C-terminal EH-domain-containing proteins: consensus for a role in endocytic trafficking, EH?

Naava Naslavsky and Steve Caplan*

Department of Biochemistry and Molecular Biology and Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA

*Author for correspondence (e-mail: scaplan@unmc.edu)

Accepted 6 June 2005
Journal of Cell Science 118, 4093-4101 Published by The Company of Biologists 2005
doi:10.1242/jcs.02595

Summary

The Eps15-homology (EH) domain is a highly conserved motif comprising ~100 residues that is found in proteins from species as diverse as yeast and mammals. Proteins that have an EH domain can carry out a variety of crucial cellular functions ranging from regulation of the actin cytoskeleton, signal transduction and transcriptional regulation to control of the endocytic pathway. EH domains bind to proteins that contain the tripeptide asparagine-proline-phenylalanine (NPF). Although EH domains are typically found at the N-terminus, mammalian cells express four highly homologous C-terminal EH-domain-containing paralogs (EHD1-EHD4), which exhibit broad amino acid conservation throughout the entire sequence. These C-terminal EH-domain-containing proteins also contain a central coiled-coil region involved in oligomerization, as well as an N-terminal nucleotide-binding motif. Recent studies have identified an array of novel binding partners for EHD1-EHD4, including NPF-containing proteins, such as the divalent Rab4/5 effector rabenosyn 5, the cell fate determinant Numb, EH-binding protein 1 (EHBP1) and syndapins I and II. Interactions with the clathrin heavy-chain and components of the internalization machinery have also been described. Indeed, C-terminal EH-domain-containing proteins appear to regulate several key endocytic steps, including internalization and recycling. EHD1 and EHD4 control recycling by regulating the transport of receptors from the recycling compartment to the plasma membrane. EHD1, EHD2 and EHD4 have also been implicated in the internalization of receptors and their transport to early endosomes.

Key words: Eps15 homology (EH) domain, Endocytosis, Recycling

Introduction

The internalization of plasma membrane proteins by mammalian cells is crucial for many essential cellular processes, such as nutrient uptake, control of ion channels, retrieval of synaptic vesicle components in neurons, and the regulated expression of signaling receptors and adhesion molecules at the cell surface (Conner and Schmid, 2003). Just as important is the ability to recycle a subset of internalized proteins to the plasma membrane to partake in additional rounds of internalization. The highly complex mechanisms regulating endocytic recycling are mediated by an array of Rab proteins, their effectors and other interacting proteins. These proteins facilitate transport of receptors through at least two distinct recycling pathways: 1) directly from the early endosome; and 2) through a transitory pericentriolar endocytic recycling compartment.

Although the precise mechanisms that control endocytic recycling are not fully understood, our knowledge of the molecular machinery regulating internalization is extensive. Interactions between clathrin, the AP-2 adaptor protein complex and the GTPase dynamin facilitate the 'pinching off' of clathrin-coated pits from the plasma membrane and the generation of clathrin-coated vesicles (Sorkin, 2004). Also recruited by AP-2 to the site of clathrin-coated pits are proteins containing Eps15 homology (EH) domains, such as the epidermal growth factor receptor tyrosine kinase substrate Eps15 (Benmerah et al., 1995). Eps15 plays a crucial role in internalization events (Benmerah et al., 1998), and EH-domain-containing (EHD) proteins along with their interaction partners form a network involved in endocytic transport (reviewed in Polo et al., 2003).

The EH domain was originally identified as a stretch of ~100 residues repeated three times at the N-terminus of Eps15 (Fazioli et al., 1993; Wong et al., 1995). EH domains are highly conserved, generally exhibiting sequence similarity of 50-60% (Wong et al., 1995). EHD proteins are expressed in single-celled organisms such as yeast, as well as multicellular organisms including nematodes, plants and mammals (reviewed in Miliaras and Wendland, 2004; Santolini et al., 1999).

NMR spectroscopy has thus far yielded closely related structures for EH domains (reviewed in Confalonieri and Di Fiore, 2002). Each EH domain contains two calcium-binding helix-loop-helix motifs known as EF-hands, which are linked by a short anti-parallel β-sheet. However, not all EF-hands are capable of calcium binding, and they have been termed either 'canonical' or 'pseudo' EF-hands, depending on their ability to bind calcium (Strynadka and James, 1989).

EH domains interact with other proteins. Probing of phage-display libraries (Paoluzi et al., 1998) and a human fibroblast expression library has identified peptides containing NPF (asparagine-proline-phenylalanine) motifs as major targets for EH-domain binding (Salcini et al., 1997). Several studies have demonstrated that NPF residues enter a conserved hydrophobic pocket within the EH domain, which allows close contact

between the asparagine residue of the tripeptide and a highly conserved tryptophan residue in the EH domain (de Beer et al., 1998; de Beer et al., 2000). Mutation of this conserved tryptophan residue dramatically impairs binding of EH domains to NPF motifs, and the mechanism of binding is thought to be conserved among most EH domains (de Beer et al., 1998).

Over 50 eukaryotic EHD proteins have been identified (reviewed in Miliaras and Wendland, 2004; Polo et al., 2003). Several of these proteins, including Eps15, the related Eps15R protein, intersectin 1 and intersectin 2 have multiple EH domains (see Fig. 1). As a general rule, most EH domains are present in the N-terminal region of the protein, and many EHD proteins have central coiled-coils, which are important for homo- and hetero-oligomerization. Other domains have been identified in various EHD proteins, including SH3 domains, pleckstrin homology (PH) domains, guanine nucleotide exchange factors for Rho, proline-rich regions and ubiquitin interaction motifs (reviewed in Polo et al., 2003).

Despite the presence of these diverse domains, EHD proteins most commonly play regulatory roles in endocytic membrane transport events. Eps15 and Eps15R are localized to assembly sites of clathrin-coated pits, where these proteins are thought to serve as molecular scaffolds. Eps15 links the epidermal growth factor receptor to the AP-2 adaptor complex (Benmerah et al., 1998) and the NPF-containing protein epsin (Chen et al., 1998). These interactions may recruit and/or stabilize clathrin, AP-2, dynamin and other proteins involved in early endocytic events at the plasma membrane (Benmerah et al., 1995; Benmerah et al., 1998; Carbone et al., 1997; van Delft et al., 1997).

