

Review

The epsin protein family: coordinators of endocytosis and signaling

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Abstract

The epsins are a conserved family of endocytic adaptors essential for cell viability in yeast and for embryo development in higher eukaryotes. Epsins function as adaptors by recognizing ubiquitinated cargo and as endocytic accessory proteins by contributing to endocytic network stability/regulation and membrane bending. Importantly, epsins play a critical role in signaling by contributing to epidermal growth factor receptor downregulation and the activation of notch and RhoGTPase pathways. In this review, we present an overview of the epsins and emphasize their functional importance as coordinators of endocytosis and signaling.

Keywords: endocytosis; epsin; notch; RhoGTPase; signaling.

Introduction

Endocytosis is the process by which cells internalize portions of the plasma membrane to uptake nutrients and to control their membrane composition and surface area. In addition to cellular homeostasis, this fundamental cellular process is crucial for the regulation of multiple signaling pathways. It is well-known that endocytosis attenuates signals by internalizing activated ligand-receptor complexes from the cell surface. However, a growing body of evidence demonstrates that endocytosis also activates signaling, for example, by contributing to the assembly of signaling endosomes from which new signaling events can be generated. This review discusses the epsin family of endocytic proteins and their role in coordinating endocytosis and signaling. For a more detailed chronological perspective and discussion of topics, such as epsin regulation, we refer the reader to an excellent review by Beverly Wendland (1).

The epsins

Brief overview of the epsin protein family

The epsins are a family of endocytic adaptor proteins conserved across evolution with crucial physiological roles. Thus, while in metazoans epsins are required for proper embryo development (2–5), in yeast, they are essential for cell viability (6–8). The founding member of the family, rat epsin-1 (Eps15 interacting protein 1) was discovered by the DeCamilli lab (Yale University) as an interaction partner of the endocytic accessory protein Eps15 (9). Sequence analysis of epsin-1 revealed striking similarities with the mitotic phosphoprotein-90 from *Xenopus laevis* (10) and two *Saccharomyces cerevisiae* proteins later identified as epsin homologs (6). Soon after, a second paralog, epsin-2 was discovered (11). Both, epsin-1 and -2 were characterized as plasma membrane-localized components of the clathrin-mediated endocytic machinery (9, 11). Although ubiquitously expressed, epsin-1 and -2 were found to be enriched in the brain (9, 11). In contrast, a third epsin paralog, epsin-3, was subsequently shown to have a very restricted expression profile that includes migrating keratinocytes (12), gastric parietal cells (13), and several carcinomas (12–15).

Saccharomyces cerevisiae and most vertebrates (including primates, rodents, and zebrafish) contain at least two epsin paralogs. In contrast, only one epsin gene is present in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. However, the single epsin gene in *D. melanogaster* produces two alternatively spliced forms (3, 16). In addition to these classical plasma membrane-localized epsins, there is a ubiquitous family of epsin-like proteins that display partial sequence similarities but possess several distinctive features. Specifically, these epsin-related (epsinR) proteins (also known as enthoprotin, clint, or epsin-4) have a different set of protein and lipid interaction partners, localize to the trans-golgi network/endosomes and participate in endosome to golgi transport (17–21). Therefore, a detailed description of this protein subfamily is beyond the scope of this review.

Domain organization of epsins

Domain organization of the epsins will be described from N- to C-terminus taking a generic mammalian epsin as a model (Figure 1). At the N-terminus, epsins bear a highly conserved, approximately 150 amino acid-long epsin

