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Signal processing by the endosomal system

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Cells need to decode chemical or physical signals from their environment in order to make decisions on their fate. In the case of signalling receptors, ligand binding triggers a cascade of chemical reactions but also the internalization of the activated receptors in the endocytic pathway. Here, we highlight recent studies revealing a new role of the endosomal network in signal processing. The diversity of entry pathways and endosomal compartments is exploited to regulate the kinetics of receptor trafficking, and interactions with specific signalling adaptors and effectors. By governing the spatio-temporal distribution of signalling molecules, the endosomal system functions analogously to a digital-analogue computer that regulates the specificity and robustness of the signalling response.

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Introduction to signalling and endocytosis

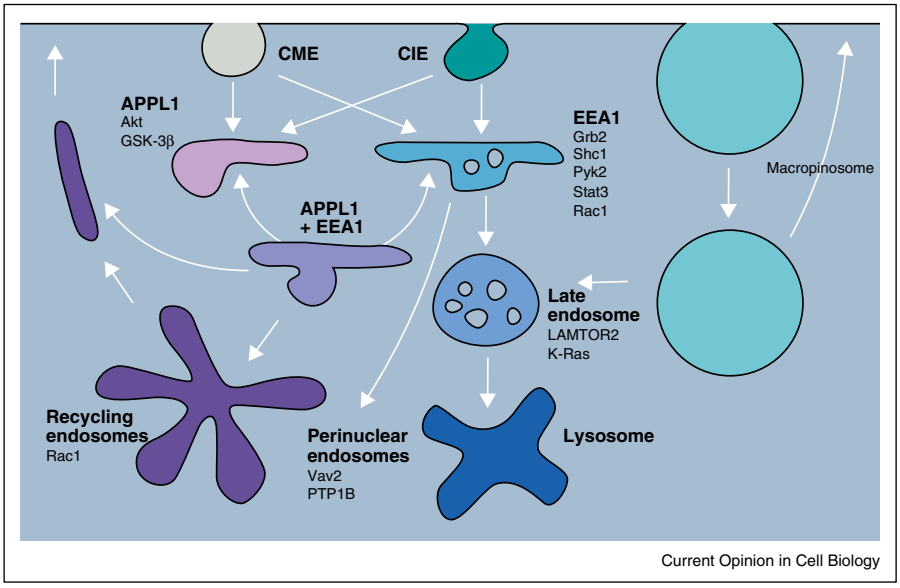
Cells constantly process information in the form of chemical or physical signals from the environment to make decisions on their fate. Despite a great deal of molecular diversity, the communication of information based on chemical signals follows general design principles that can be described with the language of information theory as source, channel, receiver, decoder and destination [1•,2]: (1) the source of signals are the cells that secrete signalling factors which are propagated by (2) the channel, the extracellular medium (e.g. blood, ECM). (3) The receiver is the receptors on the target cells, where (4) the decoder, the intracellular signalling machinery transmits and decodes the signals to (5) the destination, that is, the cell nucleus to determine cell fate. Although intracellular signal transmission and decoding occur simultaneously, they are distinct process that can be uncoupled. A well-characterized example of these processes is the family of receptor tyrosine kinases (RTKs). In this case, signalling is initiated by a ligand binding to its receptor, which leads to receptor activation and recruitment of intracellular scaffold proteins [3]. Next, intracellular signalling scaffolds and kinases amplify the signal and finally, lead to changes in target proteins that modify the cellular state [4]. For this pathway, information is transmitted and decoded/processed through phosphorylation [5], and other post-translational modifications such as ubiquitination [6,7] or SUMOylation [8]. Key properties of such a biological decoder are: (1) specificity, which is the capacity to translate a given input into a specific output; (2) multiplexing, which is the ability to process different signals through a relatively limited number of core components in order to transfer information; (3) robustness, which is the capacity to maintain signalling functions despite internal (e.g. levels of signal transducers) or external (e.g. levels of signalling molecules) fluctuations; (4) adaptation, which is the ability to adjust the signal processing network according to the cellular context (cells within their tissue) and signals that act concomitantly. How these properties arise from the cellular machinery and contribute to cell homeostasis and cell fate remain open questions.