Another related function ascribed to EHD proteins is regulation of actin dynamics (Duncan et al., 2001; Hussain et al., 2001; Tang et al., 1997; Wendland et al., 1996). Some EHD proteins (e.g. Reps1 and POB1) regulate actin microfilaments by interacting with GTPase-activating proteins (GAPs) for the Rho family GTPases Rac1 and CDC42 (Ikeda et al., 1998; Yamaguchi et al., 1997). This in turn can lead to actin assembly and the formation of membrane ruffles at the cell surface (through Rac1) and actin-rich filopodia (through CDC42) (Hall, 1998). Other EHD proteins, such as intersectin 1, regulate actin assembly by serving as guanine nucleotide exchange factors (GEFs) for CDC42 (Hussain et al., 2001) and binding to the Wiscott Aldrich Syndrome protein (WASp) (McGavin et al., 2001). WASp activates the Arp2/3 complex and stimulates nucleation of new actin filaments in response to extracellular signals (Millard et al., 2004).

EHD proteins also play various roles in signal transduction (Adams et al., 2000; Tong et al., 2000a; Tong et al., 2000b), which is not surprising considering that many contain known signaling modules, including SH3 and proline-rich domains. The intersectin SH3 domain regulates Ras activation and indirectly controls activation of MAP kinase (Tong et al., 2000a). Data also indicate that some EHD proteins act in the nucleus regulating transcription (Doria et al., 1999; Poupon et al., 2002; Vecchi et al., 2001). For example, both Eps15 and Eps15R are involved in nucleocytoplasmic shuttling of RNA and proteins via the Rev export pathway (Doria et al., 1999; Poupon et al., 2002), and this activity is independent of endocytic events (Vecchi et al., 2001).

Mammalian cells possess four highly homologous EHD proteins in which the EH domain is at the C-terminus (Mintz et al., 1999; Pohl et al., 2000) (Fig. 1). Of all known EHD proteins identified, few have C-terminal EH domains. *S. cerevisae* is a notable exception: two C-terminal EHD proteins have been identified (Irs4p and YJL085w). However, neither of these proteins shares significant sequence similarity with the mammalian C-terminal EHD proteins outside the EH domain. The few identifiable C-terminal EHD proteins in other species

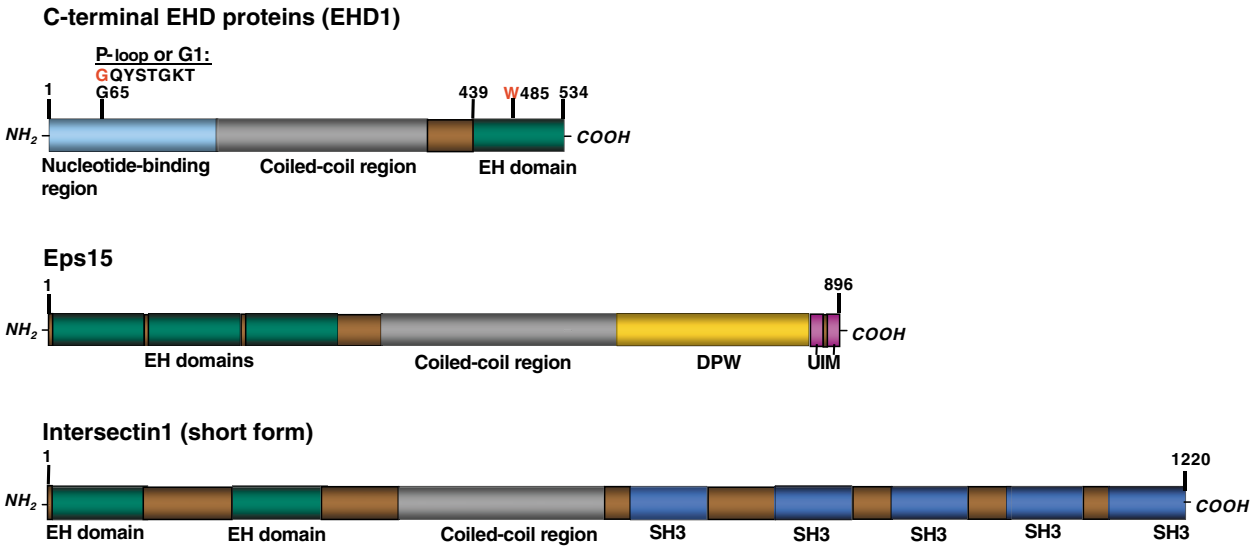

Fig. 1. Comparison of C-terminal EHD protein architecture with other EHD proteins. Mammalian C-terminal EHD proteins comprise 534-541 amino acids and have three recognizable domains: an N-terminal nucleotide-binding region (light blue), a C-terminal EH domain (green), and a central coiled-coil region (gray). The motif homologous to the P-loop (or H-Ras G1 motif) is indicated, beginning with glycine at residue 65 (shown in red). Also denoted is the conserved tryptophan (W485) essential for EH-domain-mediated binding. For comparison, the EHD proteins Eps15 and intersectin1 are shown. These contain the following domains: DPW (orange); aspartate-proline-tryptophan; ubiquitin interacting motif, UIM (purple); src homology domain 3, SH3 (dark blue).

are orthologs of the human C-terminal EHD protein family. The EHD proteins have been addressed recently in several excellent reviews (Confalonieri and Di Fiore, 2002; Miliaras and Wendland, 2004; Polo et al., 2003; Santolini et al., 1999); however, there has been very little focus on the mammalian C-terminal EHD proteins and their functions. Here, we highlight the growing consensus for functions of these C-terminal EHD proteins in endocytic transport events and outline some recent advances.

Structure and organization of mammalian C-terminal EHD proteins

C-terminal EHD orthologs and paralogs

The human C-terminal EHD proteins display very high levels of amino-acid sequence-similarity and -identity with each other, which extends throughout the proteins (Fig. 2). Interestingly, the levels of similarity and identity shared by orthologs of different species are even higher. For example, the amino acid sequence of human EHD1 (hEHD1) shares 99.6% similarity and 99.4% identity with that of its mouse ortholog counterpart. By way of comparison, hEHD1 shares only 70.3% identity with hEHD2, 86.5% identity with hEHD3 and 74.1% identity with hEHD4. This high degree of similarity/identity is maintained even in non-mammalian species, most of which contain a single C-terminal EHD protein, which best resembles hEHD1. Indeed, even the *Anopheles* mosquito and zebrafish C-terminal EHD paralogs show a higher degree of similarity to hEHD1 than does either hEHD2 or hEHD4.

