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Endocytic control of signaling at the plasma membrane

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Signaling is regulated by endocytosis at multiple levels along endocytic routes. Endocytic control of signaling starts already at the plasma membrane, where cells employ different mechanisms to finely tune the type and strength of signals emanating from the cell surface. Here, we will review some of the most recently described endocytic mechanisms controlling signaling at the plasma membrane, through the regulation of internalization dynamics and through the integration of different internalization pathways triggered by canonical chemical stimuli or physical forces.

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Introduction

Signal location is a critical parameter that cells use to decode complex signaling circuitries and to compute specific biological responses. While signaling initiates at the plasma membrane (PM), it requires membrane dynamics for its sustainment/extinction and, more importantly, for its deconvolution. Endocytosis provides this dimension through numerous mechanisms (for a review see [1]) enacted in different subcellular compartments. For instance, it provides spatial constraints to biomembrane-associated signaling molecules (e.g., PM versus endosomes) and dictates differential access to signaling effectors. Moreover, endocytosis regulates the internalization and fate (i.e., recycling versus degradation) of signaling molecules through distinct endocytic pathways and/or via endosomal sorting. Finally, endocytosis is critical in the control of membrane turnover and plasticity in fundamental cellular programs, such as mitosis, adhesion and migration, as well as in the relocalization of signaling/adhesion molecules to PM 'competent' regions [1]. Through these integrated functions, endocytosis determines signal strength and diversification of biological outputs.

In this section, we will highlight recent evidence demonstrating how endocytosis controls signaling at the PM level (for signaling control at the endosomal level see Chapter 7 of this issue), through the regulation of internalization dynamics and the integration of different internalization pathways triggered by canonical chemical stimuli or physical forces.

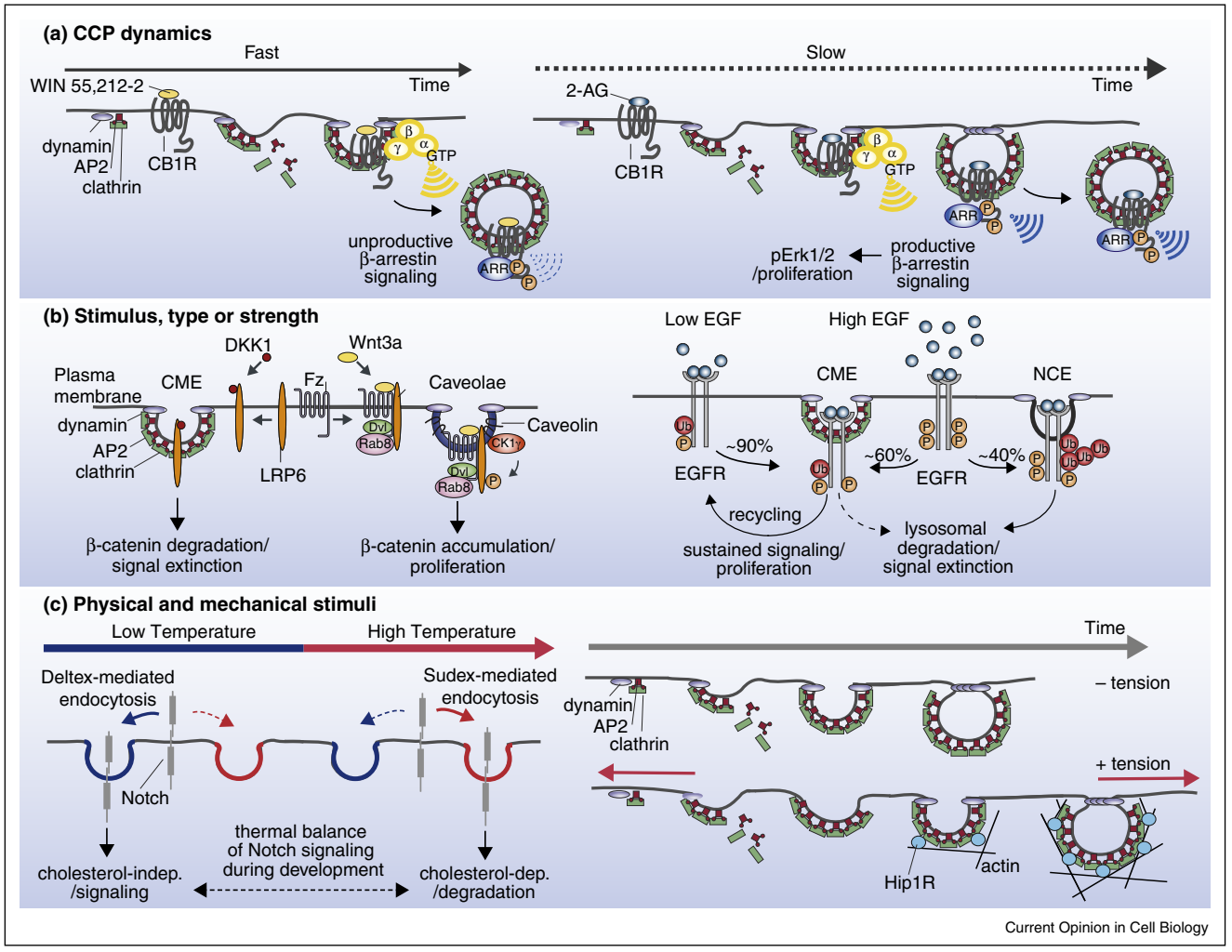
Clathrin-coated pit dynamics regulate signaling from the PM

