

A Novel Mechanism of Membrane Fission by Growth of a Membrane-active Polymer

A Thesis

Submitted in partial fulfillment of the requirements

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Doctor of Philosophy

By

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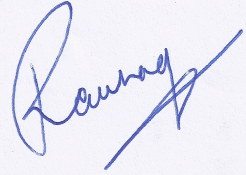


Indian Institute of Science Education and Research Pune

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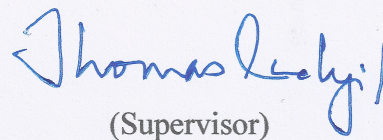
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CERTIFICATE

Certified that the work incorporated in the thesis entitled "**A Novel Mechanism of Membrane Fission by Growth of a Membrane-active Polymer**" submitted by **Raunaq Deo** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.


(Supervisor)

Date: 6.2.2019

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Table of Contents

Table of Figures	7
Synopsis	8
Chapter 1: Introduction	12
1.1 Endocytosis and Recycling Regulate Receptor Display at the Plasma Membrane.....	13
1.2 Recycling and the Endocytic Recycling Compartment.....	14
1.3 Identification of Proteins Involved in Recycling	14
1.4 Role of EHD1 in Recycling	15
1.5 Reconstitution of EHD Function.....	17
Chapter 2: Materials and Methods	18
2.1 Material and Methods.....	19
2.1.1 Cloning, Expression, and Purification of Constructs	19
2.1.2 Liposome Preparation	20
2.1.3 Liposome Co-sedimentation Assay	20
2.1.4 ATPase Assay	21
2.1.5 Expression in Cells and Microscopy	21
2.1.6 Supported Membrane Templates (SMrT).....	21
2.1.7 Microscopy and Analysis	22
2.1.8 Image and Statistical Analysis	23
2.2 Results	23
Chapter 3: EHD1 Forms Membrane Active Oligomers	25
3.1 Minimal Requirements for EHD1 Recruitment to Membranes	26
3.2 EHD1 Displays Membrane Stimulated ATPase Activity	27
3.3 A Fluorescent-tagged EHD1 Construct.....	29
3.4 Model Membranes to Visualize Protein-Membrane Dynamics	29
3.5 Nucleotide Binding Determines EHD1 Recruitment to Membranes	30
3.6 Mutations in G-domain Affect Protein Distribution in Cells.....	34
3.7 EHD1 Oligomers are Membrane Active.....	35
Chapter 4: ATPase-dependent Self-assembly of EHD1 Promotes Membrane Fission	38
4.1 ATP Hydrolysis by EHD1 Causes Membrane Fission	39
4.1.1 Membrane Remodeling with ATP.....	39

4.1.2 Rate of ATP Hydrolysis Governs Fission -----	40
4.1.3 ATP Hydrolysis Induces with Growth of EHD1 Scaffolds -----	40
4.2 Role of the N-terminus in EHD1 Function	43
4.2.1. The N-terminus Harbors a Second Conserved Membrane Binding Site -----	43
4.2.2 EHD1 ^{Δ2-9} Membrane Binding and ATPase Activity is Equivalent to Wild-type ---	44
4.2.3 EHD1 ^{Δ2-9} Cannot Catalyze Membrane Fission -----	46
Chapter 5: Insights into Membrane-active Properties of EHD2 -----	47
5.1 EHD2 Displays High Sequence Identity to EHD1.....	48
5.2 EHD2 Binds Membranes with Higher Affinity than EHD1	48
5.4 EHD2 Displays Lower ATPase Activity than EHD1	49
5.5 EHD2 Catalyzes Membrane Fission Slower than EHD1.....	50
Discussion -----	51
References -----	55

