CMLS, Cell. Mol. Life Sci. 60 (2003) 2020–2033
1420-682X/03/102020-14
DOI 10.1007/s00018-003-3010-2

CMLS Cellular and Molecular Life Sciences

Review

The odd couple: signal transduction and endocytosis

D. Teis and L. A. Huber

Institute of Anatomy, Histology and Embryology, Department of Histology and Molecular Cell Biology, University of Innsbruck, Muellerstrasse 59, 6020 Innsbruck (Austria), Fax: +43 512 507 2863, e-mail: lukas.a.huber@uibk.ac. at; david.teis@uibkac.at

Received 14 January 2003; received after revision 3 March 2003; accepted 4 April 2003

Abstract. Cell surface receptors are used to transmit extracellular information. The activation of cell surface receptors initiates signal transduction and receptor endocytosis. Signal transduction and the endosomal transport of activated receptors require precise regulation. New concepts for the integration of endocytosis and signaling arise from recent findings that suggest bidirectional interplay of these two processes. This review discusses the following questions: (i) do activated cell surface receptors modify the endosomal system to promote internalization and endosomal traffic, and (ii) do internalized cell surface receptors use specifically localized signaling complexes to generate specific biological signals?

Key words. Endocytosis; signaling; receptor tyrosine kinase; adaptor; scaffold complex; subcellular localization.

Introduction

All cells need to interpret their environment. In order to fulfill this task, extracellular information is received and translated accordingly into a specific biological response. Cell surface receptors are often used to transduce extracellular information to the cell. A great variety of ligands, representing the extracellular information, bind to and regulate the activity of cell surface receptors.

Upon ligand binding, receptor signaling is activated. Most activated receptors are efficiently cleared from the cell surface by endocytosis and sorted to lysosomes for degradation. In contrast, inactive cell surface receptors are constitutively internalized and recycled back to the cell surface. Thus, control mechanisms exist that survey the internalization and endosomal traffic of cell surface receptors.

Interestingly, the great number of ligands and their respective cell surface receptors use only a limited repertoire of signaling molecules to transduce their information, and yet signal transduction mediates a specific biological response. Therefore, signaling requires precise spatial and temporal regulation to evoke a unique biological response.

Signaling from cell surface receptors

One large family of cell surface receptors is represented by receptor tyrosine kinases (RTKs). All RTKs are monomers in the plasma membrane, with the exception of the insulin receptor [1]. Ligand-induced activation of RTKs stabilizes a receptor dimer and results in autophosphorylation of tyrosine residues within the cytoplasmic domain of the RTK [2] (fig. 1). The phosphorylated tyrosine residues serve as binding sites for a variety of signaling proteins that contain SH2, SH3 (Src homology) and/or PTB (phosphotyrosine binding) domains. The recruitment of SH2 and/or PTB domains containing proteins allows the assembly of signaling complexes on the cytoplasmic tail of the activated receptor, connecting activated receptors to the respective signal transduction cascades [3, 4].

Stimulation of all RTKs triggers the activation of the small G protein Ras. Ras activation is controlled by the

guanidine exchange factor (GEF), son of sevenless (Sos) [5, 6]. The SH2 domain of Grb2 is crucial to recruit a Sos complex from the cytoplasm to specific phosphotyrosine (pTyr) residues on the activated epidermal growth factor receptor (EGFR) [7, 8]. Alternatively, the PTB-domain-containing protein Shc is able to link the Grb2/Sos to the activated EGFR [9]. The recruitment of Sos stimulates Ras activation at the site of the activated EGFR. Activated Ras is known to interact with more then 10 different effectors, thereby modulating a multitude of downstream signaling cascades [10]. Two effector cascades, namely, the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase (PI3K), will be introduced briefly (fig. 1).

By activating Raf, Ras stimulates signaling in the MAPK cascade, which is involved in the regulation of cell survival, proliferation and differentiation. Sequential phosphorylation events within the cascade transduce signals downstream from Ras to effector proteins. Activated Ras binds to and activates Raf (the MAPKKK). Raf phosphorylates MEK (the MAPKK) on a critical serine in the activation loop. Next, MEK activates ERK (the MAPK) by phosphorylating threonine and tyrosine residues on ERK. Activated ERK, in turn, phosphorylates a great variety of targets in the cytoplasm and on membranes [11] (fig. 1). In addition, activated ERK rapidly dissociates from MEK and translocates to the nucleus to activate transcription factors (for reviews see [12, 13]).

Activation of the PI3K subunit p110 by Ras and direct interaction of the PI3K subunit p85 with the activated RTK stimulate the phosphorylation of phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2] to PtdIns(3,4,5)P_3.

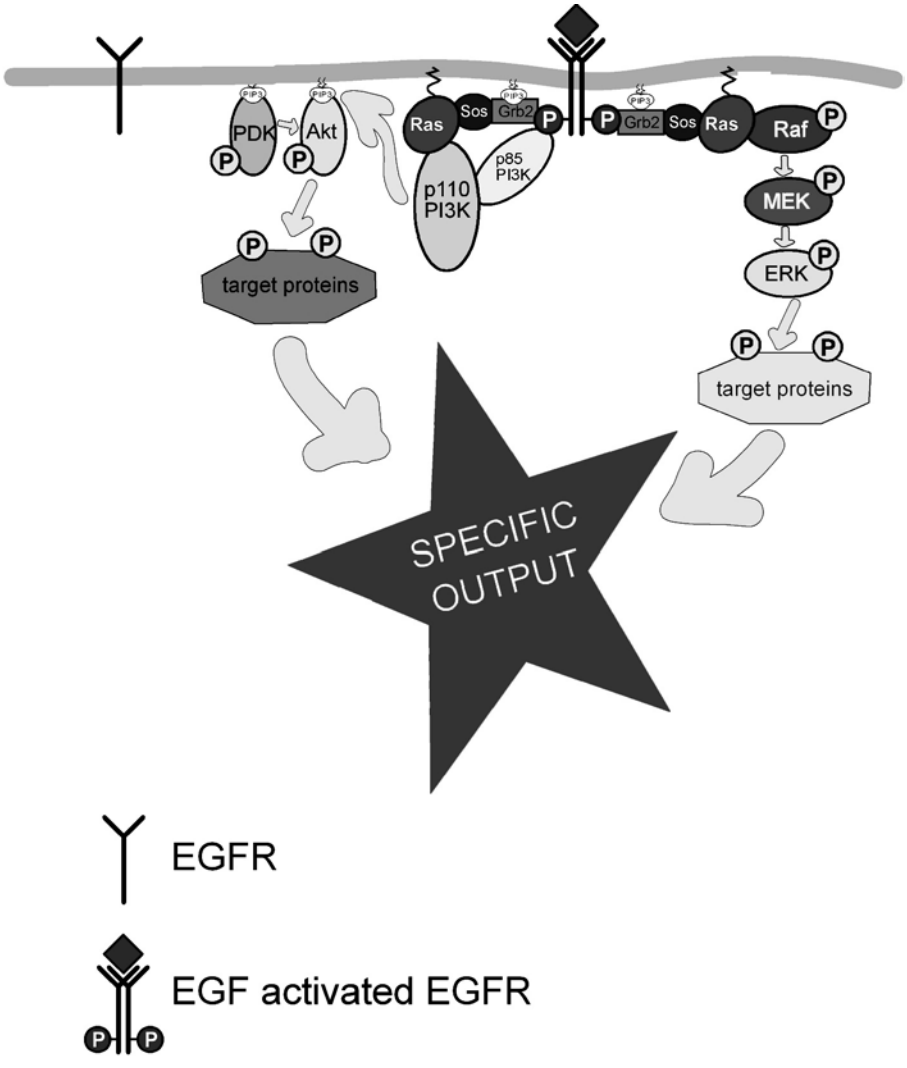

Figure 1. Activated receptor tyrosine kinases (RTKs) activate complex intracellular signaling networks. Inactive RTKs (e.g., EGFRs) are monomers in the plasma membrane. Ligand-bound RTKs (e.g., EGFRs) dimerize and are activated by autophosphorylation. The adaptor protein Grb2 binds to the activated RTK. Grb2 recruits the Ras-GEF, Sos, and thereby activates Ras. Ras, in turn, triggers signaling in the MAPK and PI3K-Akt pathways. A wide variety of target proteins are modulated upon phosphorylation.

$PtdIns(3,4,5)P_3$ serves as a high-affinity binding site for proteins containing a pleckstrin homology (PH) domain, thereby initiating the local assembly of signaling complexes and the priming of protein kinase cascades (fig. 1). Membrane translocation of Akt (protein kinase B) and phosphoinositide-dependent kinase D (PDK) brings both in close proximity and results in phosphorylation of Akt by PDK. Phospho-Akt in turn activates effector proteins that regulate cell growth, cycle and survival (for reviews see [14–16]) (fig. 1).

Although biochemical and genetic methods have identified the basic setup of signaling cascades, it is not clear how a myriad of extracellular cues employs only a limited number of signaling cascades to transduce and execute a unique biological response. Composition and compartmentalization of signaling complexes, by the use of scaffold and adaptor proteins, as well as the duration and amplitude of signaling might be crucial to define a specific biological signal.

Endocytic traffic of RTKs

Ligand-induced activation of the EGFR results in its rapid and efficient internalization [17] (fig. 2). The ligand-bound EGFR is recruited to clathrin-coated pits (CCPs). Next, the CCPs pinch off the plasma membrane to form clathrin-coated vesicles (CCVs) [18]. Upon ligand binding and activation of RTK, up to several 100–1000 CCVs or more per minute are generated for rapid internalization [19]. This exceeds by far the rate of steady-state endocytosis. Therefore, a protein network is required to precisely and efficiently regulate CCP and CCV formation at the site of receptor activation [19]. AP2, a multi-subunit adaptor protein that binds efficiently to the activated EGFR is a central player in this regulatory step. It recruits a network of essential endocytic proteins [20–23] from the cytoplasm to the activated receptor [24, 25].