Recent results suggest that both the network topology, that is, network motifs such as feedback loops [9,10], and the spatial organization of signalling components (reviewed in [11]) regulate signalling specificity and robustness. An important element of the cellular decoder is endocytosis. Concomitant with receptor activation, ligand binding also triggers the internalization of the signalling receptors from the plasma membrane into early endosomes, where receptors are sorted for degradation to late endosomes and lysosomes or recycled back to the plasma membrane to be reutilized (Figure 1). The balance among internalization, degradation, and recycling, regulates the ratio of surface receptor and intracellular pools, and, as such, the sensitivity of the cell to the concentration of ligand. This particular role of endocytosis on the levels of surface receptors and its impact on responsiveness to external signals has been extensively reviewed (e.g. see [12,13]). Here, we focus on novel insights into the role of the endocytic system as an essential part of the cellular machinery that decodes signals for a specific cell fate decision.

Compartmental organization of the endocytic network

To understand the role of the endocytic system in signal transduction, we first need to consider the diversity of

Figure 1



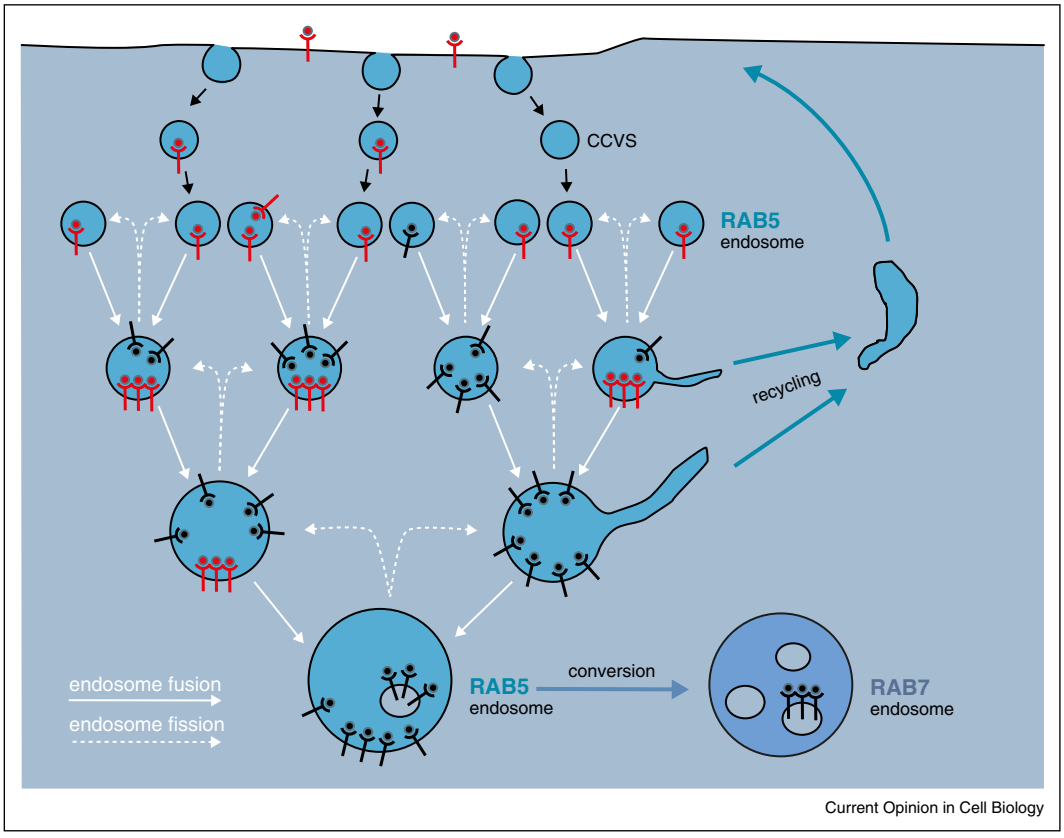
Model of basic organization of the endocytic pathway. Molecules can be internalized from the plasma membrane either by CME or various forms of CIE, including macropinocytosis. They can be transported to two distinct types of Rab5-positive early endosomes, distinguished by the presence of the Rab5 effectors APPL1 or EEA1 and PI(3)P. The arrows indicate the direction of cargo flow between different endocytic compartments. The two endosomes communicate through a double APPL1 + EEA1-positive compartment. From these endosomes, cargo can either be recycled to the plasma membrane via tubular carriers, directly or passing through perinuclear recycling endosomes, or transported to late endosomes and lysosomes. In the course of this process, cargo is incorporated in intra-luminal vesicles (ILV) and degraded. The model, which is highly simplified, emphasizes the diversity of endosomal compartments where signalling receptors can traffic, with different kinetics while encountering different adaptors and effectors.

