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Scratching the surface: Actin' and other roles for the C-T terminal Eps15 homology domain protein, EHD2

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Summary

The C-terminal Eps15 homology domain-containing (EHD) proteins participate in multiple aspects of endocytic membrane trafficking. Of the four mammalian EHD proteins, EHD2 appears to be the most disparate, both in terms of sequence homology, and in subcellular localization/ function. Since its initial description as a plasma membrane-associated protein, the precise function of EHD2 has remained enigmatic. Various reports have suggested roles for EHD2 at the plasma membrane, within the endocytic transport system, and even in the nucleus. For example, EHD2 facilitates membrane fusion/repair in muscle cells. Recently the focus has shifted to the role of EHD2 in regulating caveolae. Indeed, EHD2 is highly expressed in tissues rich in caveolae, including fat, muscle and blood vessels. This review highlights cumulative evidence linking EHD2 to actin-rich structures at the plasma membrane, where the plasma membrane-associated phospholipid phosphatidylinositol 4,5- bisphosphate controls EHD2 recruitment. Herein we examine the key pathways where EHD2 might function, and address its potential involvement in these processes.

Keywords

Actin; Eps15 homology domain protein 2; Endocytosis; Membrane trafficking; Phosphatidylinositol (4,5)-bisphosphate

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Internalization of nutrients, receptors, and lipids from the cell surface is fundamental to maintaining cellular homeostasis. As such, defects in internalization/endocytic trafficking impact cell metabolism, signaling, migration, etc., and contribute to disease (Mosesson et al., 2008; Caswell et al., 2009; Gould and Lippincott-Schwartz, 2009). Upon internalization, molecules are moved along a series of tubular/vesicular membranes collectively known as the endocytic transport system. As molecules progress through this system, they are sorted for return to the plasma membrane, lysosomal degradation, or retrograde transport to the Golgi. Coordination of endocytic trafficking requires an organized arsenal of proteins. Among the endocytic regulatory molecules are the C-terminal Eps15 homology (EH) domain-containing proteins. In mammals, there are four identified EHD proteins (EHD1-EHD4), which share 70–86% amino acid sequence identity (Naslavsky and Caplan, 2011).

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Three domains define the tertiary EHD protein structure (Fig. 1): 1) A dynamin-like G-domain binds that hydrolyzes ATP (Daumke et al., 2007), 2) A coiled-coil domain formed from two helical regions that facilitates EHD oligomerization and lipid binding (Daumke et al., 2007), and 3) A C-terminal EH domain that binds to asparagine-proline-phenylalanine (NPF) motifs in partner proteins (Salcini et al., 1997; Grant and Caplan, 2008). The EH domain is a protein interaction module found in a number of endocytic proteins, including Eps15, intersectin, Reps, and γ-synergin (Polo et al., 2003), invariably at the N-terminus. In the EHDs, which appear to be a more recent evolutionary addition and are not found in yeast, the C-terminal EH domain has a positively-charged electrostatic surface, leading to preferential binding to proteins containing NPF motifs followed by acidic residues (Henry et al., 2010; Kieken et al., 2010).

Despite their high level of homology, EHD1, EHD2, EHD3 and EHD4 localize to distinct endocytic membrane compartments and possess varied functions. Of the EHDs, the most is known about EHD1 regulation and function. EHD1 is recruited to tubular recycling endosomes through interactions with the NPF motif-containing proteins MICAL-L1 and syndapin2 (Sharma et al., 2009; Giridharan et al., 2013), where it facilitates trafficking from the endocytic recycling compartment to the plasma membrane (Lin et al., 2001; Caplan et al., 2002). The closest paralog of EHD1, EHD3, directs cargo from early endosomes to the recycling compartment (Naslavsky et al., 2006) or to the Golgi (Naslavsky et al., 2009). EHD4 is present on a subset of early endosomes, and mediates transport to the recycling compartment or to late endosomes/lysosomes (George et al., 2007; Sharma et al., 2008). In stark contrast to the tubular/vesicular membrane localization of EHD1, EHD3 and EHD4, EHD2 is found on the inner leaflet of the plasma membrane. EHD2 is the least conserved EHD family member, sharing only 70% homology with EHD1 and EHD3, and 74% homology with EHD4 (Naslavsky and Caplan, 2011). Indeed, as highlighted in this review, EHD2 is proving to be quite distinct from its EHD relatives. Despite the structural characterization of EHD2, its function has remained enigmatic. Initial findings with transferrin receptor suggested that EHD2 may function in clathrin-dependent endocytosis (Guilherme et al., 2004; Benjamin et al., 2011), and potentially in endosomal recycling (George et al., 2007; Doherty et al., 2008). However, a series of recent reports have revealed an intriguing new consensus for EHD2 function.