Regulation of receptor levels at the PM, receptor availability, and ligand accessibility are established mechanisms affecting the timing and strength of signaling responses [1]. Another critical parameter in signal regulation is clathrin-coated pit (CCP) dynamics. Recent advances in live imaging and data analysis, which allow the large-scale simultaneous detection of the endocytic machinery and cargoes, have helped to establish that CCP dynamics is directly controlled by the cargo [2••,3]. While cargoes have been long regarded as 'passengers' in internalization structures, it is now clear that — at least in some instances — they directly influence the formation and maturation of CCPs [4,5] by locally communicating with the endocytic machinery [6•]. Failure to recruit cargo generates short-lived, abortive CCPs [7–9], revealing the existence of an early checkpoint required to monitor the fidelity of CCP formation, which depends on cargo, dynamin and AP2 [10•]. For signaling receptors, this might prolong the time available for clustering at the PM and for the initiation of productive signaling.

Interestingly, different ligands can induce clustering of the same cargo receptor in dynamically distinct CCPs. This is the case of the cannabinoid receptor 1 (CB1R) that, depending on the type of agonist, is clustered into CCPs with different dwell times (i.e., the time required for the receptor to be clustered in CCPs together with the adaptor β-arrestin), which in turn affects the signaling output (Figure 1a). Indeed, pits with long dwell times elicit productive and robust β-arrestin-dependent ERK1/2 activation (Figure 1a, right), while pits with short dwell times generate scarce β-arrestin signaling (Figure 1a, left) [11••].

Similar scenarios have been uncovered for receptor tyrosine kinases (RTKs), such as the RET (REarranged during Transfection) isoforms, which are internalized in CCPs displaying different kinetics [12]. Interestingly, the different RET isoforms assemble specific signaling