The EH domains of C-terminal EHD proteins also share a higher level of sequence similarity with each other than with the EH domains of Eps15 or intersectin. Significantly, the overall identity shared by C-terminal EHD proteins is slightly higher than that shared by their EH domains alone. This raises the possibility that the conserved functions of these C-terminal EHD proteins are not exclusively dependent on the EH domain.

Domain architecture

Predictably, the four human C-terminal EHD paralogs have the same domain architecture (Fig. 1). Sequence analyses using programs such as the UniProt protein resource (http://www.ebi.uniprot.org/index.html) show that, in addition to the C-terminal EH domain, these proteins have a central region that has a high probability of coiled-coil formation and a nucleotide-binding region near the N-terminus (Fig. 1). Several studies have shown that EHD proteins form homo- and hetero-oligomers (Caplan et al., 2002; Galperin et al., 2002; Lee et al., 2005), and oligomerization appears to be mediated by the coiled-coil region (Lee et al., 2005). Although these proteins do not contain a transmembrane domain, they associate with membranes. It is not known whether the membrane association occurs through a direct interaction with lipids, or whether it is mediated by lipid-binding proteins; however, this association depends upon the ability to bind nucleotides (Grant et al., 2001; Caplan et al., 2002; Lee et al., 2005; Lin et al., 2001).

Nucleotide-binding

All mammalian C-terminal EHD proteins contain a putative P-loop motif, an ATP/GTP-binding site found in Ras-family proteins, myosin heavy-chains and other kinases (Saraste et al., 1990). A recent study has demonstrated that ATP is the primary nucleotide that binds to and is hydrolyzed by EHD1 (Lee et al., 2005), although it remains possible that in vivo EHD1 might also be capable of binding and/or hydrolyzing GTP.

The first study to demonstrate the functional significance of the predicted nucleotide-binding P-loop utilized an in vivo endocytic assay to show that growing oocytes possessing a glycine-to-arginine mutation within the conserved P-loop of the *C. elegans* Rme-1 protein (the ortholog of human EHD1) exhibit impaired uptake of the yolk protein (Grant et al., 2001). This probably resulted from impaired recycling of the yolk receptors in these mutants (Grant et al., 2001). These findings were in agreement with previous studies showing that mutations in the homologous glycine residue of the Ras P-loop decrease GTPase activity and render the protein oncogenic (Seeburg et al., 1984). The equivalent mutation (G65R) in hEHD1 causes the protein to lose its association with membranes (Caplan et al., 2002; Lin et al., 2001).

Support for EHD1 nucleotide-binding activity also came from FRAP studies in living cells (Caplan et al., 2002). EHD1 localizes to a striking array of tubular and vesicular membrane structures (Caplan et al., 2002). Following photobleaching of the tubular membranes containing GFP-EHD1 in human cell lines, the fluorescence signal returns to these structures within several minutes. This suggests that this protein cycles on and off the membranes (Caplan et al., 2002), which is a hallmark of many nucleotide-binding proteins.

Functions of mammalian C-terminal EHD proteins

Interaction partners for C-terminal EHD proteins

About 20 different direct and indirect interaction partners have been reported for the C-terminal EHD proteins (Table 1). Although the mode of binding has not been established in all cases, most appear to bind to EHD proteins through their EH domains. The Rab4/Rab5 divalent effector protein rabenosyn 5 (de Renzis et al., 2002; Nielsen et al., 2000) contains five NPF motifs, and optimal interaction with EHD1 appears to require the first two of these motifs (Naslavsky et al., 2004). The recently identified EH binding protein 1 (EHBP1) also contains five NPF motifs (Guilherme et al., 2004a). Repeated NPF motifs might strengthen the interactions with EH domains. However, various proteins containing only a single NPF motif (e.g. the cell fate adaptor protein Numb) bind to human and *D. melanogaster* EHD proteins (Smith et al., 2004). Syndapin II, a protein belonging to the syndapin/pacsin family of dynamin-binding proteins (Kessels and Qualmann, 2004), also contains a single NPF motif that binds to EHD1 (Xu et al., 2004). Similarly, the SNARE protein SNAP29/GS32 also appears to bind EHD1 with a single NPF motif (Xu et al., 2004), although it has also been shown to interact with the EHD1 coiled-coil region (Rotem-Yehudar et al., 2001).

The mode of binding is probably similar to that described for the EH domain of other EHD proteins (de Beer et al., 1998): mutation of the conserved tryptophan of EHD1 described above (W485A) impairs its binding to rabenosyn 5 (Naslavsky et al., 2004). Since this residue is conserved in all four mammalian C-terminal EHD proteins, it is probably crucial for

