Electronic Supplementary Material (ESI) for Integrative Biology

Supplementary Material for "Systems Biology and Physical Biology of Clathrin-Mediated Endocytosis"

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Table S1: Important interactions between clathrin and other endocytic proteins

Protein	**Interaction Site**	**Description**
AAK1	Multiple heavy and light-chain interactions	Assembled clathrin activates AAK1's kinase activity on AP-2, mediating AAK1phosphorylation of AP-2 on Thr156
Amphiphysin	AMPH N-terminus	Extends clathrin coat beyond immediate region of AP-2 enrichment
AP-2	AP-2 β2-hinge domain	Clusters clathrin at membrane, uniformly orients clathrin to allow polymerization and display of β-propeller domain toward membrane
AP180/CALM	Clathrin box motif	Can act as adaptor/recruiter for clathrin; CALM binding to clathrin implicated in establishing monodispersity in CCV size
Epsin	Clathrin box motif	Normally, Epsin binding to clathrin is likely secondary to AP-2 and Eps15 binding; in some conditions Epsin may function independently as clathrin adaptor
GAK/Auxilin	Clathrin-binding motif	GAK binds clathrin and Hsc70 (via J-domain) simultaneously, bringing Hsc70 into required proximity for vesicle uncoating
Synaptojanin	C-terminal domain (synaptojanin 1 only; not synaptojanin 145)	The clathrin and AP-2 binding sites on synaptojanin (only splice variant synaptojanin 1) recruit syaptojanin to the membrane, where phosphatase action on PIP_2 destabilizes clathrin coat
Numb/Dab2/ ARH/β-arrestin	Varied	Endocytic adaptor proteins involved in the CME of GPCRs (β-Arrestin [1-4]), LDL receptor (Dab2/ARH [1]), EGFR (Epsin, Eps15 [5-9]), influenza virus (epsin [10]), and Notch receptor (Numb [11]); each recruits clathrin to membrane in cargo-specific manner

Table S2: Important interactions between AP-2 and other endocytic proteins

Protein	**Interaction Site**	**Description**
AAK1	Phosphorylates AP-2 μ2 subunit	Phosphorylation of AP-2 by AAK1 causes conformational change that allows tighter cargo and membrane binding
AP180/CALM	α- and β2- subunits, binding disrupted by clathrin binding	CALM possesses inherent ability to polymerize clathrin as does AP-2, but the two work synergistically in clathrin recruitment; CALM stays in the CCP after leaving AP-2 likely by binding to PIP_2
Cargo (TfR, EGFR, some GPCRs, some viruses)	Varies depending on receptor	AP-2 binds to cargo in order to sequester it inside the CCP; for the listed motifs it binds directly while other motifs are bound by linker proteins such as arrestins, Dab, etc.
Clathrin	Clathrin β-propeller N-terminal domain binds β2-hinge and α/β2 appendages	AP-2 recruits and tethers clathrin to the plasma membrane. For some cargo (LDLR, polyubiquitinated receptors) CCP formation can occur in the absence of AP-2, but AP-2 is constitutively associated with CCPs *in vivo*
Epsin	α/β2 appendage domains with DPW motif on epsin	Epsin can recruit AP-2 to the membrane by strongly binding multiple copies AP-2 with multiple DPW motifs
Eps15/R	α/β2 appendage domains with DPF motif on Eps15/R	Eps15 (in complex with intersectin and FCHo) recruits AP-2 to the membrane to nucleate coated pits; clathrin binding to AP-2 displaces Eps15 to the rim of growing CCPs
GAK/Auxilin	α appendage domain	See Table S1: interaction is similar to GAK/clathrin interaction
Synaptojanin	C-terminal domain binds to α-subunit ear	See Table 1: interaction is similar to synaptojanin/clathrin interaction
Numb/Dab2/ARH	PTB domain	Cargo-specific adaptors bind AP-2 as an intermediate to interact with clathrin *in vivo* but also possess innate clathrin-recruiting capability (Dab2/ARH can mediate CME in the absence of AP-2)
β-arrestin	α/β2 appendage domains	Similar to above cargo-specific adaptors; however arrestins are not displaced by clathrin/AP-2 binding, serving downstream sorting function for GPCRs
PIP Kinase Type Iγ	Activated by cargo-bound AP-2	Cargo-bound AP-2 interacts with kinase core domain of PIPK Iγ to increase membrane PIP_2