Fig. 1



Endocytosis controls PM signaling through different mechanisms. **(a)** CCP dynamics control signaling. Left, the agonist WIN 55,212-2 induces recruitment and internalization of the cannabinoid receptor (CB1R) through CCPs with fast dwell times, which results in trimeric G-protein (α, β, γ) signaling at the PM, but fails to generate productive β-arrestin-dependent signaling. Right, the agonist 2-AG recruits CB1R into CCPs with slow dwell times allowing for both Gprotein and productive β-arrestin-dependent signaling, which leads to phosphorylation of Erk1/2 and cell proliferation. **(b)** Left, different agonists differentially regulate signaling by activating distinct internalization pathways. WNT3a binding to its receptor Frizzled (Fz) induces the formation of signalosomes at the PM that include LRP6, Dishevelled (Dvl) and Rab8, and which mediate internalization via caveolae. This allows phosphorylation of LRP6 by CK1γ kinase and β-catenin accumulation, leading to cell proliferation. In contrast, the Wnt pathway antagonist, DKK1, recruits LRP6 to clathrin-mediated endocytosis (CME), which ultimately leads to β-catenin degradation and signal extinction. Right, signal strength (extracellular ligand concentration) controls EGFR signaling by activating distinct endocytic pathways: CME and non-clathrin endocytosis (NCE). At low doses of EGF, ~90% of the EGFR is internalized via CME, which primarily leads to recycling and sustainment of signaling, resulting in cell proliferation. At high doses of EGF, the EGFR becomes significantly ubiquitinated, concomitantly with the activation of NCE (note, however, that CME persists). NCE targets EGFRs mainly to degradation in the lysosome causing long-term attenuation of signaling. C. Physical and mechanical stimuli, such as temperature (left) or PM tension (right), control endocytic routes and signaling. Left, in Drosophila at low temperatures, Notch is constitutively internalized mainly via Deltex-mediated endocytosis, which does not require cholesterol and allows signaling activation. At higher temperatures, Sudex-mediated endocytosis is enhanced, which is dependent on cholesterol and leads to Notch degradation. This pathway acts to compensate for the increased ligand-dependent signaling occurring at high temperatures. This dual mechanism ensures thermal balance of Notch signaling during development. Right, under low PM tension (top), assembly of the clathrin coat is sufficient to deform the PM and CME does not require the actin cytoskeleton. Bottom, under high PM tension (i.e., on the apical side of polarized cells, or in cells subjected to mechanical stretching), clathrin assembly is unable to counteract the tension force, and invaginations stall; actin polymerization then provides the energy needed to complete membrane bending. Hip1R is the link between the assembling clathrin coat and actin polymers. CCPs in this latter case have an extended lifetime, which is necessary to allow Hip1R to be recruited and actin polymerization to occur.

complexes and are connected to distinct developmental roles, raising the possibility that internalization kinetics might influence RTK biological outputs.

Endocytic routes and receptor fate

Several signaling receptors can be internalized through multiple endocytic routes, the activation of which depends on the cellular context and environmental conditions. Notably, these different endocytic routes are coupled to distinct receptor fates [1,13].

The Wnt signaling pathway is critical for development throughout evolution and is controlled by distinct endocytic pathways [14]. When Wnt binds to its receptor, Frizzled (Fz), it induces clustering of Fz and of the co-receptor LRP6 (low density lipoprotein receptor-related protein 6), followed by recruitment of Dishevelled (Dvl) and formation of signalosomes, which include scaffolding proteins (Axin1) and kinases (GSK3β and CK1γ kinases) [15]. In mammalian cells, different entry routes are used by the LRP6-Fz complex and are associated with distinct outputs (Figure 1b, left); specifically, clathrin-mediated endocytosis (CME) and caveolar endocytosis, which lead to receptor degradation and signaling, respectively [16].

The molecular mechanisms underlying the different endocytic routes are starting to be dissected. Upon WNT3a stimulation and Fz-LRP6 activation, Rab8 is recruited by Dvl to the PM, where it interacts with LRP6 and engages its guanine exchange factor (GEF) RABIN8, promoting caveolar endocytosis of the LRP6-signalosome complex (Figure 1b, left) [15,17]. Internalization of the complex promotes sequestration of GSK3β into multivesicular bodies (MVBs) and inactivation of the β-catenin destruction complex, inducing β-catenin stabilization and signaling via CK1γ [16,18••,19]. LRP6 can also bind the WNT3a antagonist, Dickkopf (DKK), which removes LRP6 from lipid rafts (where CK1γ is localized) and diverts its endocytosis from the caveolar to the CME pathway, ultimately resulting in enhanced β-catenin degradation and signal extinction (Figure 1b, left) [16,19]. There are additional layers of complexity, though, as suggested by findings that, depending on the level of the adaptor protein Dab2 in the cell, WNT3a can also direct the Fz-LRP6 complex into the CME pathway, thereby leading to signal attenuation [20•].

The above findings highlight the relevance of the cellular context in the integration of endocytosis and signaling, a notion further supported by studies in lower organisms. Here, the differential impact of endocytic pathways on Wnt signaling is less clearly defined [21,22]; however, differently from mammalian cells, CME appears to exert a positive role in the regulation of both the non-canonical (β-catenin-independent) [23–25] and canonical (β-catenin-dependent) Wnt signaling pathways [26].

