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Mechanisms of EHD/RME-1 Protein Function in Endocytic Transport

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Abstract

The evolutionarily conserved Eps15 homology domain (EHD)/receptor-mediated endocytosis (RME)-1 family of C-terminal EH domain proteins has recently come under intense scrutiny because of its importance in intracellular membrane transport, especially with regard to the recycling of receptors from endosomes to the plasma membrane. Recent studies have shed new light on the mode by which these adenosine triphosphatases function on endosomal membranes in mammals and *Caenorhabditis elegans*. This review highlights our current understanding of the physiological roles of these proteins *in vivo*, discussing conserved features as well as emerging functional differences between individual mammalian paralogs. In addition, these findings are discussed in light of the identification of novel EHD/RME-1 protein and lipid interactions and new structural data for proteins in this family, indicating intriguing similarities to the Dynamin superfamily of large guanosine triphosphatases.

Keywords

C. elegans; EH domain; EHD1; EHD2; EHD3; EHD4; endocytic trafficking; Rab effectors; recycling; RME-1

The C-terminal EHD/RME-1 proteins

The Eps15 homology domain (EHD)/receptor-mediated endocytosis (RME)-1 family of EH domain proteins is a novel group of endosomal scaffolding molecules required for receptor recycling. While much is known about the regulation of the early steps of receptor internalization, considerably less is known about how recycling from endosomes to the plasma membrane, and the sorting of recycling cargo within endosomes, is mediated. Analysis of EHD/RME-1 family function, including its interactions with important protein and lipid partners, is providing new insights into the mechanisms that promote recycling within the endosomal system.

Mammalian cells express four highly homologous C-terminal EHD paralogs, EHD1, EHD2, EHD3 and EHD4, each of which contains a single EH domain at the C-terminus. The genomes of many invertebrate organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster*, contain a single EHD family gene, most closely resembling EHD1/EHD3,

although many isoforms of the *C. elegans* EHD family protein RME-1 are known to be expressed because of alternative splicing (1). The mammalian paralogs themselves exhibit 70–86% overall identity, with EHD1 and EHD3 being the most closely related (2). Remarkably, even the *C. elegans* family member RME-1 is 67% identical to mammalian EHD1 over its entire length (1). The EH domains of human EHD1–EHD4, *C. elegans* RME-1 and *Drosophila* dEHD/PAST1 display a greater level of homology with one another than with EH domains of other proteins (Table 1). For example, the EH domains of EHD1, EHD2, EHD3 and EHD4 show between 53 and 81% amino acid identity compared with only 37% identity between each of the EH domains of the C-terminal EHDs and the most closely related N-terminal EH domain, the second of the three Eps15 EH domains (Eps15 EH-2).

Recently, new light has been shed on the domain architecture and structure of C-terminal EHDs, including the first crystal structure of a full-length family member, mouse EHD2 (3) (Figure 1A,B). This work showed that EHD2 contains highly helical regions that produce a lipid-binding surface. Like all EHD/RME-1 proteins, EHD2 contains a nucleotide-binding domain. This domain in EHD2 is remarkably similar in three dimensional structure, but not in primary sequence, to the G-domain of Dynamin, another large nucleotide-binding protein that mediates endocytosis (4). Thus, the EHD2 nucleotide-binding domain retains the nomenclature ‘G-domain’, although it binds to ATP rather than GTP (see below). These regions of the protein connect to a disordered ‘linker region’, prior to the EH domain (Figure 1B). Further functional parallels of EHD2 to Dynamin were also identified, and the authors suggested that EHD proteins could be considered members of the Dynamin-like superfamily (4). Detailed implications of the recent structural developments are discussed below.

The Structure and Function of EH Domains

The EH domain in general is a highly conserved 90–100 amino acid structure that was originally identified as a repeated domain in the endocytic adaptor protein known as the EGF receptor tyrosine kinase substrate (Eps15) (5,6). Over 500 proteins are now known to contain at least a single EH domain (SMART Architecture Analysis, <http://smart.embl-heidelberg.de>). Apparently a eukaryotic innovation, EH domains are present in diverse phyla ranging from the relatively simple budding yeast and paramecium to higher plants and metazoans such as nematodes, insects and mammals (reviewed in 7,8). Like other scaffolding proteins, EH domain proteins are generally modular, containing additional other well-characterized signaling domains such as SH3 regions, PH domains, C2 domains, proline-rich regions as well as aspartate-proline-phenylalanine (DPF) and ubiquitin interaction motifs (reviewed in 8,9). Despite the variety of functions implicated by these additional domains and motifs, regulation of endocytic trafficking appears to be a primary role for these proteins in the cell.

The first structure of an EH domain (Eps15 EH2) was solved by nuclear magnetic resonance (NMR) spectroscopy (10). Eps15 EH2 was found to contain a pair of EF-Hand type helix-loop-helix motifs that are linked by a short antiparallel β -sheet (10). Since then, several other EH domain structures have been solved, including those of C-terminal EHD proteins. The recent solution structure of the human EHD1 EH domain and the crystal structure of the complete mouse EHD2 protein have demonstrated the conservation of the basic EH domain fold and have provided new information on its function (3,11).