A theme on muscles and vessels

All four mammalian EHD proteins are highly expressed in the heart (Pohl et al., 2000; Gudmundsson et al., 2010). EHD2 mRNA is also highly expressed in fat, lung, and skeletal muscle tissue (Pohl et al., 2000; Guilherme et al., 2004), and EHD2 protein is abundant in the lung and spleen (Gudmundsson et al., 2010). Immunostaining of murine skeletal muscle (Mate et al., 2012) and kidney (George et al., 2011) showed EHD2 localized specifically to the tissue vasculature, suggesting expression in the endothelial and/or smooth muscle cells of blood vessels. Overall, the expression pattern of EHD2 points toward a prominent role in the cardiovascular and muscular systems.

Indeed, several studies demonstrate that EHD2 functions in myogenesis and muscle repair. During myogenesis, muscle fibers are formed by the fusion of myoblasts into multi-

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nucleated myotubes. Thus, membrane fusion is an important process for both myoblast fusion and plasma membrane repair. Members of the ferlin protein family, including dysferlin and myoferlin, play prominent roles in myogenesis and muscle repair. The ferlins contain multiple C2 domains, which mediate protein-protein and protein-membrane interactions. The ferlin C2 domains are similar to those found in synaptotagmin, which plays a well characterized role in fusion of synaptic vesicles with the plasma membrane (Posey et al., 2011a). Thus, ferlins are thought to facilitate the trafficking and/or fusion of intracellular vesicles at the fusion/repair site (Posey et al., 2011a). Work by McNally and colleagues showed that myoferlin and Fer-1-like-5 (Fer1L5) contain NPF motifs that bind to EHD2 (Doherty et al., 2008; Posey et al., 2011b). Consequently, EHD2 co-localized with myoferlin and Fer1L5 at discrete plasma membrane structures, and was required for myoblast fusion (Doherty et al., 2008; Posey et al., 2011b). In another study using laser wounding of primary human myotubes, Marg et al. (2012) identified EHD2 as a novel participant in muscle repair. Along with EHD2, dysferlin and the peripheral membrane-binding protein annexin A1 were also recruited to the site of membrane healing (Marg et al., 2012). Although dysferlin does not possess a canonical NPF motif, it does contain an NPxF sequence, and it is intriguing to speculate that it might interact with EHD2. Additionally, mutation of the Fer1L5 NPF motif reduced, but did not abolish EHD2 binding (Posey et al., 2011b), suggesting that alternative modes of interaction may contribute to EHD2's association with ferlin proteins. Altogether, these studies raise new questions regarding the mechanisms by which EHD2 contributes to membrane coalescence.