The nucleation of clathrin triskelions causes self-assembly into a latticelike network, thereby generating force on the plasma membrane to bud into a curvature, namely, the CCP [26–30]. The GTPase dynamin is required to promote vesicle fission from the plasma membrane [31] and is thought to act as a mechanoenzyme that tubulates the membrane. By forming ringlike structures around the neck of CCPs, CCVs pinch off the plasma membrane [32–35].

Once sorted to CCVs, the receptors enter the tubular-vesicular early endosomal compartment [36, 37] (fig. 2). Early endosomal events compromise internalization of cell surface receptors, early endosome fusion and early endosomal sorting [38, 39]. Early endosomal cargo molecules can either be quickly recycled to the plasma membrane from peripherally located early endosomes or be sorted in the late endosomal compartment, from where they are transported to lysosomes for degradation. Intriguingly, most of the activated cell surface receptors are not recycled back to the plasma membrane but rather traffic to late endosomes. Sorting toward late endosomes might be accomplished by recruiting receptors to clathrin-coated subdomains in the early endosomal compartment [40, 41] (fig. 2).

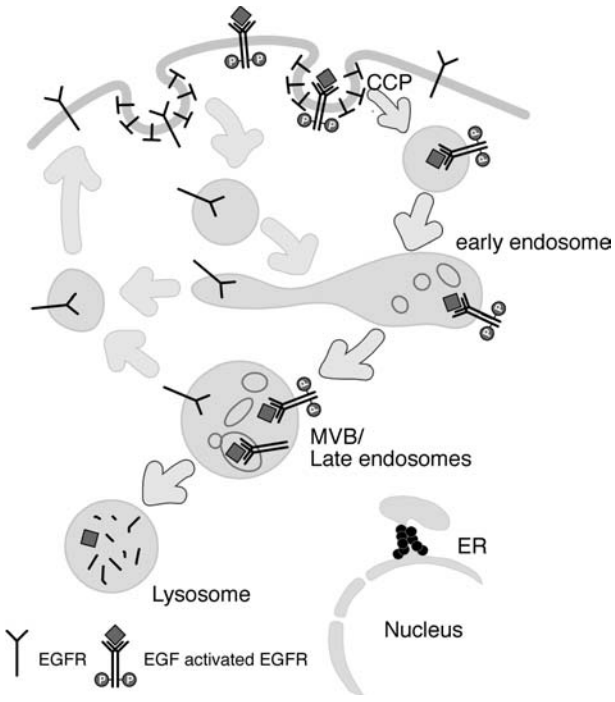

Figure 2. Endosomal traffic of the activated EGFR. The inactive EGFR localizes in steady-state equilibrium to the plasma membrane. It is internalized at low rates and efficiently recycled back via early endosomes to the plasma membrane. In contrast, the activated EGFR (yellow arrows) is efficiently internalized via CCPs. In the early endosomes they are sorted to the late endosomal/MVB compartment. Sorting into the lumen of the MVB shunts the EGFR to the lysosomes, where they are finally degraded.

Late endosomes differ from early endosomes not only in their perinuclear subcellular location [42]. Often they appear as multivesicular bodies (MVBs) composed of a limiting membrane with internal vesicles. Compared with early endosomes, late endosomes are enriched not only in specific proteins but also in lysobisphosphatidic acid (LBPA) and PtdIns(3)P [43–45]. The sorting of receptors from the limiting membrane into internal vesicles of MVBs shunts receptors to the lysosomal degradation pathway [46]. This step is controlled by interdependent mechanisms, including ubiquitination of the EGFR, recognition of the ubiquitinated receptor and invagination of the limiting membrane to form internal vesicles in the MVB.

After being sorted to internal vesicles of the MVB, the receptors are finally transported to the lysosomes for degra-

dation. Although lysosomes share many characteristics with late endosomes/MVBs in terms of protein and lipid composition, protein degradation occurs only in lysosomes [38, 47] (fig. 2).

The endocytic machinery is believed to act constitutively. However, it is clear that activated cell surface receptors are routed to lysosomes for degradation, whereas inactive receptors are constantly recycled back to the cell surface. Therefore, signaling receptors are likely to modulate an otherwise constitutively operating endosomal system to promote internalization as well as late endosomal and lysosomal sorting.

Modifications of the endocytic machinery by receptor signaling

Morphological studies have shown that the EGFR colocalizes with constantly internalizing receptors, such as the transferrin or the LDL (low-density lipoprotein) receptors in CCPs. This leads to the conclusion that the ligand-activated receptor would be internalized by a constitutively operating endocytic machinery [48]. However, there is considerable evidence that ligand-induced endocytosis involves additional regulatory events beyond the mere exposure of an internalization motive for adaptor binding.

First indications that receptor activation might influence the rate of internalization came from morphological studies showing that EGFR activation results in an increase of the CCP density at the plasma membrane [49]. In addition, ligand-bound EGFR requires tyrosine kinase activity for high internalization rates and efficient recruitment to CCPs. Tyrosine kinase-defective EGFRs are inefficiently recruited to CCPs and only poorly internalized at the rate of unbound constitutively recycling EGFRs [50, 51]. These data indicate that receptor activation might specifically modulate the endocytic machinery to efficiently internalize the activated EGFR through specialized CCPs.

EGF-induced dimerization of the EGFR generates a dimeric YXXΦ endocytic motive on the receptor's cytoplasmic tail, which results in efficient binding of the multi-subunit adaptor protein AP2 via the µ2 subunit. Thereby, AP2 links the receptor to be endocytosed (via the µ2 subunit) with molecular components that drive and regulate the formation of CCPs and CCVs. The α subunit of AP2 recruits essential regulators of the endocytic machinery, such as Eps15 and dynamin, whereas α and β2 subunits, together, link the activated receptor to the nascent clathrin coat [19, 20, 22, 25] (fig. 3).

Activation of the EGFR results in rapid tyrosine phosphorylation of the clathrin heavy chain (CHC) and in recruitment of clathrin to the plasma membrane. However,

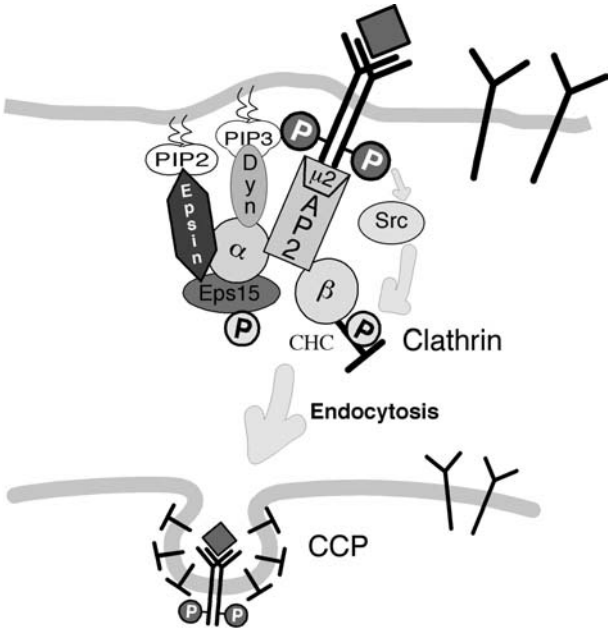

Figure 3. Activated EGFRs induce efficient internalization. The multi-subunit adaptor protein AP2 binds more efficiently to the activated EGFR. Eps15 and the CHC are phosphorylated upon EGFR activation, which results in the translocation of Eps15 and CHC to the plasma membrane, causing nucleation of the CCP. In addition, higher levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are generated at the site of EGFR activation. Dynamin, required for vesicle fission, binds to PtdIns(3,4,5)P_3. Epsin, which tubulates the plasma membrane, binds to PtdIns(4,5)P_2. Finally, activation of EGFR signaling results in efficient internalization by recruiting the endocytic machinery to the site of receptor activation.

the tyrosine kinase activity of the receptor, although necessary, is not sufficient to phosphorylate the CHC. Receptor-induced activation of the tyrosine kinase Src is required and sufficient to phosphorylate a critical tyrosine residue on the CHC. This CHC phosphorylation promotes rapid clathrin recruitment and assembly at the plasma membrane. Cells from mice that are deficient for Src or treated with Src inhibitors show delayed internalization of the EGFR upon ligand binding [52, 53] (fig. 3). Src-dependent phosphorylation of the CHC might also account for the efficient internalization of the G-protein coupled receptor (GPCR) since the GPCR also activates Src [54].

Another important regulator of EGF-induced endocytosis is Eps15. Eps15 was initially i dentified to be a major cytoplasmic substrate of the activated EGFR. Phosphorylation upon EGF stimulation results in plasma membrane translocation of Eps15 and binding to the α-adaptin ear domain of AP2 [55, 56]. Interestingly, the tyrosine phosphorylation of Eps15 is required exclusively for the EGF-induced internalization of the EGFR. Mutations in the phosphorylation site affect only internalization of the EGFR but do not affect the constitutive inter-

nalization of the transferrin receptor [57]. In addition, overexpression of Eps15 resulted in transformation of fibroblasts, suggesting an involvement in mitogenic signaling [58]. Furthermore, Eps15 contains three EH (Eps15 homology) domains that form a dual EF hand (binding of Ca^{2+}) [59, 60]. The EH domains bind to proteins that contain an Asn-Pro-Phe (NPF) domain [61, 62]. The NPF domain is present mainly in endocytic proteins such as epsin, synaptojanin and numb. Thus, Eps15 recruits an endocytic protein network to control the generation of CCVs [63] (fig. 3). Recently it has been shown that numb generates asymmetric cell division in the *Drosophila melanogaster* sensory organ precursor cells by polarizing the α-adaptin distribution. The asymmetric distribution of α-adaptin, which depends on numb, might cause polarized endocytosis of notch, thereby directing the cell-fate decision [64].