A variety of experimental methods, including phage display screens (12) and the screening of a human fibroblast expression library, have demonstrated that EH domains bind to peptides containing an asparagine-proline-phenylalanine (NPF) sequence (13). An explanation of the structural basis for EH-NPF interactions was first provided by the solution structure of Eps15 EH2 bound to NPFL or NPLR (10,14). These studies led to the conclusions that interacting NPF residues, in the conformation of a type I β -turn, fit into a hydrophobic pocket on the surface

of the EH domain, between the α B; and the α C helices (14). Moreover, these studies precisely defined the conserved hydrophobic patch that accommodates the proline of the NPF, permitting the phenylalanine to insert deeply within the conserved pocket [(14) and reviewed in 15]. A recent study has also described an additional hydrophobic pocket within Eps15 EH2 (16) and raises the possibility that other EH domains may similarly be capable of additional modes of NPF binding.

Structural Insights into the EH Domain of EHD/RME-1 Proteins: NPF and Lipid Binding

An interesting question relates to how the specificity of individual EH domains, for particular NPF motifs in target proteins, is attained. For instance, a search of the predicted *C. elegans* proteome reveals over 800 proteins containing at least one NPF sequence (S. Pant and B. G., unpublished data). The answer, at least in part, appears to derive from differential preferences of specific EH domains for flanking residues surrounding the target NPF. Previous work showed that each EH domain has its own inherent preference for NPF-flanking residues (12). Indeed, the elegant work of Paoluzi et al. showed that the first and third EH domains of Eps15R prefer peptides with an arginine immediately following the NPF motif (12).

In contrast, our analysis, and that of Braun et al. (17), indicates that the EH domains of EHD proteins prefer acidic residues following the NPF (Figure 2). Although these data are not derived from a comprehensive phage display, the comparisons shown in this study in Figure 2 suggest that proteins ranked as strong binders to EHD proteins [i.e. Rabenosyn-5 NPFs 1 and 2 (18), Rab11-FIP2 (19), SNAP29 (20) and Syndapins (17,20)] have a strong acidic consensus in positions +1, +2 and +3. While not all NPFs implicated in EHD/RME-1 binding are surrounded by acidic residues in their primary sequence (Figure 2), those that lack this feature have only been found in proteins that contain multiple EHD/RME-1-binding sites (17-19,21). In fact, some interactors appear to require multiple intact NPFs for binding, suggesting a preference for interaction with assembled EHD oligomers (17-19).

Recent structural advances have allowed new insights into the likely mechanisms by which the EHD EH domain produces binding specificity (3,11,22). The recent NMR solution structure of the EH domain of EHD1 has provided some important clues as to the reason that C-terminal EH domains preferentially bind to NPF motifs followed by a cluster of acidic residues (Figure 3). The electrostatic surface potential of the EH domain of EHD1 is highly positive, possibly facilitating interactions with the aspartate and glutamate residues that follow the NPF motifs of binding proteins (11). Indeed, upon comparison of the six residues of the EHD1 EH domain modeled into the NPF-binding pocket, these amino acids display a net electrostatic charge of +4, whereas the homologous residues in the Eps15 EH2 exhibit a net electrostatic charge of -3 (Figure 3). This analysis supports the hypothesis that positive electrostatic surface potential is an important mechanism to fine-tune the C-terminal EH domains and ensure that they interact with a select group of NPF-containing interaction partners, particularly those containing acidic clusters following their NPF motif.

One of the interesting features of EHD/RME-1 proteins is their distinctive localization to long tubular endosomal membranes (up to 10 μ m), in addition to punctate endosomes (23). A recent study has also shed new light on this localization, suggesting that EH domain interaction with phosphoinositide lipids is important for interaction with, or formation of, such extended tubular endosomal domains (22). Chemical shift experiments, using two-dimensional NMR, detected perturbation of select residues of the EHD1 EH domain upon titration of phosphoinositides. A conserved, positively charged amino acid, lysine 483, was hypothesized to be involved in the EH domain/lipid binding (22). Indeed, mutation of lysine 483 to glutamic acid did not alter the overall structure of the EH domain but caused an altered subcellular distribution of the mutant

EHD1, from tubulovesicular membranes to entirely punctate structures. This phenotype resembles truncation of the entire EH domain, indicating that this residue, and therefore EH phosphoinositide interactions, is critical for EHD-mediated tubulation (22).

Oligomerization, tubulation and nucleotide hydrolysis

It was recently demonstrated that all four full-length EHDs can bind to various phosphoinositides (24). However, because both the EH domain truncation and the lysine-483-to-glutamic acid mutant are nonetheless capable of interacting with punctate endosomal membranes, these data implicated the involvement of an additional region of the protein as the primary determinant of membrane association. The recent crystal structure of mouse EHD2 identified this binding site as a polybasic cluster of residues in close proximity to the tip of helix $\alpha 9$ (3). The dimerization of EHD2 allows the formation of a highly curved, scissor-like, membrane interface composed of the lipid-binding sites of the two helical domains (3). Further oligomerization of dimers to form rings around lipid tubules and the ability to tubulate negatively charged liposomes in isolation suggest that EHD2 and by extension the entire EHD family are functionally similar to Dynamin (3). Importantly, these results suggest that like Dynamin, EHD proteins may function to tubulate membranes and/or detach budding vesicles or tubules from endosomal membranes (promote fission). Further analysis will be required to determine how far the Dynamin analogy holds.