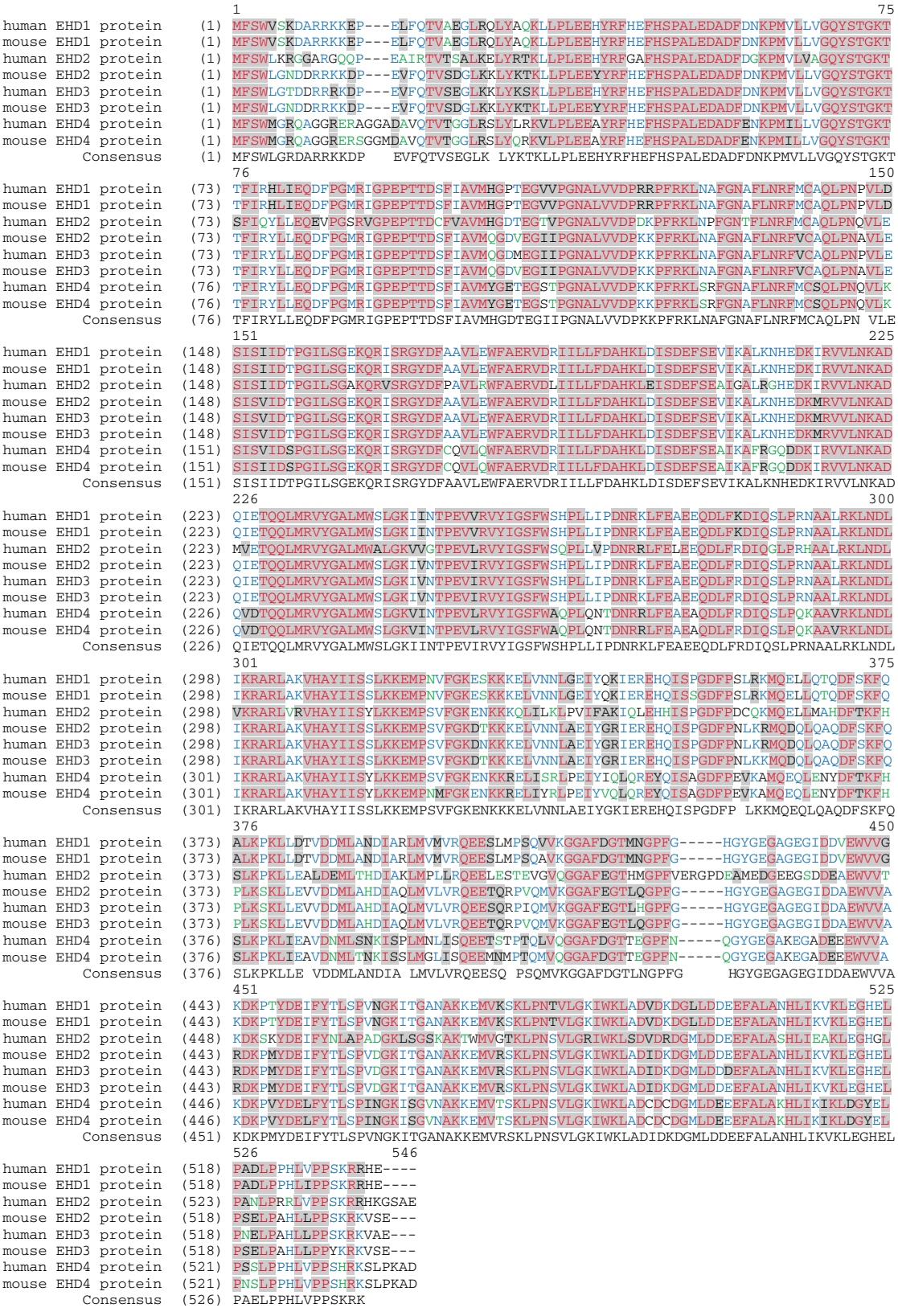

Fig. 2. Alignment of human and mouse C-terminal EHD proteins. Full-length amino acid sequences of human and mouse C-terminal EHD proteins were aligned by the ClustalW Multiple Sequences Alignment in the Vector NTI software program (Invitrogen, Carlsbad, CA). Shaded red letters indicate an identical match between all eight sequences, unshaded blue letters denote identity with the consensus sequence, while shaded black letters indicate similarity (but not identity) with the same consensus sequence. Unshaded green letters denote amino acids with little similarity to the consensus sequence. Unshaded black letters are used when there is no consensus residue.

Table 1. C-terminal EHD interacting proteins

C-terminal EHD protein	Interacting partner	Mode of interaction	References
EHD1	Insulin-like growth factor 1 receptor		(Rotem-Yehudar et al., 2001)
EHD1	SNAP29/GS32	Coiled-coil (Rotem-Yehudar et al., 2001) EH domain (Xu et al., 2004)	(Rotem-Yehudar et al., 2001; Xu et al., 2004)
EHD1	AP-2 α-adaptin		(Rotem-Yehudar et al., 2001)
EHD1	Clathrin heavy chain		(Rotem-Yehudar et al., 2001)
EHD1	Syndapin I	EH domain	(Xu et al., 2004; Braun et al., 2005)
EHD1	Rabenosyn-5	EH domain	(Naslavsky et al., 2004)
EHD1	Epsin	EH domain	(our unpublished observations)
EHD1	Stonin2	EH domain	(our unpublished observations)
EHD1	Syndapin II	EH domain	(Braun et al., 2005)
Rme-1 (*C. elegans*)	Reticulon-C protein	C-terminal region	(Iwahashi et al., 2002)
Drosophila EHD1	Numb	EH domain	(Smith et al., 2004)
EHD2	GLUT4		(Park et al., 2004)
EHD2	AP-1μ1		(Park et al., 2004)
EHD2	AP-2μ2		(Park et al., 2004)
EHD2	CALM		(Park et al., 2004)
EHD2	EHBP1	EH domain and NPF motifs	(Guilherme et al., 2004a)
EHD2	Arp2/3	Acidic region prior to the EH domain	(Guilherme et al., 2004b)
EHD2	Rabenosyn-5	EH domain	(Naslavsky et al., 2004)
EHD3	Rabenosyn-5	EH domain	(Naslavsky et al., 2004)
EHD3	Syndapin I and Syndapin II	EH domain	(Braun et al., 2005)
EHD4	Numb	EH domain	(Smith et al., 2004)
EHD4	Type VI collagen		(Kuo et al., 2001)
EHD4	Syndapin I and Syndapin II	EH domain	(Braun et al., 2005)

interacting with all NPF-containing binding partners for these proteins.

It is noteworthy that there are instances in which C-terminal EHD proteins can bind to the same interaction partners as the other EHD proteins. The NPF-containing protein stonin 2 interacts with Eps15, Eps15R, intersectin 1 (Martina et al., 2001) and, at least in vitro, EHD1 and EHD3. Numb interacts with Eps15 (Salcini et al., 1997), as well as with EHD4 (Smith et al., 2004). However, whether other NPF-containing proteins bind promiscuously to C-terminal and other EHD proteins remains to be seen.

Roles for C-terminal EHD proteins in endocytic transport and recycling

Given the number of C-terminal-EHD-interacting proteins known to have roles in endocytosis, the regulation of endocytic events is probably a major function of these proteins (Fig. 3). EHD1 localizes to endocytic structures and binds to various components of the endocytic machinery, including the clathrin heavy-chain and AP-2 (Mintz et al., 1999). Furthermore, genetic screens in *C. elegans* identified Rme-1 as an important mediator of yolk receptor recycling, as previously mentioned (Grant et al., 2001). Mammalian EHD1 was also found to regulate the distribution of the endocytic recycling compartment (ERC) and control exit of transferrin and its receptor from the ERC (Lin et al., 2001). In addition to regulating clathrin-dependent transport, EHD1 controls the endocytic recycling and transport of receptors internalized through clathrin-independent pathways. For example, the recycling of major histocompatibility complex class I (MHC-I) proteins is regulated by EHD1 (Caplan et al., 2002), and overexpression of EHD4 stimulates clathrin-independent macropinocytosis of the nerve growth factor receptor (TrkA) in PC12 rat adrenal pheochromocytoma cells (Shao et al., 2002).