Importantly, activation of the EGFR results in elevated PtdIns(3,4,5)P_3 and, remarkably, in increased PtdIns (4,5)P_2 levels at the plasma membrane [65]. Many endocytic proteins have been found to bind to either PtdIns (3,4,5)P_3 or PtdIns(4,5)P_2. However, treatment of cells with the PI3K inhibitor wortmannin does not inhibit EGFR endocytosis but rather blocks EGFR sorting to the internal lumen of the MVB [66–68]. Dynamin, the GTPase that is essential for membrane fission at the site of CCPs, uses its PH domain for membrane localization. Dynamin is also associated with the AP2 network [69, 70]. Consequently, dynamin mutants that lack the PH domain do not localize to the membrane and block endocytosis [71] (fig. 3).

The µ2 subunit of AP2 binds not only directly to the YXXΦ endocytic motive on the activated receptor but also to PtdIns(4,5)P_2. Mutations in the PtdIns(4,5)P_2 binding site of µ2 affect the membrane localization of µ2 and inhibit endocytosis [72].

Epsin interacts with AP2 and Eps15 [73]. In addition, the epsin N-terminal homology (ENTH) domain binds strongly to PtdIns(4,5)P_2 [74]. Epsin modifies the plasma membrane curvature in conjunction with clathrin polymerization, thereby stimulating endocytosis [75]. Mutations in the ENTH domain, which abolish the binding of epsin to PtdIns(4,5)P_2, result in an inability to modify the membrane curvature and inhibit endocytosis of the activated EGFR [73–75] (fig. 3).

c-Cbl is a multi-adaptor protein involved in ligand-induced down-regulation of RTKs. On the one hand, c-Cbl acts as a ubiquitin ligase to sort the EGFR into the degradation pathway. On the other hand, c-Cbl seems to exert a ubiquitin ligase-independent function at the plasma membrane. In response to EGF stimulation, c-Cbl becomes phosphorylated and interacts with the CIN-85 endophilin complex [76]. This interaction drives the membrane localization of the CIN-85 endophilin complex to the site of receptor activation [77].

Endophilin is a SH3 domain-containing protein whose major binding partners are synaptojanin and dynamin [78, 79]. By acting as a lysophosphatidic acid acyl transferase, endophilin is required for the formation of endocytic vesicles [80]. Synaptojanin is an inositol 5-phosphatase and is required for the uncoating of CCVs, since its enzymatic function would eliminate PtdIns(4,5)P_2 and, subsequently, all PtdIns(4,5)P_2 binding coat components from CCVs [81]. Indeed, mice deficient for synaptojanin have elevated levels of PtdIns(4,5)P_2 and show an accumulation of clathrin-coated vesicles [82].

Taken together, ligand binding to the EGFR and EGFR signaling activity exert regulatory influence on the endocytic machinery to generate CCPs and CCVs (fig. 3). Thereby, activated EGFRs are efficiently internalized into the early endosomal system (fig. 3).

Early endosomal sorting and signaling

Not only internalization but also transport and sorting of the EGFR within the endosomal system appears to be controlled by EGFR signaling events (fig. 4).

The small GTPase rab5 is a key regulator of early endosomal function, including internalization, recycling and endosome fusion [38, 39]. So far, more than 20 rab5 effectors have been identified that bind to rab5 and modify its function [83]. A considerable number of rab5 effectors are directly or indirectly regulated by signaling molecules.

EEA1 (early endosomal autoantigen 1) is a major effector of the rab5 function that is required for endosome fusion events [83]. EEA1 is thought to tether endosomal membranes and to thereby promote membrane fusion [84, 85]. EGF stimulation simultaneously increases recruitment of EEA1 to rab5 domains and rab5 GTPase activity, which stimulates membrane fusion events in the early endosomal compartment [86]. The early endosomal localization of EEA1 is mediated by its FYVE domain, which binds specifically to PtdIns(3)P [87, 88]. Thus, the localization and function of EEA1 rely on the PtdIns(3)P metabolism. Importantly, rab5 interacts with a special isoform of the PI3K, namely, the hVPS34, the mammalian homologue of yeast VPS34. hVPS34 catalyzes PtdIns(3)P, regulating the association of EEA1 with early endosomal membranes [89–91] (fig. 4).

RN-tre is a rab5 GTPase-activating protein (GAP) whose activity is regulated by the EGFR. Eps8, a substrate of the EGFR [92], interacts via its SH3 domain with RN-tre. Upon EGF stimulation, Eps8 becomes phosphorylated. Phospho-Eps8 interacts with RN-tre and sequesters the GAP from rab5, thereby affecting rab5 GTPase activity [93] (fig. 4).

RIN is a GEF for rab5. It interacts upon EGF stimulation with Ras, which leads to the potentiation of the GEF ac-

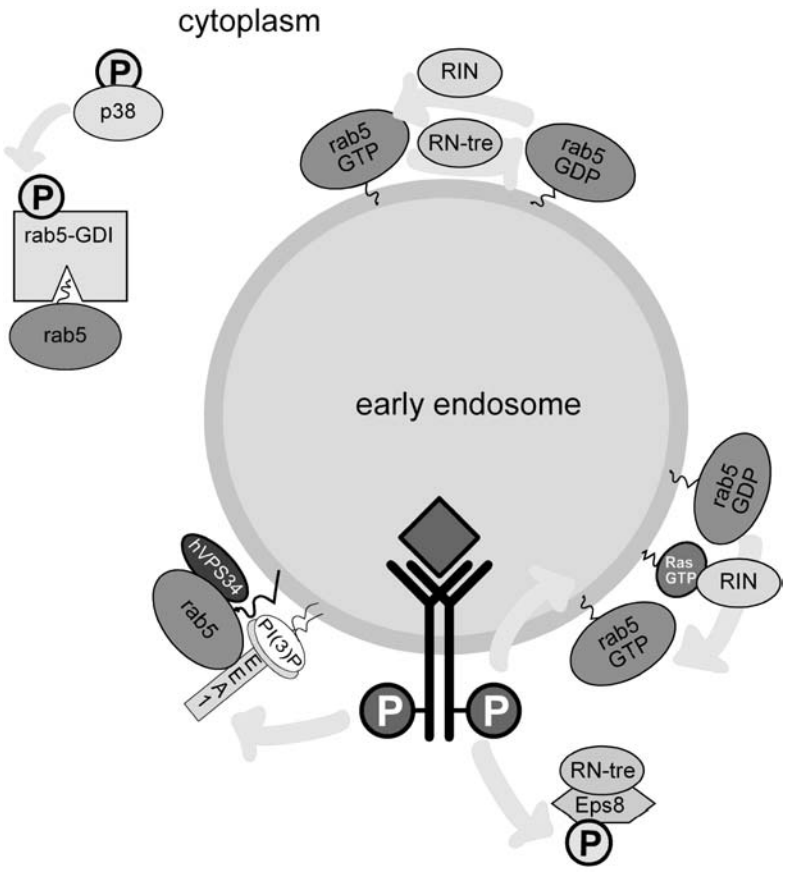

Figure 4. The function of rab5 effectors is modulated by EGFR signaling. EGFR signaling results in enhanced association of EEA1 with early endosomes. RN-tre (a rab5 GAP) is sequestered from rab5 by phosphorylated Eps8. Eps8 is phosphorylated upon EGFR activation. RIN (a rab5 GEF) interacts with activated Ras, which results in enhanced GDP to GTP exchange on rab5. Inhibition of RN-tre (GAP) and promotion of RIN (GEF) cause enhanced rab5 activity. The stress MAPK p38 phosphorylates Rab5 – GDI, thereby modulating the membrane association of rab5.

tivity on rab5, resulting in enhanced rab5 GTPase activity, endosome fusion and EGFR internalization [94]. Thus, RIN (Ras and Rab interactor) bridges the activities of Ras and rab5 by serving as a sensor for Ras activation and an activator of rab5-mediated endocytic events (fig. 4).

Additionally, rab5 cycles between a membrane-bound and a cytoplasmic state. The guanyl-nucleotide dissociation inhibitor (GDI) proteins are known to regulate the rab5 membrane association by sequestering rab5 into the cytoplasm [95–97]. Rab5–GDI extracts rab5 from membranes more efficiently when phosphorylated by the stress MAPK p38. Thus, under stress conditions activation of p38 controls the rate of endocytosis by regulating the localization of rab5 [98] (fig. 4).