In addition to identifying the dimeric lipid-binding interface, the crystal structure of EHD2 also produced several somewhat surprising findings. For example, the authors identified a conserved GPF motif in the linker that separates the EH domain from the central helical region and were able to predict that in solution, the EH domains of the dimer would be adjoined, with each EH domain occupied by the conserved linker GPF motif (3). The authors further propose that in the course of oligomerization, the EH domains shift from their interaction with the linker GPF motif to interact with a disordered loop sequence XPFRKLNPF localized within the G-domain (3). Interestingly, while the XPFRKL motif is conserved among mammalian EHDs, the NPF motif itself is not found in EHD1, EHD3 or EHD4 (bold sequences indicate residues proposed to make contact with the EH domain binding pocket). Furthermore, the EHD homologs from some other species, such as isoform D of *C. elegans* RME-1, lack amino terminal XPF motifs, yet can fully rescue a null mutant lacking all RME-1 isoforms (21). These differences leave open the question of whether EHD1, EHD3, EHD4 and RME-1 are capable of the same proposed EH domain movement from the linker region to the side site of the G-domain upon higher order oligomerization. One possibility is that NPF-containing binding partners provide this function 'in trans' for these EHD proteins, promoting conformational changes in the EHD that in turn promote its polymerization around membrane tubules.

Based on sequence alignments, it was predicted that EHD proteins contain a nucleotide-binding site (1,23) most similar to the site found in guanosine triphosphatases (GTPases) such as Ras and Dynamin (25). The first evidence demonstrating the functional significance of this nucleotide-binding site came from *in vivo* endocytic assays showing that oocytes possessing a glycine-to-arginine mutation within the conserved nucleotide-binding site of the *C. elegans* RME-1 protein exhibited impaired recycling of yolk receptors and in fact behaved as dominant negatives (1). A somewhat surprising finding, which has been strongly supported by the recent crystal structure of EHD2 in the presence of a non-hydrolysable ATP analog (3), is that the G-domain of the EHD1 protein displays a strong preference for ATP binding over GTP binding, with a K_m of about 80 μ m for ATP (26). A structural explanation for this specificity was provided by the evidence that arginine 536 of EHD2 forms a hydrogen bond with aspartate 222 of the conserved NKxD motif in the G-domain (3), thus disrupting the typical function of such aspartates in GTP recognition and binding in GTPases (27). Furthermore, the EHD2

structure showed that a methionine at position 223 in EHD2 would likely sterically interfere with GTP binding (3).

An important feature of EHD proteins is their ability to oligomerize. EHD2 dimerization has been localized to a highly hydrophobic interface spanning about 2100 Å² within the G-domain (3). EHD2 adenosine triphosphatase activity is stimulated by about 10-fold in the presence of liposomes and correlates with the apparent assembly of EHD2 rings around the membranes. This behavior is highly reminiscent of the assembly-stimulated GTPase activity characteristic of Dynamin (28-31). It remains to be determined if EHD/RME-1 family proteins contain an intrinsic assembly stimulated GTPase-activating protein (GAP) domain, as is known to be the case for Dynamin family proteins (31,32). There is no apparent homology between EHD2 and the proposed GAP domain (GED) of Dynamin, suggesting that the stimulation of nucleotide hydrolysis is achieved differently. Furthermore, the EHD2 structure indicates that the beta and gamma phosphate of the ATP are capped, preventing direct insertion of a catalytic residue to promote ATP hydrolysis (3).

While it has been demonstrated that EHD proteins are found in large complexes (23), the stoichiometry of the EHD paralogs interacting with one another has been difficult to resolve. Various laboratories have demonstrated *in vitro* with purified proteins, by two-hybrid analysis and by overexpression in cells that EHDs are capable of hetero-oligomerizing (19,26,33,34), but thus far, the only *in vivo* interaction documented has been between EHD1 and EHD4 (35). Such oligomerization has functional significance as depletion of either EHD1 or EHD4 dramatically alters the subcellular localization of its binding partner (35). Moreover, oligomerization may regulate the binding of EHDs to NPF-containing binding partners (19). The main exception to this is EHD2, which appears not to hetero-oligomerize with other EHDs *in vivo*, even when co-overexpressed in the same cells (34,35). Future studies are likely to provide exciting new information on the mode by which oligomerization and lipid binding affect the functional roles of EHD proteins.