Table S3: Important interactions between Eps15 and other endocytic proteins		
Protein	**Interaction Site**	**Description**
AP-2		See Table S1
AP180/CALM	EH domain of Eps15 to CALM NPF motif	CALM functions as an alternative adaptor in CME; Eps15 plays similar role in CALM clustering and AP-2 clustering
Epsin	EH domain of Eps15 with Epsin NPF motifs	Epsin and Eps15 are both involved in sequestering polyubiquitinated CME cargo; interaction between Epsin and Eps15 may help cluster both at the membrane
EGFR	Activated EGFR phosphorylates Eps15 on Tyr850	EGFR phosphorylation of Eps15 is required for EGFR clathrin-dependent internalization; phosphorylation is dependent on ubiquitination of EGFR
FCHo ½		FCHo proteins colocalize with Eps15 and intersectin-1 in puncta prior to CCP formation; this complex is required for productive CCP nucleation
Intersectin	Multiple sites	Intersectin (Dap160) is a major binding partner of Eps15, forming a multimeric complex with 14 association points; intersectin links Eps15 to dynamin (via SH3 binding) and to mitogenic signaling pathways
Synaptojanin	EH-domain binding to synaptojanin	Synaptojanin may bind to Eps15 as a way to localize its activity to specific parts of the plasma membrane
Dynamin	via Intersectin SH3 domain	Eps15 regulates Dynamin localization via the Eps15-Intersectin complex, increasing dynamin recruitment to the plasma membrane early on in the CCP lifetime

Section S1. Epidermal Growth Factor Receptor Internalization via Endocytosis

Here, we specifically discuss EGFR endocytosis as an example a cargo-specific endocytic pathway. We propose a mechanism of EGFR sorting and endocytosis that has heretofore been inadequately elucidated.

S1.1 Specific Proteins for EGFR Endocytosis

EGFR is a receptor tyrosine kinase (RTK) that plays various signaling roles in cellular migration and proliferation as well as transcriptional regulation. Dysregulation of EGFR signaling is further implicated in the development of certain types of cancers, and endocytic degradation is essential to controlling the strength and duration of EGF-stimulated EGFR stimulation [12]. Normal endocytosis of EGFR is initiated by the activation of the receptor by EGF stimulation. Upon ligand (EGF) binding, EGFR undergoes dimerization and subsequent autophosphorylation [13]; this initiates the multiple EGFR signal transduction cascades while also beginning an EGF concentration-dependent mobilization of the EGFR internalization machinery [2, 14].

Cbl family proteins: in mammalian cells, two major Cbl family proteins, c-Cbl and Cbl-b, play major roles in EGFR endocytosis [13] while a third member of this group- Cbl-3-has been recently identified as playing a role in EGFR endosomal signaling [15]. Both c-Cbl and Cbl-b possess RING domains with E3 ubiquitin ligase activity, which is effectively 'switched on' by EGFR phosphorylation of the Cbl protein upon initial binding [16, 17]. Phosphorylation also targets the Cbl proteins for ubiquitination and proteasomal degradation, limiting total throughput for EGFR ubiquitination [18]. Indeed, the Src oncogene attenuates EGFR internalization by rapidly phosphorylating Cbl proteins [19].

The major difference between the two endocytic Cbl proteins is the ability of Cbl-b to bind ubiquitinated EGFR via its ubiquitin-associated (UBA) domain; the UBA domain of c-Cbl presents a different structure and is unable to mediate this binding. Functionally, while c-Cbl may only monoubiquitinate naked EGFR (at multiple sites), Cbl-b possesses the additional ability to bind monoubiquitinated EGFR and attach additional ubiquitin moieties to generate a polyubiquitin tail on the RTK. This additional binding capacity also affords Cbl-b an extra EGFR binding site. While direct binding of c-Cbl to EGFR is dependent on the phosphorylation of EGFR 1045Y, Cbl-b possesses an alternate binding site N-terminal to EGFR 957 [13].