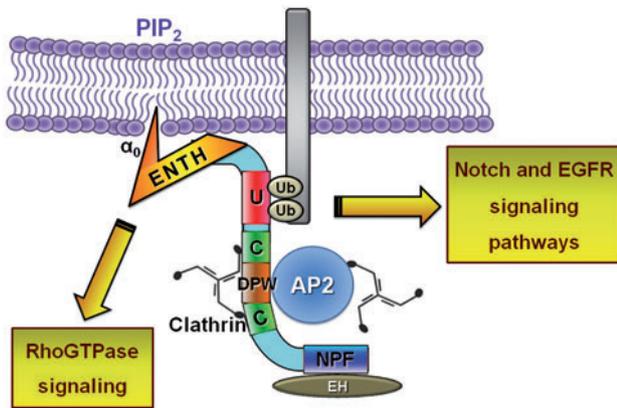


Figure 1 Schematic representation of a generic mammalian epsin. Cartoon represents typical epsin domain organization, known interaction partners, and signaling links. PIP₂, phosphatidylinositol-4,5-bisphosphate; ENTH, epsin N-terminus homology domain; α_0 , helix 0; Ub, ubiquitin; U, ubiquitin-interacting motif; C, clathrin-binding motif; DPW (or DPF), AP2-binding aspartate-proline-tryptophan (or aspartate-proline-phenylalanine) motifs; NPF, asparagine-proline-phenylalanine motif that binds to EH domain-containing proteins, such as Eps15 and intersectin; EH, Eps15 homology domain; AP2, clathrin assembly or adaptor protein 2. Figure is not drawn to scale; not all possible interactions are depicted.

N-terminal homology (ENTH) domain (22) [reviewed in (23)]. The structure of the mammalian ENTH domain has been solved by X-ray crystallography as well as by NMR spectroscopy (24–27), and the emerging structural consensus revealed a superhelical fold comprised of seven α helices followed by an eighth helix misaligned with the superhelical axis. Structurally, the ENTH domain is most similar to the VHS domain characteristic of the Vps27, Hrs, and STAM trafficking proteins, and it also contains *armadillo* and HEAT repeat segments found in β -catenin and karyopherin- β , respectively (24). The ENTH domain binds phosphatidylinositol 4,5-bisphosphate, a lipid enriched at different regions within the plasma membrane including endocytic sites (25, 27, 28) [reviewed in (29)]. Importantly, an elegant study from the McMahon lab demonstrated that this interaction triggers a dramatic conformational change that induces the formation of an additional N-terminal alpha-helix, helix0 (α_0) (27). Owing to its amphipathic nature, α_0 inserts into the cytoplasmic leaflet of the plasma membrane and promotes membrane curvature (27, 28, 30–32). This remarkable process is believed to be one of the initial steps of membrane invagination toward the formation of clathrin-coated pits (CCPs) and eventually of clathrin-coated vesicles [reviewed in (33–36)].

Following the discovery of ubiquitin interacting motifs (UIM) in a subunit of the 26S proteasome (37), the consensus UIM sequence was identified in several proteins involved in receptor-mediated endocytosis, including epsin (38). Most epsins contain two or three UIM copies that interact with ubiquitinated cargo and are located C-terminal to the ENTH domain (39–41) [reviewed in (1)]. Importantly, cooperative binding of the UIMs is required for high avidity and

ubiquitin-dependent recruitment of the yeast epsins to biological membranes (41). Furthermore, it has been proposed that multiple monoubiquitination or limited polyubiquitination (up to approximately four ubiquitin units) of cargo is essential for achieving sufficient interaction avidity for epsin recruitment (42–45) [reviewed in (46)].

The region between the UIMs and the very C-terminus of epsin is mostly unstructured (47) and displays high interspecies sequence divergence. However, this region also includes several conserved short signature motifs for binding various components of the endocytic machinery (corresponding to 5–13% of this region's sequence). Specifically, mammalian epsins possess two clathrin-binding motifs (CBM) that closely follow the consensus L ϕ Z ϕ Z motif (where L is leucine, ϕ and Z are amino acids with bulky hydrophobic and polar side chains, respectively) and bind to the terminal domain of clathrin heavy chain (48, 49) [reviewed in (50)]. These clathrin-binding sequences flank a cluster of three to eight DP[W/F] (aspartate-proline-tryptophan/phenylalanine) repeats that bind to the adaptor complex AP2 (9, 51–54). In fact, the resulting avidity of these AP2-binding motifs is so high that overexpression of an epsin-1 fragment containing its DP[W/F] motifs leads to severe impairment of AP2-mediated internalization by a dominant-negative mechanism (9).

C-terminal to the CBM-DPW cassette, two or three NPF (asparagine-proline-phenylalanine) tripeptide repeats are found. These motifs are recognized by the eps15-homology (EH) domain of proteins like eps15, intersectin, and POB1 (9, 55, 56).

This general architecture (Figure 1) is mostly preserved in higher eukaryotes with minor variations in the number of some binding motifs. In contrast, yeast epsins display significant differences. For example, the AP2-binding DP[W/F] motifs are absent, and a single clathrin-binding domain is present at their very C-terminus (6) (see Figure 2). Other deviations from this general architecture are present in the epsin ortholog of *Dictyostelium discoideum*. This epsin lacks UIMs but interacts with clathrin and AP2 at the plasma membrane (57) (Figure 2). In *Trypanosoma brucei*, the single ENTH domain-containing protein, TbEpsinR, displays features common to both epsin and epsinR. Similar to epsinR, TbEpsinR lacks UIMs, and a fraction is partially localized at endosomal membranes; however, unlike epsinR, a second population contributes to clathrin-dependent endocytosis at the plasma membrane (58).