Functional Roles of EHD Proteins in the Regulation of Endocytic Recycling

The first evidence for the role of an EHD family protein in controlling receptor recycling from endosomes to the plasma membrane, and its effects on the morphology of the endocytic recycling compartment (ERC), was provided by *C. elegans* genetics (1) and parallel studies utilizing mammalian Chinese hamster ovary cells (36) (Table 2). In *C. elegans*, the RME-1 protein is required for yolk receptor recycling, which in turn is required for efficient yolk uptake by oocytes (1). Many additional cell types show endocytic defects in *rme-1* mutants, and the RME-1 protein is expressed in all cells, localizing to recycling endosomes and the plasma membrane (1). Interestingly, RME-1 displays a preference in the *C. elegans* intestine for basolateral recycling endosomes, and *rme-1* mutants accumulate baso-laterally endocytosed fluid in grossly enlarged endosomes. Likewise perturbation or loss of EHD1 in mammalian cells interferes with the recycling of the transferrin receptor, major histocompatibility complex class I, Cystic fibrosis transmembrane conductance receptor, long-term potentiation AMPA receptors and β 1 integrins to name a few known EHD/RME-1-dependent cargo proteins [(23,36-40) and Table 2]. Notably, EHD1 is important for the recycling of transmembrane cargo that is internalized by clathrin-dependent and clathrin-independent mechanisms (23). Recent evidence suggests that this dual requirement for recycling both types of cargo is also true in *C. elegans* (21,41).

While the role of RME-1/EHD1 appears to be well conserved between *C. elegans* and mammalian cells, the roles of the other three mammalian EHD paralogs, EHD2, EHD3 and EHD4, have not been as well characterized, and there are some partially conflicting reports with regards to their function. To some extent, such studies have been hampered by the high

level of identity between these proteins and difficulties in generating specific antibodies directed against each paralog. However, recent studies have begun to unravel the functional differences between the EHD family proteins (Figure 4).

EHD2 is the least homologous paralog, displaying about 70% amino acid identity with EHD1. While the crystal structure of EHD2 has led to some very interesting observations, the precise function of this protein remains vague. Studies by Guilherme et al. demonstrated that small interfering RNA (siRNA)-based depletion of EHD2 (or its calponin homology domain interaction partner, EHBPI) inhibits the internalization of transferrin receptor and GLUT4 in adipocytes (42). Consistent with these findings, Park et al. have identified interactions between EHD2 and μ 1 and μ 2 subunits of the clathrin adaptor complexes AP-1 and AP-2, respectively (43). However, EHD2 has also been depicted as a regulator controlling the exit of vesicular cargo from the ERC, similar to the function described for EHD1 (34). Additional experiments will be needed to definitively assess whether EHD2 is primarily involved in the internalization of receptors or their recycling.

EHD3 is the most homologous paralog to EHD1, showing 85% residue identity. Along with this high level of sequence identity, studies have shown that overexpressed EHD3 displays a strong colocalization with EHD1 and a similar tubulovesicular distribution pattern (19,24,33, 34). However, unlike EHD1, depletion of EHD3 from HeLa cells causes internalized cargo to accumulate in enlarged peripheral early endosomes rather than at the centralized ERC (19, 34). This function in the early endosome may be a vertebrate innovation because *rme-1* null mutants in *C. elegans* do not appear to have any early endosomal defects (1).

Perhaps, the most conflicting reports have surfaced with regards to the function of EHD4. Despite displaying 74% identity to EHD1, and the lack of a secretory signal sequence, EHD4 was first described as a component of the extracellular matrix (44). Subsequently, EHD4 was characterized as an intracellular regulator of nerve growth factor receptor (TrkA) internalization (45). Intriguingly, TrkA does contain an NPF motif, but a direct interaction between these proteins has not been reported. One recent study has proposed that EHD4 is not involved in trafficking through endosomes but rather suggests that EHD4 functions in other highly specialized transport processes (24). A second study provides evidence that EHD4 does play a role in transport at the early endosome (34). In support of this, endogenous EHD4 has now been shown to colocalize with markers of the early endosome (35). This study also demonstrates the formation of enlarged early endosomal structures upon EHD4 depletion and the accumulation of internalized cargo in these compartments, which are enriched in active, GTP-bound, Rab5 (35). Moreover, the interaction described between endogenous EHD4 and EHD1, as well as the dramatic effects of depleting one EHD protein on the localization of the other, indicates a functional role for the interaction between these two paralogs (35).

Co-ordination of EHDs and Rab Proteins in Membrane Trafficking

Over 60 different mammalian Rab proteins have been described that participate in various aspects of regulating subcellular trafficking (46). One of the important questions arising from studying the function of EHD proteins is how they are able to co-ordinately regulate endocytic trafficking events with the Rab family of small GTP-binding proteins. For example, both EHD1 and Rab11 have been ascribed a role in regulating the exit of recycling cargo from the perinuclear ERC to the plasma membrane (1,23,36,47), and both EHD4 and Rab4 have been localized to peripheral early endosomes and implicated in controlling the recycling of receptors from this organelle (35,48). These findings have led to the hypothesis that EHD proteins and Rab proteins may interact with one another to co-ordinately regulate endocytic trafficking events. However, despite extensive colocalization between some EHDs and Rab proteins, to date, no direct interactions between these proteins have been reported.

