO1A,	Myosins (IA, VI, and XVA)	601478	Autosomal dominant nonsyndromic deafness. (607841, 606346, 600316)	176, 313, 816
MYO6,	Molecular motors	600970		
MYO15A		602666		
MYO5A	Myosin VA	160777	Griscelli syndrome type 1 (214450)	515
	Molecular motor			
MYO7A	Myosin VIIA	276903	Usher syndrome type I (276900)	4
	Molecular motor			
NDRG1	N-myc downstream regulated 1	605262	Charcot-Marie-Tooth disease, type 4d (601455)	337, 376
	RAB4A effector protein involved in E-cadherin recycling			
NPC1	Niemann-Pick disease, type C1 and C2	607623	Niemann-Pick type C (NPC). Approximately 95% of cases are caused by mutations in the NPC1 gene (257220); 5% are caused by mutations in the NPC2 gene (607625)	798

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
NPC2	Related proteins that reside in the limiting membrane of endosomes and lysosomes and mediate intracellular cholesterol trafficking via binding of cholesterol to their NH_2- terminal domain	601015		
OTOF	Otoferlin	603681	Neurosensory nonsyndromic recessive deafness 9 (601071)	799
	Otoferlin is the key calcium ion sensor involved in the Ca^{2+}-triggered synaptic vesicle-PM fusion and in the control of neurotransmitter release at these output synapses			
PLEKHM1	Pleckstrin homology domain containing, family M (with RUN domain) member 1	611466	Autosomal recessive osteopetrosis 6 (611497)	797
	PLEKHM1 colocalizes with RAB7 to late endosomal/lysosomal vesicles, and may have critical function in vesicular transport			
PSEN1	Presenilin 1	104311	Early-onset familial Alzheimer disease-3 (607822)	713
	Presenilins regulate APP and NOTCH processing through their effects on -γ-secretase			
PTRF (CAVIN1)	Polymerase I and transcript release factor	603198	Lipodystrophy, congenital generalized, type 4 (613327)	297
	The PTRF gene encodes cavin, an essential factor in the biogenesis of caveolae			
RAB7A	RAB7A, member RAS oncogene family	602298	Charcot-Marie-Tooth Type 2B (602298)	752
	Small GTPase of the RAB subfamily			
RAB23	RAB23, member RAS oncogene family	606144	Carpenter syndrome (201000)	363
	Small GTPase of the RAB subfamily			
RAB27A	RAB27A, member RAS oncogene family	603868	Griscelli syndrome type 2 (607624)	(6
	Small GTPase of the RAB subfamily			
RAB39B	RAB39B, member RAS oncogene family	300774	*X*-linked mental retardation (300271)	253
	Small GTPase of the RAB subfamily			
RAB3GAP1	RAB3 GTPase activating protein subunit 1 (catalytic)	602536	Warburg Micro syndrome (600118)	8
	Catalytic subunit of a RAB GTPase activating protein; it specifically regulates the activity of members of the RAB3 subfamily			
RIMS1	Regulating synaptic membrane exocytosis 1	603649	Cone-rod dystrophy-7 (603649)	367
	RAB3-interacting protein molecule 1. Likely functions as protein scaffolds that help regulate vesicle exocytosis during short-term plasticity			

