Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs

Linton M. Traub[1,*] and Gergely L. Lukacs[2]

[1]Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, 3500 Terrace Street, Pittsburgh, PA 15261 USA
[2]Program in Cell and Lung Biology, Hospital for Sick Children Research Institute and Department of Laboratory Medicine and Pathobiology, University of Toronto, 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada
*Author for correspondence (e-mail: traub@pitt.edu; glucas@sickkids.ca)

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Summary

Cargo selectivity is a hallmark of clathrin-mediated endocytosis. A wide range of structurally unrelated internalization signals specify the preferential clustering of transmembrane cargo into clathrin coats forming on the plasma membrane. Intriguingly, the classical endocytic adaptor AP-2 appears to recognize only a subset of these endocytic sorting signals. New data now reveal the molecular basis for recognition of other internalization signals, including post-translationally appended ubiquitin, by clathrin-coat-associated sorting proteins (CLASPs). Curiously, structurally related ubiquitin-recognition modules are shared by select CLASPs and the 26S proteasome, and recent work indicates that both display similar requirements for ubiquitin binding. During endocytosis, these modules engage oligoubiquitylated cargo in the form of polyubiquitin chains and/or multiple single ubiquitin molecules appended to different acceptor lysines. Functional separation between clathrin-mediated endocytosis and proteasome-dependent proteolysis is probably ensured by temporally regulated, local assembly of ubiquitin-tagged membrane cargo at sorting stations on the cell surface, shielding ubiquitin sorting signals from the proteasome. Thus, an expanded repertoire of CLASPs couples the process of clathrin-coat assembly with high-fidelity incorporation of assorted, cargo-specific sorting signals.

Key words: Clathrin, Cargo sorting, Ubiquitin, Adaptor, CLASP

Introduction

The original conception of clathrin-mediated endocytosis was beguilingly straightforward (Brown and Goldstein, 1979; Pearse and Robinson, 1990). Dedicated vesicular shuttles control the efficient conveyance of macromolecular nutrients into the cell interior or regulate the abundance and/or activity of certain transmembrane proteins with bound ligands at the cell surface. In this process, cargo molecules are gathered into a membrane specialization marked at the cytosolic face by a distinctive open polyhedral clathrin lattice. Rearrangement or introduction of pentagons into a planar hexagonal clathrin coat promotes invagination, mechanically deforming the underlying lipid bilayer and clustered cargo molecules into a nascent transport vesicle. Here, AP-2, the heterotetrameric plasma membrane adaptor (Ohno, 2006), executes the pivotal task of connecting the clathrin scaffold to the designated cargo being concentrated within the membrane-bound vesicle.

The operative trio of clathrin-adaptor-cargo still represents the core of contemporary models for clathrin-mediated endocytosis (Ehrlich et al., 2004; Ohno, 2006), bolstered by the findings that targeted homozygous disruption or mutation of genes encoding AP-2 subunits is lethal in *C. elegans* (Kamikura and Cooper, 2003; Shim and Lee, 2000), *Drosophila* (Gonzalez-Gaitan and Jackle, 1997) and mice (Mitsunari et al., 2005). However, we now appreciate that, in addition to these three chief components, at least 20 other proteins contribute to the assembly of clathrin-coated vesicles (Lafer, 2002; Perrais and Merrifield, 2005; Slepnev and De Camilli, 2000; Traub, 2005). Much effort has focused on unraveling the contribution(s) of these factors, often termed endocytic 'accessory' proteins. Some are catalytic and perform regulatory functions: adaptor-associated kinase 1 (AAK1) phosphorylates AP-2 directly to ensure local cargo loading only at assembling buds (Höning et al., 2005; Smythe, 2002); dynamin, a large GTPase, assembles into an oligomeric spiral that enwraps the base of deeply invaginated coated buds to promote scission upon concerted GTP hydrolysis (Praefcke and McMahon, 2004); and synaptojanin 1, a phosphoinositide polyphosphatase, hydrolyzes phosphorylated lipid head groups to terminate the coat assembly process (Di Paolo and De Camilli, 2006). Others, such as Hip1R (Engqvist-Goldstein et al., 2004), cortactin (Cao et al., 2003; Zhu et al., 2005) and a family of extended FCH (EFC or F-BAR) domain proteins (Itoh et al., 2005; Tsujita et al., 2006), appear to couple Arp2/3-dependent dendritic actin polymerization to the process of bud invagination and subsequent movement of the vesicle away from the plasma membrane following the fission event (Brett and Traub, 2006; Kaksonen et al., 2006; Perrais and Merrifield, 2005). But what a sizable number of the proteins massed at endocytic clathrin structures appear to do is govern precisely what cargo is gathered into the nascent vesicle. Elucidation of the biochemical features and operation of these clathrin-coat-associated sorting proteins (CLASPs) has clarified how distinct cargo types can be gathered non-competitively during the fabrication of these remarkable sorting nanomachines.

Congregation of transmembrane cargo within clathrin assembly zones is an active process, requiring a positive sorting signal. Numerous internalized proteins display a crucial cytosol-oriented, tyrosine-based endocytic signal such as YxxΦ (Φ representing a bulky hydrophobic residue) that binds directly to the μ2 subunit of AP-2 (Bonifacino and Traub, 2003; Owen et al., 2004). AP-2 also binds to a non-tyrosine-based internalization signal, the [DE]xxxL[LI] motif (Bonifacino and Traub, 2003; Höning et al., 2005), probably through an interaction involving the α and σ2 subunits (Coleman et al., 2005; Janvier et al., 2003). Yet other peptide-based endocytic sorting signals do not appear to associate directly with AP-2 (Bonifacino and Traub, 2003) or interfere with YxxΦ recognition (Warren et al., 1998) and, perhaps somewhat unexpectedly, do not cease to be internalized when AP-2 is extinguished by RNA interference (RNAi) (Hinrichsen et al., 2003; Keyel et al., 2006; Maurer and Cooper, 2006; Motley et al., 2003). Endocytosis of proteins bearing these alternative sorting signals is nonetheless clathrin dependent, clearly indicating that the coat has cargo-recognizing components other than AP-2.

Alternate CLASPs include the β-arrestins, which direct most, but not all (Van Koppen and Jakobs, 2004), activated G-protein-coupled receptors (GPCRs) to preexisting clathrin coats on the cell surface (Santini et al., 2002; Scott et al., 2002). Ligand-bound GPCRs are multiply phosphorylated by GPCR kinases (GRKs), often within the cytosol-oriented C-terminal segment; this creates a docking surface for β-arrestins (Lefkowitz and Shenoy, 2005). Tandemly arrayed binding motifs for clathrin and AP-2 in the β-arrestin C-terminus become exposed following engagement of a phosphorylated GPCR (Edeling et al., 2006; Gurevich and Gurevich, 2004; Kim and Benovic, 2002; Laporte et al., 2000; Schmid et al., 2006), driving rapid clustering of GPCRs in clathrin lattices already assembled on the plasma membrane (Santini et al., 2002; Scott et al., 2002). Effective downregulation of liganded GPCRs by β-arrestins depends on the AP-2- and clathrin-binding motifs and also on the ability of β-arrestins to engage phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and the GPCR cargo (Moore et al., 2007).