One potential link between Rab proteins and EHD proteins is through Rab effectors. Each Rab protein is known to mediate its functional effects in the cell through binding to a series of 'Rab effector proteins'. With few exceptions, these effectors bind exclusively to the GTP-bound Rab proteins, and each effector is thought to provide specific transport promoting functions. Two recent studies have linked Rab effectors with EHD proteins. First, the divalent Rab4/5 effector, Rabenosyn-5, was identified as an EHD interaction partner (18). Indeed, siRNA-mediated depletion of Rabenosyn-5 led to a delay in receptor recycling similar to that observed upon depletion of EHD1. However, whereas receptors affected by EHD1 depletion accumulated in the cell at the perinuclear ERC, knockdown of Rabenosyn-5 caused their accumulation in enlarged peripheral endosomes (18). These findings led to the proposition that Rabenosyn-5 interactions with EHD1 facilitate the transport of internalized receptors from peripheral sorting endosomes to the ERC. It is not yet known if Rabenosyn-5 interacts with early endosomal EHD3 or EHD4. A second study has identified the Rab11 effector, Rab11-FIP2, as an interaction partner for EHD proteins, suggesting a possible mechanism by which Rab11 and EHD1 might regulate the exit of receptors from the ERC to the plasma membrane (19). Additionally, Rab8a and Myosin Vb have been identified on EHD1/3 tubules (49), although no physical interaction between EHDs and Rab8a has been identified thus far. Similarly, *C. elegans* RME-1 acts downstream of RAB-10 in the basolateral recycling pathway of the intestine, but no direct link has been identified between the two proteins (41). Recent work suggests that Rab8 mediates Golgi-to-recycling endosome transport in polarized Madin-Darby Canine Kidney cells, while Rab10 mediates early endosomes-to-recycling endosome transport in such cells (50-52). Thus, Rab8 and Rab10 may regulate entry of distinct cargos into the EHD-regulated ERC.

In addition to the direct interactions between EHD proteins and Rab effectors, EHD proteins may be capable of regulating Rab activity. For example, depletion of EHD4 leads to the activation of Rab5, producing enhanced levels of GTP-bound Rab5 associated with endosomal membranes (35). Although the precise manner by which this interplay between Rab5 and EHD4 occurs has not yet been deciphered, it is clear that Rab and EHD proteins have evolved a coordinated strategy for the regulation of endocytic events.

Interaction Partners of EHD Proteins

The mode of interaction of Rabenosyn-5, Rab11-FIP2 and another endocytic regulator, Syndapin/Paccin, with EHD proteins was found to be through EH domain/NPF motifs, and other EHD partners interact through similar mechanisms (Table 3). Rabenosyn-5, Rab11-FIP2 and Syndapin II all contain multiple NPF motifs (human Rabenosyn-5 carries five NPFs, mouse Rabenosyn-5 has six NPFs, mammalian Rab11-FIP2 has three NPF motifs and Syndapin II contains three NPF motifs). Other interaction motifs have also been observed aside from the typical NPF-EH interface. One such atypical interactor is the *C. elegans* protein ALX-1/ALIX, the V domain of which binds to a YPXL motif in the extreme C-terminus of RME-1. This strong V-domain-YPXL interaction requires augmentation *in vivo*, provided by an NPF-EH interface (21). This work uncovered a novel role for ALX-1/ALIX in the regulation of receptor recycling in worms and mammalian cells, with specificity for cargo taken up independently of clathrin (21). Bro1p/ALIX was already well known for its role in membrane protein degradation through interaction with endosomal sorting complex required for transport-III (ESCRT-III) on multivesicular endosomes in yeast and mammals, a function that is also conserved in *C. elegans* (21,53-56). One exciting possibility is that this dual role of ALX-1/ALIX, in recycling and degradation, indicates co-ordinate regulation of the decision to degrade or recycle cargo after endocytosis.

Much remains to be learned regarding the role for interaction of EHD/RME-1 proteins with diverse partners and how they contribute to endocytic traffic. As alluded to above, some of

these partners may regulate the assembly and/or nucleotide state of EHD oligomers. In addition, these proteins may act to link EHD/RME-1 proteins to the actin cytoskeleton. The Syndapin/Pacsin interaction with EHDs on recycling endosomes may be particularly relevant to such discussions (17) because Syndapin also interacts with Dynamin during receptor internalization and activates actin polymerization through N-Wasp (57). Thus, association with Syndapin further extends the EHD/Dynamin parallel and suggests that like Dynamin, EHD proteins may help link membrane remodeling to actin dynamics, a process suggested to generate membrane tension during scission events (58,59). Likewise, EHD/RME-1-interacting proteins EHBP1, Numb and ALIX are also associated with actin binding or remodeling (21,42,60-62), and EHD/RME-1 colocalizes with the known actin regulators Arf6 and Cdc42 on endosomal tubules (23,63).