Sorting to lysosomes and signaling

During the passage through the endosomal compartment, only activated EGFRs are sorted for the degradation pathway (fig. 5). Inactive receptors are recycled back to the plasma membrane [99, 100]. The sorting decision for the degradation pathway is controlled by sequentially acting mechanisms: ubiquitination of the EGFR, recognition of the ubiquitinated receptor and invagination of the limiting membrane to form internal vesicles in the MVB. Defects in these sorting mechanisms result not only in aberrant morphology of MVBs but also in defective signaling [101–104].

c-Cbl acts as an E3 ubiquitin ligase that ubiquitinates the EGFR [105]. Upon EGF stimulation, c-Cbl becomes phosphorylated [76] and translocates from the cytoplasm to the plasma membrane and to endosomes, where it binds to and ubiquitinates the activated EGFR [106–108]. Interestingly, binding of the transforming growth factor-α (TGFα), an alternative ligand to the EGFR, also results in activation of MAPK signaling and internalization of the EGFR. However, TGFα-activated EGFR recycles back to the plasma membrane because TGFα dissociates from the EGFR upon endocytosis [109, 110]. The early dissociation results in loss of EGFR

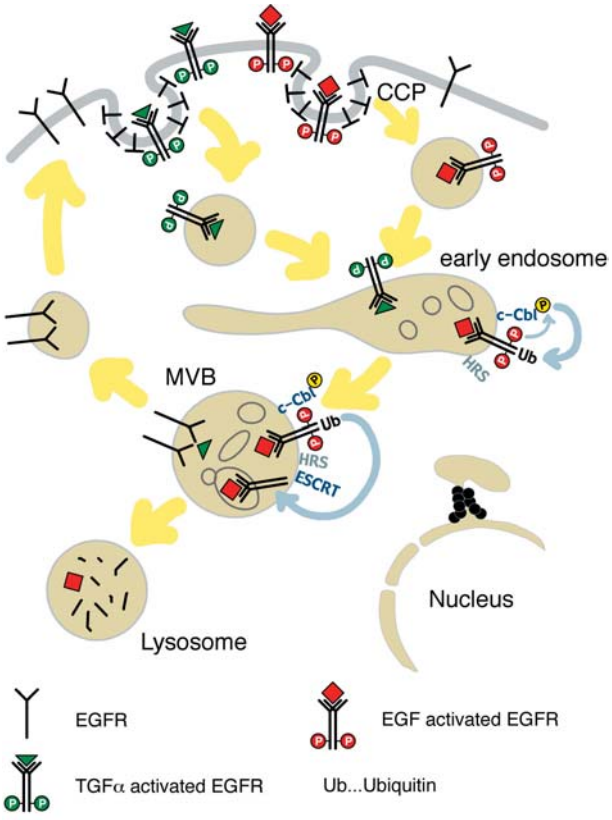

Figure 5. c-Cbl, HRS and ESCRT sort the EGF-activated EGFR to the degradation pathway. TGFα dissociates from the EGFR in endosomes, and TGFα-activated EGFR recycles back to the plasma membrane, where it can undergo a new round of stimulation. In contrast, EGF induces prolonged phosphorylation of c-Cbl. c-Cbl transfers ubiquitin onto EGF-activated EGFRs on endosomes. HRS contains a UIM, which is required for the sorting of active EGFRs into the internal lumen of MVBs. The ESCRT is also critically involved in the sorting step. Sorting of activated EGFRs into MVBs and subsequent delivery to lysosomes might disconnect the receptor from its cytoplasmic downstream signaling molecules, thereby regulating signaling duration.

dimers, loss of EGFR signaling activities and recycling. In contrast, EGF does not dissociate from the EGFR; therefore dimers and signaling activity (see below) are preserved in endosomes, which leads to sorting of the EGFR for degradation in lysosomes [111, 112] (fig. 5).

The sustained activity of the EGFR upon EGF but not upon TGFα stimulation increases the presence of tyrosine-phosphorylated c-Cbl on endosomes. Active EGFRs are ubiquitinated in a c-Cbl-dependent manner on endosomes. This c-Cbl-mediated ubiquitination of the EGFR in response to EGF is a crucial sorting signal to the internal lumen of MVBs [108]. Thus, c-Cbl acts as a negative regulator of RTK signaling [113]. Importantly, overexpression of c-Cbl does not result in enhanced internalization but enhances the degradation of the EGFR. In contrast, overexpression of viral Cbl (v-Cbl), which lacks the ring-finger domain, enhances the recycling of the EGFR. The lack of the ring-finger interferes with the ubiquitination activity of v-Cbl, thereby preventing the degradation of the activated EGFR, which finally results in cell transformation [106, 107] (fig. 5).

The hepatocyte growth-factor regulated tyrosine kinase substrate (HRS) mediates membrane invagination on endosomes. It localizes to the endosomal compartment by virtue of a FYVE domain and is, therefore, also sensitive to changes in the phosphoinositide metabolism. HRS-deficient mice died around embryonic day 11 because of several severe developmental defects. Biological analysis of cells from HRS-deficient mice revealed that these cells contain enlarged endosomes [114]. Mutants of HRS in *D. melanogaster* cannot invaginate the limiting membrane of the late endosome; subsequently, they do not terminate RTK signaling [115]. HRS contains a ubiquitin interacting motive (UIM) [116] by which HRS recruits ubiquitinated cell surface receptors to the clathrin-containing microdomains on endosomes that would invaginate to form MVBs [41]. Thus, ubiquitinated receptors are sorted for degradation and do not recycle (fig. 5).

The endosomal sorting complex required for transport (ESCRT) was identified to be essential for the ubiquitin-dependent sorting to internal membranes of MVBs in yeast. Vps23 is an essential component of this protein complex since this protein contains a ubiquitin-conjugating-like domain by which it recognizes ubiquitinated cargo proteins [117]. The mammalian homologue of Vps23 is the protein encoded by the tumor susceptibility gene 101 (TSG101). TSG101 has been demonstrated to function in late endosomal trafficking. In fact, in TSG101 mutant cells, activated EGFR was rapidly recycled back to the cell surface and inefficiently degraded [118].

In a concerted action, ESCRT-II and ESCRT-III concentrate ubiquitinated cargo on the limiting membrane of the MVB to finally cause inward budding into the lumen of the MVB. The AAA(-ATPases associated to a variety of cellular activities)-ATPase Vps4 releases the ESCRTs after the budding is complete to finish the ESCRT sorting process [119, 120]. Thereby, late endosomal sorting is completed, and receptors can be sorted for degradation in the lysosomes (fig. 5).

Sorting steps at the MVB might determine the duration of receptor signaling. At the limiting membrane of the MVB, activated receptors would still be in contact with the cytoplasmic signaling machinery. Sorting to the internal membrane of the MVB would sequester activated receptors from their cytoplasmic downstream signaling cascades. This sorting step would terminate the signaling activity of the receptor and finally result in degradation of the activated receptor in lysosomes.

Mutations in c-Cbl, HRS and TSG101 that interfere with their function in endosomal cargo sorting result in delayed or defective degradation of activated RTKs and, notably, in overactivation of MAPK signaling [115, 118, 121, 122] (fig. 5).

The endocytic traffic of activated cell surface receptors is a tightly regulated process. Many endocytic processes appear to be modulated upon activation of cell surface receptors. Activated cell surface receptor and downstream signaling molecules act on the endosomal machinery that executes the regulated receptor internalization and controlled sorting events in the endosomal system. The controlled endocytic traffic of activated cell surface receptors might regulate the signal duration by defining the half-life of the receptor.

Signaling from endosomes

There is a good amount of evidence that RTKs signal from endosomes. First, it has been demonstrated that the internalized EGFR is signaling. [111]. Second, the EGFR remains bound to EGF within the endosomal compartment [123, 124], whereas TGFα dissociates from the receptor already in early endosomes [108, 112]. Therefore, EGF-stimulated EGFRs preserve their dimerization and their kinase activity within endosomes, whereas TGFα-stimulated EGFRs do not. Furthermore, kinase-dead EGFRs recycle back to the plasma membrane and are not degraded [99, 100, 125]. In addition to biochemical methods [126], imaging techniques such as fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) were used to localize the activated EGFR. Notably, tyrosine-phosphorylated EGFR was detected at the plasma membrane and on the membranes of endosomes [126–128] (fig. 5). Consistently, adaptor proteins like Grb2 and Shc, which interact with the activated EGFR, have been found to reside at the plasma membrane and also on endosomes [127, 129, 130].

The localization of Grb2 and Shc to endosomes suggests that the small GTPase Ras would also be active on endosomes. Indeed, Ras and its GEF, Sos, have been found to copurify with and to localize on endosomes [126, 129, 131]. Upon EGF stimulation, Ras becomes activated at the plasma membrane. However, after endocytosis of the activated EGFR, Ras activity persists on endosomes, as demonstrated by the use of FRET technique [130, 132]. Furthermore, all components of the Ras-MAPK signaling cascade, namely, Raf, MEK, and ERK have been found to reside on endosomes, either by biochemical or immunofluorescence methods [133–136].

Internalized EGFRs show all the characteristics of receptors that are activated at the plasma membrane. They are bound to EGF, exist as dimers, are tyrosine-phosphorylated and interact with SH2 and PTB domain-containing adaptor proteins to connect receptor activation to downstream effectors. Furthermore, all components of downstream signaling cascades have been found to reside not only at the plasma membrane but, notably, also on endosomes. It appears likely that signaling from the EGFR is not terminated upon internalization. Internalized receptors continue to signal until they are sorted to internal vesicles of MVBs. This sorting step would disconnect the receptor from its 'cytoplasmic' downstream effectors, and signaling would be shut off.

Function of signaling endosomes

Cell surface receptors are active at the plasma membrane and in the endosomal system. Therefore, activated cell surface receptors could engage a variety of different signaling complexes in a sequential manner, first at the plasma membrane, then on early endosomes and, finally, on the limiting membrane of MVBs. The activation of diverse signaling complexes by activated receptors along the endocytic pathway would increase the signaling potential. Receptor signaling from endosomes might provide a window of opportunity that could contribute to the spatial and temporal regulation of signal transduction. Thus, signal transduction could be compartmentalized at the plasma membrane and on endosomes.