EHD1 is itself regulated by the small GTPase Arf6 (Caplan et al., 2002). EHD1 colocalizes with Arf6, and overexpression of Arf6 mutants dramatically alters the subcellular localization of EHD1. Arf6 is the most divergent member of the Arf family, and its dynamic cycling between GDP- and GTP-bound states is thought to regulate membrane trafficking and recycling and to control transport of receptors internalized in a clathrin-independent manner (Donaldson, 2003). A recent study supports the connection between C-terminal EHD proteins and Arf6, demonstrating that EHD4 and the cell-fate determinant Numb both colocalize with Arf6. Expression of a GTP-locked Arf6 mutant causes both EHD4 and Numb to exhibit altered subcellular distribution patterns (Smith et al., 2004), which is similar to the effects on EHD1 described above. Numb and EHD4 might thus regulate recycling from the ERC to the plasma membrane.

C-terminal EHD proteins regulate the recycling of a wide array of proteins. The recycled cargo includes transferrin receptors (Lin et al., 2001; Naslavsky et al., 2004; and see also Fig. 4), MHC-I proteins (Caplan et al., 2002), the cystic fibrosis transmembrane conductance regulator (Picciano et al., 2003), the insulin-regulated GLUT4 glucose transporter (Guilherme et al., 2004b), HIV Nef (Larsen et al., 2004) and long-term potentiation AMPA-type glutamate receptors at post-synaptic membranes (Park et al., 2004a). Overall, these studies indicate a key role for mammalian C-terminal EHD proteins in endocytic recycling.

Although the precise mechanism underlying the mode by which C-terminal EHD proteins regulate recycling is not yet clear, a role for EHD1 has been established in controlling transport out of the ERC (Fig. 3). This is a function that has also been attributed to Rab11 and some of its effectors (Hales et al., 2002; Mammoto et al., 1999; Prekeris et al., 2000; Ren et al., 1998; Ullrich et al., 1996; Wilson et al., 2005). While there is no evidence suggesting a connection between Rab11 and C-terminal EHD proteins, it is tempting to speculate that these two proteins coordinately regulate transport out of the

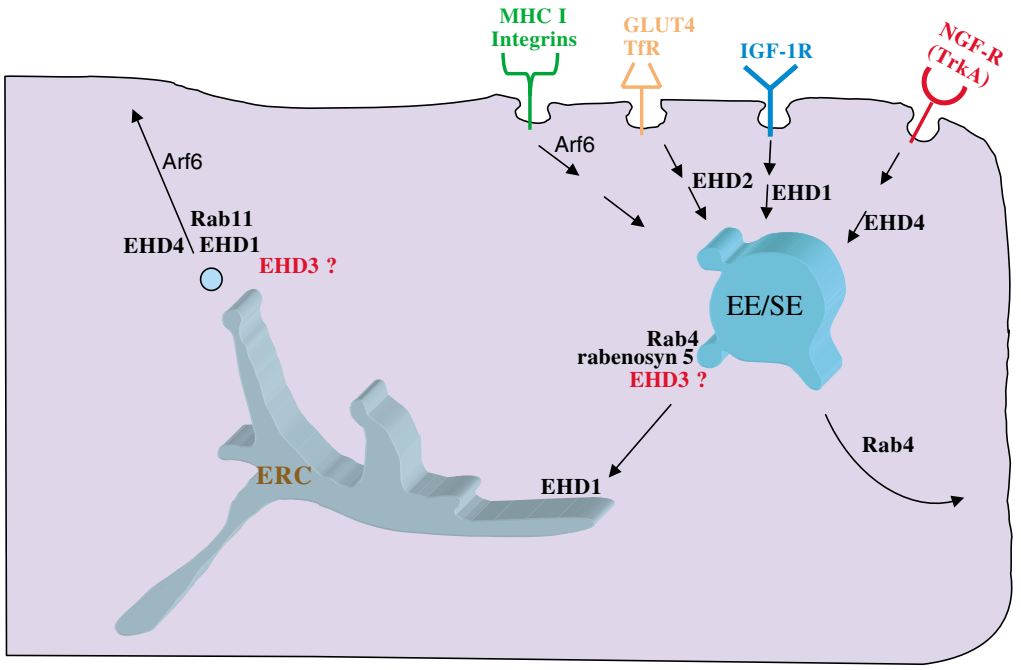

Fig. 3. Involvement of mammalian C-terminal EHD proteins in endocytic pathways. Mammalian EHD proteins affect a wide range of endocytic events. EHD1 plays a central role in regulating the recycling of various receptors from the perinuclear recycling compartment to the plasma membrane. These include receptors that have been internalized either through clathrin-coated pits (e.g. the transferrin receptor, TfR) or independently of clathrin [e.g. major histocompatability complex class I molecules (MHC-I) and integrins]. The latter are regulated by the small GTPase Arf6. An endocytic regulatory role similar to that of EHD1 has been proposed for EHD4, and both EHD proteins are involved in the Arf6 recycling pathway. However, EHD1 has also been implicated at earlier stages of the endocytic pathway, including the internalization of insulin-like growth factor 1 receptor (IGF-1R). Both EHD2 and EHD4 have also been linked to early endocytic events, regulating internalization of transferrin and nerve growth factor receptors (NGF-R, TrkA), respectively. The function of EHD3 has not yet been elucidated. The relationship between key Rab-family members and EHD proteins remains an open question. EE/SE, early endosome/sorting endosome; ERC, endocytic recycling compartment; GLUT4, glucose transporter isoform 4.

ERC and on to the plasma membrane. EHD1 may, however, regulate additional endocytic transport steps. For example, there is evidence that it acts in concert with rabenosyn 5 and possibly other EHD paralogs to regulate the transport step from early endosomes to the ERC (Naslavsky et al., 2004) (Fig. 3). In addition, EHD1 controls earlier, pre-endosomal transport events in the case of insulin-like growth factor 1 (IGF-1) receptor (Rotem-Yehudar et al., 2001). On the basis of its homology to EHD1 and the interaction between the two proteins, EHD3 is predicted to play a role in endocytic transport and regulate transport at the early endosome and/or ERC. However, the function of this protein has not been elucidated yet.