Other functionally related CLASPs likewise use combinatorial interactions involving AP-2, clathrin, PtdIns(4,5)P_2 and cargo to promote non-competitive uptake within clathrin-coated vesicles. For example, an FxNPxY signal ensures efficient internalization of the LDL receptor and other members of the LDL receptor superfamily (Chen et al., 1990). It is one type of tyrosine-based sorting signal not recognized by AP-2, and instead, associates with either of two phosphotyrosine-binding domain (PTB) proteins, Disabled-2 (Dab2) and the autosomal recessive hypercholesterolemia (ARH) protein, which localize to clathrin-coated structures in vivo (He et al., 2002; Keyel et al., 2006; Maurer and Cooper, 2006; Mishra et al., 2002a; Mishra et al., 2002b; Morris and Cooper, 2001). A somewhat different endocytic signal is post-translational modification with ubiquitin, which can be reversibly conjugated to target transmembrane cargo proteins, marking them for recognition by CLASPs. This locally-appended signal obeys many of the general principles that govern endocytosis of intrinsic peptide signals such as YxxΦ and FxNPxY, but it offers several advantages. The role of this particular signal in internalization and recent work that has shed light on the mechanisms involved are discussed below.

Ubiquitin – a reversible sorting signal

Ubiquitin is a 76-residue polypeptide reversibly conjugated to the ϵ-amino group of lysine residues via a cascade of at least three enzymatic reactions. Following the initial thioester linkage to the E1 ubiquitin-activating enzyme, ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. Substrate specificity is usually conferred by an E3 ubiquitin-protein ligase complex that either directly conjugates ubiquitin, or facilitates its transfer from the cognate E2 enzyme (Hicke et al., 2005; Hurley et al., 2006; Pickart, 2001). Additional cycles of ubiquitylation at one or more of the seven potential acceptor sites (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63) within the first ubiquitin molecule can generate structurally and functionally distinct polyubiquitin chains (Peng et al., 2003) (Fig. 1). Alternatively, a target can be monoubiquitylated at either a single or multiple sites (Haglund et al., 2003b) (Fig. 1C). In addition to the originally recognized role of Lys48-linked polyubiquitin chains in proteasome-mediated proteolytic degradation, it is now widely accepted that mono- and polyubiquitin conjugation controls a plethora of cellular processes, including the activity of signaling cascades, DNA replication and repair, protein targeting and virus budding (Hicke, 2001). The reversibility of ubiquitylation is ensured by a large group of deububiquitylating enzymes, which have important regulatory functions (Amerik and Hochstrasser, 2004; Nijman et al., 2005).

Post-translational attachment of ubiquitin to polypeptides has a fundamental role modulating the protein composition of the plasma membrane and the maintenance of cellular homeostasis. Lys48-based polyubiquitylation prevents the delivery of non-native membrane proteins to the surface by signalling their proteasome-dependent degradation at the ER or targeting them for vacuolar/lysosomal proteolysis from the Golgi compartment (Bonifacino and Traub, 2003; Ellgaard and Helenius, 2003). Ubiquitylation is also an endocytic and vacuoloar/lysosomal sorting signal for transmembrane proteins (Di Fiore et al., 2003; Dupre et al., 2004; Haglund et al., 2003b; Hicke and Dunn, 2003; Mosesson et al., 2003).

The first indications that ubiquitin signals the internalization of plasma membrane proteins came from *Saccharomyces cerevisiae*, in studies of the mating pheromone **a**-factor transporter Ste6p (Kolling and Hollenberg, 1994) and Ste2p, a GPCR for α-factor (Hicke and Riezman, 1996). Endocytosis-defective yeast strains accumulate the ubiquitylated forms of Ste6p and Ste2p, and genetic ablation of the ubiquitylation machinery is associated with stabilization of Ste2p and Ste6p. Subsequent studies showed that tightly regulated ubiquitylation of numerous transporters (for example, the uracil permease Fur4p, the general amino acid permease Gap1p and the tryptophan permease Tat2p) is indispensable for metabolic adaptation in yeast and targets transporters for vacuolar degradation via endocytosis from the cell surface or transport directly from the trans-Golgi network (TGN) (Bonifacino and Traub, 2003; Dupre et al., 2004).

In metazoans, ligand-induced downregulation of plasma membrane signaling molecules, including tyrosine kinase receptors and some of the GPCRs [for example, the epidermal growth factor (EGF), growth hormone (GH) and hepatocyte