Grb2: as an EGFR-specific adaptor, Grb2 plays a putative role in initially recruiting Cbl family proteins to activated EGFR. Once Cbl proteins are localized at the plasma membrane, they may bind directly to the EGFR or indirectly via this Grb2 interaction [20]. In the latter case, Grb2 associates simultaneously with the Cbl RING domain and one of two phosphotyrosine residues (1068Y or 1086Y) on the C-terminal tail of the EGFR. Multiple studies have shown the initial Grb2 recruitment of Cbl to be essential for normal EGFR internalization, while the Grb2-mediated indirect association between Cbl and EGFR makes possible a fraction of Cbl ubiquitin ligase activity [14].

CIN85: this Cbl-interacting protein complexes additionally with endophilin and may be responsible for certain modes of EGFR internalization [21]. Recent experimental evidence has cast doubt on the necessity of the CIN85-dependent internalization pathway, and reframed it as a redundant pathway that functions when the Cbl-EGFR 1045Y association is disrupted [17].

p38 MAP kinase: p38 is a mitogen-activated protein kinase (MAPK) involved in signal transduction relating to cellular stress and apoptosis. The initial ubiqutination of EGFR by c-Cbl or Cbl-b is dependent upon p38 association with the EGFR-Cbl complex, which allows EGFR to phosphorylate Cbl and activate its E3 ubiquitin ligase ability [22, 23]. In the absence of p38 (due to siRNA knockdown, mutation or pathological condition), activated EGFR cannot be ubiquitnated and instead is endocytosed via the Ub-independent rescue pathway (discussed below).

Other proteins: multiple other proteins are involved in mediating EGFR signaling and endocytic downregulation, but are outside the immediate scope of this review. Briefly, these include hSpry2 (human Sprouty) which prevents Cbl-EGFR interaction until hSpry2 phosphorylation by activated EGFR,

and Cdc42 which prevents sequesters Cbl to prevent its association with EGFR [24]. While these interactions are important members of the EGFR signaling pathway, their marginal relevance to the endocytic machinery precludes their further coverage.

S1.2 Multiple Modes of EGFR Internalization

EGFR polyubiquitination/recycling (pUb) pathway: polyubiquitination of activated EGFR requires the joint actions of c-Cbl and Cbl-b, and targets the RTK to the recycling endosome and eventually back to the plasma membrane. This pathway extends the signaling duration of the EGFR, and *in vitro* experiments have demonstrated that, at low levels of EGF stimulation, this pathway dominates EGFR endocytosis. At higher levels, the recycling pathway is saturated and excess activated EGFR is monoubiquitinated and degraded [8].

The initial steps of the two ubiquitin-dependent endocytic pathways are congruent. EGF docking at its receptor causes the RTK to dimerize, activating its kinase functionality. Multiple C-terminal tyrosines are autophosphorylated, and Grb2 docks at the activated EGFR via its SH3 or SH2 domain [14, 20]. The PTB protein recruits both c-Cbl and Cbl-b to the cargo site, although the temporal activity profile of the two Cbl proteins differs [13]. c-Cbl binds EGFR first, either directly or indirectly through Grb2, is phosphorylated and proceeds to monoubiquitinate the EGFR at multiple locations. Here, the two ubiquitin-dependent pathways diverge. In pUb, Cbl-b binds the monoubiquitinated EGFR via its UBA domain, and proceeds to attach a string of ubiquitin to the RTK. The polyubiquitinated EGFR is then bound by Ub-specific adaptors Epsin [7] and Eps15[25], and sequestered in a growing CCP. Since unmodified Eps15 cannot persist within a CCP, the phosphorylation of Eps15 850Y is required for this sequestration [26], suggesting that a PTB protein likely binds the EGFR-associated Eps15 to the CCP machinery. Aside from the Ub-specific adaptors, CME proceeds as described earlier in the review, and upon arrival at the early endosome, EGFR is targeted to the recycling endosome and sent back to the plasma membrane.