EHD2 plays an endocytic role in adipocytes, where it serves to connect endocytic events at the plasma membrane with the actin cytoskeleton through its interaction with EH-binding protein 1 (EHBP1) (Guilherme et al., 2004a). EHBP1 is an actin-binding protein, and its overexpression or that of EHD2 causes extensive actin reorganization. Internalization of transferrin or its transport during the early steps of the endocytic pathway en route to the early endosome is impaired in cells overexpressing either wild-type EHD2 or an EHD2 mutant that lacks the EH domain, and cells in which EHD2 is knocked-down by RNAi (Guilherme et al., 2004a). In agreement with a role for EHD proteins in internalization are studies showing that they interact with components of the internalization machinery. EHD2 binds to the μ1 and μ2 subunits of the AP-1 and AP-2 adaptor complexes (Park et al., 2004b), and EHD1 interacts with clathrin and the α-adaptin subunit of AP-2 (Rotem-Yehudar et al., 2001). Moreover, treatment with IGF-1 leads to the colocalization of EHD1 with IGF-1 receptors at the plasma membrane (Rotem-Yehudar et al., 2001) and presumably the recruitment of AP-2. Interestingly, Eps15 also binds to the α-adaptin subunit of AP-2 (Benmerah et al., 1996) and stimulation of epidermal growth factor receptors leads to recruitment of Eps15 and AP-2 to the these receptors (van Delft et al., 1997). However, it remains unclear whether Eps15 and C-terminal EHD proteins carry out similar scaffolding tasks for different receptors, or whether they compete for binding to α-adaptin and NPF-containing proteins.

Perspectives

The recent developments in the field of C-terminal EHD proteins have led to a consensus for their involvement in the regulation of endocytic transport. The findings are in agreement with the scaffolding functions described for other EHD proteins, such as Eps15 and intersectins. Several features distinguish C-terminal EHD proteins from other EH-domain-containing proteins. First, C-terminal EHD proteins have only a single EH domain, positioned at the C-terminus. Second,

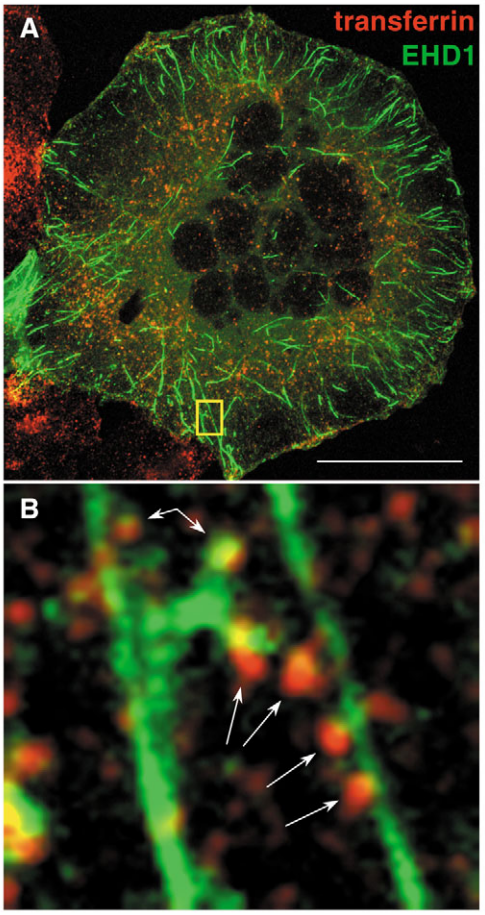

Fig. 4. Colocalization of internalized transferrin vesicles with EHD1. Human HeLa cells were transfected with GFP-EHD1 and subjected to a 10-minute pulse with labeled transferrin (transferrin 568 nm). As depicted in A, transferrin is observed in a range of peripheral and perinuclear vesicles, whereas EHD1 localizes to tubulo-vesicular membranes. The merged image shows a partial colocalization of internalized transferrin with vesicular EHD1-containing structures. The yellow box in A marks the inset depicted as B. Examples of transferrin-containing vesicular structures aligned with EHD1-containing tubular membranes are marked by white arrows. Bar, 10 μm.

these proteins contain a nucleotide-binding motif. Third, C-terminal EHD proteins lack other identifiable domains and motifs that are commonly found on other EHD proteins. Finally, C-terminal EHD proteins (EHD1 and EHD3) display a remarkable tubular and vesicular subcellular localization pattern, concentrations of the protein being localized to the perinuclear ERC and these distributions differ from those of Eps15 and intersectin. Although evidence suggests that C-terminal EHD proteins control the internalization of certain receptors in a manner similar to other EHD proteins, EHD1-EHD4 appear to be unique among EHD proteins in their ability to regulate recycling events.

EHD1 has been the most extensively studied C-terminal EHD protein. To determine the specific functions of the other mammalian paralogs, it will be important to generate specific antibodies for each of the EHD proteins. While this goal has been complicated by the high degree of sequence identity between the proteins, the first step in understanding their function and the significance of EHD oligomerization will be determining whether they are all simultaneously expressed in the same cell types. Once this has been achieved, RNAi technology should allow us begin to address the functional differences between members of this family.

Many questions concerning the mechanisms by which C-terminal EHD proteins control endocytic transport remain. Among these are the significance of homo- and hetero-oligomerization, nucleotide binding, and interactions with binding partners. One of the key issues is understanding the mode by which C-terminal EHD proteins coordinately regulate recycling with Rab proteins. As noted, Rab4 and Rab11 play crucial roles in endocytic recycling. C-terminal EHD proteins have been linked to Rab4-mediated transport via the EHD1–rabenosyn-5 interaction (Naslavsky et al., 2004). However, thus far no attempts have been made to discover how C-terminal EHD proteins coordinate transport out of the ERC with Rab11 and its effectors. The identification of new interacting partners is likely to enhance our understanding of this complex mode of coordinate regulation.

We thank E. Haas at the UNMC Genetic Sequence Analysis Facility for helpful advice and C. Arighi (Georgetown Protein Information Resource) for advice and critical reading of the manuscript. We also thank R. G. MacDonald, R. E. Lewis, R. C. Aguilar, and the members of the Caplan laboratory for their critical reading of the manuscript. This work was supported by NIH Grant number P20 RR018759 from the National Center for Research Resources, and American Heart Association Grant number 0460001z, and the State of Nebraska, Dept of Health and Human Services.

References

Adams, A., Thorn, J. M., Yamabhai, M., Kay, B. K. and O'Bryan, J. P. (2000). Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways. *J. Biol. Chem.* **275**, 27414-27420.

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

Benmerah, A., Begue, B., Dautry-Varsat, A. and Cerf-Bensussan, N. (1996). The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein. *J. Biol. Chem.* **271**, 12111-12116.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A. and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* **140**, 1055-1062.