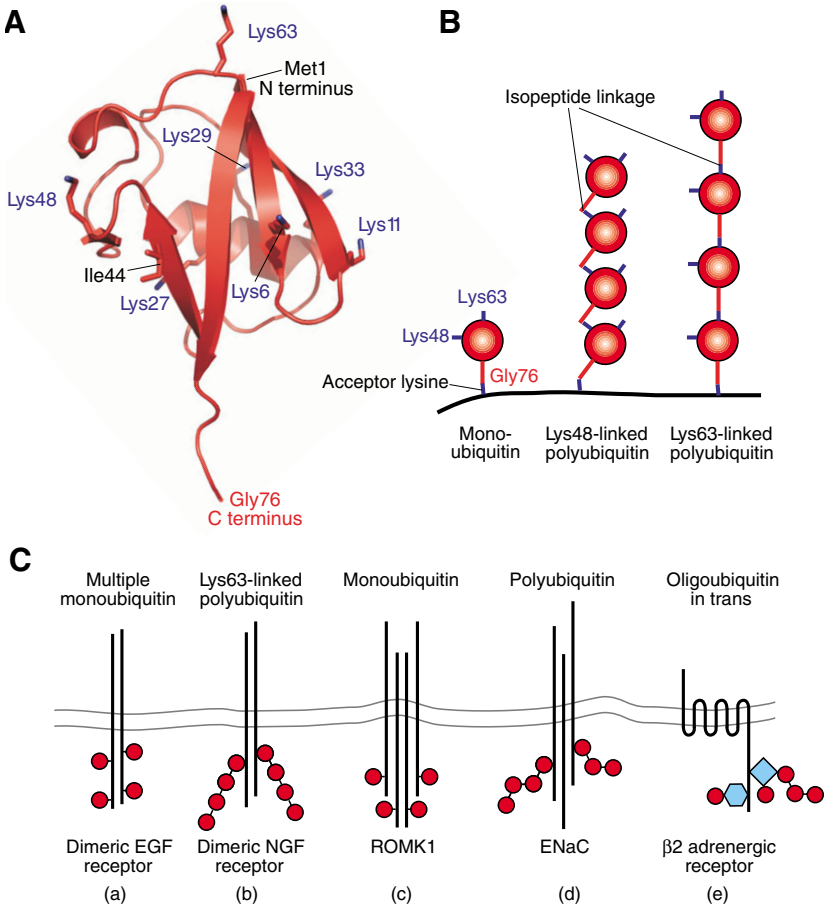

Fig. 1. Ubiquitin, isopeptide linkage and chain conformation variations. (A) Ribbon diagram of the main chain of the ubiquitin molecule (Protein Data Bank ID: 1UBQ) with the seven lysine side chains and Ile44 shown in stick representation. Ubiquitin is composed of a single antiparallel β-sheet curved over an adjacent α-helix. Solvent-exposed Ile44 demarcates the chief contact surface of the ubiquitin molecule (Hicke et al., 2005; Hurley et al., 2006), and the majority of the ubiquitin lysine residues are located in a circumferential band relative to this key residue. Lys63, however, is positioned opposite the extended C terminus and Gly76 necessary for isopeptide linkage to a target lysine side chain. (B) Schematic representation of monoubiquitylation, Lys48-linked or Lys63-linked polyubiquitylation of target lysine residues. Note that the differential positioning of the lysine within the acceptor ubiquitin dictates different polyubiquitin chain conformations and geometries. We use the term oligoubiquitylation to indicate the presence of either multiple monoubiquitins, a polyubiquitin chain(s), or a combination of these modifications. (C) Examples of different ubiquitin configurations in plasma membrane proteins internalized in mammalian cells. (a) multiple monoubiquitylation (dimeric EGF receptors, for example), (b) polyubiquitylation with Lys63 linkage (for example, dimeric NGF receptor TrkA), (c) monoubiquitylation of subunits of homooligomeric complexes (for example, ROMK1), (d) polyubiquitylation of heterooligomeric complexes (for example, ENaC), (e) oligoubiquitylation in trans (for example, β-arrestin 1/2 in complex with β_2 adrenergic receptor or insulin-like growth factor-1). Given the available evidence, we believe monoubiquitylation to be an inefficient endocytic signal in higher eukaryotes and therefore do not include it.

growth factor (HGF) receptors, CXCR4 and the β_2 adrenergic receptor] represents an important mechanism for signal attenuation and termination. This is achieved by the accelerated internalization and/or lysosomal targeting of ubiquitylated receptors (Katzmann et al., 2002; Raiborg et al., 2003). Those receptors that undergo constitutive internalization are downregulated by rerouting of the activated receptor from the recycling pathway to lysosomes for degradation by the ubiquitin-dependent endosomal sorting machinery, which consists of a network of ESCRT complexes (Katzmann et al., 2002; Raiborg et al., 2003). Most transmembrane proteins are ubiquitylated in cis – that is, one or more receptor lysines are coupled directly to ubiquitin through an isopeptide linkage. Yet some receptors, such as the GH and β_2 adrenergic receptors, are downregulated by ubiquitylation in trans: they associate with endocytic machinery that is ubiquitylated (Hicke and Dunn, 2003) (Fig. 1C). For example, ubiquitylation of non-neuronal β-arrestin 1/2 by the Mdm2 E3 ligase is essential for agonist-induced internalization and degradation of the β_2 adrenergic receptor (Shenoy et al., 2001). Here, the modification adds an additional layer of regulation and ensures prompt GPCR–β-arrestin uptake and signal modulation.

Ubiquitylation also plays a pivotal role in the endocytic trafficking of transporters and ion channels (such as the AMPA-type glutamate receptor, the glycine receptor, the ClC-5 chloride channel and the epithelial sodium channel, ENaC) modulating a plethora of cellular functions, including synaptic and postsynaptic plasticity, and transepithelial water and salt movements (Abriel and Staub, 2005; Hicke and Dunn, 2003). A variety of immune molecules (for example, MHC class I, B7-2 and CD4) are also downregulated by virally encoded E3 ligases, as part of a strategy to evade the immune response (Lehner et al., 2005).

In the examples above, the relevant E2-E3 complex recognizes small sequence motifs within the target. For instance, α-factor-induced serine phosphorylation of the Ste2p receptor cytoplasmic domain promotes binding of the E3 ligase Rsp5p and subsequent ubiquitylation. Growth factors provoke the tyrosine phosphorylation of their cognate receptors [the EGF, platelet-derived growth factor (PDGF) and HGF receptors, for example], which recruits the Cbl E3 enzyme through its SH2 domain. Recognition of proline rich (PPxY) motifs (as found in the TGF-β receptor, occludin, Roundabout, the ClC-5 channel and ENaC) is central to binding of WW-domain-containing E3 ligases (such as Smurf1/2, Nedd4 and WWP2) (d'Azzo et al., 2005; Staub and Rotin, 2006). In each case, interference with ubiquitylation impairs substrate turnover, triggers the accumulation of the target polypeptide and, ultimately, can cause severe human diseases. For example, accumulation of ENaC at the cell surface of renal epithelia upon deletion of its PPxY motif leads to Liddle syndrome, characterized by dysregulated renal ion transport that results in augmented Na^+ resorption and severe hypertension. The uncontrolled proliferation that can occur in cells expressing a mutant Cbl E3 that has defective ligase activity is a further example (Staub and Rotin, 2006).

Conformational destabilization has been proposed to induce oligoubiquitylation of plasma membrane proteins, and this may be related to a similar process that occurs in the ER (Laney and Hochstrasser, 1999). Ubiquitylation of mutant cystic fibrosis transmembrane conductance regulator (CFTR) (Sharma et al., 2004) and a yeast H^+-ATPase mutant (*pma1-10*) (Liu and Chang, 2006) in post-Golgi compartments is attributed to their partial unfolding. This would account their for preferential downregulation by lysosomal/vacuolar proteolysis and could involve ubiquitin-binding proteins that function in the endocytic pathway (for example Hrs, STAM1 and TSG101) (Liu and Chang, 2006; Sharma et al., 2004). Thus a peripheral quality control mechanism that exploits the ubiquitin-dependent internalization and endocytic sorting machinery could overcome the topological problem that degrading misfolded plasma membrane proteins presents. Such a mechanism might account for the expression defect and severe clinical manifestation typical of C-terminally truncated CFTR mutants (Sharma et al., 2004).

Monoubiquitin versus polyubiquitin as an internalization signal

Initial work showed that monoubiquitylation can drive uptake of Ste2p (Terrell et al., 1998) as well as the yeast maltose and galactose plasma membrane transporters (Horak and Wolf, 2001; Lucero and Lagunas, 1997). Subsequent studies showed, however, that attachment of a Lys63-linked polyubiquitin chain to Fur4p and Gap1p, although not an absolute requirement for endocytosis, significantly accelerates internalization (Dupre and Haguenauer-Tsapis, 2001; Galan and Haguenauer-Tsapis, 1997). Further analysis reveals multiple ubiquitin moieties are attached to Ste2p, the **a**-factor receptor (Ste3p) and the zinc transporter (Ztr1p), implying that polyubiquitin chains promote more efficient cargo recognition and/or retention than monoubiquitin (Dupre et al., 2004). Ste6p, the **a**-factor transporter, is also multiply ubiquitylated and five acceptor lysine residues are known (Kelm et al., 2004). It has also been demonstrated very convincingly that Rsp5p, the E3 ligase that governs ubiquitylation of numerous transmembrane proteins at the yeast plasma membrane (Dunn and Hicke, 2001a; Dupre et al., 2004), is able to generate (Lys63-linked) polyubiquitin chains in a robust fashion (Kee et al., 2006; McNatt et al., 2007; Oestreich et al., 2007). This clearly raises the possibility that oligoubiquitin may be the predominant endocytic sorting signal in yeast. Still, the contrary opinion that a single ubiquitin molecule drives efficient internalization and that monoubiquitylation is, in fact, the standard form of the ubiquitin-based sorting signal in all eukaryotes is widely held (d'Azzo et al., 2005; Di Fiore et al., 2003; Duan et al., 2004; Hicke and Dunn, 2003; Hicke et al., 2005; Mosesson and Yarden, 2006; Polo et al., 2003).