Shining the spotlight into "little caves"

At the plasma membrane, endogenous EHD2 localizes to discrete, punctate structures that coalesce in patches along cell edges (Fig. 2). Recent studies have identified these EHD2-containing structures as caveolae. Caveolae (Latin for "little caves") are small (50–100 nm) plasma membrane invaginations comprised of integral membrane caveolin proteins and a coat of soluble cavin proteins. Based on their lipid content (enriched in cholesterol, sphingolipids, and phosphatidylinositol 4,5- bisphosphate (PI(4,5)P2)), caveolae are classified as lipid rafts. Proteomic analysis of caveolae isolated from adipocytes first identified EHD2 as a caveolar component (Aboulaich et al., 2004; Brasaemle et al., 2004). Hansen et al. (2011) subsequently established that transfected GFP-EHD2 localized to caveolae. More recently, two concurrent studies demonstrated that endogenous EHD2 co-localizes with caveolar markers, and defined a role for EHD2 in restraining caveolar dynamics (Moren et al., 2012; Stoeber et al., 2012). Notably, caveolae are abundant in vascular endothelial cells, smooth muscle cells, adipocytes, and fibroblasts (Parton and Simons, 2007), which likely pertains to EHD2's observed tissue expression as discussed above.

Since their discovery, multiple functions have been attributed to caveolae, including regulation of cell signaling, cholesterol homeostasis, endocytosis, and mechanosensation (Bastiani and Parton, 2010; Nassoy and Lamaze, 2012). Although initially thought to be dynamic endocytic carriers, live cell imaging studies demonstrated that caveolae are stationary structures that are normally confined to the plasma membrane (Thomsen et al., 2002; Kazazic et al., 2006). Both Moren et al. (2012) and Stoeber et al. (2012) found that in

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the absence of EHD2, caveolae became more dynamic, exhibiting increased motility (visualized using live cell imaging) (Stoeber et al., 2012) and enhanced budding (seen by electron microscopy) (Moren et al., 2012). Additionally, both studies suggested that EHD2 was not required for the biogenesis of caveolae (Moren et al., 2012; Stoeber et al., 2012).

Interestingly, Moren et al. (2012) posited that the mechanism by which EHD2 restricts caveolar mobility may pertain to recent findings regarding EHD proteins and the GTPase dynamin (Jakobsson et al., 2011). Dynamin facilitates scission of endocytic vesicles (including caveolae) from the plasma membrane. To achieve vesicle scission, GTP-bound dynamin assembles into spirals around the neck of membrane invaginations; upon GTP hydrolysis, the dynamin coil constricts, pinching the endocytic vesicle off from the plasma membrane (Ferguson and De Camilli, 2012). Using river lampreys (which express a single EHD protein), Jakobsson et al. (2011) showed that EHD binds and regulates dynamin spiral formation. Thus, EHD2 might control dynamin activity at the caveolar neck to maintain stable invaginations. In support of this hypothesis, blocking dynamin activity in EHD2-depleted cells by expression of dominant-negative dynamin (Stoeber et al., 2012) or treatment with the dynamin inhibitor dynasore (Moren et al., 2012) restored caveolae to their static state.

In addition to EHD2, the F-BAR domain-containing protein syndapin2 (also known as pacsin2) is recruited to caveolae (Hansen et al., 2011). The crescent-shaped BAR domain dimers bind to membranes through their concave surface, thereby sensing and promoting membrane curvature. In keeping with this property, syndapin2 is necessary for shaping caveolar invaginations (Hansen et al., 2011; Senju et al., 2011). Syndapin2 also contains an NPF motif that mediates binding to EHD2 (Braun et al., 2005; Moren et al., 2012). However, as syndapin2 is not required for targeting EHD2 to caveolae (Moren et al., 2012; Simone, et al., 2013), the role of the EHD2-syndapin2 interaction remains undefined. Syndapin proteins are known to bind dynamin through the syndapin SH3 domain. As such, binding of syndapin2 to EHD2 could promote EHD2 regulation of dynamin. However, while EHD2 is a ubiquitous caveolar component, syndapin2 is present in only a subset of caveolae (Hansen et al., 2011; Moren et al., 2012). As noted by Moren et al. (2012), this suggests that the EHD2-syndapin2 association may function at discrete stages of caveolar dynamics.