Compartmentalization would provide high flexibility to an otherwise limited number of signaling cascades to transduce specific signals from a plethora of signaling cues. There is, in fact, accumulating evidence that signaling from endosomes could be used as a means to compartmentalize signal transduction [47, 137] (fig. 6).

The use of dominant-negative dynamin revealed that the EGF-stimulated EGFR must be internalized to fully activate ERK1/2 and PI3K signaling. In contrast, phospholipase Cγ (PLCγ) and Shc are hyperphosphorylated. Thus, normal endocytic trafficking of activated EGFR might play a critical role in establishing and controlling specific signaling pathways [134, 138].

The use of a recently established experimental protocol allows endosomal, but not cell surface, activation of the EGFR. Using this protocol, it has been demonstrated that endosomal activation of EGFR signaling is sufficient to activate the Ras-MAPK and the PI3K cascades. The endosomal activation of theses effector cascades protected cells from serum-withdrawal-induced apoptosis [139].

Interestingly, Ras isoforms do not localize equally to endosomes. Upon EGF stimulation, K-Ras is found predominantly at the plasma membrane, whereas H-Ras is internalized into endosomes together with the EGFR [130, 131]. Moreover, MAPK signaling from H-Ras, but not from K-Ras, appears to depend on endocytosis, because inhibition of endocytosis blocked H-Ras but not K-Ras-mediated PC12 cell differentiation [131].

Rap1, a Ras-related small GTPase, is crucial for the regulation of MAPK signaling in PC12 cells. In contrast to Ras, Rap1 is located mainly to endosomes but not to the plasma membrane [140]. Consistently, the Rap1 adaptor protein CRK and the respective GEF, C3G, are located to

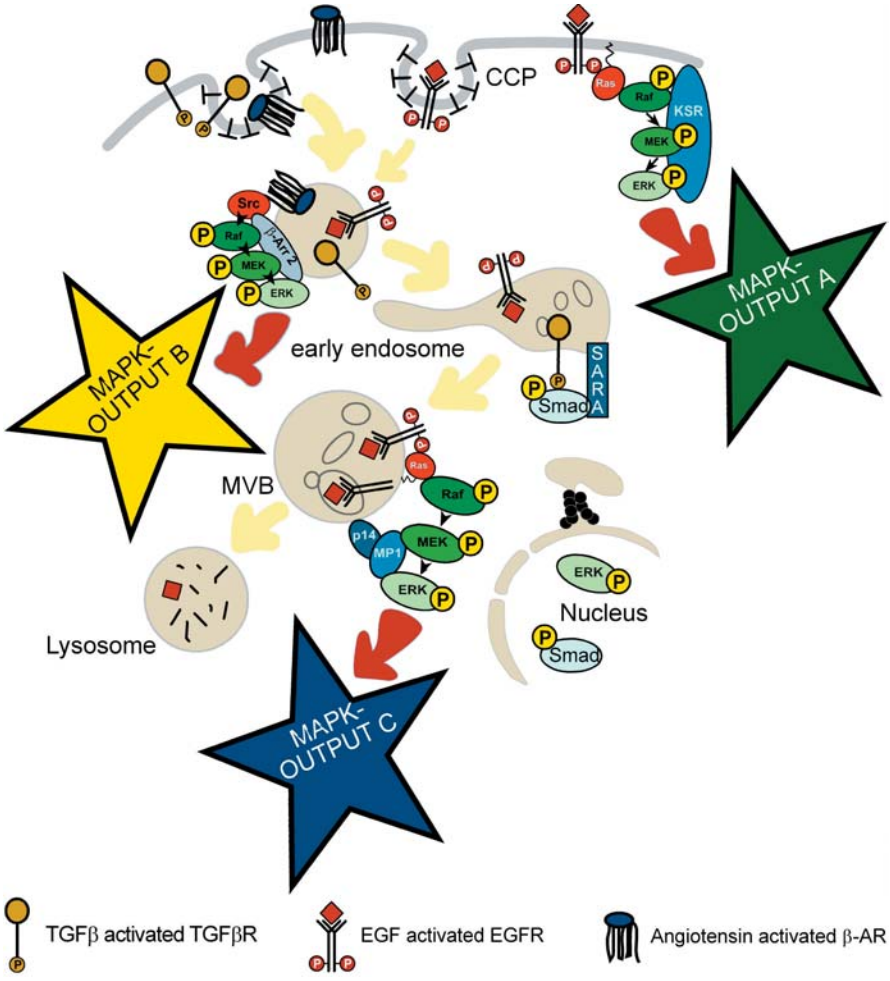

Figure 6. Subcellular compartmentalization of signaling complexes. Different scaffold complexes reside at different subcellular compartments. The use of the KSR-scaffold module at the plasma membrane could result in the MAPK signaling output A. β-Arrestin 2 (β-Arr 2) assembles another MAPK signaling complex on early endosomes and might define MAPK signaling output B. The late endosomal p14/MP1 scaffold module generates yet another MAPK signaling complex, which might regulate a MAPK signaling output C. Thus, the MAPK pathway might be compartmentalized by a variety of scaffold proteins. This compartmentalization of signaling modules might be used to define a specific biological signal. In addition to the scaffold modules, other signaling adaptor modules (like the SARA/Smad module) have been found to localize on endosomes.

endosomes [136]. Whereas Ras is activated at the plasma membrane and remains active on endosomes, Rap1 is predominantly activated on endosomes upon NGF (nerve growth factor)-induced activation of the neurotrophin receptor TrkA (another RTK) [132]. The activation of Rap1 on endosomes requires the endocytosis of the TrkA receptor, whereas Ras activation does not depend on TrkA endocytosis [141].

Importantly, NGF-induced activation of Rap1 and Ras results in sustained MAPK signaling, ultimately causing PC12 cell differentiation. EGF stimulation also triggers Ras, but not Rap1, activation and results in transient MAPK signaling, which drives PC12 cells into proliferation. Apparently, the activation of Rap1 on endosomes upon NGF stimulation switches the MAPK signaling specificity to cause differentiation instead of proliferation [132, 142].

The specification of the signaling pathways can be achieved by organizing signaling components into multiprotein complexes [143]. Scaffold proteins are used to organize specific signaling units [12, 144]. Interestingly, different scaffold proteins have been found to reside at the plasma membrane and on endosomes.

KSR (kinase suppressor of Ras) and MP1 (MEK1 partner) serve as MAPK-scaffold proteins to regulate signal transduction in higher eukaryotes. The MAPK-scaffold protein KSR mediates the interaction between Raf and MEK [145–147] and is required for signal transduction in the Ras-MAPK pathway [148–150]. In concert with C-TAK1, KSR regulates the formation of a MAPK signaling unit at the plasma membrane upon EGF stimulation. Mutant KSR, which cannot be phosphorylated by C-TAK1, localizes constitutively to the plasma membrane, thereby causing overactivation of MAPK signaling [145, 146] (fig. 6).

MP1 also functions as a scaffold protein in the Ras-MAPK pathway. MP1 binds specifically to MEK1 and ERK1, thereby facilitating signaling in the MAPK cascade [151]. However, unlike KSR, MP1 localizes to late endosomes [152]. The adaptor protein p14 is essential to mediate the localization of MP1 to endosomes. The endosomal localization of the p14/MP1 complex is exclusively required for the activation of ERK at endosomes but not for ERK activation at the plasma membrane [153] (fig. 6).

β-arrestin 2 is a key molecule in GPCR signaling [154]. On the one hand, β-arrestin 2 interacts with clathrin to promote the internalization of the GPCR, in particular, the β2-adrenergic receptor (β2-AR) [155, 156]. On the other hand, β-arrestin 2 is required to recruit Src for the activation of MAPK signaling from β2-AR [157]. β-arrestin 2 acts as a scaffold protein, directing Raf and ERK to endosomes after angiotensin stimulation [158] (fig. 6). β-arrestin 2 recruits jun-kinase 3 (JNK3) and specific upstream activators to endosomes [159]. Thus, β-arrestin 2 combines the features of a protein, which is critical for receptor internalization, and a scaffold protein, which is used to form a signaling complex at endosomes.

The family of Smad proteins represents transcription factors that mediate transforming growth factor-β (TGFβ) signaling from transmembrane serine-threonine receptor kinases (TGFβ receptors) to the nucleus. Upon activation, TGFβ receptors internalize into endosomes and phosphorylate Smads, which are tethered to endosomes by the association with the Smad anchor for receptor activation (SARA). SARA is an early endosomal FYVE-domain protein and functions by recruiting Smad2 to endosomes. On endosomes, Smad2 becomes phosphorylated by the activated TGFβ receptor. Phosphorylated Smads translocate from endosomes into the nucleus to activate the transcription of specific target genes. Notably, the disruption of the early endosomal localization of SARA, as well as blockage of the TGFβ receptors' internalization, inhibits Smad activation on early endosomes [160, 161] (fig. 6).

Thus, scaffold, anchor and adaptor proteins might bring the spatial distribution and activation of specific signaling complexes under the control of activated cell surface receptors. At the plasma membrane, KSR would assemble a specific scaffold complex. On endosomes, β-arrestin 2, the p14/MP1 complex or SARA would orchestrate the composition of the respective scaffold complex. The subcellular distribution of scaffolded signaling complexes of one signal transduction pathway (fig. 6, e.g., MAPK pathway) might finally contribute to the generation of different specific signaling outputs (fig. 6, e.g., MAPK output A, B, C). The formation of such membrane-based scaffold complexes would be ideal for the propagation of signals with high spatial as well as temporal resolution and specificity within cells [162, 163].