The notion that monoubiquitin is an endocytic signal in mammalian cells is primarily based on studies showing reactivity with the monoclonal antibody FK1, which detects polyubiquitin chains but not monomeric ubiquitin (Fujimuro et al., 1994), and/or on studies of the internalization of fusion proteins containing a ubiquitin moiety attached to a truncated EGF, PDGF or IL-2α receptor (Haglund et al., 2003b; Mosesson et al., 2003; Nakatsu et al., 2000). Since these receptors can homo- and/or heterooligomerize and may undergo multiple ubiquitylation-deubiquitylation cycles at the plasma membrane/endosomes (McCullough et al., 2004) owing to the physical proximity of E3 ligases and deubiquitinylating enzymes (Kee et al., 2005; Kee et al., 2006; Nijman et al., 2005), local presentation of oligomeric ubiquitin adduct(s) to the endocytic machinery cannot be ruled out. Indeed, accumulating evidence indicates that a single unextendible ubiquitin is insufficient to signal efficient internalization in higher eukaryotes (Barriere et al., 2006; Duncan et al., 2006; Geetha et al., 2005; Hawryluk et al., 2006).

Whereas fusion of one ubiquitin to a truncated IL-2α receptor, CD4 or the invariant chain, which associates with newly synthesized MHC molecules, dramatically increases their endocytosis, an Arg-for-Lys-substituted ubiquitin (UbAllR) that cannot undergo ubiquitin chain elongation, is unable to signal internalization (Barriere et al., 2006; Hawryluk et al., 2006). However, the defective endocytosis of the UbAllR chimera can be rescued by reintroduction of a single lysine residue at one of the several positions normally occupied by lysine (Barriere et al., 2006). Furthermore, formation of non-covalent oligomers of CD4-UbAllR by antibody crosslinking or by genetic means fully restores endocytic activity (Barriere et al., 2006). Indeed, ligand-induced internalization of the nerve growth factor receptor TrkA depends on formation of Lys63-linked adducts (Geetha et al., 2005). Here, numerous ubiquitins are conjugated to a single lysine reside (Lys483) in TrkA by the E3 ligase TRAF 6 (Geetha et al., 2005). Available evidence reveals that several activated receptor tyrosine kinases (such as EGF, PDGF and HGF receptors) undergo both multiple monoubiquitylation and extensive polyubiquitylation (Haglund and Dikic, 2005; Haglund et al., 2003b; Huang et al., 2006). This suggests that oligoubiquitylation is the rule rather than an exception in endocytosis. Although some membrane proteins do apparently undergo only monoubiquitylation (according to immunochemical detection), their multimerization ensures presentation of multiple ubiquitin molecules – for example, in the case of tetramerized ROMK1 potassium channels (Lin et al., 2005). Finally, monoubiquitin has a poor ability to promote endocytosis in *Drosophila*; this is evidenced by the markedly impaired downstream signalling of Delta in vivo following the replacement of its cytosolic domain with a single unextendible UbAllR (Wang and Struhl, 2004).

The best physiological evidence for natural polyubiquitylation orchestrating the highly efficient uptake of receptors from the cell surface comes from studies of immune cells. In immature dendritic cells, the β-chain of the MHC class II complex is endogenously ubiquitylated; a ladder of β-chain bands indicates conjugation of one to five ubiquitin monomers (Ohmura-Hoshino et al., 2006; Shin et al., 2006; van Niel et al., 2006). Because a single lysine residue (Lys225) within the cytosolic sector of the β-chain is entirely responsible for MHC ubiquitylation, the modification must be in the form of a polymeric ubiquitin chain (Ohmura-Hoshino et al., 2006; Shin et al., 2006; van Niel et al., 2006). Mature dendritic cells halt MHC class II internalization to facilitate antigenic peptide presentation. They do so by preventing the ubiquitylation of the β-chain specifically, rather than inhibiting endocytic uptake in general: non-ubiquitylatable MHC class II stagnate at the cell surface (Shin et al., 2006; van Niel et al., 2006). The ligase that catalyses its polyubiquitylation is MIR [also termed

MARCH (membrane-associated RING-CH)-VIII] (Ohmura-Hoshino et al., 2006), a cellular relation of the viral modulator of immune recognition 1 and -2 (MIR1 and MIR2, also termed K3 and K5, respectively) proteins, a pair of E3-ubiquitin ligases encoded by the Kaposi-sarcoma-associated herpesvirus (Coscoy et al., 2001; Hewitt et al., 2002). These E3 ligases clear several immune recognition molecules, such as MHC class I, B7.2, ICAM-I, CD1d and the interferon γ receptor 1 (Li et al., 2007), off the surface of infected B lymphocytes or other host cells as a way of evading detection by cytotoxic T lymphocytes (Lehner et al., 2005). Again, electrophoretic analysis of the MIR1/2-modified membrane receptors reveals a ladder of bands indicative of polyubiquitylation (Cadwell and Coscoy, 2005; Coscoy et al., 2001; Duncan et al., 2006; Hewitt et al., 2002). Lys63-linked polyubiquitylation of MHC class I molecules is indispensable for their downregulation by MIR1/2, and preventing the formation of the Lys63 linkage or restricting the modification to monoubiquitin severely compromises MHC class I endocytosis (Duncan et al., 2006). MIR1 can even polyubiquitylate lysine-less targets on cysteine residues, and a single cytosolic cysteine residue is sufficient (Cadwell and Coscoy, 2005). The fact that virally encoded E3 ligases preferentially generate ubiquitin chains to clear host molecules off the cell surface argues strongly for the polymer being a highly effective sorting signal.

Distinct structural requirements for ubiquitin-dependent endocytosis may thus exist in yeast and higher eukaryotes. A possible explanation for favored use of monoubiquitin as an internalisation signal in yeast might be that endocytic CLASPs have a higher affinity for ubiquitin in yeast compared with mammalian cells, but this speculation awaits experimental verification. Notably, the ubiquitin-binding adaptors have surprisingly flexible recognition capability in higher eukaryotes in vivo, capturing both polymeric and oligomeric forms of ubiquitin that display different topologies (see above). This might explain the equally efficient internalization of oligomeric and monomeric plasma membrane proteins subject to monoubiquitylation, multiple-monoubiquitylation and/or polyubiquitylation, respectively; for example in ROMK1 (Lin et al., 2005), ENaC (Staub et al., 1997), EGF receptor (Haglund et al., 2003a; Huang et al., 2006; Mosesson et al., 2003), β_2 adrenergic receptor–β-arrestin complex (Shenoy et al., 2001), CD4 (Bartee et al., 2004), and MHC class I and B7.2 (Coscoy et al., 2001) (Fig. 1).