To determine the mode by which EHD2 associates with caveolae, Stoeber et al. (2012) and Moren et al. (2012) assessed the localization of multiple EHD2 mutants. From these studies, Stoeber et al. (2012) found that the ability of EHD2 to bind lipids through conserved lysine residues (Fig. 1) was necessary for targeting EHD2 to punctate caveolar structures. Both groups also found that binding of ATP to the EHD2 G domain was required for caveolar targeting (Moren et al., 2012; Stoeber et al., 2012). Additionally, Moren et al. (2012) showed that ATP hydrolysis-deficient EHD2 mutants caused distortion of caveolar membranes into tubules. Thus, nucleotide hydrolysis appears to temper the membrane remodeling activity of EHD2 on caveolae. Finally, both Stoeber et al. (2012) and Moren et al. (2012) found that the EHD2 KPFxxxNPF motif (Fig. 1) (which is involved in EHD2 oligomerization (Daumke et al., 2007)) was necessary for EHD2 caveolar localization. Furthermore, using a KPF mutant that also lacked the EH domain, Moren et al. (2012)

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concluded that EHD2 oligomerization via the KPF motif was not necessary for caveolar binding. This suggests that the KPF motif is directly involved in caveolar targeting, perhaps through binding to another protein. In sum, these works provide a detailed foundation to unveil the precise role of EHD2 at caveolae.

Connections with the actin cytoskeleton and PI(4,5)P2

In multiple cell types, immunostaining has shown that EHD2-containing structures are associated with the actin cytoskeleton. For instance, in wounded muscle fiber, EHD2 co-localizes with filamentous actin at the membrane repair site (Marg et al., 2012). In adipocytes, EHD2 is found in ring-shaped structures beneath the plasma membrane that are closely associated with cortical actin (Guilherme et al., 2004). And in fibroblast-like COS-1 cells, EHD2 is found in actin-rich membrane ruffles (Guilherme et al., 2004). Also, over-expressed EHD2 is observed in filopodia (adipocytes and COS-1 cells) (Guilherme et al., 2004) and microspikes (HeLa cells) (George et al., 2007) protruding from the plasma membrane. Finally, EHD2-containing caveolae are aligned along actin filaments in primary human fibroblasts (Stoeber et al., 2012).

EHD2 may be connected to the actin cytoskeleton through several modes, including through actin-binding partner proteins such as EHBP1 (EHD2-binding protein 1). EHBP1 contains 5 closely-spaced NPF motifs that mediate association with EHD2 (Guilherme et al., 2004). Additionally, EHBP1 contains a C-terminal CAAX motif suggested to target it to membranes through prenylation, and a calponin homology (CH) domain that can bind and cross-link actin filaments (Guilherme et al., 2004). In addition to describing the EHBP1-EHD2 interaction, Guilherme et al. (2004) noticed a conserved cluster of acidic residues spanning amino acids 428–444 of EHD2 that terminates in a tryptophan (Fig. 1). As noted by Guilherme et al. (2004), similar sequences in actin-regulatory proteins (including WASP and cortactin) mediate association with the Arp2/3 complex, which nucleates actin filaments. Thus, as suggested by Guilherme et al. (2004), EHD2 may potentially play an active role in actin dynamics through recruitment of Arp2/3.

Another recent study suggests that EHD2 may regulate actin remodeling through its effects on Rac1 activity (Benjamin et al., 2011). In this study, EHD2 bound to the serine/threonine kinase Nek3 (Benjamin et al., 2011), which activates members of the Vav protein family (Miller et al., 2005, 2007). The Vav proteins in turn activate small Rho GTPases, including Rac1. Indeed, Benjamin et al. (2011) found that EHD2 associated with Vav1 (presumably through Nek3), and overexpression of EHD2 led to reduced Rac1 activity. Notably, the Rac proteins are key cytoskeletal regulatory proteins involved in lamellipodium and membrane ruffle formation. As such, future studies examining the role of EHD2 in lamellipodium extension/membrane ruffling may be warranted.