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
RIN2	RAS and RAB interactor 2	610222	Macrocephaly, alopecia, cutis laxa, and scoliosis (MACS) syndrome (613075)	48
	Member of the RIN family of RAS interaction-interference proteins, which are binding partners to RAB5. It functions as a RAB5GEF			
ROBLD3	Roadblock domain containing 3	610389	Primary immunodeficiency (610798)	70
(MAPBPIP/P14)	Associated with the cytoplasmic face of late endosomes and lysosomes. Interacts with MAPK scaffold protein 1. Possible role in endosome biogenesis			
SEC23A	Sec23 homolog A	610511	Craniolenticulosutural dysplasia (607812)	81
	Essential component of coat protein complex II (COPII)-involved in ER to GA transport			
SNAP25	Synaptosomal-associated protein, 25 kDa	600322	Attention deficit-hyperactivity disorder (143465)	214
	Involved in vesicle membrane docking and fusion (SNARE)			
SNAP29	Synaptosomal-associated protein, 29 kDa	604202	Cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma (CEDNIK) syndrome (609528)	754
	Member of the SNAP25 family (SNAREs), involved in vesicle membrane docking and fusion			
SPG20	Spastic paraplegia 20 (Troyer syndrome)	607111	Troyer syndrome or autosomal recessive spastic paraplegia 20 (275900)	588
	This protein contains a MIT (microtubule interacting and trafficking molecule) domain, and it is implicated in regulating endosomal trafficking and mitochondria function. Also shown to function in the degradation and intracellular trafficking of EGFR			
STX11	Syntaxin 11	605014	Familial hemophagocytic lymphohistiocytosis 4 (603552)	669
	Involved in vesicle membrane docking and fusion (SNARE)			
STXBP1	Syntaxin binding protein 1	602926	Early infantile epileptic encephalopathy 4 (612164)	677
	Neural-specific, syntaxin-binding protein			
STXBP2	Syntaxin binding protein 2	601717	Familial hemophagocytic lymphohistiocytosis 5 (613101)	137
	Member of the STXBP/UNC-18/SEC1 family. Involved in protein trafficking from the Golgi apparatus to the PM. STXBP2 interacts with STX11			
SYN1	Synapsin I	313440	Epilepsy, *X*-linked, with variable learning disabilities and behavior disorders (300491)	248
	Member of the synapsin family, neuronal phosphoproteins which associate with the cytoplasmic surface			

Gene Symbol	Gene Name/Protein Function	OMIM Mutation	Genetic Syndrome (OMIM number)	Reference Nos.
	of synaptic vesicles and modulate neurotransmitter release			
SYNJ1	Synaptojanin 1	604297	Chromosome 21q22-linked bipolar disorder (125480)	676
	A phosphoinositide phosphatase that regulates levels of membrane PIP_2			
TSC2	Tuberous sclerosis 2	191092	Tuberous sclerosis complex (TSC). ~10-30% of cases of TSC are due to mutations in the TSC1 gene, the remainder to mutations in the TSC2 gene (613254)	120
	Regulator of the MTOR pathway			
VAMP7	Vesicle-associated membrane protein 7	300053	Bipolar disorder, an *X*-linked form of manic-depressive illness that affects females and causes a deficiency of male-to-male transmission (309200)	541
	SNARE protein. Localizes to late endosomes and lysosomes and is involved in the fusion of transport vesicles to their target membranes			
VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	605704	ALS8, an atypical form of ALS (amyotrophic lateral sclerosis) (608627)	557
	Member of the vesicle-associated membrane protein (VAMP)-associated protein (VAP) family. Interacts with VAMP1 and VAMP2 (SNAREs) and may be involved in vesicle trafficking			
VPS13B	Vacuolar protein sorting 13 homolog B	607817	Cohen syndrome (216550)	403
	Possible role in vesicle-mediated sorting and intracellular protein trafficking			
VPS33B	Vacuolar protein sorting 33 homolog B	608552	ARC syndrome (arthrogryposis, renal dysfunction, and cholestasis) (208085)	356
	Member of the Sec-1 domain family, homologous to the yeast class C Vps33 protein. Predominantly associated with late endosomes/lysosomes, may mediate vesicle trafficking steps in the endosome/lysosome pathway			
WASP (*)	Wiskott-Aldrich syndrome protein	300392	Wiskott-Aldrich syndrome (301000)	159, 162, 809
	The Wiskott-Aldrich syndrome (WAS) family of proteins are involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton.		Thrombocytopenia 1 (313900)	
			X-linked recessive congenital neutropenia (300299)	