Table of Figures

Figure 2.1 Schematic depiction of a liposome co-sedimentation assay -----	20
Figure 2.2 Schematic depicting preparation of SMrT-----	22
Figure 2.3 Purification of EHD1 -----	24
Figure 3.1 EHD1 can bind membranes and can hydrolyse ATP -----	27
Figure 3.2 EHD1-EGFP is functional -----	28
Figure 3.3 Two regions on membrane templates -----	31
Figure 3.4 EHD1-EGFP binding with different nucleotide analogs -----	32
Figure 3.5 EHD1 distribution with ATP _γ S-----	34
Figure 3.6 G-domain mutations affect EHD1 distribution -----	35
Figure 3.7 Nucleotide bound EHD1 remodels membranes -----	37
Figure 4.1 EHD1 with ATP remodels membrane and causes fission-----	39
Figure 4.2 Rate of ATP hydrolysis governs fission -----	42
Figure 4.3 Growing EHD1 scaffolds induce membrane fission -----	43
Figure 4.4 N-terminus of EHD1 is required for fission-----	46
Figure 5.1 EHD2 is different from EHD1 -----	49
Figure 6.1 Schematic representation of remodelling of membrane tube by EHD1 -----	52

Synopsis

Eukaryotic cells actively internalize substances at the plasma membrane. This process is known as endocytosis. It encompasses diverse processes such as engulfment of pathogens (phagocytosis) or nutrients and other fluid phase material (macropinocytosis), and even directed uptake of ligands bound to receptors (receptor-mediated endocytosis) that trigger various signaling cascades inside the cell. Extensive endocytosis would deplete the reservoir of lipids and proteins at the plasma membrane. This does not happen, as receptor recycling mechanisms work to restore membrane receptors and lipids back to the plasma membrane.

It is via receptor recycling that membrane proteins and lipids are returned majorly back to the plasma membrane. This allows the cell to reuse them for subsequent rounds of endocytosis. Some mammalian cells display an ability to regulate the recycling of membrane receptors, by directing the trafficking of these receptors to a perinuclear endocytic recycling compartment (ERC). Disruption of receptor recycling affects a multitude of cellular processes, including nutrient uptake, antigen presentation, cell migration, maintenance of cell polarity and cytokinesis. Studies have shown that depletion of EHD1 (EPS15 homology-domain containing protein 1) in mammalian cells affects the recycling of different membrane receptors.

A characteristic feature of EHD1 depletion in mammalian cells is the retention of cargo (membrane receptors) at the ERC. Thus, these experiments suggest that EHD1 is required for the exit of these receptors from the ERC. Structural studies on EHD proteins have revealed that they possess a dynamin-like G-domain that hydrolyzes ATP and are also membrane-active. Moreover, EHD2, another mammalian EHD paralog has been shown to bind and tubulate liposomes with ATP. While these experiments offer an insight into EHD function, it is difficult to glean any mechanistic details of this process. Using a system of supported membrane templates (SMrT), this work aims to reconstitute EHD1 activity. Using a combination of fluorescence microscopy and biochemical reconstitution the membrane-active functions of EHD1 are investigated. Furthermore, this work elucidates a unique mechanism by which EHD1 remodels lipid membranes.

Chapter 1 introduces the processes of endocytosis and recycling. It outlines the process of recycling and its role in cells. A screen in *C.elegans* identified receptor-mediated endocytosis 1 (*rme-1*) to be required for basolateral recycling in the worm. Its mammalian counterpart is the EHD family proteins. Amongst the 4 highly homologous EPS15 homology-domain (EHD) proteins in mammals, EHD1 displays the highest sequence identity to RME-1. EHD1 depletion experiments in mammalian cells display trapping of receptors at ERC and

changes in ERC morphology suggesting a membrane-active role for EHD1 in the endocytic recycling process. Despite evidence for the need for EHD1 in recycling, crucial mechanistic details about EHD1's membrane-activity are still not known.

Chapter 2 talks about the materials and methods used in this study. It describes the dual tag tandem affinity purification of EHD1 and the setup of assays used to validate and test the membrane-active functions of the purified protein.