Epsin and endocytosis

Epsins are proteins capable of fulfilling different roles at nascent endocytic sites. On one hand, as these proteins bear UIMs, they act as endocytic adaptors by directly binding to ubiquitinated cargo. On the other hand, epsins also interact with other elements of the endocytic machinery including the hubs of the endocytic network, i.e., AP2 and clathrin. Therefore, epsins function as an accessory protein by consolidating and regulating the endocytic network.

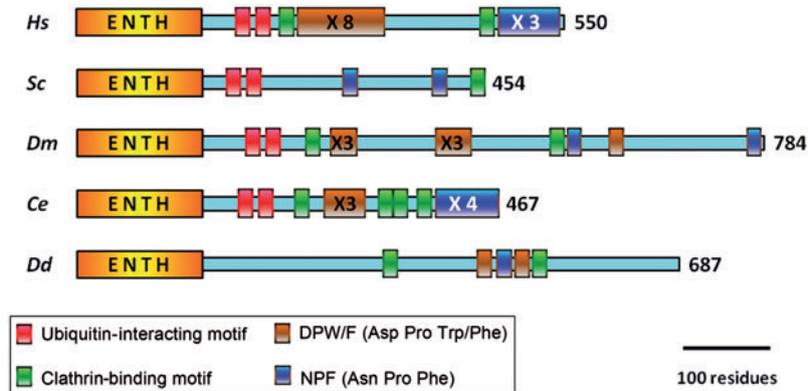


Figure 2 Comparison of domain architecture of epsins from different organisms.

Hs, *Homo sapiens* (NP_037465.2); *Sc*, *S. cerevisiae* (NP_010120); *Dm*, *D. melanogaster* (AAF05113); *Ce*, *C. elegans* (NP_510459); *Dd*, *D. discoideum* (XP_635269).

Epsin-specific cargoes

Although yeast epsins are known to participate in the internalization of several membrane proteins, such as the alpha-factor receptor Ste2 (40, 59), they are not essential for their endocytosis, i.e., to date, no physiological cargo has been found to be epsin specific. In higher eukaryotes, epsin is required for endocytosis of notch ligands (4, 60–62), and a body of evidence strongly implicates this adaptor in epidermal growth factor receptor (63–66) and epithelial sodium channel (67–69) internalization.

Notch ligands The notch signaling pathway is a conserved signaling module involved in cell fate determination [reviewed in (70)]. While genetic evidence obtained in *D. melanogaster* suggests that endocytosis is required in both signal-sending and -receiving cells for notch activation (71), other studies indicated that *Drosophila* epsin, liquid facets (Lqf) is only required in the signal-sending cell (60, 61). These observations imply that epsin contributes to notch activation by regulating/facilitating the endocytosis of the notch ligands delta/serrate/lag2 (DSL) (4, 60–62). Introduction of a tyrosine-based internalization signal (from the LDL receptor) in the cytoplasmic domain of delta (DI) could bypass the need for epsin in notch signaling (61), supporting the notion that the endocytic adaptor function of epsin is critical for this pathway activation. Moreover, the endocytosis and signaling of DI was found to be epsin specific, as disruption of AP2 had no significant impact (72). DI and serrate accumulate at the plasma membrane in cells deficient in mindbomb or neuralized, suggesting that these E3 ligases ubiquitinate DSL ligands for epsin-mediated internalization (61, 62, 73–77). Although the exact mechanism by which epsin-mediated endocytosis triggers notch activation is not fully understood, it is clear that it requires ubiquitination, epsin binding, and internalization of notch ligands. Details on the signaling relevance of epsins for this pathway are described in the section on ‘Epsin controls notch signaling activation via endocytosis’.