Recognition of ubiquitylated cargo by CLASPs

The ubiquitin-interacting motif (UIM) was identified computationally (Hofmann and Falquet, 2001), following prior mapping of the ubiquitin-binding region of the S5a subunit (Rpn10p in *S. cerevisiae*) of the 26S proteasome (Young et al., 1998). It is a conserved ΦxxAxxxSxxAc peptide sequence (Ac is an acidic residue) that forms a single α-helix to contact ubiquitin across the vital Ile44-containing surface (Hicke et al., 2005; Hofmann and Falquet, 2001; Hurley et al., 2006; Swanson et al., 2003; Wang et al., 2005). Multiple UIMs are tandemly arrayed in the endocytic proteins epsin 1/2 and eps15/eps15R (epidermal growth factor receptor substrate) and in the yeast epsin orthologues Ent1p and Ent2p (Hofmann and Falquet, 2001) (Fig. 2). The *S. cerevisiae* eps15 orthologue, Ede1p, lacks UIMs but instead has a single UBA (ubiquitin associated) domain at the extreme C terminus (Fig. 2). Since the discovery of the UIM, it has been presumed that the ubiquitin endocytic sorting signal is recognized by epsin/eps15/eps15R in metazoans and by Ent1p/Ent2p and Ede1p in yeast (Aguilar et al., 2003; Di Fiore et al., 2003; Dupre et al., 2004; Polo et al., 2002; Shih et al., 2002; Wendland, 2002). These CLASPs associate with phospholipids, AP-2, clathrin and each other through conserved domains and peptide signals (Fig. 2) (Aguilar et al., 2003; Owen et al., 2004). Therefore, they probably oversee the assembly of heterooligomeric protein complexes and clathrin coats in a similar manner to the heterotetrameric AP-2 adaptor. This makes them prime candidates for proteins that link ubiquitylated cargo to the clathrin-dependent endocytic pathway. Internalization of ubiquitylated cargo is indeed unaffected by loss or genetic inactivation of AP-2 subunits (Hinrichsen et al., 2003; Huang et al., 2004; Huang et al., 1999; Motley et al., 2003; Nesterov et al., 1999; Yeung et al., 1999), which is consistent with the idea that it is AP-2-independent.

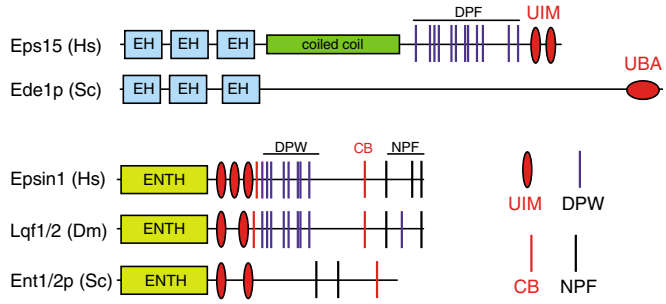

Fig. 2. Ubiquitin-binding CLASPs. Schematic illustration of the domain organization of eps15/15R and epsin 1 from human (Hs), *S. cerevisiae* (Sc) and *Drosophila* (Dm). The ubiquitin-interacting motif (UIM), ubiquitin associated (UBA) domain, AP-2 binding Asp-Pro-Trp/Phe (DP[WF]) sequences, clathrin-binding (CB) sequences and EH-domain-binding Asn-Pro-Phe (NPF) motifs are indicated. The epsin N-terminal homology (ENTH) binds to PtdIns(4,5)P_2.

The functional relevance of ubiquitin-binding CLASPs has been directly demonstrated by genetic deletion and RNA interfernce (RNAi) experiments. Internalization of Ste2p is severely inhibited in an *ent1Δent2Δede1Δ S. cerevisiae* strain, and complementation with an Ent1p mutant lacking the UIMs is inefficient (Shih et al., 2002). The *Drosophila* epsin orthologue, Liquid facets, is also indispensable for internalization of and downstream signaling by ubiquitylated Delta (Fischer et al., 2006; Overstreet et al., 2004; Wang and Struhl, 2005) (see below). In mammalian cells, simultaneous gene silencing of eps15/eps15R and epsin retains ubiquitylated EGF receptors at the cell surface (Sigismund et al., 2005). RNAi directed at eps15/eps15R or epsin 1 profoundly impedes the internalization of the CD4-ubiquitin chimera, activated β_2 adrenergic receptor, as well as the MARCH-IV-ubiquitylated CD4 (Barriere et al., 2006). Clathrin-dependent uptake of Lys63-polyubiquitylated MHC class I is reduced by epsin depletion (Duncan et al., 2006), as is internalization of Nedd4-ubiquitylated dopamine transporter (DAT) upon epsin/eps15/eps15R gene silencing (Sorkina et al., 2006). Thus the relative contribution of eps15/eps15R and epsin to ubiquitin-conjugated membrane protein recognition and

internalization may have cargo-, ubiquitin-chain- and cell-specific characteristics.

Biochemical analysis shows that the UIMs of mammalian endocytic adaptors have surprisingly limited affinity for monoubiquitin in vitro. The N- and C-terminal UIMs of eps15 have low to negligible affinity for monoubiquitin ($K_D \approx 0.9$ mM and >10 mM, respectively) measured by surface plasmon resonance (Fisher et al., 2003), whereas isolated GST-UIM fusion proteins show the opposite relative affinities in pull-down assays (Polo et al., 2002). By contrast, the UIMs of Ent1p can bind to GST-ubiquitin that, owing to the tendency of GST to homodimerize, probably represents dimeric ubiquitin (Shih et al., 2002). Thus the substrate specificity of eps15 and epsin UIMs appears to reflect their inability to recognize monoubiquitin and instead depends on avidity-dependent recognition of oligoubiquitin in vivo. Both epsin and eps15 UIMs preferentially associate with polyubiquitin chains over monoubiquitin (Hawryluk et al., 2006; Miller et al., 2004; Polo et al., 2002; Sugiyama et al., 2005), as do the UIMs of the S5a proteasome subunit (Wang et al., 2005; Young et al., 1998). Furthermore, these UIMs are virtually unable to discriminate between Lys63- and Lys48-linked polyubiquitin chains in vitro (Hawryluk et al., 2006; Wang et al., 2005). Importantly, selective recognition of tetraubiquitin chains by a UIM also occurs in vivo; an intramolecular association between a UIM and a Lys48-linked chain of at least four ubiquitins appended to a single lysine is typical of Met4p, a *S. cerevisiae* transcription factor (Flick et al., 2006).