EHD2's lipid-binding properties may also contribute to the linkage between EHD2-containing membrane structures and the actin cytoskeleton. EHD2 membrane-binding is mediated through a conserved polybasic sequence comprised of lysine residues at positions 324 and 327–329 along with phenylalanine 322 (Fig. 1) (Daumke et al., 2007). Additionally, in vitro studies show that EHD2 binds to PI(4,5)P2, which is predominately found at the

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plasma membrane (Blume et al., 2007; Daumke et al., 2007). Indeed, recent work from our lab demonstrates that PI(4,5)P2 plays a critical role in targeting EHD2 to the plasma membrane in vivo (Simone et al., 2013). The actin cytoskeleton is connected to the plasma membrane in part through cytoskeletal proteins that bind to PI(4,5)P2 such as WASP, cofilin and vinculin (Zhang et al., 2012). Thus, the ability of EHD2 to bind PI(4,5)P2 likely underpins the spatial/functional relationship between EHD and actin.

The functional consequences of EHD2's association with and/or impact on the actin cytoskeleton remain inchoate. Stoeber et al. (2012) implicated EHD2 in actively tethering caveolae to the actin cytoskeleton, which may contribute to the stabilizing effect of EHD2 on caveolar dynamics. Along these lines, cortical actin likely confines caveolae to the plasma membrane (Mundy et al., 2002), and caveolar internalization involves dynamic actin remodeling (Pelkmans et al., 2002; Parton and Simons, 2007). Similarly, both Guilherme et al. (2004) and Benjamin et al. (2011) suggested that the association between EHD2 and actin controls the early stages of clathrin-dependent and/or caveolar endocytosis. Clearly, more work is required to fully understand the interplay between EHD2 and the actin cytoskeleton and the outcomes of their interaction.

Taking center stage

A bipartite nuclear localization sequence (NLS) (KK-X10-KKK) beginning at amino acid position 315 (Fig. 1) was first noted upon cloning and sequence analysis of EHD2–4 (Pohl et al., 2000). In addition to being conserved in all four mammalian EHDs, the bipartite NLS is also present in EHD orthologs expressed by C. elegans, Drosophila, and Xenopus. Notably, lysines 327–329, which comprise the second polybasic motif of the NLS, also function in EHD membrane targeting (Daumke et al., 2007). Despite the existence of the NLS in all four mammalian EHD paralogs, only EHD2 has been observed in the nucleus (Pekar et al., 2012). Similarly, the two plant EHD orthologs (AtEHD1 and AtEHD2) contain a bipartite NLS located near the C-terminus; however, only AtEHD2 was observed in plant nuclei (Bar et al., 2008, 2009).

Modification of EHD2 by SUMOylation at lysine 315 (LKKE consensus sequence, also conserved in EHD1, EHD3 and EHD4) was implicated in EHD2 nuclear export (Pekar et al., 2012). To probe the function of nuclear EHD2, Pekar et al. (2012) used luciferase reporter assays, which suggested that EHD2 acts as a transcriptional co-repressor. These studies also identified an interaction between EHD2 and MoKA, an F-box-containing protein that stimulates the KLF7 transcription factor (Pekar et al., 2012). While these initial findings are intriguing, the in vivo consequences of EHD2 nuclear function await future analysis. Of note, EHD2 was upregulated in lineage-restricted human primordial germ cells, suggesting that EHD2 may contribute to the unipotent nature of these cells (Pashai et al., 2012). Thus, while plasma membrane-localized EHD2 could impact cell signaling, one may also envision that nuclear EHD2 could more directly influence the cellular transcriptome. Finally, the reason for the exclusive nuclear localization of EHD2 (and not EHD1, EHD3 or EHD4) remains an open question.

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The curtain call

Since the characterization of EHD2 as a plasma membrane-associated protein, its function has remained enigmatic. As evidenced in this review, multiple studies have begun to unravel the roles of EHD2 at the plasma membrane, and possibly within the nucleus (Fig. 3). EHD2 now has a firmly established function in stabilizing caveolae (Moren et al., 2012; Stoeber et al., 2012). The prominent expression of EHD2 in blood vessels and muscle cells indicates an important role in these tissues. Consistent with this observation, EHD2 participates in membrane fusion/resealing of muscle cells (Doherty et al., 2008; Marg et al., 2012). Altogether, these functions of EHD2 are quite disparate from the classical roles of EHD1, EHD3 and EHD4 in endocytic recycling.