List of endocytic, trafficking, and organelle-associated genes found mutated and causative of specific disease. We searched for mutations in genetic syndromes of 277 “endocytic genes” (the list was manually curated and is available upon request) encoding for: structural/accessory endocytic proteins, ARF GTPases and their effectors; endocytic and nonendocytic RABs; proteins belonging to the ESCRT complexes; SNARE proteins; sorting nexins and synaptotagmins; proteins associated to lysosomes or endosomes and important for their biogenesis or function, and of 62 genes encoding actin regulator/dynamics proteins [these latter genes are identified by (*) in the “Gene symbol” column]. Databases searched were the OMIM and the GENE database at NCBI (http://www.ncbi.nlm.nih.gov/omim and http://www.ncbi.nlm.nih.gov/gene). Shown are the official gene symbol and gene name, the protein function (as from the OMIM and GENE databases, supplemented with information derived from literature), and the OMIM numbers for the mutation and the genetic syndrome.

Table 3
Alterations of endocytic/trafficking proteins and of actin regulators in cancer

Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
ABI1 (*)	Abl-interactor 1	Loss/downregulation in gastric and prostate cancer. Overexpression in breast and ovarian cancer.	Possible tumor suppressor activity: downregulation correlates with the progression of gastric cancer. Oncogenic properties: overexpression associates with early recurrence and worse survival in breast and ovarian cancers.	121, 145, 489, 817
	ABI1 forms a complex with EPS8/SOS1, and is involved in signaling from RAS to RAC. It is also a critcal component of the WAVEs(WASFs)-actin nucleator promoting complex.			
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	Overexpression in breast cancer.	Necessary for v-SRC-induced transformation including tumor formation in nude mice.	5
	GEF for RHO-GTPases			
C3ORF10 (*)	Chromosome 3 open reading frame 10	Overexpressed in node positive lung squamous cell carcinoma. Genetic loss is protective in clear cell carcinoma.	Genetic loss or inhibition of C3ORF10 is likely to be protective against tumor development due to proliferation and motility defects in affected cells. Loss of the HSPC300 gene confers protection against renal clear cell carcinoma.	96, 109, 197
	C3ORF10, also known as HSPC300 or BRK1, is a component of the WAVEs (WASFs) actin nucleator promoting complex involved in actin polymerization in migratory cells.			
CAPG (*)	Gelsolin-like capping protein	Overexpressed in ovarian cancer, oral squamous cell carcinoma, breast cancer, ocular melanomas, glioblastomas, and pancreatic ductal adenocarcinomas.	Overexpression enhances the invasiveness and metastasizing potential of cancer cells.	562, 584, 771, 795
	Actin capper, controls actin-based motility in nonmuscle cells			
CAPZA2 (*)	Capping protein (actin filament) muscle Z-line, alpha 2	Overexpressed in breast cancer and possibly in glioblastomas.	Unclear	540, 576
	Alpha subunit of the barbed-end actin binding protein Cap Z.			
CAV1	Caveolin 1	Downregulation and sporadic mutations in breast cancer. Upregulation in multiple cancers types; positively correlates with high tumor grade and poor clinical outcome. Amplification in aggressive breast carcinomas.	Expression inversely correlates with cell cycle progression and transformation. However, ectopic expression suppresses oncogene-induced apoptosis and confers resistance to anoikis.	705
	Essential component of caveolae			
CBL	Cas-Br-M ecotropic retroviral transforming sequence	Point mutations (e.g., R420Qin RING), insertions,	Inhibits ubiquitination and downregulation of several	1, 99, 231, 680, 685, 765

Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
		deletions and fusions in AML. Mutations in NSCLC.	receptor protein tyrosine kinases.	
	E3 UB ligase			
CLTC	Clathrin, heavy chain	CHC-ALK fusion in IMT and large B-cell lymphoma. CHC-TFE3 fusion in pediatric renal carcinoma.	Constitutive activation of ALK. Aberrant transcription factor activity.	85
	Principal coat protei			
CTTN	Cortactin	Amplification at gene locus (11q13) and protein overexpression in primary breast carcinomas and head and neck squamous carcinomas.	Overexpression inhibits ligand-induced EGFR endocytosis; knockdown accelerates receptor downregulation in HNSCC cell lines.	91
	Coordinates actin polymerization at endocytic sites; ARP2/3 activator; binds F-actin and dynamin			
CYFIP1 (*)	Cytoplasmic FMR1 interacting protein 1	Deleted in epithelial cancers.	Deletion of CYFIP1 alters normal epithelial morphogenesis in vitro and cooperated with oncogenic RAS to produce invasive carcinomas in vivo.	723
	Interacts with RAC1 and is a key component of the WAVEs(WASFs)-actin nucleator promoting complex.			
DAB2	Disabled homolog 2	Downregulation in ovarian, prostate, bladder, breast, esophageal, and colorectal carcinomas.	Increased expression suppresses growth of choriocarcinoma and prostate cancer cells.	381
	Cargo-selective clathrin adaptor; recruits myosin VI to clathrin-coated structures			
ENAH (*)	Enabled homolog	Overexpressed in breast cancer.	Promotes tumor invasion.	264, 664
	Belongs to the ENA/VASP family of proteins that bundle and elongate linear actin filaments. It potentiates EGF-induced membrane protrusion and increases the matrix degradation activity of tumor cells.			
EPS8 (*)	Epidermal growth factor receptor pathway substrate 8	Overexpressed in colon, pancreatic, ovarian cancer and oral squamous cell carcinoma.	Promotes cell proliferation in colon carcinoma, and SRC-transformed cells. Enhances chemoresistance in cervical cancer patients. Promotes cell migration and invasion of ovarian and oral squamous cell carcinoma.	121, 123, 469, 487, 503, 819, 870
	Participates in both EGFR signaling through RAC and EGFR trafficking through RAB5. It also acts as an actin capping protein when bound to ABI1, and as a bundler when it is associated with BAIAP2.			
EPS15	Epidermal growth factor receptor pathway substrate 15	EPS15–MLL fusion in AML.	EPS15 coiled-coil domain mediates oligomerization of MLL, a DNA-binding histone methyltransferase.	737
	Endocytic accessory protein			

Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
EVL (*)	Enah/Vasp-like	Overexpressed in breast cancer.	EVL may be implicated in invasion and/or metastasis of breast cancer.	331
	Belongs to the ENA/VASP family of proteins.			
FLNA (*)	Filamin A, alpha	Overexpression together with MET in adenocarcinomas.	FLNA is one of the important regulators of MET signaling and HGF induced tumor cell migration.	880
	FLNA is an actin-binding protein that crosslinks actin filaments and links actin filaments to membrane glycoproteins.			
FMNL2 (*)	Formin-like 2	Overexpression in metastatic cell lines and colorectal carcinoma.	FMNL2 is involved in epithelial-mesenchymal transition (EMT) maintenance in human colorectal carcinoma cells.	452, 882
	FMNL2 is a member of diaphanous-related formins that control actin-dependent processes such as cell motility and invasion by promoting linear filament elongation.			
FNBP1L (*)	Formin binding protein 1-like	Fusion partner of MLL in acute myeloid leukemias (AML).	Unclear	233
	It promotes CDC42-induced actin polymerization.			
GSN (*)	Gelsolin	Downregulated in breast, stomach, colon, bladder and lung cancers	May function as a tumor suppressor by regulating a G2 checkpoint function of cancer cells through phosphoinositol lipid metabolism.	25, 179, 673
	Caps the “plus” ends of actin monomers and filaments to prevent monomer exchange and regulates both assembly and disassembly of actin filaments.			
IQSEC1	IQ motif and Sec7 domain 1	Overexpression in invasive ductal carcinomas of the breast.	Knockdown inhibits metastasis formation by breast cancer cells in nude mice.	536
	ARF6 GEF; interacts with activated EGFR			
HAX1	HCLS1 associated protein X-1	Overexpression in advanced oral carcinoma.	Knockdown inhibits endocytosis of integrin $\alpha v\beta 6$ and migration of oral carcinoma cells.	635
	Regulates clathrin-mediated integrin endocytosis			
HIP1 HIP1R	Huntingtin interacting protein 1 Huntingtin interacting protein 1-related	HIP1-PDGFBR fusion in CMML. Overexpression in primary epithelial tumors and gliomas.	Induce cytokine-independent growth. Transform mouse fibroblasts to induce colonies in soft agar and tumors in nude mice.	636, 661
	Coordinate actin remodeling during formation of clathrin-coated vesicles			
MTSS1 (*)	Metastasis suppressor 1	Downregulated in breast and ovarian cancer. Overexpressed in colorectal cancer.	Overexpression of MTSS1 suppresses the invasive, migratory, growth and adherence properties of a	441, 467, 583, 818

Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
			human breast cancer cell line. High levels of MTSS1 correlated with an increased patient overall survival and disease-free survival in breast cancer. Overexpression of MTSS1 in colorectal cancer tissues was significantly correlated to poor differentiation, tissue invasion, lymph node metastasis and high TNM stage. Loss of expression is significantly associated with poorly differentiated tumors, large tumor size, deep invasion level, nodal metastases and advanced disease stage in gastric cancer.	
	Possesses an I-BAR domain that deforms the PM and binds actin through its WH2 domain. Overexpression of Mtss1 causes formation of abnormal actin structures.			
NUMB	Numb homolog (*Drosophila*)	NUMB expression is lost in about 50% of human mammary carcinomas and nonsmall cell lung carcinomas.	NOTCH antagonist. Tumor suppressor activity attributed to stabilization of TP53.	131, 593, 838
	Regulates internalization and recycling of several PM-resident proteins			
PARD3	Par-3 partitioning defective 3 homolog (*C. elegans*)	Downregulation in HCC.	Associations with tumor suppressors (VHL and PTEN) and oncogenes (e.g., ERBB2) impinge on regulation of cell polarity.	210
	PARD3 proteins, which were first identified in *C. elegans*, are essential for asymmetric cell division and polarized growth. PARD3 controls endocytosis and recycling in clathrin-dependent and independent pathways			
PRKCDBP (CAVIN3)	Protein kinase C, delta binding protein	Epigenetic inactivation.	Epigenetic inactivation of PRKCDBP, due to aberrant promoter hypermethylation, is a common event and might be implicated in human ovarian tumorigenesis (possible tumor suppressor).	776
	Cavin-3, a component of caveolae			
RAB25	RAB25, member RAS oncogene family	Amplification of genomic locus (1q22) in advanced ovarian and breast cancers.	Overexpression promotes increased anchorage-independent growth and tumor cell invasion.	124
	Small GTPase of the RAB subfamily. Regulates receptor recycling. Promotes invasion by delivery of integrin $\alpha 5\beta 1$ to the leading edge			
SCIN (*)	Scinderin	Lack of expression in megakaryoblastic leukemia cells, but is present in normal megakaryocytes and platelets.	Cell proliferation and cell ability to form tumors in nude mice are inhibited by the expression of scinderin.	888

Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
	Scinderin is a Ca^{2+}-dependent actin-severing and -capping protein.			
SH3GL1	SH3-domain GRB2-like 1 (EEN, endophilin II)	EEN-MLL fusion in AML.	EEN coiled coil-dependent dimerization and oncogenic activation of MLL.	464
	Endocytic accessory protein, induces membrane curvature during vesicle formation			
SNAP91	Synaptosomal-associated protein, 91 kDa homolog	SNAP91-AF10 fusion in ALL and AML.	Fusion comprising clathrin-binding domain of SNAP91and putative transcription factor AF10.	21
	Clathrin-binding adaptor; involved in assembly of clathrin coats			
SPRY1 SPRY2	Sprouty homolog 1, antagonist of FGF signaling and Sprouty homolog 2	Deregulation of SPRY1 and SPRY2 in breast and prostate cancers.	As a tumor suppressor it acts as an antagonist of RAS-ERK pathway: SPRY1 and SPRY2 overexpression in osteosarcoma and prostate cancer cells, respectively, inhibits cell proliferation and invasion. As a putative oncogene it functions as an inhibitor of EGFR downregulation by targeting both the CBL and CIN85 pathways.	283,473
	SPRY1 and SPRY2 inhibit the transcriptional events mediated by growth factor signaling and the induction of FOS. They compete with RTKs for CBL binding and prevent receptor degradation			
TNK2 (ACK1)	Tyrosine kinase, nonreceptor, 2	Gene amplification in advanced-stage primary tumors and metastases derived from prostate and breast.	Enhances tumorigenesis in nude mice. Promotes degradation of tumor suppressor protein WWOX.	794
	Binds clathrin and activated EGFR; promotes receptor degradation			
TRIP10 (*)	Thyroid hormone receptor interactor 10	TRIP10 is hypermethylated in brain tumor and breast cancer, but hypomethylated in liver cancer.	TRIP10 regulates cancer cell growth and death in a cancer type-specific manner. Differential DNA methylation of TRIP10 can either promote cell survival or cell death in a cell type-dependent manner.	328
	It is a F-BAR-containing protein involved in CDC42, Dynamin and WASP-dependent endocytic processes.			
VIL1 (*)	Villin 1	Overexpressed and amplified in cervical adenocarcinomas.	Cervical carcinomas show variability in the expression and genomic copy number of Villin1 (VIL1). Kaplan-Meier survival curves revealed worse disease-free survival in VIL1-positive tumors.	549
	Encodes a member of a family of calcium-regulated actin-binding proteins that can cap, sever, or bundle actin filaments.			