Caplan, S., Naslavsky, N., Hartnell, L. M., Lodge, R., Polishchuk, R. S., Donaldson, J. G. and Bonifacino, J. S. (2002). A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. *EMBO J.* **21**, 2557-2567.

Carbone, R., Fre, S., Iannolo, G., Belleudi, F., Mancini, P., Pelicci, P. G., Torrisi, M. R. and Di Fiore, P. P. (1997). eps15 and eps15R are essential components of the endocytic pathway. *Cancer Res.* **57**, 5498-5504.

Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P. and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394**, 793-797.

Confalonieri, S. and Di Fiore, P. P. (2002). The Eps15 homology (EH) domain. *FEBS Lett.* **513**, 24-29.

Conner, S. D. and Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.

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

de Beer, T., Hoofnagle, A. N., Enmon, J. L., Bowers, R. C., Yamabhai, M.,

Kay, B. K. and Overduin, M. (2000). Molecular mechanism of NPF recognition by EH domains. *Nat. Struct. Biol.* **7**, 1018-1022.
de Renzis, S., Sonnichsen, B. and Zerial, M. (2002). Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. *Nat. Cell. Biol.* **4**, 124-133.
Donaldson, J. G. (2003). Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J. Biol. Chem.* **278**, 41573-41576.
Doria, M., Salcini, A. E., Colombo, E., Parslow, T. G., Pelicci, P. G. and Di Fiore, P. P. (1999). The eps15 homology (EH) domain-based interaction between eps15 and hrb connects the molecular machinery of endocytosis to that of nucleocytosolic transport. *J. Cell Biol.* **147**, 1379-1384.
Duncan, M. C., Cope, M. J., Goode, B. L., Wendland, B. and Drubin, D. G. (2001). Yeast Eps15-like endocytic protein, Pan1p, activates the Arp2/3 complex. *Nat. Cell Biol.* **3**, 687-690.
Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T. and Di Fiore, P. P. (1993). eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol. Cell. Biol.* **13**, 5814-5828.
Galperin, E., Benjamin, S., Rapaport, D., Rotem-Yehudar, R., Tolchinsky, S. and Horowitz, M. (2002). EHD3: a protein that resides in recycling tubular and vesicular membrane structures and interacts with EHD1. *Traffic* **3**, 575-589.
Grant, B., Zhang, Y., Paupard, M. C., Lin, S. X., Hall, D. H. and Hirsh, D. (2001). Evidence that RME-1, a conserved C. elegans EH-domain protein, functions in endocytic recycling. *Nat. Cell Biol.* **3**, 573-579.
Guilherme, A., Soriano, N. A., Bose, S., Holik, J., Bose, A., Pomerleau, D. P., Furcinitti, P., Leszyk, J., Corvera, S. and Czech, M. P. (2004a). EHD2 and the Novel EH Domain Binding Protein EHBP1 Couple Endocytosis to the Actin Cytoskeleton. *J. Biol. Chem.* **279**, 10593-10605.
Guilherme, A., Soriano, N. A., Furcinitti, P. S. and Czech, M. P. (2004b). Role of EHD1 and EHBP1 in perinuclear sorting and insulin-regulated GLUT4 recycling in 3T3-L1 adipocytes. *J. Biol. Chem.* **279**, 40062-40075.
Hales, C. M., Vaerman, J. P. and Goldenring, J. R. (2002). Rab11 family interacting protein 2 associates with Myosin Vb and regulates plasma membrane recycling. *J. Biol. Chem.* **277**, 50415-50421.
Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.
Hussain, N. K., Jenna, S., Glogauer, M., Quinn, C. C., Wasiak, S., Guipponi, M., Antonarakis, S. E., Kay, B. K., Stossel, T. P., Lamarche-Vane, N. et al. (2001). Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. *Nat. Cell Biol.* **3**, 927-932.
Ikeda, M., Ishida, O., Hinoi, T., Kishida, S. and Kikuchi, A. (1998). Identification and characterization of a novel protein interacting with Ral-binding protein **1**, a putative effector protein of Ral. *J. Biol. Chem.* **273**, 814-821.
Kessels, M. M. and Qualmann, B. (2004). The syndapin protein family: linking membrane trafficking with the cytoskeleton. *J. Cell Sci.* **117**, 3077-3086.
Larsen, J. E., Massol, R. H., Nieland, T. J. and Kirchhausen, T. (2004). HIV Nef-mediated major histocompatibility complex class I down-modulation is independent of Arf6 activity. *Mol. Biol. Cell* **15**, 323-331.
Lee, D. W., Zhao, X., Scarselletta, S., Schweinsberg, P. J., Eisenberg, E., Grant, B. D. and Greene, L. E. (2005). ATP Binding regulates oligomerization and endosome association of RME-1 family proteins. *J. Biol. Chem*. **280**, 17213-17220.
Lin, S. X., Grant, B., Hirsh, D. and Maxfield, F. R. (2001). Rme-1 regulates the distribution and function of the endocytic recycling compartment in mammalian cells. *Nat. Cell Biol.* **3**, 567-572.
Mammoto, A., Ohtsuka, T., Hotta, I., Sasaki, T. and Takai, Y. (1999). Rab11BP/Rabphilin-11, a downstream target of rab11 small G protein implicated in vesicle recycling. *J. Biol. Chem.* **274**, 25517-25524.
Martina, J. A., Bonangelino, C. J., Aguilar, R. C. and Bonifacino, J. S. (2001). Stonin **2**, an adaptor-like protein that interacts with components of the endocytic machinery. *J. Cell Biol.* **153**, 1111-1120.
McGavin, M. K., Badour, K., Hardy, L. A., Kubiseski, T. J., Zhang, J. and Siminovitch, K. A. (2001). The intersectin 2 adaptor links Wiskott Aldrich Syndrome protein (WASp)-mediated actin polymerization to T cell antigen receptor endocytosis. *J. Exp. Med.* **194**, 1777-1787.
Miliaras, N. B. and Wendland, B. (2004). EH Proteins: Multivalent Regulators of Endocytosis (and Other Pathways). *Cell Biochem. Biophys.* **41**, 295-318.
Millard, T. H., Sharp, S. J. and Machesky, L. M. (2004). Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem. J.* **380**, 1-17.
Mintz, L., Galperin, E., Pasmanik-Chor, M., Tulzinsky, S., Bromberg, Y., Kozak, C. A., Joyner, A., Fein, A. and Horowitz, M. (1999). EHD1 – an EH-domain-containing protein with a specific expression pattern. *Genomics* **59**, 66-76.
Naslavsky, N., Boehm, M., Backlund, P. S., Jr and Caplan, S. (2004). Rabenosyn-5 and EHD1 Interact and Sequentially Regulate Protein Recycling to the Plasma Membrane. *Mol. Biol. Cell* **15**, 2410-2422.
Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B. and Zerial, M. (2000). Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. *J. Cell Biol.* **151**, 601-612.
Paoluzi, S., Castagnoli, L., Lauro, I., Salcini, A. E., Coda, L., Fre, S., Confalonieri, S., Pelicci, P. G., Di Fiore, P. P. and Cesareni, G. (1998). Recognition specificity of individual EH domains of mammals and yeast. *EMBO J.* **17**, 6541-6550.
Park, M., Penick, E. C., Edwards, J. G., Kauer, J. A. and Ehlers, M. D. (2004a). Recycling endosomes supply AMPA receptors for LTP. *Science* **305**, 1972-1975.
Park, S. Y., Ha, B. G., Choi, G. H., Ryu, J., Kim, B., Jung, C. Y. and Lee, W. (2004b). EHD2 interacts with the insulin-responsive glucose transporter (GLUT4) in rat adipocytes and may participate in insulin-induced GLUT4 recruitment. *Biochemistry* **43**, 7552-7562.
Picciano, J. A., Ameen, N., Grant, B. D. and Bradbury, N. A. (2003). Rme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol. Cell Physiol.* **285**, C1009-C1018.
Pohl, U., Smith, J. S., Tachibana, I., Ueki, K., Lee, H. K., Ramaswamy, S., Wu, Q., Mohrenweiser, H. W., Jenkins, R. B. and Louis, D. N. (2000). EHD2, EHD3, and EHD4 encode novel members of a highly conserved family of EH domain-containing proteins. *Genomics* **63**, 255-262.
Polo, S., Confalonieri, S., Salcini, A. E. and Di Fiore, P. P. (2003). EH and UIM: endocytosis and more. *Sci STKE* **2003**, re17.
Poupon, V., Polo, S., Vecchi, M., Martin, G., Dautry-Varsat, A., Cerf-Bensussan, N., Di Fiore, P. P. and Benmerah, A. (2002). Differential nucleocytoplasmic trafficking between the related endocytic proteins Eps15 and Eps15R. *J. Biol. Chem.* **277**, 8941-8948.
Prekeris, R., Klumperman, J. and Scheller, R. H. (2000). A Rab11/Rip11 protein complex regulates apical membrane trafficking via recycling endosomes. *Mol. Cell* **6**, 1437-1448.
Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M. and Sabatini, D. D. (1998). Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proc. Natl. Acad. Sci. USA* **95**, 6187-6192.
Rotem-Yehudar, R., Galperin, E. and Horowitz, M. (2001). Association of insulin-like growth factor 1 receptor with EHD1 and SNAP29. *J. Biol. Chem.* **276**, 33054-33060.
Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G. and Di Fiore, P. P. (1997). Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* **11**, 2239-2249.
Santolini, E., Salcini, A. E., Kay, B. K., Yamabhai, M. and Di Fiore, P. P. (1999). The EH network. *Exp. Cell Res.* **253**, 186-209.
Saraste, M., Sibbald, P. R. and Wittinghofer, A. (1990). The P-loop – a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430-434.
Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V. and Levinson, A. D. (1984). Biological properties of human c-Ha-ras1 genes mutated at codon 12. *Nature* **312**, 71-75.
Shao, Y., Akmentin, W., Toledo-Aral, J. J., Rosenbaum, J., Valdez, G., Cabot, J. B., Hilbush, B. S. and Halegoua, S. (2002). Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. *J. Cell Biol.* **157**, 679-691.
Smith, C. A., Dho, S. E., Donaldson, J., Tepass, U. and McGlade, C. J. (2004). The Cell Fate Determinant Numb Interacts with EHD/Rme-1 Family Proteins and Has a Role in Endocytic Recycling. *Mol. Biol. Cell* **15**, 3698-708.
Sorkin, A. (2004). Cargo recognition during clathrin-mediated endocytosis: a team effort. *Curr. Opin. Cell Biol.* **16**, 392-399.
Strynadka, N. C. and James, M. N. (1989). Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* **58**, 951-998.
Tang, H. Y., Munn, A. and Cai, M. (1997). EH domain proteins Pan1p and End3p are components of a complex that plays a dual role in organization of the cortical actin cytoskeleton and endocytosis in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **17**, 4294-4304.
Tong, X. K., Hussain, N. K., Adams, A. G., O'Bryan, J. P. and McPherson,