The extent to which EHD2 localizes to plasma membrane structures apart from caveolae remains undetermined. Thus, it will be important for future studies to define the extent to which EHD2 operates in non-caveolar compartments. Along these lines, dysferlin interacts with caveolin-3 via a DysF domain (Patel et al., 2008), which is also present in myoferlin and Fer1L5. However, the role of EHD2 in membrane resealing may be distinct from its function at caveolae, since EHD2 and caveolin-3 did not co-localize at the membrane repair site (Marg et al., 2012).

Caveolae also regulate integrin adhesion receptors, thereby impacting cell attachment to the extracellular matrix (Caswell et al., 2009; Bastiani and Parton, 2010). For instance, $\alpha v\beta 3$ integrin is recruited to caveolae and can be internalized through caveolae (Galvez et al., 2004). Additionally, EHD2 localizes to structures involved in cell motility, including membrane ruffles and filopodia. Altogether, these findings point towards potential involvement of EHD2 in regulating cell adhesion and/or motility. Notably, dynamin also localizes to membrane ruffles and lamellipodia (Ferguson and De Camilli, 2012). Thus, EHD2 could be poised to regulate dynamin activity at multiple cellular structures.

Finally, EHD2's high expression in fat cells invites the possibility that EHD2 may have a role in lipid storage. As much as 40% of the cell surface of adipocytes is comprised of caveolae, which are thought to facilitate fatty acid uptake (Bastiani and Parton, 2010). Both EHD2 (Brasaemle et al., 2004) and caveolin are found on intracellular lipid storage droplets (Brown, 2001), and EHD1 impacts lipid droplet size (Naslavsky et al., 2007). Thus, the extent to which EHD2 participates in lipid metabolism, either at the plasma membrane or on lipid droplets is an intriguing question. Overall, future studies will undoubtedly reveal new and exciting functions of EHD2.

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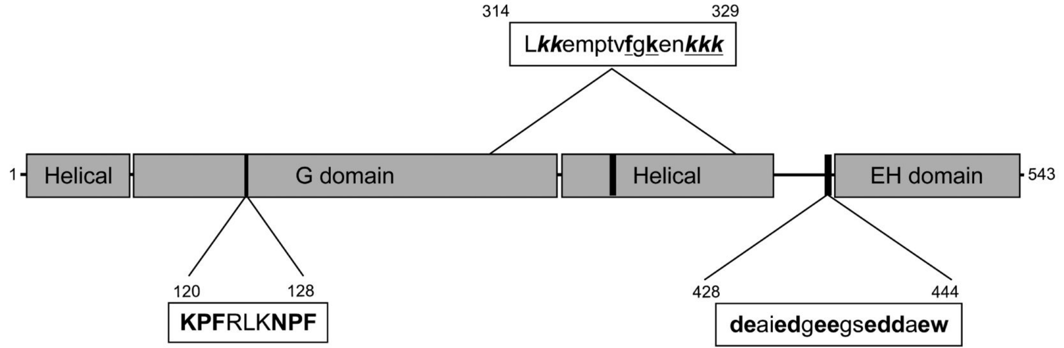