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Gene Symbol	Gene Name/Protein Function	Type of Alteration	Oncogenic Properties	Reference Nos.
VPS37A	Vacuolar protein sorting 37 homolog A	Downregulation in hepatocellular carcinoma.	Knockdown strongly stabilizes EGFR.	863
	Component of ESCRT-I complex; promotes down-regulation of ubiquitinated receptors			
WASF2 (*)	WAS protein family, member 2	Overexpression of WAVE2 (WASF2) was seen in node-positive as well as in moderately and poorly differentiated breast cancer, and in colon cancer with respect to normal colonic epithelial cells.	High levels of WAVE2 expression were associated with death due to disease in breast cancer patients. Colocalization of Arp2 and WAVE2 has been found as an independent risk factor for liver metastasis of colorectal carcinoma.	216, 353
	Forms a WAVEs (WASFs) actin nucleator promoting complex that links receptor kinases to actin dynamics.			
WASF3 (*)	WAS protein family, member 3	Overexpression in prostate cancer.	WAVE3 is pivotal in controlling the invasiveness of prostate cancer cells.	217
	This gene encodes a member of the Wiskott-Aldrich syndrome protein family and has similar function to its homologues WASF1 and 2			

We searched for mutations or deregulation in cancer in a list of 277 “endocytic genes” and of 62 genes encoding actin regulator/dynamics proteins [these latter genes are identified by (*) in the “Gene symbol” column] (same as in Table 2). Databases searched were the OMIM and the GENE database at NCBI (http://www.ncbi.nlm.nih.gov/omim and http://www.ncbi.nlm.nih.gov/gene), supplemented with ad hoc literature searches. Shown are the official gene symbol and gene name, the protein function (as from the OMIM and GENE databases, supplemented with information derived from literature), the type of alteration detected in cancer and a description of the oncogenic properties of the protein (as obtained from the literature).

Table 4

Mutations in genes encoding endocytic/trafficking proteins and actin regulators in cancer in the COSMIC database