An additional level of control, conserved in evolution, is exerted by the ZNFR3/RNF43 transmembrane Ub ligases [27]. These enzymes promote the continuous ubiquitination, constitutive endocytosis and lysosomal degradation of Fz, thus regulating receptor availability at the PM [28,29]. ZNFR3 and RNF43 are also direct transcriptional targets of β-catenin in the WNT3a signaling pathway, thus constituting a negative-feedback loop [30,31•]. This loop can, in turn, be inhibited by secreted R-Spondins [32] that antagonize ZNFR3/RNF43, stabilizing Fz receptors and increasing Wnt signaling strength [30,31•]. In summary, the emerging picture is that Wnt signaling is finely tuned by endocytosis, through mechanisms regulated by the cellular context and environmental cues.

In the case of the transforming growth factor receptor β (TGFβR) and epidermal growth factor receptor (EGFR), CME and various forms of non-clathrin endocytosis (NCE) are associated with distinct receptor fates, although with opposite outcomes as compared with LRP6-Fz in mammalian cells. For these receptors, CME is predominantly associated with receptor recycling and sustainment of signaling, while NCE mainly directs receptors to degradation and signal extinction [33,34]. For the EGFR, NCE occurs through different mechanisms [35–37]. In all cases, however, NCE is activated only when the ligand is present at high, nearly saturating, doses (>10 ng/ml) (Figure 1b, right). One mechanism involves the conversion of a linear gradient of EGF into an almost all-or-nothing EGFR ubiquitination (Ub) response, which in turn leads to the sharp activation of NCE and to receptor degradation [38]. Thus, NCE likely protects cells from overstimulation under conditions of excess ligand. A combination of mathematical modeling and wet-lab experiments revealed that EGFR ubiquitination — and its recruitment into NCE — is critically controlled by EGFR levels [39•]. Indeed, in cells displaying high EGFR levels — a hallmark of some cancer cells–the receptor is inefficiently ubiquitinated while being highly phosphorylated/activated [39•]. The prediction here is that, under these conditions, EGFR would escape internalization through NCE and the ensuing signal extinction, thereby providing a proliferative advantage to cancer cells.

Of note, a second level of analogical-to-digital control of EGFR signaling intensity occurs in the endosomal compartment, where increasing EGF concentrations induce a proportional increment in the number of endosomes, so that the number of active EGFRs/endosome remains constant. A linear EGF gradient is thus converted into 'quanta' of signaling receptor. In addition to the EGFR, other receptors, such as hepatocyte growth factor receptor (HGFR) or nerve growth factor receptor (NGFR), can induce 'quanta' of different magnitudes, which correlate with distinct biochemical and biological outputs in

specific cellular context ([40••], see also Chapter 7 of this issue).

Similarly, endocytosis-controlled modalities of analogical-to-digital conversion of signals have been described in the establishment/decoding of morphogen gradients during development, as well as in the control of collective cellular motility in physiology and in cancer [41]. For instance, it has recently been shown how malignant lymphocytes, traditionally regarded as individual movers, have an intrinsic tendency to gather into clusters that display unique migratory and chemotactic properties [42]. These collective entities, at variance with single cells, display chemotactic prowess in shallow chemokine gradients and are resistant to the chemorepulsion that normally results from increases in gradient steepness. Not surprisingly, endocytic dynamics are at the core of these processes, although the precise molecular mechanisms remain to be elucidated [42].

Physical stimuli and regulation of endocytic pathways

Physical stimuli, such as temperature or mechanotension, are emerging as important and specific activators/regulators of endocytic routes. Notch signaling during *Drosophila* development is maintained across a wide range of temperatures through a compensation mechanism relying on distinct internalization pathways [43••]. Ligand-independent endocytosis of Notch occurs through two routes with distinct lipid and temperature requirements (Figure 1c, left). At low temperatures, Notch is mainly internalized via Deltex-mediated endocytosis, which occurs through glycosylphosphatidylinositol (GPI)-negative endosomes in a cholesterol-independent manner, and leads to Notch signaling activation. At higher temperatures, Sudex-mediated endocytosis is activated, characterized by GPI-positive endosomes and cholesterol sensitivity. This pathway dampens signaling by targeting receptors for lysosomal degradation, thereby counterbalancing the increased ligand-receptor binding kinetics occurring at high temperature [43••]. The net result is thermal robustness of Notch signaling. Moreover, temperature might control the cholesterol-enriched pathway by directly influencing membrane fluidity and tension (see also below). These regulatory mechanisms are clearly relevant in ectothermic organisms; it will be interesting to investigate whether they apply also to endothermic organisms, to specific organs/tissues or under inflammatory conditions.