Conclusions/Perspectives

Since the turn of the millennium, rapid advances have been made in elucidating the function of EHD/RME-1 proteins. Despite the high levels of homology between the four mammalian paralogs, it is clear that in addition to partially overlapping functions, each of the EHDs also carries out distinct endocytic regulatory roles. The identification of novel EHD/RME-1 interactors through genetic and biochemical approaches, and the recent structural advances, have continued to highlight the pivotal role of EHDs in endocytic traffic and promise to further the understanding of the molecular mechanisms by which these proteins act.

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A C-terminal EHD proteins

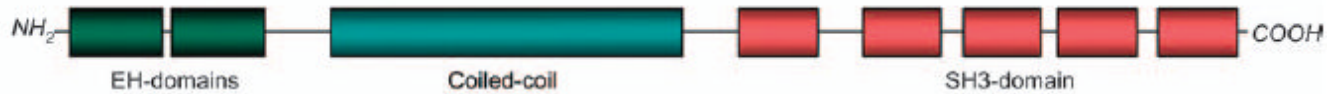


N-terminal EHD proteins

Eps15



Intersectin (short isoform)



Reps1



B

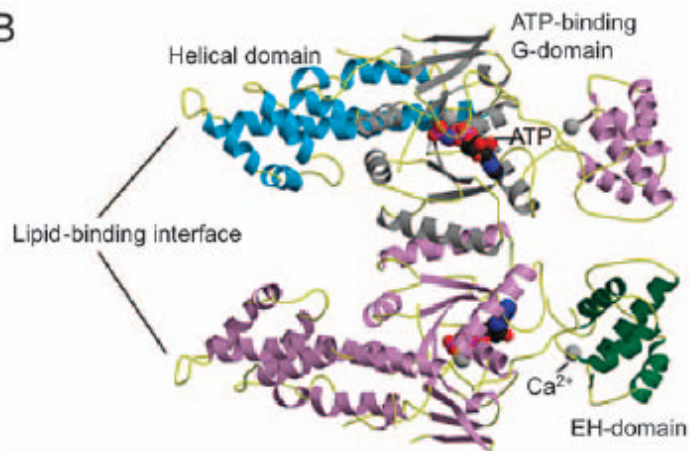


Figure 1. Domain architecture and structure of C-terminal EHD proteins

A) Comparison of the domain architecture of C-terminal/RME-1 proteins and N-terminal EHD proteins. UIM, ubiquitin interaction motif. B) The crystal structure of mouse EHD2 dimer. EHD2 is shown by a ribbon-type representation. The top monomer is colored to match the domain architecture depicted in (A).

Strong or moderate binding to C-terminal EHDs

```

Rabenosyn-5 NPF1  EGPSLNPFDEEDLSSPMEEA---
• Rab11-FIP2 NPF3  --PDSNPFDATAGYRSLTYEE--
• SyndapinII NPF3  ---DSNPFDDDATSGTEVRVRAL
Rabenosyn-5 NPF2  ---EYNPFEEEEDEEEEAVAG---
• SyndapinII NPF1  --SSYNPFEDDDTGSTVSE---
• Rab11-FIP2 NPF1  ---KKNPFEESSSETWDSS-----
• SyndapinI NPF2  ---GANPFEDDSKGVRVRA-----
• SNAP29 NPF      ---SYNPFDDGGEDEGARPA---
  NUMB NPF        ---PTNPFSSDLQKTFE
• syndapinI NPF1  ---SGNPFGGSETNGGA-----
• SyndapinII NPF2  ---SNNPFSSTDANGDS-----
Rab11-FIP2 NPF2  ---STNPF TAKFRASNIMPS---
• Consensus      S NPFDDD

```

Presence of acidic residues at +1 to +3 (or +4) and lack of hydrophobic residues at +1 to +4

Weak or no binding to C-terminal EHDs

```

APPL1 NPF  ---RTNPFGESGGSTKSETE
• Dab2 NPF1 ---RENPF LTNGITSCSLP-
• Dab2 NPF2 ---SPNPFVGSPPKGLSIQN
• Epsin1 NPF2 ---VTNPFQPAPPATLTLN-
• Dab2 NPF3 ---LGNPFQSNIFPAPAV--
• Dab2 NPF4 DNAFENPFKDSFGSSQASV
• Dab2 NPF5 RDPFGNPF A--
• Epsin1 NPF1 ---ASNPF LPGGGPATGPSV
• Epsin1 NPF3 ---NTNPF LL-----
• Consensus  NPF

```

Presence of hydrophobic residues at +1 and prolines at +2 to +6

Figure 2. Selectivity of C-terminal EH domains for NPF motifs flanked by acidic residues
 Alignment of NPF motifs and surrounding residues that exhibit weak or strong binding with EHD proteins. NPF motifs are indicated by red shading, whereas flanking acidic residues are denoted by light blue shading.