Epidermal growth factor receptor (EGFR) This receptor tyrosine kinase belongs to the erythroblastic leukemia viral oncogene homolog (ErbB) family consisting of four closely related members: ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4 (also designated as HER1–4 for human epidermal growth factor receptor). This protein family, particularly ErbB1, has been the focus of extensive research due to its role in cancer. Indeed, EGFR expression is frequently upregulated in many solid tumors where it promotes cancer progression [recently reviewed in (78)], thus making the use of EGFR inhibitors a powerful therapeutic approach. Importantly, deficient internalization of EGFR also leads to receptor hypersignaling and, in consequence, to malignant transformation (79, 80) [reviewed in (81)]. Therefore, the study of EGFR endocytosis has captured considerable interest. Classically, binding of ligands to the extracellular domain of their receptors not only induces downstream signal transduction but also promotes receptor endocytosis [reviewed in (82, 83)]. The ubiquitin-ligase Cbl is believed to initiate the internalization of EGFR (63) by promoting mono-, multi-, and polyubiquitination (63, 66, 84). However, whether ubiquitination is necessary and sufficient for internalization still remains to be fully established [reviewed in (85)].

Although there is some result variability, likely associated with knock-down efficiency and diversity of cell lines used, it is broadly accepted that internalization of ubiquitinated-EGFR is dependent on epsin and requires the integrity of its UIMs (43, 64–66). Whether this process is clathrin dependent or not has been a topic of debate. On one hand, it has been proposed that upon exposure to high (but still physiological) concentrations of ligand, EGFR undergoes extensive ubiquitination (64). Under these conditions, in addition to the classical clathrin-dependent internalization mechanism, a clathrin-independent, caveolae-, Eps15-, and epsin-dependent internalization route has been reported to occur for EGFR endocytosis (64). Interestingly, another report indicated that epsin binding to ubiquitin negatively affected its interaction with clathrin (86). Further, it was proposed that the

clathrin-independent pathway of EGFR internalization leads to degradation of cargo (i.e., to signaling termination), whereas the clathrin-dependent route promotes persistent signaling as well as recycling of EGFR (64, 87). In contrast, other studies have indicated that epsins are able to interact with both clathrin and ubiquitin simultaneously (43, 63, 65, 66) and that EGFR does not significantly colocalize with caveolar structures (88). These seemingly contradictory results likely suggest that EGFR endocytosis is a more complex process than anticipated. In fact, other posttranslational modifications besides ubiquitination have recently been found to contribute to EGFR uptake (84), raising the possibility of multiple endocytic routes and additional adaptors besides epsin (yet to be identified) being involved in the process.

Epithelial sodium (Na) channel (ENaC) This amiloride-sensitive sodium channel is primarily found in polarized epithelia in lung, kidney, colon, sweat ducts, and salivary glands. ENaC is a heteromer composed of two alpha, one beta, and one gamma subunit. This channel functions to maintain electrolyte balance and fluid movement across the epithelia [reviewed in (89)]. It is known that the ubiquitin-ligase neural precursor cell expressed, developmentally downregulated protein 4-2 (Nedd4-2) ubiquitinates and promotes ENaC internalization (90–93). In Liddle's syndrome where ENaC recognition by Nedd4-2 is affected, the channel activity is increased resulting in hypertension (94, 95).

ENaC was found in clathrin-coated vesicles along with epsin, and these proteins efficiently co-immunoprecipitated together in an epsin UIM-dependent manner (68). Furthermore, coexpression of ENaC with Nedd4-2 or epsin decreased ENaC-dependent currents, in contrast to a dominant-negative dynamin mutant, which increased such currents (67). This evidence implicates epsin in the downregulation of this sodium channel. Interestingly, the same authors reported that epsin was not implicated in the endocytosis of the K⁺ channel (68), renal outer medullary potassium channel (ROMK), even when it is also known to be subjected to ubiquitination (96).

In addition to the abovementioned cargoes, endocytosis of a number of other proteins seems to be dependent on epsins. For example, epsins play a role in the uptake of influenza virus bound to its receptor in mammalian cells (97). Epsin is also important for the clathrin-mediated internalization of the antigen-presenting MHC class I molecules following ubiquitination by a Kaposi's sarcoma-associated herpesvirus ubiquitin ligase (45, 98). Furthermore, epsin is involved in the internalization of insulin receptor (42), and a large-scale RNA interference study revealed that epsin contributes to the internalization of dopamine transporter in neurons (99). Additionally, epsin was shown to mediate the internalization of ubiquitinated G protein-coupled receptor protease activated receptor 1 (PAR1) (100). The *Drosophila* epsin homolog Lqf was reported to be required for the receptor-mediated endocytosis of *Drosophila* larval serum proteins into larval fat body cells from the hemolymph (101).