It is plausible that in vivo ubiquitin-binding specificity and affinity are influenced by sequences flanking the UIMs, and/or by homo- and hetrooligomerization, lipid binding, phosphorylation and ubiquitylation of adaptors (Fisher et al., 2003; Hoeller et al., 2006). Although the ubiquitin-binding properties of endocytic adaptors have not yet been systematically compared with respect to these variables, studies of recombinant CLASPs attached to liposomes suggest that the selectivity of the UIMs for polyubiquitin chains over monoubiquitin is preserved (Hawryluk et al., 2006). Moreover, complexes of epsin 1 with AP-2 and clathrin exhibit a high selectivity for polyubiquitin (Hawryluk et al., 2006), which apparently rules out the possibility that binding of epsin 1 to ubiquitin and binding to AP-2 and clathrin are mutually exclusive (Chen and De Camilli, 2005). Finally, HeLa-cell-derived or recombinant eps15 preferentially engages polymeric ubiquitin over monoubiquitin, which is in accord with results obtained with the isolated UIM domains (Barriere et al., 2006; Hawryluk et al., 2006).

The internalization pathway of ubiquitylated cargo proteins

Most plasma membrane proteins that undergo ligand-induced or constitutive oligo/polyubiquitylation, including CD4, MHC class I, β_2 adrenergic receptor, ENaC, the anthrax receptor, EGF, GH and PDGF receptors (Bonifacino and Traub, 2003; Hicke and Dunn, 2003) as well as the FcγIIRA (Mero et al., 2006) are internalized within clathrin-coated vesicles. Both caveolin- and clathrin-dependent mechanisms, however, have been invoked for uptake of ubiquitylated EGF and PDGF receptors (Chen and De Camilli, 2005; Dupre et al., 2004; Johannessen et al., 2006; Sigismund et al., 2005; Stang et al., 2004). Nevertheless, recent morphological, biochemical, pharmacological and genetic approaches indicate that the internalization pathway of three different ubiquitin-chimeras is independent of caveolin but clathrin-mediated (Barriere et al., 2006; Hawryluk et al., 2006). These results agree with numerous reports that epsin 1 and eps15/eps15R localize to clathrin-coated buds and vesicles (Benmerah et al., 1995; Chen et al., 1998; Chen et al., 1999; de Melker et al., 2004; Drake et al., 2000; Stang et al., 2004; Tebar et al., 1996; van Delft et al., 1997) but are excluded from caveolae (Hawryluk et al., 2006). The conflicting data on the EGF receptor may simply reflect the fact that, at high EGF concentrations, EGF receptors (and other receptor tyrosine kinases) are internalized en masse by a clathrin-independent macropinocytic process (Orth et al., 2006; Sigismund et al., 2005; Yamazaki et al., 2002).

Ubiquitylated adaptors

The molecular mechanisms governing ubiquitin-dependent endocytosis are anticipated to be highly dynamic and reversible, ensuring not only clustering of cargo within clathrin-coated structures but also its rapid release upon coat disassembly and arrival at the intracellular destination. Since ubiquitylation of both cargo and UIM-containing adaptors occurs in vivo and the UIM itself directs monoubiquitylation of UIM-harboring CLASPs (Chen et al., 2003; Miller et al., 2004) (Fig. 3A), it is technically challenging to unravel the overall dynamics. One possible way to generate polyvalent ubiquitin-UIM interactions is through multimerization of eps15/eps15R and epsin. Eps15/eps15R homooligomerize using the coiled-coil domain (Cupers et al., 1997) and associates with epsin through the EH domains (Chen et al., 1998). Homo- or heterooligomerization as well as autoactivation of adaptors may be attained by their monoubiquitylation; promoting deubiquitylation would then facilitate disassembly of the complexes (Fig. 3B). The latter occurs upon elevation of cytoplasmic Ca^{2+} concentration at the synapse (Chen et al., 2003). The autoactivation of UIMs upon monoubiquitylation of eps15/eps15R and epsin could function allosterically (Fig. 3B) (Di Fiore et al., 2003; Hicke et al., 2005). Indeed, inhibiting ubiquitylation by depleting the Rsp5p E3 ligase in yeast (Dunn and Hicke, 2001b) or inactivating the E1 enzyme in ts20 cells (Barriere et al., 2006) attenuates cargo uptake, which is consistent with the notion that ubiquitylation of components of the endocytic machinery itself facilitates recruitment of ubiquitylated cargo. Note, however, that a scenario in which deubiquitylation is inhibited by Rsp5p or E1 inactivation cannot be formally ruled out.

Monoubiquitylation of soluble adaptors may also inhibit recognition of ubiquitylated cargo by inducing intramolecular UIM-ubiquitin interactions (Fig. 3C). This occurs in the case of subset of adaptors for EGF receptor endocytosis (Sts1, Sts2, eps15 and Hrs) following overexpression or in-frame fusion of ubiquitin (Hoeller et al., 2006). It leads to the stabilization of activated EGF receptors by impeding endocytic sorting (Hoeller et al., 2006). Establishing the physiological significance of such adaptor autoinhibition will require additional studies. Naturally, formation of adaptor networks through ubiquitin-independent intermolecular interactions involving peptide motifs, lipid-binding or auxiliary binding proteins probably complements this and enhances the avidity for oligoubiquitylated cargo (Aguilar et al., 2003) (Fig. 3D).

Ubiquitin-dependent endocytosis

What advantage does posttranslational conjugation of ubiquitin adducts provide in vivo and why are there so many CLASPs? Studies of the developmental patterning of external mechanosensory bristles in *Drosophila* provide a good illustration. These organs comprise four differentiated cellular structures: an externally visible socket and shaft, and an internal sheath cell and neuron. During development, each cell arises after three rounds of asymmetric cell division of a sensory organ precursor (SOP) cell (Emery and Knoblich, 2006; Fischer et al., 2006; Le Borgne et al., 2005) (Fig. 4A). In the first round of division, one cell adopts the pIIa fate because the transmembrane receptor Notch drives a transcriptional response within it. The adjacent daughter cell, fated to become the neural precursor pIIb, promotes Notch signaling in the pIIa-fated cell by acting as the signal-sending cell but downregulates its own ability to transduce Notch signals. This coupled process is intimately regulated by clathrin-mediated endocytosis (Emery and Knoblich, 2006; Fischer et al., 2006; Le Borgne et al., 2005).

pIIb, the signal-sending cell, presents a transmembrane Notch ligand, Delta, to pIIa (Fig. 4B). Ligand engagement triggers proteolytic cleavage of the extracellular region of Notch, and then the pIIb cell internalizes the cleaved Notch-Delta pair. This is necessary for further proteolytic processing of Notch within pIIa and subsequent signal transduction. Internalization of Notch-Delta is clathrin/dynamin dependent, and governed by two functionally redundant but differentially expressed RING-type E3 ligases, Neuralized (Lai et al., 2001; Yeh et al., 2001) and Mind bomb (Lai et al., 2005; Wang and Struhl, 2005), together with the *Drosophila* epsin, Liquid facets (Cadavid et al., 2000). The E3 ligases ubiquitylate the cytosolic domain of Delta to promote internalization; when a short segment of the LDL receptor cytosolic domain encompassing the FxNPxY sorting signal replaces the native Delta cytosolic domain, Liquid-facets-independent Notch signaling ensues (Wang and Struhl, 2004). Still, a single ubiquitin molecule appears insufficient for maximal Notch activity in this system as well, because in-frame fusion of one UbAllR molecule in place of the cytosolic portion of Delta drives only exceptionally weak transcriptional activity of Notch targets in comparison with either wild-type Delta or the Delta-LDL receptor chimera (Wang and Struhl, 2004).