GENE SYMBOL	BR	CNS	HAE	KID	COL	LIV	LUN	OVA	PAN	MEL	URI	TOT	MEND.
CBL		2 (447)	133 (2322)				9 (318)					144	X
VPS13B	1 (48)	2 (23)					3 (11)	6 (7)	1 (2)	4 (6)		17	X
CUBN	4 (48)	1 (22)		1 (1)	1 (37)			6 (9)	2 (2)			15	X
LYST	4 (30)	2 (22)						5 (6)				11	X
TSC2		4 (22)					3 (338)	2 (22)				9	X
CYFIP1 (*)						1 (1)		4 (4)	3 (2)			8	
FLNB (*)	6 (49)							2 (3)				8	X
RIMS1	1 (30)							5 (7)	1 (2)			7	X
FLNC (*)	2 (33)				2 (32)			2 (2)				6	X
ALS2	1 (30)									4 (6)		5	X
FMN2 (*)					1 (32)			2 (4)	2 (3)			5	
KIF16B	1 (48)			1 (101)	1 (38)		1 (11)			1 (6)		5	
OTOF	2 (48)							3 (3)				5	X
AP1M1	1 (48)							3 (3)				4	
BIN1		2 (447)						1 (2)		1 (6)		4	X
DIAPH2 (*)							1 (11)	3 (3)				4	
FHOD3 (*)	2 (30)				1 (32)			1 (1)				4	
HIP1		1 (447)						1 (1)	2 (2)			4	
HPS3					1 (37)			3 (5)				4	X
MYO15A		1 (22)						1 (2)	1 (1)	1 (1)		4	X
SEC23A								4 (4)				4	X
SNX19							2 (11)	2 (2)				4	
SNX25	2 (30)							2 (2)				4	
SVIL (*)								4 (5)				4	
SYT6		1 (22)						2 (3)	1 (1)			4	
AMPH						1 (1)		2 (3)				3	
AP3B2								2 (3)	1 (1)			3	
DAAM2 (*)								2 (2)		1 (1)		3	
EVL (*)					2 (37)			1 (1)				3	
FLNA (*)	3 (30)											3	X
GGA1	2 (48)							1 (1)				3	
GSN (*)	2 (48)							1 (1)				3	X
ITSN2				2 (412)				1 (1)				3	
MYO5A		1 (22)						2 (3)				3	X
MYO7A	1 (30)							2 (3)				3	X
NDRG1								3 (4)				3	X

GENE SYMBOL	BR	CNS	HAE	KID	COL	LIV	LUN	OVA	PAN	MEL	URI	TOT	MEND.
SNX13		2 (447)		1 (101)								3	
SNX4	2 (13)							1 (1)				3	
SYT3	1 (48)							2 (2)				3	
DIAPH3 (*)								2 (2)				2	
EPS8 (*)								2 (2)				2	
EPS8L3 (*)								2 (2)				2	
FHOD1 (*)	2 (48)											2	
FMNL2 (*)		2 (446)										2	
FMNL3 (*)									2 (1)			2	
NCKAP1 (*)								2 (2)				2	
SCIN (*)								2 (2)				2	
WASF2 (*)								1 (1)		1 (2)		2	
AP1G1								2 (2)				2	
AP1M2								2 (2)				2	
AP2A1								2 (2)				2	
CHMP4A								2 (2)				2	
CHMP4C								2 (2)				2	
GGA3	1 (48)							1 (1)				2	
HPS5								1 (1)	1 (1)			2	X
LAMP1		2 (446)										2	
LDLR								2 (2)				2	X
MYO1A								1 (2)			1 (2)	2	X
RAB36		1 (23)						1 (1)				2	
RAB3C		1 (23)						1 (1)				2	
RAB5C					1 (38)					1 (6)		2	
SH3GL3		1 (22)							1 (1)			2	
SNX21	1 (48)								1 (1)			2	
SNX27								2 (2)				2	
SNX7								2 (2)				2	
STAM					1 (37)			1 (1)				2	
STAMBP								1 (1)	1 (1)			2	
STX12	1 (48)	1 (22)										2	
STX3								2 (1)				2	
STX5	1 (48)							1 (1)				2	
SYNJ1								2 (1)				2	
SYT14								1 (1)		1 (1)		2	X
SYT7								2				2	
SYTL4								2				2	
ABI1 (*)		1 (447)										1	