P. S. (2000a). Intersectin can regulate the Ras/MAP kinase pathway independent of its role in endocytosis. *J. Biol. Chem.* **275**, 29894-29899.

Tong, X. K., Hussain, N. K., de Heuvel, E., Kurakin, A., Abi-Jaoude, E., Quinn, C. C., Olson, M. F., Marais, R., Baranes, D., Kay, B. K. et al. (2000b). The endocytic protein intersectin is a major binding partner for the Ras exchange factor mSos1 in rat brain. *EMBO J.* **19**, 1263-1271.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135**, 913-924.

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

Vecchi, M., Polo, S., Poupon, V., van de Loo, J. W., Benmerah, A. and Di Fiore, P. P. (2001). Nucleocytoplasmic shuttling of endocytic proteins. *J. Cell Biol.* **153**, 1511-1517.

Wendland, B., McCaffery, J. M., Xiao, Q. and Emr, S. D. (1996). A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J. Cell Biol.* **135**, 1485-1500.

Wilson, G. M., Fielding, A. B., Simon, G. C., Yu, X., Andrews, P. D., Hames, R. S., Frey, A. M., Peden, A. A., Gould, G. W. and Prekeris, R. (2005). The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis. *Mol. Biol. Cell* **16**, 849-860.

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

Xu, Y., Shi, H., Wei, S., Wong, S. H. and Hong, W. (2004). Mutually exclusive interactions of EHD1 with GS32 and Syndapin II. *Mol. Membr. Biol.* **21**, 269-277.

Yamaguchi, A., Urano, T., Goi, T. and Feig, L. A. (1997). An Eps homology (EH) domain protein that binds to the Ral-GTPase target, RalBP1. *J. Biol. Chem.* **272**, 31230-31234.