Conclusion

Taken together, a variety of cell surface receptors, including RTKs, GPCRs and serine-threonine receptor kinases, use endosomes as a compartment for the activation of specialized signaling complexes. Receptor signaling appears to control the endocytic machinery, which in a feedback loop reciprocates back to regulate endosomal traffic of cell surface receptors. By regulating the internalization and endosomal traffic of the activated receptors, and also by controlling the location of signaling complexes, a vast number of possible receptor-signaling cascade interactions could be envisioned at different subcellular compartments. Compartmentalization of signaling would serve the purpose of directing the spatial and temporal specifications in signal transduction. The activation of defined signaling complexes at the plasma membrane and on endosomes might indeed contribute to the spatial and temporal regulation of signal transduction (fig. 6). This would ultimately assign a unique biological response to specific extracellular information.

Although it appears clear that endosomes are used as signaling platforms, the biological function of endosomal signaling remains elusive. Whether signaling from endosomes results in a specific biological response in vivo, remains to be demonstrated. Furthermore, it needs to be determined whether signaling from endosomes generates a biological output that differs from the biological output generated by signaling from the plasma membrane. To address these questions, it will be important to interfere with the function of proteins that have been shown to specifically regulate plasma membrane or endosomal signaling. Therefore, it will be crucial to use genetically modifiable model organisms to measure the biological endpoints of plasma membrane or endosomal signaling in vivo. With the findings currently at hand, signal transduction and endocytosis have to be considered as intimately linked cell biological functions.

Acknowledgments. We are grateful to Bernhard Hampoelz and Yan Sun for critically discussing the manuscript. We wish to thank Hannes Tkadletz for his invaluable support in designing the figures. This work was supported by Boehringer Ingelheim and by a European Union shared-cost project (QLG-CT-2001-02278) to L.A.H.

1 Jiang G. and Hunter T. (1999) Receptor signaling: when dimerization is not enough. Curr. Biol. **9:** R568–571
2 Schlessinger J. (1988) Signal transduction by allosteric receptor oligomerization. Trends Biochem. Sci. **13:** 443–447
3 Pawson T. and Gish G. D. (1992) SH2 and SH3 domains: from structure to function. Cell **71:** 359–362
4 van der Geer P. and Pawson T. (1995) The PTB domain: a new protein module implicated in signal transduction. Trends Biochem. Sci. **20:** 277–280
5 Bar-Sagi D. and Hall A. (2000) Ras and Rho GTPases: a family reunion. Cell **103:** 227–238
6 Schlessinger J. (2000) Cell signaling by receptor tyrosine kinases. Cell **103:** 211–225

7 Li N., Batzer A., Daly R., Yajnik V., Skolnik E., Chardin P. et al. (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature **363:** 85–88
8 Rozakis-Adcock M., Fernley R., Wade J., Pawson T. and Bowtell D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature **363:** 83–85
9 Margolis B. (1999) The PTB domain: the name doesn't say it all. Trends Endocrinol. Metab. **10:** 262–267
10 Shields J. M., Pruitt K., McFall A., Shaub A. and Der C. J. (2000) Understanding Ras: 'it ain't over 'til it's over'. Trends Cell Biol. **10:** 147–154
11 Lewis T. S., Hunt J. B., Aveline L. D., Jonscher K. R., Louie D. F., Yeh J. M. et al. (2000) Identification of novel MAP kinase pathway signaling targets by functional proteomics and mass spectrometry. Mol. Cell. **6:** 1343–1354
12 Kolch W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J. **351 (Pt 2):** 289–305
13 Widmann C., Gibson S., Jarpe M. B. and Johnson G. L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. **79:** 143–180
14 Cantley L. C. (2002) The phosphoinositide 3-kinase pathway. Science **296:** 1655–1657
15 Czech M. P. (2000) PIP2 and PIP3: complex roles at the cell surface. Cell **100:** 603–606
16 Wymann M. P. and Pirola L. (1998) Structure and function of phosphoinositide 3-kinases. Biochim. Biophys. Acta **1436:** 127–150
17 Haigler H., Ash J. F., Singer S. J. and Cohen S. (1978) Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. Proc. Natl. Acad. Sci. USA **75:** 3317–3321
18 Larkin J. M., Brown M. S., Goldstein J. L. and Anderson R. G. (1983) Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. Cell **33:** 273–285
19 Marsh M. and McMahon H. T. (1999) The structural era of endocytosis. Science **285:** 215–220
20 McPherson P. S., Kay B. K. and Hussain N. K. (2001) Signaling on the endocytic pathway. Traffic **2:** 375–384
21 Owen D. J. and Evans P. R. (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. Science **282:** 1327–1332
22 Owen D. J., Vallis Y., Noble M. E., Hunter J. B., Dafforn T. R., Evans P. R. et al. (1999) A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. Cell **97:** 805–815
23 Robinson M. S. (1994) The role of clathrin, adaptors and dynamin in endocytosis. Curr. Opin. Cell Biol. **6:** 538–544
24 Gallusser A. and Kirchhausen T. (1993) The beta 1 and beta 2 subunits of the AP complexes are the clathrin coat assembly components. EMBO J. **12:** 5237–5244
25 Owen D. J., Vallis Y., Pearse B. M., McMahon H. T. and Evans P. R. (2000) The structure and function of the beta 2-adaptin appendage domain. EMBO J. **19:** 4216–4227
26 Crowther R. A. and Pearse B. M. (1981) Assembly and packing of clathrin into coats. J. Cell Biol. **91:** 790–797
27 Heuser J. E., Keen J. H., Amende L. M., Lippoldt R. E. and Prasad K. (1987) Deep-etch visualization of 27S clathrin: a tetrahedral tetramer. J. Cell Biol. **105:** 1999–2009
28 Peeler J. S., Donzell W. C. and Anderson R. G. (1993) The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits. J. Cell Biol. **120:** 47–54
29 Unanue E. R., Ungewickell E. and Branton D. (1981) The binding of clathrin triskelions to membranes from coated vesicles. Cell **26:** 439–446
30 Ungewickell E. and Branton D. (1981) Assembly units of clathrin coats. Nature **289:** 420–422
31 Damke H., Baba T., Warnock D. E. and Schmid S. L. (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J. Cell Biol. **127:** 915–934
32 Hinshaw J. E. and Schmid S. L. (1995) Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature **374:** 190–192
33 Marks B., Stowell M. H., Vallis Y., Mills I. G., Gibson A., Hopkins C. R. et al. (2001) GTPase activity of dynamin and resulting conformation change are essential for endocytosis. Nature **410:** 231–235
34 Sweitzer S. M. and Hinshaw J. E. (1998) Dynamin undergoes a GTP-dependent conformational change causing vesiculation. Cell **93:** 1021–1029
35 Warnock D. E. and Schmid S. L. (1996) Dynamin GTPase, a force-generating molecular switch. Bioessays **18:** 885–893
36 Hopkins C. R., Gibson A., Shipman M. and Miller K. (1990) Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. Nature **346:** 335–339
37 Tooze J. and Hollinshead M. (1991) Tubular early endosomal networks in AtT20 and other cells. J. Cell Biol. **115:** 635–653
38 Gruenberg J. (2001) The endocytic pathway: a mosaic of domains. Nat. Rev. Mol. Cell. Biol. **2:** 721–730
39 Zerial M. and McBride H. (2001) Rab proteins as membrane organizers. Nat. Rev. Mol. Cell. Biol. **2:** 107–117
40 Sachse M., Urbe S., Oorschot V., Strous G. J. and Klumperman J. (2002) Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. Mol. Biol. Cell **13:** 1313–1328
41 Raiborg C., Bache K. G., Gillooly D. J., Madshus I. H., Stang E. and Stenmark H. (2002) Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. **4:** 394–398
42 Gruenberg J. and Maxfield F. R. (1995) Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. **7:** 552–563
43 Kobayashi T., Beuchat M. H., Lindsay M., Frias S., Palmiter R. D., Sakuraba H. et al. (1999) Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. Nat. Cell Biol. **1:** 113–118
44 Kobayashi T., Gu F. and Gruenberg J. (1998) Lipids, lipid domains and lipid-protein interactions in endocytic membrane traffic. Semin. Cell Dev. Biol. **9:** 517–526
45 Kobayashi T., Stang E., Fang K. S., de Moerloose P., Parton R. G. and Gruenberg J. (1998) A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature **392:** 193–197
46 Piper R. C. and Luzio J. P. (2001) Late endosomes: sorting and partitioning in multivesicular bodies. Traffic **2:** 612–621
47 Sorkin A. and Von Zastrow M. (2002) Signal transduction and endocytosis: close encounters of many kinds. Nat. Rev. Mol. Cell Biol. **3:** 600–614
48 Carpentier J. L., Gorden P., Anderson R. G., Goldstein J. L., Brown M. S., Cohen S. et al. (1982) Co-localization of 125I-epidermal growth factor and ferritin-low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. J. Cell Biol. **95:** 73–77
49 Connolly J. L., Green S. A. and Greene L. A. (1984) Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. J. Cell Biol. **98:** 457–465
50 Lamaze C. and Schmid S. L. (1995) Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. J. Cell Biol. **129:** 47–54
51 Prywes R., Livneh E., Ullrich A. and Schlessinger J. (1986) Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. EMBO J. **5:** 2179–2190

52 Sorkina T., Huang F., Beguinot L. and Sorkin A. (2002) Effect of tyrosine kinase inhibitors on clathrin-coated pit recruitment and internalization of epidermal growth factor receptor. J. Biol. Chem. **277:** 27433–27441