entry routes and endosomal compartments that could bestow regulation for the decoder. Receptors at the cell surface can be internalized through several distinct endocytic routes and mechanisms ([14•] and Di Fiore *et al.*, this issue). The best-characterized pathway in molecular terms is Clathrin-mediated endocytosis (CME). CME requires the GTPase Dynamin. Reagents that block its GTPase activity inhibit CME. Note, however, that Dynamin is dispensable for other forms of endocytosis. In addition to the well-known macropinocytosis and phagocytosis, several Clathrin-independent endocytosis (CIE) mechanisms have been discovered that differ on the basis of morphological features, cargo specificity, and molecular requirements [14•]. These include Dynamin-dependent mechanisms such as caveolae and RhoA-dependent vesicles but also a variety of Dynamin-independent vesicles, such as Clathrin-independent tubulovesicular carriers CLIC/GEEC or vesicles that are differentially regulated either by Cdc42, Arf6 and Flotillin [14•]. Recently, the protein endophilin, a component of CME, has been implicated in a form of rapid CIE (Fast endophilin-mediated endocytosis (FEME); [15]). CIE pathways can contribute to RTK internalization. For example, RTKs can be internalized by caveolae [16] and FEME [15]. The different internalization pathways could lead to changes in signalling output. For a detailed discussion on the mechanisms of endocytosis and their role in signalling see Di Fiore's review in the same issue.

All internalization pathways converge at the early endosome, where cargo is sorted and transported to subsequent compartments (Figure 1). It is important to bear in mind that these functions result from the collective properties of several hundreds of individual endosomes that form a dynamic network. One can view the early endosome network as a funnel-like system [17,18] (Figure 2). Endocytic cargo such as RTKs progressively flow from small endosomes at the cell periphery to large endosomes in the centre, where eventually early endosomes that are positive for the small GTPase Rab5 convert into Rab7-positive late endosomes [19,20]. Progressively, the internalized receptors are incorporated into intra-luminal vesicles (ILV), leading to degradation. The endosomes undergo fusion and fission reactions, which shape the distribution (in space, number and size) of the organelles and, thus, the kinetics and fate of cargo transport in the network.

This organization already provides a simple means to regulate cargo flow through the network by modulating the kinetics of transport and, consequently, the duration of signalling before degradation [21•]. However, recent

Figure 2



Funnel-like organization of the early endosomal network and signalling *quanta*. Endocytic cargo such as EGF-EGFR complexes internalized from the surface, for example, via clathrin-coated vesicles CCVs, are transported to Rab5-positive early endosomes (see Figure 1). By tracking individual endosomes as well as endosomal populations, it was shown that cargo progressively flows from many small early endosomes at the cell periphery to few large endosomes at the centre, resembling a funnel-like shape distribution of endosomes. This is because early endosomes progressively undergo fusion and fission reactions, propagating with time cargo to larger endosomes. The kinetics of these two processes as well as of membrane recycling to the surface shape the distribution of endosomes in terms of number, size and location. The few large Rab5-positive early endosomes that accumulate in the perinuclear region eventually convert into Rab7-positive late endosomes leading to cargo degradation. Due to these features, the early endosomal network functions as a digital-analogue computer that regulates specificity and robustness of the signalling response. Upon internalization, active p-EGFR (single EGF-EGFR red) is distributed and packaged in early endosomes as small clusters or *quanta* of p-EGFR (triple EGF-EGFR red), whereas unphosphorylated EGFR is distributed randomly. With time, the number of endosomes with p-EGFR *quanta* decreases due to p-EGFR dephosphorylation (EGF-EGFR black) and sequestration into intra-luminal vesicle (ILV), leading to degradation in late endosomes/lysosomes (EGF-EGFR grey).