Epsin as an accessory protein

Besides its role in the internalization of ubiquitinated cargo, epsins are believed to participate in the stabilization of the endocytic network. An interesting study from the Schmid laboratory (Scripps Institute) demonstrated that CCPs undergo maturation into functional clathrin-coated vesicles only if they successfully pass through an 'endocytic checkpoint' (102). The checkpoint verifies, via protein-protein interactions, a critical mass of cargo, endocytic components, and other factors (102). Failure to reach this critical mass leads to destabilization of the immature CCPs, which subsequently abort (102). This observation is in agreement with a previous study that demonstrated that stochastically initiated CCPs undergo rapid collapse if conditions for stabilization are not met (103). Epsin-1, along with CALM and SNX9, contributes to this endocytic checkpoint, and even a partial depletion of epsin-1 (by 40%) leads to an increase in the proportion of abortive CCPs (104). Epsins in yeast are also recruited during the early stages of clathrin coat formation (105) and participate in the assembly of clathrin-coated areas (106). Furthermore, the yeast epsins play a similar role in expanding and sustaining the endocytic network via protein-protein interactions mediated by their UIM and NPF motifs (59, 107). In addition, epsins might be involved in other regulatory mechanisms affecting the endocytic network, for example, in *Dictyostelium*, epsin has been implicated in facilitating phosphorylation of the actin-binding adaptor protein huntingtin-interacting protein 1 related (Hip1r) (108, 109).

Epsin and signaling

The process of endocytosis plays a dual role in signaling regulation. It not only desensitizes cells by downregulating activated receptors (e.g., EGFR) but is also required for the activation of other signaling pathways (e.g., via internalization of notch ligands). Importantly, epsins are functional in both the modes of signaling regulation. The role of epsins in signal termination is directly linked to their ability to support endocytosis of receptors (as described in the 'Epsin and endocytosis' section) targeted for lysosomal degradation. The function of epsins in signaling activation has been linked to the notch developmental pathway and RhoGTPase signaling.

Epsin controls notch signaling activation via endocytosis

Endocytosis is critical for the activation of the notch signaling pathway. It has been established that Lqf is essential for proper signaling by the DSL ligands (60, 61). *lqf*⁻ clones showed no overall disruption of global endocytosis, but DI was unable to signal to receiving cells (61). In agreement, epsins' relevance for embryogenesis and development has been clearly demonstrated: Lqf null mutation is embryonic lethal (2), and flies overexpressing epsin or expressing mutant epsin alleles display severe eye, wing, and leg morphology defects (3, 110). Similarly, epsin mutations, in both

D. melanogaster and *C. elegans*, led to defects in germline and heart development due to deficiencies in notch signaling (4). A detailed study on the phenotypes of double epsin-1/-2 knockout (KO) mice revealed that embryos did not survive beyond E10.5, while single epsin KO pups were healthy (5). However, embryonic fibroblasts taken from double epsin KO embryos did not show abnormalities in general clathrin-mediated endocytosis. The major cause for the lethality of the embryos was the disruption of the extraembryonic structures, defects in cardiovascular development, somitogenesis, and neural tube differentiation of the embryo (5). Interestingly, these phenotypes are highly comparable to those produced by impairment of the notch pathway. These studies suggest that although epsin is dispensable for general endocytosis, it may be critical for cargo-specific signaling functions.

Epsin contributes to RhoGTPase signaling activation

Cdc42 is a highly conserved member of the RhoGTPase family that acts as the master regulator of cell polarity [reviewed in (111)]. This signaling protein was first identified in yeast through mutants that led to a cell division arrest and loss of actin cytoskeleton polarization (112). Indeed, Cdc42 regulates the actin and microtubule cytoskeletons [reviewed in (111)] and modulates membrane trafficking [reviewed in (113)] among other functions. Complementary to their endocytic role, epsins are involved in the regulation of this key cell polarity protein (8). Mechanistically, the epsin's ENTH domain interacts with and sequesters/inhibits yeast Cdc42 GTPase-activating proteins (Cdc42 GAPs) (8). This interaction has been proposed to relieve the GAP-induced Cdc42 inhibition and thereby to promote Cdc42 signaling activation. Interestingly, this function is analogous to the one described for the epsin-interacting protein intersectin that sequesters/inhibits a Cdc42 GAP, CdGAP (114), and displays GEF activity toward Cdc42 (115).