Grant and Caplan

A

Surface residues within the NPF-binding pocket	N-terminal EH domains					C-terminal EH domains									
	hEps15 EH-1	hEps15 EH-2	hEps15 EH-3	hITSN EH-1	hITSN EH-2	hEHD1	hEHD2	hEHD3	hEHD4	<i>X. laevis</i>	<i>D. melanogaster</i>	<i>C. elegans</i>	<i>D. rerio</i>	<i>S. japonicum</i>	
EHD1 residue 461	R	F	F	F	H	K	K	K	K	K	K	Y	K	K	
EHD1 residue 465	S	D	L	D	P	A	S	A	V	A	A	R	A	E	
EHD1 residue 469	A	N	E	N	T	K	T	K	K	K	Q	E	K	K	
EHD1 residue 473	K	N	K	Q	Q	K	G	R	T	K	K	K	K	K	
EHD1 residue 479	L	D	T	P	A	T	S	S	S	T	S	S	N	S	
EHD1 residue 486	D	E	S	A	N	K	K	K	K	K	K	K	K	I	
Relative electrostatic surface potential	+1	-3	-1	-1	0	+4	+2	+4	+3	+4	+3	+2	+4	+2	

B

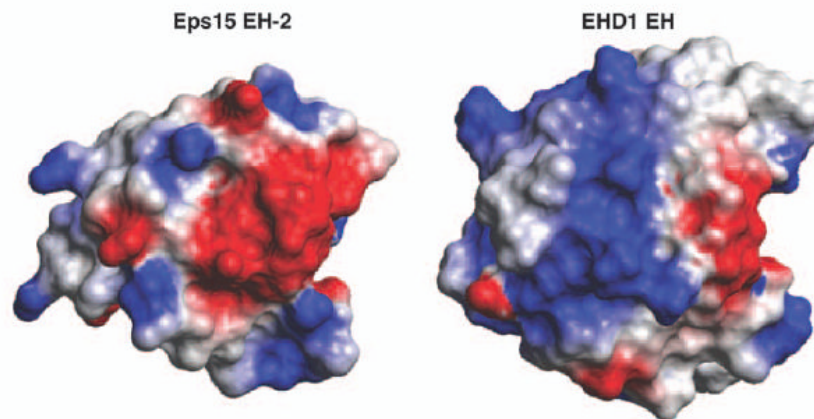


Figure 3. C-terminal EH domains contain positively charged surface residues within the NPF-binding pocket

A) Comparison of the relative electrostatic surface potential of C-terminal and N-terminal EH domains. B) Comparison of the relative electrostatic surface potential of the human EHD1 EH domain and Eps15 EH-2. Blue represents residues with positively charged side chains, whereas red represents negatively charged residues.