evidence further suggests that early endosomes are a heterogeneous population of distinct organelles in communication with each other. The canonical early endosome is defined by the presence of Rab5, and its effectors, such as EEA1, that are recruited to the membrane in a phosphatidylinositol 3-phosphate PI(3)P-dependent fashion [20,22]. Some Rab5-positive early endosomes contain the effectors APPL1 and APPL2. These endosomes are enriched in the sub-cortical region of the cell, and are devoid of EEA1 and PI(3)P [23,24]. A fraction (~15–30%) of endosomes contain both APPL1 and EEA1 (APPL1 + EEA1 endosomes [23,24]). It has been proposed that, similar to Rab5-to-Rab7 conversion [19], APPL1 vesicles are transport intermediates in the generation of EEA1-positive early endosomes [25,26]. Recent work, however, has provided evidence for APPL1- and APPL1 + EEA1 and EEA1 endosomes representing distinct populations of early endosomes with distinct spatial distributions and cargo sorting activity. The presence of distinct classes of early endosomes offers additional modes of signalling regulation by RTKs, because each endosome class can sort cargo specifically and provides a distinct membrane platform for signalling adaptors and effectors, before transport to late endosomes and lysosomes.

Signalling from endosomes

There is an ongoing debate whether signalling from endosomes is actually required for the signalling response. Can signals propagate directly from the plasma

membrane to the nucleus? Are endosomes essential for signal decoding? Studies using Dynamin knock-out (KO) cells showed that reduced endocytosis did not prevent MAPK signalling [27], suggesting that endocytosis is not an absolute requirement for signal transmission to the nucleus. However, stimulation with low (physiological) concentrations of EGF leads to different Erk activation kinetics in Dynamin KO compared to control cells [27]. Inhibition of receptor CME or ubiquitination changes the EGF-induced transcriptional response, similar to EGFR overexpression [28]. Interestingly, stimulating cells with PMA, a compound known to rewire the signal decoding network [10], causes substantial changes in the temporal pattern of the transcriptional response [28], similar to those produced by inhibition of receptor internalization. The effect of Clathrin and Dynamin-2 knockdown is less pronounced than that of PMA, most likely because the block of EGFR CME can be compensated by CIE mechanisms [14•,29]. By contrast, reduction of EGFR degradation by silencing components of the ESCRT complex (TGS101, Alix) does not affect the EGF-dependent transcriptional response [28], arguing that regulation of signalling transmission is not due to degradation but takes place at a previous stage (see below). By blocking (dynamin-dependent) endocytosis, the levels of active receptors aberrantly increase at the cell surface. In such an artificial situation, both the precise temporal activation pattern of downstream signalling molecules [27] and the magnitude of the transcriptional response [28] are altered. These changes can result in incorrect signal decoding since it is known that altering signalling activation kinetics leads to signalling network rewiring and signal misinterpretation [10]. We conclude from these studies that, although endocytosis may not be *strictly* necessary for signal transmission *per se*, it appears to be required for *correct* signal decoding.

Numerous studies have provided evidence that, under normal conditions, signalling continues following receptor endocytosis in endosomes. Early biochemical work on EGFR and insulin signalling revealed receptor recruitment of adaptor proteins (SHC, GRB2 and mSOS) in endosomes [30,31]. Recently, different quantitative techniques have confirmed these initial observations. Live-cell imaging revealed sustained localization of the fluorescently-tagged signalling adaptor Grb2 to endosomes [32••]. Phosphorylation of c-Met in endosomes leads to recruitment of the guanine nucleotide exchange factor Vav2 and sustained activation of Rac1 [33]. Work using a conformation specific intracellular nanobody showed that activation of the β2-adrenoceptor GPCR is sustained in endosomes, contributing to the overall cyclic AMP response [34••]. A recent study using quantitative immunofluorescence and FRET showed that a sub-population of EGFR remains activated in early endosomes where it recruits the signalling adaptor Shc [35••]. Importantly, this study also showed that EGFR was dephosphorylated before sequestration into ILVs, arguing that signal quenching (receptor dephosphorylation) and degradation can be uncoupled. By contrast to degradation, de-phosphorylation is a reversible process and can be exploited to fine-tune signalling through the endocytic system. Altogether, these findings support the concept that signalling is not limited to the plasma membrane but continues in endosomes, which act as signalling platforms.