Delta and Notch are coexpressed on the surface of both the pIIa and pIIb cells (Fig. 4C). Each daughter can then, in principle, act as a signal-sending cell; what makes pIIb refractory to Delta on the abutting pIIa cell? Again, clathrin-mediated endocytosis is the key. During SOP cell division, several endocytic components localize cortically in an asymmetric crescent at metaphase and, consequently, are inherited unequally by the daughter cells. The E3 ligase Neuralized and the CLASP Numb are two of these asymmetrically inherited proteins, partitioning selectively into the neurogenic pIIb cell (Emery and Knoblich, 2006; Fischer et al., 2006) (Fig. 4C). One general advantage of the ubiquitin sorting signal is thus that only cells expressing the appropriate E3 ligase can drive endocytosis of particular cargo.

Other signaling receptors that are ubiquitylated, such as the EGF receptor, are targeted to multivesicular bodies/late endosomes for eventual lysosomal degradation. One reason for the preferential use of ubiquitin as an internalization signal might be that it might selectively route cargo to the late endosomal compartment. Indeed, selection of numerous cargo by the Hrs/STAM-positive bilayered clathrin coat – an atypical, non-budding clathrin structure, that was first described (Rosenbluth and Wissig, 1964) the same year endocytic clathrin-coated structures were identified (Roth and Porter, 1964) and promotes generation of intralumenal membranes within maturing multivesicular bodies – is ubiquitin dependent (Gruenberg and Stenmark, 2004). Both Hrs and STAM contain UIMs, and, in *Drosophila*, *hrs*-mutant alleles cause numerous, structurally distinct, signaling receptors to accumulate with abnormal concentrations of ubiquitin in enlarged endosomal structures that fail to progress properly to the lysosomal compartment (Jekely and Rorth, 2003).

Yet, the existence of only a single, dedicated trafficking itinerary for ubiquitylated cargo cannot be entirely correct. For example, ENaC depends on E3-mediated ubiquitin attachment and epsin 1 for rapid entry into an endosomal compartment (Wang et al., 2006), but can recycle back to the surface

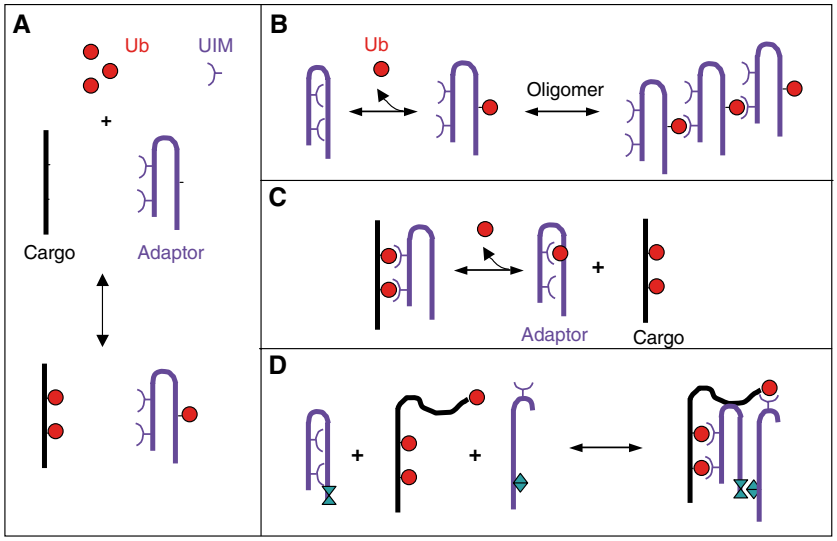

Fig. 3. Putative modulatory mechanisms of ubiquitylated cargo recognition by endocytic adaptors harboring UIM domains. (A) Ubiquitylation of both cargo and relevant UIM-containing endocytic adaptors (e.g. eps15/eps15R and epsin) may influence the internalization efficiency. (B) Adaptor autoactivation and formation of a signal relay network by adaptor ubiquitylation. Ubiquitin allosterically activates the UIMs. In addition, increased local concentration may facilitate the assembly of a polyvalent adaptor network at the cell surface through intermolecular ubiquitin-UIM interactions, manifesting in higher avidity for oligoubiquitylated cargo. (C) Adaptor autoinhibition by ubiquitylation. Monoubiquitylation-induced intramolecular interaction between ubiquitin and UIM attenuates adaptor affinity for ubiquitylated cargo. (D) Substrate-induced stabilization of heterooligomeric ubiquitin-binding adaptors. Peptide interactions may facilitate the association of ubiquitin-adaptors and synchronous presentation of multiple UIMs, increasing the avidity towards oligoubiquitylated substrate.