In yeast, the ENTH domain is necessary and sufficient to sustain cell viability upon epsin deletion (6, 8). Furthermore, the specific residues required for this essential function of the ENTH domain were also identified. As expected, ENTH domain mutations in these residues (ENTH^{Y100R} and ENTH^{T104D}) led to defects in Cdc42 activation, reduced viability, and actin cytoskeleton depolarization (8). In agreement, in *Dictyostelium discoideum*, T107, the residue analogous to the yeast T104, was essential for normal actin dynamics at the membrane (109) as well as for the development of normal spore morphology (57). Interestingly, yeast epsin Ent2 has also been found to have physical and genetic interactions with the only *S. cerevisiae* Cdc42 GEF (116, 117). In addition, the ENTH domain of Ent2 bears an additional activity in cell division signaling pathways (118) that is dependent on its ability to interact strongly with one of the yeast Cdc42 GAPs: bud emergence 3 (Bem3). Bem3 is also one of the key players in the assembly of the septin cytoskeleton, a family of scaffolding proteins essential for proper cell division (119, 120). In fact, overexpression of the ENTH domain of Ent2, but not of Ent1, induces severe abnormalities in septin organization (118).

Taken together, this evidence indicates that the endocytic protein epsin is involved in Cdc42 regulation, probably coordinating the processes of establishment of cell polarity and endocytosis in time and space.

Importantly, epsin involvement in Rho GTPase signaling function is conserved in mammals. Specifically, epsins via their ENTH domain were found to interact with the Cdc42/Rac1 GAP and Ral effector, Ral-binding protein 1 (RalBP1) (121). Indeed, siRNA-mediated knockdown of epsins led to Rac1 activation deficiencies (121). Importantly, this interaction was shown to be essential for another polarity-dependent process: cell migration. Interestingly, epsins are enriched in the leading edge of migratory cells (121, 122), and epsin-3 is selectively expressed in migratory keratinocytes (12). Further, overexpression of epsins enhanced cell migration and invasion, with epsin-3 being the most potent paralog for induction of this behavior (121). Interestingly, upregulation of epsins has been reported in skin, breast, and lung cancers (12–14, 123). In addition, our lab also identified epsin-3 expression in mouse pancreatic cancer models and in human pancreatic cancer cell lines, such as BxPC-3 and Panc-1 (15, 121). Furthermore, overexpression of epsin-2 and -3 in Madin Darby canine kidney cells induced morphological changes characterized by extended lamellipodia that prompted the epsin-transfected cells to migrate out of epithelial colonies (15). Therefore, it is tempting to speculate that epsin upregulation can contribute to enhance cancer cell invasion *in vivo* by RhoGTPase (Rac1) and notch signaling hyperactivation, and it constitutes an interesting possibility to be investigated.

Nonclassical functions of epsins

Apart from its role in endocytosis and signaling, epsin seem to be important for autophagy in *Drosophila*, as larvae lacking Lqf are unable to form autophagosomes (101). Further, epsin has been reported to accumulate in the nucleus following inhibition of nuclear export and to be involved in regulation of transcription (24, 124). Additionally, the ability of epsin to induce membrane curvature is important for proper mitotic membrane organization (125). Interaction of epsin with microtubules has also been reported (126).

Perspectives

Epsin is a multifaceted protein that apart from its classical role in cargo recognition and endocytic network stabilization, plays a crucial role in signaling. Epsin is required for the activation of the notch pathway, and it affects RhoGTPase signaling. Therefore, we propose that epsins are proteins that coordinate endocytosis and signaling in time and space.

This coordination between endocytosis and signaling is of utmost importance because efficient functioning of one requires precise regulation by the other. For example, work in yeast has demonstrated that the polarized distribution of signaling molecules, such as Cdc42 regulates membrane trafficking, which in turn reinforces polarization (127, 128)

[recently reviewed in (129)]. The establishment and maintenance of such polarized regions is crucial for eukaryotic cellular organization and impacts central functions, such as cell viability, cell fate specification, and cellular migration.

Thus, it is not surprising that epsin is required for cell viability (yeast), proper embryo development (flies and mice), as well as establishment of cell polarity and functions associated with it (e.g., cytokinesis, cell migration, and invasion). Unlike other endocytic proteins [reviewed in (130)], the epsins (particularly epsin-3) are found to be upregulated in cancer cells. Although a direct link to carcinogenesis remains to be established, the role of epsins in promoting cancer invasion is a promising direction to be explored.

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Conflict of interest statement

The author stated that there are no conflicts of interest regarding the publication of this article.

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