Qualitative regulation of signalling specificity by endocytosis

A large fraction of signalling adaptors identified by mass-spectrometry [36,37] is localized to endosomes (see Table 1). A recent review of protein databases identified nearly 50 endosomal scaffolds that could regulate multiple signalling pathways including Wnt, Notch and MAPK [38]. Furthermore, these scaffolds are localized to specific endosomal compartments. For example, the kinase PYK2 is recruited specifically to early endosomes, where it sustains EGFR-mediated STAT3 activation [39]. The LAMTOR complex is restricted to late endosomes, where it regulates mTOR and MAPK signalling [40]. This organization allows for different mechanisms of signalling regulation. For example, sorting of the same receptor to different compartments can give rise to substantially different signalling responses and cellular outcomes. This is the case for the c-Met receptor, which triggers acute Rac1 activation when transported to early endosomes but leads to PI3K-dependent and Vav2-dependent sustained Rac1 activation only from perinuclear endosomes [33]. Table 1 summarizes the localization of signalling molecules recently found in endosomes.

Several studies have reported interactions of APPL1 with specific receptors and components of signalling pathways including adiponectin receptors [41], TRAF2

Table 1

Summary of recently discovered signalling proteins localized to distinct endosome compartments

Protein	Signalling pathway	Endosome compartment	Reference
Akt	PI3K-Akt	APPL+ endosomes	[44]
GSK-3b	PI3K-Akt, Wnt	APPL+ endosomes	[44]
GRB2	MAPK (EGFR)	Early endosomes	[32••]
SHC1	MAPK (EGFR)	Early endosomes	[35••]
PYK2	JAK/STAT	Early endosomes	[39]
LAMTOR2	MAPK, mTOR	Late endosomes	[40]
PTP1B	Broad spectrum phosphatase	ER-late or recycling endosome contacts	[46,47,49••]
VAV2	PI3K-Rac (c-Met)	Uncharacterized perinuclear endosomes	[33]
KRas	MAPK	Late endosomes	[64]
STAT3	JAK/STAT	Early endosomes	[65]
Rac1	RTK	Early endosomes	[66]
Adenylyl Cyclase	cAMP (TSHR)	Uncharacterized endosomes	[67]

in the NF-kb pathway [42], Akt [43,44], and TAK1 in the p38 MAPK pathway [45]. These results suggest that APPL1 endosomes play a specialized function as part of the signal decoder. In addition, RTKs entering APPL1 endosomes can be temporarily protected from degradation, as shown by the enhanced ERK activation downstream of EGFR [26]. Changing the proportion of cargo flow between APPL1-early and EEA1-early endosomes could thus result in changes in signalling.

The endosomal system as an analogue-digital computer

The cytoplasm is not homogeneous, and the concentration of molecules can change in space. This means that the spatial distribution of receptors in the cytoplasm can modulate signalling. For example, deactivation of signalling receptors is regulated by compartmentalization. PTP1B, a well-characterized phosphatase of EGFR, localizes to the ER [46]. This results in a gradient of PTP1B from the periphery to the perinuclear area of the cell where the termination of signalling is higher in late [47] or recycling endosomes [48]. Recent studies on both EphA2 [48] and EGFR [49••] showed that ligand-independent autonomous activation was suppressed via transport to the phosphatase-rich perinuclear area, whereas ligand-bound receptors were transported to early endosomes where signalling was prolonged. This mechanism could have a key role in signalling robustness as it allows high sensitivity while suppressing autonomous activation.

Nano-clusters (less than ten molecules) of activated receptors or of Ras at the plasma membrane regulate intracellular signal transduction [50,51,52•,53]. Recent developments in quantitative microscopy revealed that active (phosphorylated) EGFR forms small clusters of ~80 molecules on early endosomes [35••]. Increasing the EGF concentration does not produce larger clusters but a higher number of clusters. Remarkably, increasing the number of clusters through a mild reduction in endosome fusion is sufficient to change the signalling outcome. These clusters can be considered as *quanta* of signalling information that confer regulation and robustness to the cellular response against fluctuations in ligand or receptor expression [35••].