53 Wilde A., Beattie E. C., Lem L., Riethof D. A., Liu S. H., Mobley W. C. et al. (1999) EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. Cell **96:** 677–687

54 Ahn S., Kim J., Lucaveche C. L., Reedy M. C., Luttrell L. M., Lefkowitz R. J. et al. (2002) Src-dependent tyrosine phosphorylation regulates dynamin self-assembly and ligand-induced endocytosis of the epidermal growth factor receptor. J. Biol. Chem. **277:** 26642–26651

55 Benmerah A., Gagnon J., Begue B., Megarbane B., Dautry-Varsat A. and Cerf-Bensussan N. (1995) The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2. J. Cell Biol. **131:** 1831–1838

56 van Delft S., Schumacher C., Hage W., Verkleij A. J. and van Bergen en Henegouwen P. M. (1997) Association and colocalization of Eps15 with adaptor protein-2 and clathrin. J. Cell Biol. **136:** 811–821

57 Confalonieri S., Salcini A. E., Puri C., Tacchetti C. and Di Fiore P. P. (2000) Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. J. Cell Biol. **150:** 905–912

58 Fazioli F., Minichiello L., Matoskova B., Wong W. T. and Di Fiore P. P. (1993) eps15, a novel tyrosine kinase substrate, exhibits transforming activity. Mol. Cell Biol. **13:** 5814–5828

59 Whitehead B., Tessari M., Carotenuto A., van Bergen en Henegouwen P. M. and Vuister G. W. (1999) The EH1 domain of Eps15 is structurally classified as a member of the S100 subclass of EF-hand-containing proteins. Biochemistry **38:** 11271–11277

60 Wong W. T., Schumacher C., Salcini A. E., Romano A., Castagnino P., Pelicci P. G. et al. (1995) A protein-binding domain, EH, identified in the receptor tyrosine kinase substrate Eps15 and conserved in evolution. Proc. Natl. Acad. Sci. USA **92:** 9530–9534

61 de Beer T., Carter R. E., Lobel-Rice K. E., Sorkin A. and Overduin M. (1998) Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. Science **281:** 1357– 1360

62 de Beer T., Hoofnagle A. N., Enmon J. L., Bowers R. C., Yamabhai M., Kay B. K. et al. (2000) Molecular mechanism of NPF recognition by EH domains. Nat. Struct. Biol. **7:** 1018–1022

63 Salcini A. E., Confalonieri S., Doria M., Santolini E., Tassi E., Minenkova O. et al. (1997) Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. Genes Dev. **11:** 2239–2249

64 Berdnik D., Torok T., Gonzalez-Gaitan M. and Knoblich J. A. (2002) The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in *Drosophila*. Dev. Cell **3:** 221–231

65 Honda A., Nogami M., Yokozeki T., Yamazaki M., Nakamura H., Watanabe H. et al. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell **99:** 521–532

66 Futter C. E., Collinson L. M., Backer J. M. and Hopkins C. R. (2001) Human VPS34 is required for internal vesicle formation within multivesicular endosomes. J. Cell Biol. **155:** 1251–1264

67 Chen X. and Wang Z. (2001) Regulation of intracellular trafficking of the EGF receptor by Rab5 in the absence of phosphatidylinositol 3-kinase activity. EMBO **Rep 2:** 68–74

68 Chen X. and Wang Z. (2001) Regulation of epidermal growth factor receptor endocytosis by wortmannin through activation of Rab5 rather than inhibition of phosphatidylinositol 3-kinase. EMBO **Rep 2:** 842–849

69 Wang L. H., Sudhof T. C. and Anderson R. G. (1995) The appendage domain of alpha-adaptin is a high affinity binding site for dynamin. J. Biol. Chem. **270:** 10079–10083

70 David C., McPherson P. S., Mundigl O. and de Camilli P. (1996) A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. Proc. Natl. Acad. Sci. USA **93:** 331–335

71 Vallis Y., Wigge P., Marks B., Evans P. R. and McMahon H. T. (1999) Importance of the pleckstrin homology domain of dynamin in clathrin-mediated endocytosis. Curr. Biol. **9:** 257–260

72 Rohde G., Wenzel D. and Haucke V. (2002) A phosphatidylinositol (4,5)-bisphosphate binding site within µ2-adaptin regulates clathrin-mediated endocytosis. J. Cell Biol. **158:** 209–214

73 Chen H., Fre S., Slepnev V. I., Capua M. R., Takei K., Butler M. H. et al. (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature **394:** 793–797

74 Itoh T., Koshiba S., Kigawa T., Kikuchi A., Yokoyama S. and Takenawa T. (2001) Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science **291:** 1047–1051

75 Ford M. G., Mills I. G., Peter B. J., Vallis Y., Praefcke G. J., Evans P. R. et al. (2002) Curvature of clathrin-coated pits driven by epsin. Nature **419:** 361–366

76 Galisteo M. L., Dikic I., Batzer A. G., Langdon W. Y. and Schlessinger J. (1995) Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. J. Biol. Chem. **270:** 20242–20245

77 Soubeyran P., Kowanetz K., Szymkiewicz I., Langdon W. Y. and Dikic I. (2002) Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. Nature **416:** 183–187

78 Micheva K. D., Kay B. K. and McPherson P. S. (1997) Synaptojanin forms two separate complexes in the nerve terminal. Interactions with endophilin and amphiphysin. J. Biol. Chem. **272:** 27239–27245

79 Ringstad N., Nemoto Y. and De Camilli P. (1997) The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. Proc. Natl. Acad. Sci. USA **94:** 8569–8574

80 Schmidt A., Wolde M., Thiele C., Fest W., Kratzin H., Podtelejnikov A. V. et al. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature **401:** 133–141

81 McPherson P. S., Garcia E. P., Slepnev V. I., David C., Zhang X., Grabs D. et al. (1996) A presynaptic inositol-5-phosphatase. Nature **379:** 353–357

82 Cremona O., Di Paolo G., Wenk M. R., Luthi A., Kim W. T., Takei K. et al. (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell **99:** 179–188

83 Christoforidis S., McBride H. M., Burgoyne R. D. and Zerial M. (1999) The Rab5 effector EEA1 is a core component of endosome docking. Nature **397:** 621–625

84 Dumas J. J., Merithew E., Sudharshan E., Rajamani D., Hayes S., Lawe D. et al. (2001) Multivalent endosome targeting by homodimeric EEA1. Mol. Cell. **8:** 947–958

85 McBride H. M., Rybin V., Murphy C., Giner A., Teasdale R. and Zerial M. (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell **98:** 377–386

86 Barbieri M. A., Roberts R. L., Gumusboga A., Highfield H., Alvarez-Dominguez C., Wells A. et al. (2000) Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a. J. Cell Biol. **151:** 539–550

87 Kutateladze T. G., Ogburn K. D., Watson W. T., de Beer T., Emr S. D., Burd C. G. et al. (1999) Phosphatidylinositol 3-