Mammalian cells are constantly subjected to environmental mechanical forces that regulate PM tension [44]. A tight bidirectional regulation exists between membrane tension, the extracellular matrix (ECM) and endocytosis (see Section 3 of this issue), which can be harnessed by cancer cells to establish invasive programs [45]. Through membrane recycling and turnover, endocytosis can influence the physical properties of the PM. In addition, through trafficking of adhesion molecules, endocytosis regulates cellular communication with the ECM [46]. In turn, endocytosis is regulated by external forces through the activation of specific internalization pathways [44], as exemplified by the endocytic dynamics of integrins. Integrins display different endocytic responses to mechanical forces, which influence their signaling [47]. Endocytosis of integrin-beta3 is controlled by ECM-originated cues: RGD (Arg-Gly-Asp) ligands immobilized on supported lipid membranes cannot generate traction on the engaged integrin nor promote its clustering and removal from the PM via CME. Conversely, the increase in force, obtained by using rigid RGD ligands immobilized on glass, induces the reinforcement of the actomyosin network and the recruitment of focal adhesion adaptors, such as talin, resulting in CME inhibition and focal adhesion formation [48•]. Thus, mechanical forces control the balance of adhesion signaling versus integrin turnover.

The cortical actin cytoskeleton functions both as a sensor and as a transducer of membrane tension. During phagocytosis, a bidirectional crosstalk between membrane tension and the actin cytoskeleton allows for pseudopodia extension and particle engulfment. Upon particle engagement, Rac1 is activated and the actin cytoskeleton pushes the PM forward [49•]. As a consequence of PM stretching, membrane tension increases, leading to inactivation of Rac1 and to its redistribution from the pseudopodia to the cell center, thereby promoting actin reorganization in the pseudopodia and induction of actin-mediated exocytic events at the site of engagement. The newly exocytic-delivered membranes relieve membrane tension and allow wrapping of the particle [49•]. Thus, continuous communication exists between the PM, deformed by particle engagement, and the actin machinery required for phagocytosis to progress.

Also in CME, the action and recruitment of the actin cytoskeleton is regulated by membrane tension (Figure 1c, right) [50••]. At variance with yeast, in mammalian cells CME proceeds also in absence of actin polymerization [51]. However, the scenario changes under conditions of high membrane tension in which actin recruitment via the clathrin-light-chain adaptor Hip1R becomes essential. Mechanistically, membrane tension opposes clathrin polymerization and hinders the closure of CCPs by varying the membrane budding energy [52•]. This provides enough time for assembly of actin filaments via Hip1R, rescuing the stalled coat (Figure 1c, right) [50••]. Since actin-dependent and -independent CCPs have distinct lifetimes [50••], it is tempting to speculate that they might influence the retention time of signaling molecules and receptors, thus regulating signaling outputs.

Outlook: mechanotransduction, cell context and endocytosis at the system level

Membrane tension is a critical stimulus to which cells packed in a tissue, or a culture dish, are subjected [53]. Recent studies showed that endocytic pathways are directly controlled by local cell density in culture [54••]. This is achieved through a PM-based mechanism, centered on focal adhesion kinase (FAK), which senses local crowding and responds by controlling membrane lipid composition, via a feedback loop that does not require the exchange of a 'chemical signal' between cells [55]. These data show that local crowding (and, most likely, mechanical forces) contributes to the generation of single-cell heterogeneity of endocytic pathways [55].

Single-cell heterogeneity has been previously described for different cellular processes (e.g., gene transcription [56]) and cell signaling pathways influenced by endocytosis, including calcium signaling [57], and NF-kB [58] and Erk signal transduction pathways [59]. Interestingly, when single-cell variability is taken into account in the analysis of endocytosis data from perturbation screenings, it improves the statistical significance of the results [60,61,62•,63].

Overall, these studies highlight the importance of monitoring endocytic and signaling events at the system level, coupling single cell measurements to quantitative computational analysis of large datasets and mathematical modeling, to untangle the impact of the endocytic machinery on cell regulation.

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