Experimental data suggest that *quanta* formation is an emergent property of positive and negative feedback loops between receptor activation and de-phosphorylation. The positive feedback loop is due to the catalytic auto-activation of receptors in a cluster [54,55••]. The activation of EGFR also leads to phosphotyrosine-mediated recruitment and local activation of phosphatases (e.g. PTPN11/SHP2) in the clusters, thus forming a negative feed-back loop [35••,56]. Mathematical simulations showed that such an auto-inhibitory loop, together with fast phosphatase diffusion into the cytosol can give rise to self-organized clusters of activated receptors of a characteristic size [55••], a mechanism first described by Turing [57]. Since it is known that multiple receptors can recruit phosphatases to their cytoplasmic tails, *quanta* formation is in all likelihood a general property of signalling receptors in endosomes. The formation of p-EGFR *quanta* on endocytic membranes by Turing mechanism has interesting consequences. The endosomal *quanta* behave as a digital-analogue computer. Clustering of activated EGFR on endosomes is equivalent to an analogue-to-digital conversion of signalling. The basic properties of the endosomal network, such as endosome fusion, fission and sequestration of receptors into ILV (Figure 2) allow performing calculus operations (addition and subtraction) on *quanta*. For example, the Turing mechanism ensures that the fusion of two endosomes each containing a p-EGFR cluster does not result in two *quanta* or a cluster of double size, but keeps only one *quantum* per endosome. This is similar to the bits addition in single-bit processors (digital summation by module 2). Therefore, the kinetics of endosome fusion and fission (the analogue component) regulates the number and lifetime of the *quanta*, which in turn modulates signal decoding [35••].

Feed-back regulation of endocytosis

The previous examples underscore the importance of endosomal sorting for signal transduction. An additional level of regulation comes from the feedback that signalling pathways exert on the endosomal system itself (reviewed in [58]). Phospho-proteomic analysis of EGFR downstream targets identified multiple endosomal proteins [37]. Phosphorylation of endosomal proteins can lead to substantial changes in cargo uptake and sorting. For example, Akt-mediated phosphorylation of PIKFYVE regulates EGFR degradation [59], whereas p38-mediated phosphorylation of EEA1 promotes early endosome fusion [60]. But even more impressive is the extent to which signalling pathways regulate the endocytic system. Genomic surveys by RNA interference revealed a profound influence on endocytosis and the endosomal network by a variety of signalling pathways (e.g. MAP Kinase, Notch, Wnt, TGFb, among others.) [18,61]. Signalling molecules can thus modify the endosomal system by causing specific changes in endosome fusion and fission, which imply changes in number, size and intracellular position of the endosomes, and consequently, in the trafficking fate of signalling molecules themselves [35••,62]. The combined action of simultaneous signalling pathways can lead to a specific spatio-temporal organization of endosomes that, in turn, can regulate signalling specificity and responsiveness. This means that re-adjustment of the endocytic system depending on the cell state can modify the decoding of signals. Through this mechanism, endosomes allow for multiplexing, that is, a weighted integration of different types of signals which share a common down-stream signalling molecular machinery, resulting in a unique cell fate decision.

Concluding remarks

Our understanding of signalling endosomes, first described using biochemical techniques, has been greatly expanded due to the advancement in high-resolution quantitative microscopy applied to single cells or subcellular structures. A better understanding of these mechanisms will require fast live-cell imaging to study spatiotemporal dynamics with sufficient precision but also resolving molecular nano-clusters below the diffraction limit. A current limitation, however, is that signalling molecules on endosomes have been explored mainly in cell culture systems. In the context of multicellular systems, the bidirectional regulation of endocytosis and signalling may lead to a more complex signal decoding than appreciated so far. For example, the spatial regulation of VEGFR endocytosis by PKC in retinal vasculature leads to an endocytosis gradient that is required for vessel patterning [63•]. Therefore, a major challenge will be to study the role played by endosomal signalling *quanta* and other regulatory mechanisms in physiological processes *in vivo*.

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