Fig. 1.
EHD2 domain architecture. The EHD protein secondary structure is comprised of four domains: 2 helical regions, a G domain, and a C-terminal EH domain. The EH domain facilitates protein-protein interactions through binding to NPF motifs followed by acidic residues. The G domain of EH D proteins shares similarity with the dynamin G domain, but binds to ATP rather than GTP (Daumke et al., 2007). The G domain also contains a KPFxxxNPF motif involved in EHD oligomerization (Daumke et al., 2007) and caveolar targeting (Moren et al., 2012; Stoeber et al., 2012). In the tertiary protein structure, the two helical domains fold together to form a coiled-coil domain that mediates EHD oligomerization and lipid binding. In the second helical domain, F322, K324, K327, K328 and K329 (shown bolded and underlined) facilitate membrane binding (Daumke et al., 2007). A bipartite nuclear localization sequence comprised of basic residues at positions 315–316 and 327–329 (shown bolded and italicized) partially overlaps with the lipid-binding motif. Additionally, studies suggest that SUMOylation at the LKKE consensus sequence (residues 314–317) functions in nuclear export of EHD2 (Pekar et al., 2012). An acidic sequence followed by a tryptophan residue (spanning amino acids 428–444) has been proposed to function in actin dynamics through potential binding to Arp2/3 (Guilherme et al., 2004).

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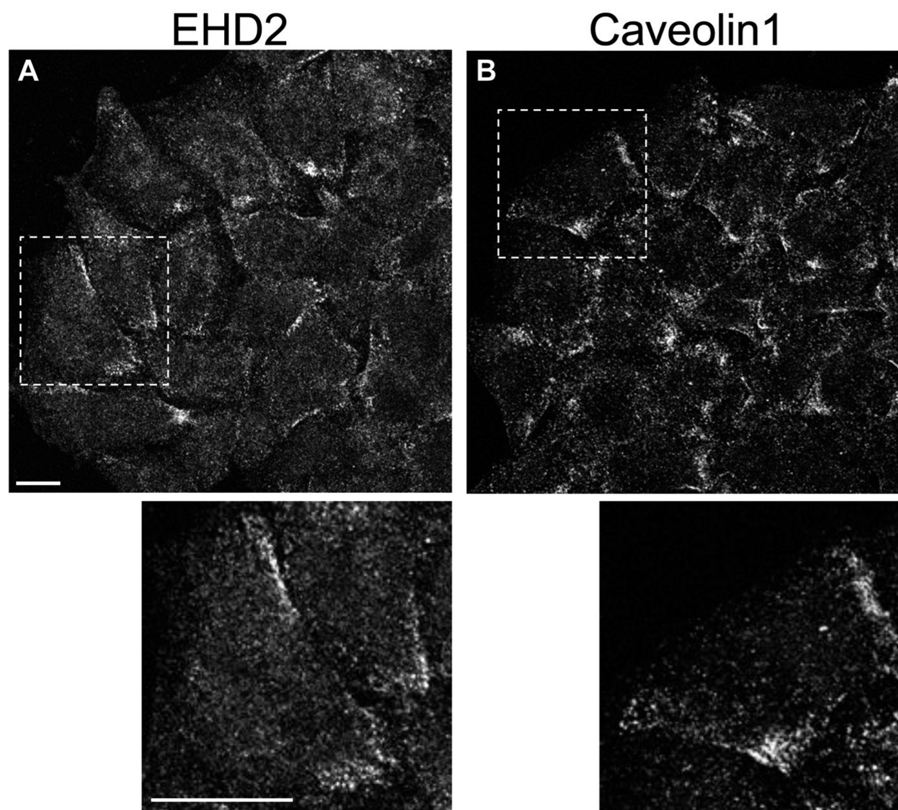

Fig. 2.

EHD2 and caveolin1 subcellular distribution. Endogenous EHD2 and caveolin1 localize to fine, punctate structures at the plasmamembrane. HeL a cells growing on coverslips were fixed and stained with rabbit anti-EHD2 antibody (A) or with rabbit anti-caveolin1 antibody (Cell Signaling Technology) (B). Following incubation with primary antibody, the cells were washed and stained with fluorescently-labeled goat anti-rabbit IgG (Molecular Probes). The cells were then washed, mounted onto slides, and analyzed using a Zeiss LSM 5 Pascal confocal microscope with a 63x, 1.4 numerical aperture objective with appropriate filters. Bar: 10 μm.