GENE SYMBOL	BR	CNS	HAE	KID	COL	LIV	LUN	OVA	PAN	MEL	URI	TOT	MEND.
ACTG1 (*)								1 (1)				1	X
AP1B1								1 (1)				1	
AP1G2								1 (1)				1	
AP2B1								1 (1)				1	
ARF5								1 (1)				1	
AVIL (*)		1 (446)										1	
BAIAP2L (*)1								1 (1)				1	
BAIAP2L2 (*)									1 (1)			1	
CAPZA3 (*)								1 (1)				1	
CHM								1 (1)				1	X
CHMP4B								1 (1)				1	X
CHMP6								1 (1)				1	
CYFIP2 (*)								1 (1)				1	
DAAM1 (*)								1 (1)				1	
DNM1								1 (1)				1	
DNM2								1 (1)				1	X
EEA1								1 (1)				1	
EPS8L2 (*)									1 (1)			1	
FMN1 (*)								1 (1)				1	
ITSN1								1 (1)				1	
LAMP2								1 (1)				1	X
MCOLN1	1 (48)											1	X
MLPH								1 (1)				1	X
MTSS1 (*)								1 (1)				1	
MURC								1 (1)				1	
MYO6									1 (1)			1	X
NPC1								1 (1)				1	X
PDCD6IP										1 (6)		1	
PICALM								1 (1)				1	
PSEN1				1 (102)								1	X
PTRF	1 (48)											1	X
RAB10								1 (1)				1	
RAB15								1 (1)				1	
RAB28								1 (1)				1	
RAB2B								1 (1)				1	
RAB31										1 (6)		1	
RAB37								1 (1)				1	
RAB38					1 (38)							1	
RAB3B								1 (1)				1	

GENE SYMBOL	BR	CNS	HAE	KID	COL	LIV	LUN	OVA	PAN	MEL	URI	TOT	MEND.
RAB3D								1 (1)				1	
RAB41								1 (1)				1	
RAB43							1 (200)					1	
RAB4A								1 (1)				1	
RAB4B										1 (6)		1	
RAB6C							1 (200)					1	
RAB7L1								1 (1)				1	
RAB8A										1 (6)		1	
RAB8B				1 (101)								1	
RAB9A								1 (1)				1	
RIN1							1 (188)					1	
RIN2								1 (1)				1	X
SDPR									1 (1)			1	
SH3GL1								1 (1)				1	
SNAP91								1 (1)				1	
SNX16								1 (1)				1	
SNX2				1 (101)								1	
SNX20								1 (1)				1	
SNX29								1 (1)				1	
SNX5					1 (38)							1	
SNX8					1 (38)							1	
STAM2								1 (1)				1	
STX11								1 (1)				1	X
STX16								1 (1)				1	
STX17					1 (37)							1	
STX6								1 (1)				1	
STXBP2		1 (22)										1	X
SYN1								1 (1)				1	X
SYT1									1 (1)			1	
SYT10								1 (1)				1	
SYT11								1 (1)				1	
SYT12								1 (1)				1	
SYT13								1 (1)				1	
SYT16		1 (1)										1	
SYT17								1 (1)				1	
SYT2								1 (1)				1	
SYT9					1 (37)							1	
SYTL1								1 (1)				1	
SYTL2					1 (32)							1	

GENE SYMBOL	BR	CNS	HAE	KID	COL	LIV	LUN	OVA	PAN	MEL	URI	TOT	MEND.
TRIP10 (*)								1 (1)				1	
USP8							1 (11)					1	
VIL1 (*)								1 (1)				1	
VPS24		1 (22)										1	
VPS4B								1 (1)				1	
WAS (*)								1 (1)				1	
WASF3 (*)									1 (1)			1	
Total	46	32	133	8	17	2	23	184	26	19	1	491	

We searched for mutations in cancer (in the COSMIC database, Version 53, www.sanger.ac.uk/perl/genetics/CGP/cosmic) in a list of 277 "endocytic genes" and of 62 genes encoding actin regulator/dynamics proteins [these latter genes are identified by (*)] (same as in Table 2). Genes are ranked by the total number of mutations found. Shown are the official gene symbol and frequency of mutations (total number of mutations and number of analyzed cases in parentheses) within each tumor type. Silent mutations were not computed. Type of tumor: BR, breast; CNS, central nervous system; HAE, tumors of hematological and lymphoid tissues; KID, kidney; COL, colon-rectum; LIV, liver; OVA, ovary; PAN, pancreas; MEL, melanomas; URI, urinary tract. In column "MEND," we report (by an X) whether the listed genes are also mutated in Mendelian diseases (as from Table 2).