phosphate recognition by the FYVE domain. Mol. Cell. **3:** 805–811
88 Stenmark H., Aasland R., Toh B. H. and D'Arrigo A. (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. J. Biol. Chem. **271:** 24048–24054
89 Christoforidis S., Miaczynska M., Ashman K., Wilm M., Zhao L., Yip S. C. et al. (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat. Cell Biol. **1:** 249– 252
90 Nielsen E., Christoforidis S., Uttenweiler-Joseph S., Miaczynska M., Dewitte F., Wilm M. et al. (2000) Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. J. Cell Biol. **151:** 601–612
91 Simonsen A., Lippe R., Christoforidis S., Gaullier J. M., Brech A., Callaghan J. et al. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature **394:** 494–498
92 Fazioli F., Minichiello L., Matoska V., Castagnino P., Miki T., Wong W. T. et al. (1993) Eps8, a substrate for the epidermal growth factor receptor kinase, enhances EGF-dependent mitogenic signals. EMBO J. **12:** 3799–3808
93 Lanzetti L., Rybin V., Malabarba M. G., Christoforidis S., Scita G., Zerial M. et al. (2000) The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. Nature **408:** 374–377
94 Tall G. G., Barbieri M. A., Stahl P. D. and Horazdovsky B. F. (2001) Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev. Cell **1:** 73–82
95 Sasaki T., Kikuchi A., Araki S., Hata Y., Isomura M., Kuroda S. et al. (1990) Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. J. Biol. Chem. **265:** 2333– 2337
96 Ullrich O., Stenmark H., Alexandrov K., Huber L. A., Kaibuchi K., Sasaki T. et al. (1993) Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. J. Biol. Chem. **268:** 18143–18150
97 Ullrich O., Horiuchi H., Bucci C. and Zerial M. (1994) Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature **368:** 157–160
98 Cavalli V., Vilbois F., Corti M., Marcote M. J., Tamura K., Karin M. et al. (2001) The stress-induced MAP kinase p38 regulates endocytic trafficking via the GDI:Rab5 complex. Mol. Cell. **7:** 421–432
99 Felder S., Miller K., Moehren G., Ullrich A., Schlessinger J. and Hopkins C. R. (1990) Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. Cell **61:** 623–634
100 Futter C. E., Felder S., Schlessinger J., Ullrich A. and Hopkins C. R. (1993) Annexin I is phosphorylated in the multivesicular body during the processing of the epidermal growth factor receptor. J. Cell Biol. **120:** 77–83
101 Di Fiore P. P. and De Camilli P. (2001) Endocytosis and signaling. an inseparable partnership. Cell **106:** 1–4
102 Di Fiore P. P. and Gill G. N. (1999) Endocytosis and mitogenic signaling. Curr. Opin. Cell Biol. **11:** 483–488
103 Waterman H. and Yarden Y. (2001) Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. FEBS Lett. **490:** 142–152
104 Wiley H. S. and Burke P. M. (2001) Regulation of receptor tyrosine kinase signaling by endocytic trafficking. Traffic **2:** 12–18
105 Joazeiro C. A., Wing S. S., Huang H., Leverson J. D., Hunter T. and Liu Y. C. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2- dependent ubiquitin-protein ligase. Science **286:** 309–312
106 Levkowitz G., Waterman H., Ettenberg S. A., Katz M., Tsygankov A. Y., Alroy I. et al. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol. Cell. **4:** 1029– 1040
107 Levkowitz G., Waterman H., Zamir E., Kam Z., Oved S., Langdon W. Y. et al. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. **12:** 3663–3674
108 Longva K. E., Blystad F. D., Stang E., Larsen A. M., Johannessen L. E. and Madshus I. H. (2002) Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. J. Cell Biol. **156:** 843–854
109 Ebner R. and Derynck R. (1991) Epidermal growth factor and transforming growth factor-alpha: differential intracellular routing and processing of ligand-receptor complexes. Cell Regul. **2:** 599–612
110 Sorkin A. and Waters C. M. (1993) Endocytosis of growth factor receptors. Bioessays **15:** 375–382
111 Cohen S. and Fava R. A. (1985) Internalization of functional epidermal growth factor:receptor/kinase complexes in A-431 cells. J. Biol. Chem. **260:** 12351–12358
112 French A. R., Tadaki D. K., Niyogi S. K. and Lauffenburger D. A. (1995) Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. J. Biol. Chem. **270:** 4334– 4340
113 Yoon C. H., Lee J., Jongeward G. D. and Sternberg P. W. (1995) Similarity of sli-1, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene c-cbl. Science **269:** 1102–1105
114 Komada M. and Soriano P. (1999) Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. Genes Dev. **13:** 1475–1485
115 Lloyd T. E., Atkinson R., Wu M. N., Zhou Y., Pennetta G. and Bellen H. J. (2002) Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in *Drosophila*. Cell **108:** 261–269
116 Polo S., Sigismund S., Faretta M., Guidi M., Capua M. R., Bossi G. et al. (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature **416:** 451–455
117 Katzmann D. J., Babst M. and Emr S. D. (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell **106:** 145–155
118 Babst M., Odorizzi G., Estepa E. J. and Emr S. D. (2000) Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. Traffic **1:** 248–258
119 Babst M., Katzmann D., Estepa-Sabal E., Meerloo T. and Emr S. (2002) Escrt-III. An endosome-associated heterooligomeric protein complex required for mvb sorting. Dev. Cell **3:** 271
120 Babst M., Katzmann D., Snyder W., Wendland B. and Emr S. (2002) Endosome-Associated Complex, ESCRT-II, Recruits Transport Machinery for Protein Sorting at the Multivesicular Body. Dev. Cell **3:** 283
121 Meisner H., Daga A., Buxton J., Fernandez B., Chawla A., Banerjee U. et al. (1997) Interactions of *Drosophila* Cbl with epidermal growth factor receptors and role of Cbl in R7 photoreceptor cell development. Mol. Cell. Biol. **17:** 2217–2225
122 Pai L. M., Barcelo G. and Schupbach T. (2000) D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in *Drosophila* oogenesis. Cell **103:** 51–61
123 Sorkin A. D., Teslenko L. V. and Nikolsky N. N. (1988) The endocytosis of epidermal growth factor in A431 cells: a pH of microenvironment and the dynamics of receptor complex dissociation. Exp. Cell Res. **175:** 192–205

124 Zapf-Colby A. and Olefsky J. M. (1998) Nerve growth factor processing and trafficking events following TrkA- mediated endocytosis. Endocrinology **139:** 3232–3240

125 Futter C. E., Pearse A., Hewlett L. J. and Hopkins C. R. (1996) Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J. Cell Biol. **132:** 1011–1023

126 Burke P., Schooler K. and Wiley H. S. (2001) Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol. Biol. Cell **12:** 1897–1910

127 Sorkin A., McClure M., Huang F. and Carter R. (2000) Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. Curr. Biol. **10:** 1395–1398

128 Wouters F. S. and Bastiaens P. I. (1999) Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells. Curr. Biol. **9:** 1127–1130

129 Di Guglielmo G. M., Baass P. C., Ou W. J., Posner B. I. and Bergeron J. J. (1994) Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. EMBO J. **13:** 4269–4277

130 Jiang X. and Sorkin A. (2002) Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. Mol. Biol. Cell **13:** 1522–1535

131 Roy S., Wyse B. and Hancock J. F. (2002) H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. Mol. Cell. Biol. **22:** 5128–5140

132 Mochizuki N., Yamashita S., Kurokawa K., Ohba Y., Nagai T., Miyawaki A. et al. (2001) Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. Nature **411:** 1065–1068

133 Howe C. L., Valletta J. S., Rusnak A. S. and Mobley W. C. (2001) NGF signaling from clathrin-coated vesicles. Evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. Neuron **32:** 801–814

134 Kranenburg O., Verlaan I. and Moolenaar W. H. (1999) Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. J. Biol. Chem. **274:** 35301–35304

135 Pol A., Calvo M. and Enrich C. (1998) Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. FEBS Lett. **441:** 34–38

136 Wu C., Lai C. F. and Mobley W. C. (2001) Nerve growth factor activates persistent Rap1 signaling in endosomes. J. Neurosci. **21:** 5406–5416

137 Seto E. S., Bellen H. J. and Lloyd T. E. (2002) When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev. **16:** 1314–1336

138 Vieira A. V., Lamaze C. and Schmid S. L. (1996) Control of EGF receptor signaling by clathrin-mediated endocytosis. Science **274:** 2086–2089

139 Wang Y., Pennock S., Chen X. and Wang Z. (2002) Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol. Cell Biol. **22:** 7279–7290

140 Pizon V., Desjardins M., Bucci C., Parton R. G. and Zerial M. (1994) Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex. J. Cell Sci. **107:** 1661–1670

141 York R. D., Molliver D. C., Grewal S. S., Stenberg P. E., McCleskey E. W. and Stork P. J. (2000) Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol. Cell Biol. **20:** 8069–8083

142 York R. D., Yao H., Dillon T., Ellig C. L., Eckert S. P., McCleskey E. W. et al. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature **392:** 622–626

143 Pawson T. and Scott J. D. (1997) Signaling through scaffold, anchoring, and adaptor proteins. Science **278:** 2075–2080

144 Garrington T. P. and Johnson G. L. (1999) Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr. Opin. Cell Biol. **11:** 211–218

145 Muller J., Ory S., Copeland T., Piwnica-Worms H. and Morrison D. K. (2001) C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol. Cell. **8:** 983– 993

146 Roy F., Laberge G., Douziech M., Ferland-McCollough D. and Therrien M. (2002) KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev. **16:** 427–438

147 Roy F. and Therrien M. (2002) MAP kinase module: the Ksr connection. Curr. Biol. **12:** R325–327

148 Kornfeld K., Hom D. B. and Horvitz H. R. (1995) The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. Cell **83:** 903–913

149 Sundaram M. and Han M. (1995) The *C. elegans* ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:** 889–901

150 Therrien M., Chang H. C., Solomon N. M., Karim F. D., Wassarman D. A. and Rubin G. M. (1995) KSR, a novel protein kinase required for RAS signal transduction. Cell **83:** 879–888

151 Schaeffer H. J., Catling A. D., Eblen S. T., Collier L. S., Krauss A. and Weber M. J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science **281:** 1668–1671

152 Wunderlich W., Fialka I., Teis D., Alpi A., Pfeifer A., Parton R. G. et al. (2001) A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J. Cell Biol. **152:** 765–776

153 Teis D., Wunderlich W. and Huber L. A. (2002) Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell **3:** 803–814

154 Miller W. E. and Lefkowitz R. J. (2001) Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. Curr. Opin. Cell Biol. **13:** 139–145

155 Goodman O. B. Jr, Krupnick J. G., Santini F., Gurevich V. V., Penn R. B., Gagnon A. W. et al. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2- adrenergic receptor. Nature **383:** 447–450

156 Laporte S. A., Oakley R. H., Zhang J., Holt J. A., Ferguson S. S., Caron M. G. et al. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc. Natl. Acad. Sci. USA **96:** 3712–3717

157 Luttrell L. M., Ferguson S. S., Daaka Y., Miller W. E., Maudsley S., Della Rocca G. J. et al. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science **283:** 655–661

158 Luttrell L. M., Roudabush F. L., Choy E. W., Miller W. E., Field M. E., Pierce K. L. et al. (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proc. Natl. Acad. Sci. USA **98:** 2449– 2454

159 McDonald P. H., Chow C. W., Miller W. E., Laporte S. A., Field M. E., Lin F. T. et al. (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science **290:** 1574–1577

160 Hayes S., Chawla A. and Corvera S. (2002) TGFbeta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. J. Cell Biol. **158:** 1239–1249

161 Tsukazaki T., Chiang T. A., Davison A. F., Attisano L. and Wrana J. L. (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell **95:** 779–791

162 Kholodenko B. N. (2002) MAP kinase cascade signaling and endocytic trafficking: a marriage of convenience? Trends Cell Biol. **12:** 173–177

163 Kholodenko B. N., Hoek J. B. and Westerhoff H. V. (2000) Why cytoplasmic signalling proteins should be recruited to cell membranes. Trends Cell Biol. **10:** 173–178