The quick saturation of the recycling pathway intrinsically provides the cell with a method of controlling the duration of EGFR signaling, and additionally affords predictions about wild-type biochemical parameters. Since both Cbl proteins are required for polyubiquitination, it is likely that either Cbl-b is present at far lower levels than c-Cbl, or that the rate constant for Cbl-b/EGFR-Ub association is lower than that for either Cbl or naked EGFR. The former hypothesis is bolstered by the fact that c-Cbl knockdown drastically increases the amount of EGFR trafficked via an ubiquitin-independent recycling pathway. Were Cbl-b more plentiful, an increase in EGFR monoubiquitination would be predicted upon c-Cbl knockdown.

EGFR monoubiquitination/degradation (mUb) pathway: in the mUb pathway, EGFR is monoubiquitinated at multiple sites, endocytosed in a clathrin-independent manner, and targeted for lysosomal degradation [23]. In wild-type cells, monoubiquitination occurs when EGFR is bound by only one of the two endocytic Cbl proteins. Since Cbl association with EGFR leads to Cbl phosphorylation and eventual proteasomal degradation, it is highly unlikely that one Cbl-b molecule can polyubiquitinate multiple copies of EGFR-Ub [18]. Thus, saturation of Cbl-b with EGFR-Ub will cause some EGFR to remain monoubiquitinated. Alternatively, if Cbl-b levels match or exceed those of c-Cbl, concentrated EGF stimulation could saturate both Cbl proteins, or saturate only c-Cbl leaving Cbl-b to monoubiquitinate naked EGFR.

While the mUb pathway utilizes the same adaptors, namely Epsin and Eps15 as pUb, the internalization of EGFR-Ub occurs via a clathrin-independent route involving caveolae. This distinct internalization mechanism is likely the basis for differential sorting once EGFR reaches the early endosome [25]; while pUb leads to receptor recycling, mUb causes EGFR to be sorted into the late endosome, to the multivesicular bodies and finally to the lysosome for its degradation.

In light of the above results, the saturability of the two ubiquitin-dependent pathways must be emphasized when interpreting cell studies of EGFR endocytosis. Often, the EGF concentration used to stimulate cells is far beyond the saturation point of the pUb pathway. Since the maximum throughput of the mUb pathway is much greater, the experimental protocol can cause results to be drastically skewed

toward mUb-based mechanisms. This problem may be compounded when transfected cell lines are used which display supraphysiological levels of relevant receptors and other proteins.

EGFR Ubiquitin-independent rescue (Ui) pathway(s): knockdown and mutation studies of Cbl proteins have shown that neither Cbl association nor ubiquitination of EGFR is required for its efficient internalization, suggesting that ubiquitous rescue pathways exist for EGFR internalization. If Cbl association with EGFR is preserved but ubiquitination is not, the likely rescue pathway involves interaction between Cbl and CIN85 (CIN85-dependent endocytosis is not ubiquitin-dependent but requires the association of Cbl). In the absence of any Cbl association, the persistence of EGFR internalization must be explained another way.

Since the EGFR possesses multiple YXXϕ motifs, it is feasible that the RTK may be endocytosed using AP-2 as an adaptor [11, 27]. In this case, EGFR would associate with the AP-2 μ2 subunit and be internalized in a similar manner to transferrin (Tf) receptor. Positive results from *in vitro* binding assays between EGFR and AP-2 support this result, as does the observation that vesicles from Cbl-independent EGFR internalization do not contain Eps15 [17]. This is consistent with the traditional, non-adaptor role of Eps15 in which it is displaced to the edge of the growing clathrin coat and fails to incorporate into budding vesicles.

The ubiquitin-independent rescue pathway may also be activated in the absence of EGF stimulation of the EGF receptor. When faced with cellular stress which hyperactivates the p38 MAP kinase cascade, EGFR can be endocytosed at high rates without Cbl interaction and ubiquitination. This p38-induced internalization does not result in EGFR degradation [22, 23], consistent with other observations of the rescue pathway as a recycling rather than a degradative endocytic route. The multifarious nature of EGFR internalization makes the interpretation of experimental results somewhat tricky, but we hope that the mechanisms described here will aid in the analysis of cell biological data in the framework of a quantitative signaling and trafficking module.

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