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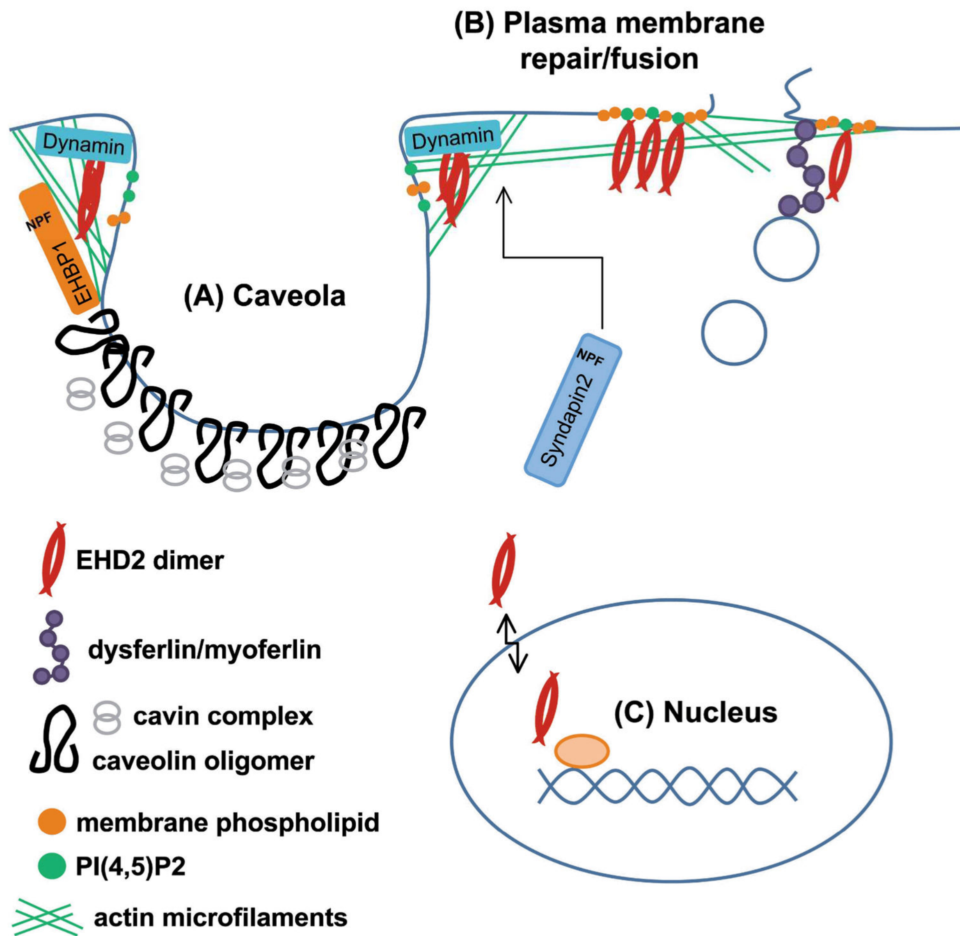

Fig. 3.

Model of EHD2 subcellular localization. EHD2 oligomers primarily localize to the inner leaflet of the plasmamembrane, where they associate with actin-rich structures. The phospholipid PI(4,5)P2 is enriched on the plasma membrane, and plays a key role in stabilizing EHD2 within this subcellular compartment (Simone et al., 2013). (A) EHD2 stably associates with caveolae (Aboulaich et al., 2004; Hansen et al., 2011; Moren et al., 2012; Stoeber et al., 2012), where it could potentially regulate dynamin-induced endocytosis. The EHD2- binding partner syndapin2 is also a transient component of caveolae (Hansen et al., 2011; Stoeber et al., 2012). Interaction between EHD2 and EHBP1 might tether caveolae to the actin cytoskeleton. (B) In muscle cells, EHD2 works in concert with members of the ferlin family to facilitate plasma membrane repair and muscle cell fusion (Doherty et al., 2008; Marg et al., 2012; Posey et al., 2011b). (C) Apart from its role at the plasmamembrane, EHD2 translocates to the nucleus where it may impact transcription (Pekar et al., 2012).