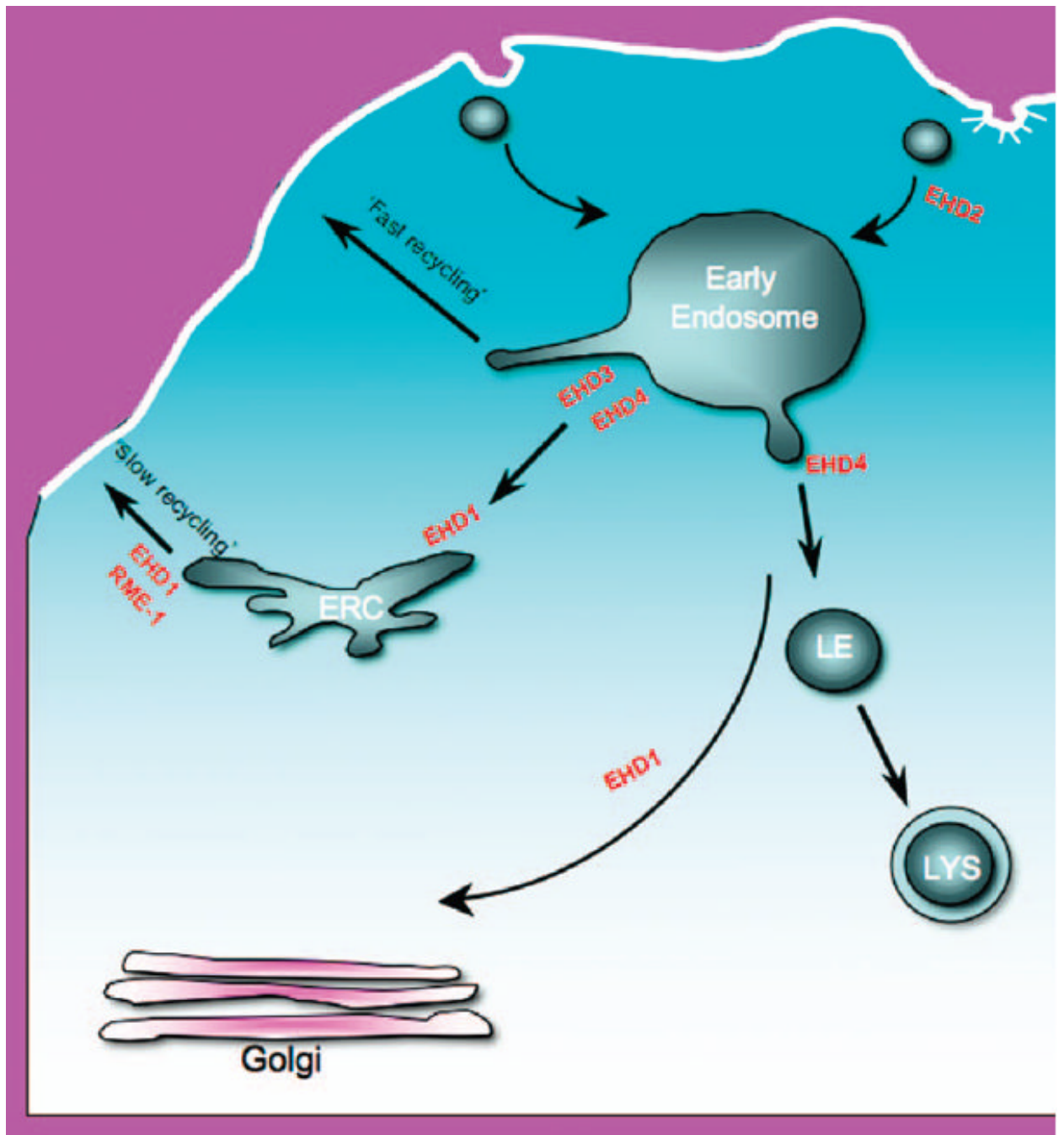


Figure 4. Schematic diagram showing the proposed roles for EHD proteins in the regulation of endocytic transport

EHD2 has also been reported to function at the ERC (34), while EHD4/pincher controls the internalization of nerve growth factor receptor (45). LE, late endosome; LYS, lysosome.

Table 1

Comparison of amino acid sequence identity between C-terminal EH domains and the second Eps15 EH domain

Comparison of EH domains	Percent identity
EHD1 EH/EHD2 EH	59
EHD1 EH/EHD3 EH	81
EHD1 EH/EHD4 EH	68
EHD2 EH/EHD3 EH	57
EHD2 EH/EHD4 EH	53
EHD3 EH/EHD4 EH	67
Eps15 EH-2/EHD1 EH	37
Eps15 EH-2/EHD2 EH	37
Eps15 EH-2/EHD3 EH	37
Eps15 EH-2/EHD4 EH	37

Table 2

Functional roles for EHD1/RME-1

Function	Model	Reference
Yolk receptor recycling	<i>C. elegans</i>	(1)
Basolateral fluid recycling	<i>C. elegans</i>	(1)
Transferrin receptor recycling	mammalian cells	(36)
Major histocompatibility complex class I recycling	mammalian cells	(23)
Transferrin receptor recycling	<i>Ehd1^{-/-} MEF</i> cells	(40)
Cystic fibrosis transmembrane conductance regulator recycling	Mammalian cells	(39)
Insulin-regulated GLUT4 glucose transporter recycling	Mammalian cells	(60)
Long-term potentiation AMPA type glutamate receptor recycling	Mammalian cells	(38)
β 1 integrin recycling	Mammalian cells	(37)
Low-density lipoprotein receptor endocytosis/cholesterol and lipid droplet homeostasis	Mammalian cells	(64)
Endosome-to-Golgi retrieval	Mammalian cells	(65)

Table 3

Interactions between EHD proteins and binding partners

EHD protein	Interaction partner	Mode of EHD interaction	Reference
Rme-1 (<i>C. elegans</i>)	Reticulon-C protein	C-terminal region	(66)
Rme-1 (<i>C. elegans</i>)	Alix/ALX-1	1) Rme-1 EH domain binds ALX-1 NPF motif 2) Rme-1 YPSL motif binds ALX-1 V-domain	(21)
EHD1 (<i>D. melanogaster</i>)	Numb	EH domain	(62)
EHD1	Insulin-like growth factor 1 receptor	Not characterized	(67)
EHD1	SNAP29/GS32	EH domain	(20)
EHD1	AP-2 α -adaptin	Not characterized	(67)
EHD1	Clathrin heavy chain	Not characterized	(67)
EHD1	Rabenosyn-5	EH domain	(18)
EHD1	Syndapin I	EH domain	(17,20)
EHD1	Syndapin II	EH domain	(17)
EHD1	Rab11-FIP2	EH domain	(19)
EHD1	Retromer subunits	Not through EH domain	(65)
EHD1	Epsin1	EH domain	(our unpublished data)
EHD1	Stonin2	EH domain	(our unpublished data)
EHD2	GLUT4	Not characterized	(43)
EHD2	AP-1 μ 1	Not characterized	(43)
EHD2	AP-2 μ 2	Not characterized	(43)
EHD2	CALM	Not characterized	(43)
EHD2	EHP1	EH domain	(42)
EHD2	Arp2/3	Acidic region prior to EH domain	(42)
EHD2	Rabenosyn-5	EH domain	(18)
EHD3	Syndapin I and II	EH domain	(17)
EHD3	Rabenosyn-5	EH domain	(18)
EHD3	Rab11-FIP2	EH domain	(19)
EHD4	Numb	EH domain	(62)
EHD4	Syndapin I and II	EH domain	(17)
EHD4	Type VI collagen	Not characterized	(44)