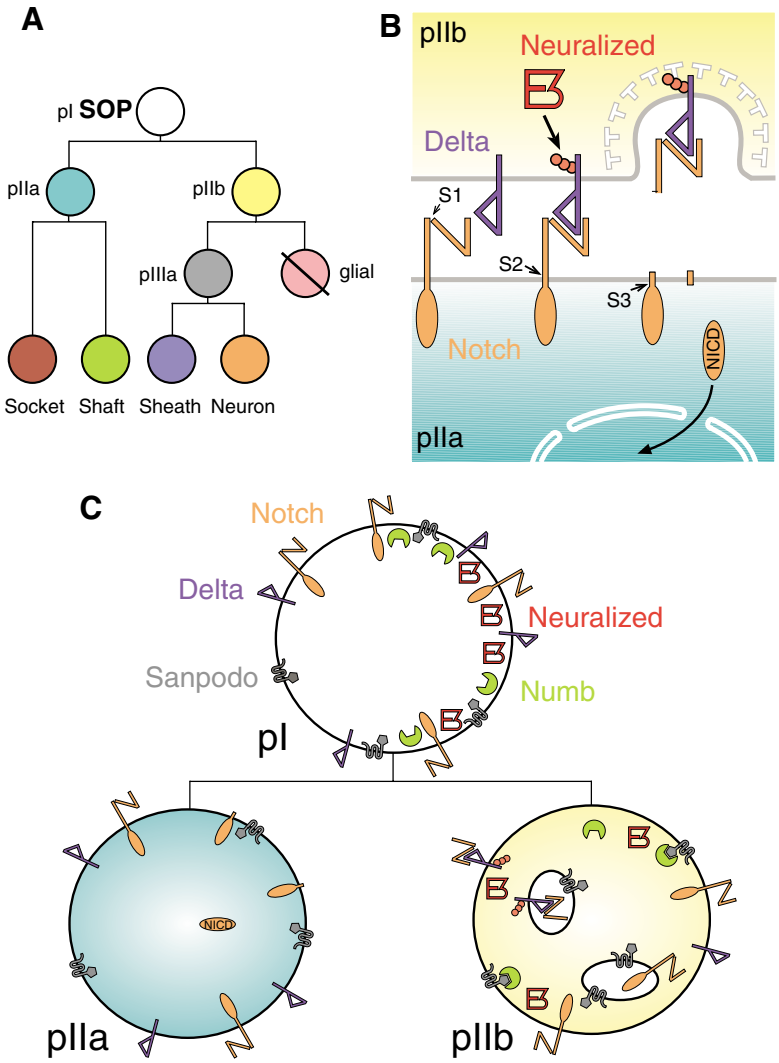

Fig. 4. Endocytic regulation of asymmetric cell division and cell fate determination in *Drosophila*. (A) Lineage of the four cell types, which together form the functional *Drosophila* sensory bristle, all derived from a single pI SOP precursor. One daughter from pIIb mitotic division undergoes apoptosis. (B) Notch receptor signal transduction in adjacent pII cells. The transmembrane Notch ligand, Delta, is presented at the surface of the signal-sending pIIb cell. During trafficking along the biosynthetic pathway, the extracellular domain of Notch is cleaved at the S1 site by furin. At the pIIa cell surface, ligand (Delta) engagement triggers the S2 site proteolytic processing event by an ADAM metalloprotease and coupled or subsequent endocytosis of the extracellular portion of Notch still bound to Delta. Uptake of the Delta-Notch complex depends upon Delta ubiquitylation by the E3 ligase Neuralized and packaging into clathrin-coated vesicles assembling at the surface of the pIIb by the CLASP Liquid facets (epsin). In the signal-receiving pIIa, the S2 cleavage promotes S3 site cleavage by γ-secretase to release the Notch intracytoplasmic domain (NICD), which then translocates to the nucleus and acts as a transcriptional co-activator. (C) Notch deactivation in the signal-sending pIIb. At metaphase, certain proteins, including Neuralized and Numb (PTB domain CLASP related to Dab2), localize to one pole of the cell. Following cytokinesis, only one sister, the pIIb, inherits these endocytic components, whereas both express Notch, Delta and Sanpodo. Numb promotes the clathrin-mediated internalization of Sanpodo, interfering with signal transduction of Notch on the pIIb cell surface, whereas Neuralized and Liquid facets drive clathrin-dependent endocytosis of the Delta-Notch binary complex. The overall effect of this endocytic activity is differential signal transduction in the pIIa and pIIb, leading to different cell fates and morphological characteristics.

following deubiquitylation (Butterworth et al., 2005). β-arrestins, too, are polyubiquitylated, and the precise site and duration of ubiquitylation determines the intracellular trafficking itinerary of the bound GPCR (Shenoy and Lefkowitz, 2005). For example, the β_2 adrenergic receptor is a so-called class A receptor that interacts selectively, but only transiently, with β-arrestin 2 following ligand activation, and recycles back to the cell surface promptly (Lefkowitz and Shenoy, 2005). By contrast, the type 1 angiotensin II (AT_1) and the type 2 vasopressin (V_2) receptors are class B GPCRs, which display a protracted association with β-arrestin 1/2 and return to the surface only slowly (Lefkowitz and Shenoy, 2005). β_2 adrenergic receptor stimulation promotes rapid but quickly reversed polyubiquitylation of β-arrestin 2 by the E3 ligase Mdm2 (Perroy et al., 2004; Shenoy and Lefkowitz, 2003; Shenoy et al., 2001), whereas V_2 receptor activation results in rapid and prolonged polyubiquitylation of β-arrestin (Perroy et al., 2004; Shenoy and Lefkowitz, 2003). The sustained presence of ubiquitin on the GPCR–β-arrestin complex is correlated with trafficking to spherical recycling endocytic structures (Shenoy and Lefkowitz, 2003); engineering β-arrestin ubiquitylation to be rapidly reversible converts the trafficking itinerary of the AT_1 receptor from class B to class A (Shenoy and Lefkowitz, 2005). Different trafficking outcomes can therefore also result from the plasticity of the signal; ongoing ubiquitin conjugation preferentially relays cargo to distal endosomal elements (Huang et al., 2006; Longva et al., 2002; Shenoy and Lefkowitz, 2005), whereas deubiquitylation favors prompt reinsertion into the plasma membrane.

In fact, it is now appreciated that E3-ubiquitin ligases and deubiquitylating enzymes coevolved, and can exist as specific co-complexes (Kee et al., 2005; Kee et al., 2006; Nijman et al., 2005). The interplay between these counterbalancing enzymatic pairs and the target probably generates different spatio-temporal ubiquitin signals on endocytic cargo and, thereby, discrete sorting decisions. For instance, controlled deubiquitylation probably accounts for the intracellular accumulation of internalized TRPV4 Ca^{2+}-channels following the oligoubiquitylation, catalyzed by the AIP4 E3 enzyme, that is required to downregulate the channel from the cell surface (Wegierski et al., 2006). Highly regulated oscillations of ubiquitin conjugation, removal and, perhaps, conformational rearrangements at precise intracellular locales also obviate a requirement for fundamentally distinct ubiquitin tags for proteasomal degradation and protein trafficking. So, although

it has now emerged that the biochemical features of the ubiquitin signal required for proteasomal degradation and membrane trafficking are tantalizingly similar (Kirkpatrick et al., 2006), in normal operation, endocytic ubiquitin signals are ordinarily inaccessible to the proteasome.

Perspective

Recent work has paired numerous internalization signals with cognate CLASPs, and mounting functional and genetic evidence provides strong support for the concept of an expanded spectrum of endocytic hardware that coordinates uptake but avoids deleterious competition or segregation. Beside the obvious advantage of enabling concentration of diverse and unrelated sorting signals within single nascent coated vesicles, CLASPs allow the system to be fine-tuned by regulating uptake of certain cargo independently. Ubiquitylation and other post-translational modifications add layers of complexity. The interrelationship between epsin and ubiquitin is clearly intricate. Not only does the UIM region facilitate polyubiquitin binding and monoubiquitylation of epsin 1 and -2 (and eps15) (Polo et al., 2002), Liquid facets is regulated by Fat facets, a deubiquitylating isopeptidase (Cadavid et al., 2000; Chen et al., 2002). In this instance, Fat facets appears to buffer the intracellular epsin concentration by salvaging proteasome destined, polyubiquitylated Liquid facets by deubiquitylating the protein (Chen et al., 2002). In fact, expression of a single extra copy of *Liquid facets* abolishes the requirement for Fat facets in the developing compound eye (Cadavid et al., 2000). These *Drosophila* data suggest then that during development, the intracellular abundance of epsin is crucial, presumably to effect the timely endocytosis of certain membrane proteins/receptors (like Delta), thereby facilitating appropriate cell fate determination (Cadavid et al., 2000; Chen et al., 2002) and underpinning whole developmental programs. This is in full accord with the idea of CLASPs operating as essential cargo-selective endocytic adaptor proteins. Next, in order to fully understand how CLASP activity is programmed spatially and temporally, we must delineate the molecular basis and biologic consequences of various regulatory inputs.

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