Chapter 3 describes the membrane binding and distribution of EHD1. Using a liposome co-sedimentation and ATPase assay, activity of bacterially-expressed EHD1 is tested and confirmed. A fluorescently tagged EHD1 construct is generated to visualize its distribution on membrane templates and study dynamics of the reaction. EHD1 displays ATP-binding dependent recruitment to membranes. In the ATP-bound state, EHD1-EGFP forms clusters on planar supported lipid bilayers (SLBs) and highly curved membrane tubes. Membrane binding is highest on thinner tubes than thicker tubes. EHD1 displays an intrinsic ability to bind curved membrane templates over planar SLBs. Consistent with this, ATP hydrolysis allows disassembly of the protein leading to loss of protein fluorescence from the membrane. G-domain mutants, which affect EHD-ATP binding, or display a defect in ATP hydrolysis are expressed in cells. On expression of these mutants in the cells, there is a change in the steady-state distribution of EHD1 suggesting the role of ATP in governing EHD1 distribution. Remarkably, further analysis of EHD1-EGFP distribution on membrane tubes reveals that the protein oligomers are capable of “bulging” the underlying membrane in the ATP-bound state. Importantly, to conserve the finite volume of the membrane, bulging by EHD1 scaffolds causes thinning of regions adjacent to the scaffold.

Chapter 4 details membrane remodeling by EHD1 upon ATP hydrolysis. Addition of EHD1 with ATP to SMrT templates causes extensive remodeling of membrane tubes leading to fission. Membrane remodeling is observed to be more prominent on thinner tubes over thicker tubes, and thinner tubes display a higher probability of fission. Moreover, membrane fission is dependent on ATP hydrolysis. EHD1^{T94A}, a mutant defect in ATP hydrolysis displays lesser fission and also displays a dominant negative phenotype over wild-type protein. Analyzing EHD1^{T94A} revealed that it hydrolyzes ATP slower than WT. In agreement with this, adding the protein with ATP on membrane tubes and incubating for long time points displays a steady rise in the number of tubes cut. EHD1-EGFP oligomers assembled on membrane tube display distinct scaffolds with helical symmetry when observed using STED. Flowing in

EHD1-EGFP at a lower concentration with ATP allows observation of dynamics of assembly on membrane tubes. The protein nucleates on membrane tubes and by self-assembly, protein scaffolds grow bi-directionally along the length of the tube. Growing EHD1 scaffolds lead to a concomitant extension of the bulge under the scaffold. This, in turn, causes more thinning of the membrane between bulges, leading to membrane fission. Fission seem to occur at the thinned membrane regions. The N-terminus residues of EHD1 are critical for this process. Deleting the N-terminus results in the protein being arrested in the bulged state. Thus deleting the N-terminus reduces fission.

Chapter 5 attempts to understand functional differences between EHD1 and EHD2. When tested and compared under the same conditions, EHD2 displays higher binding to membranes than EHD1. However, EHD2 hydrolyzes ATP at a 40-fold slower rate than EHD1. Following this rationale, EHD2 displays membrane remodeling only at late time points, showing very little fission. Hence, despite sharing high sequence identity, the differences between EHD1 and EHD2 become apparent when compared under the same experimental conditions. This suggests that EHD2 and EHD1 may perform different functions in cells.

Discussion describes a novel mechanism of membrane remodeling by EHD proteins. In the ATP-bound state, EHD1 is recruited to membrane tubes and bulges the underlying membrane. It requires ATP hydrolysis for growth and elongation of protein scaffolds along the length of the tube, via self-assembly. Growing EHD1 scaffolds expand the underlying bulge, thinning the intervening membrane. This thinning leads to membrane fission. Thus EHDs mediate a unique membrane remodeling reaction unlike that seen with other membrane scaffolding proteins like BAR domain protein family or dynamin family members. Since depletion of EHD1 results in expansion of the recycling compartment and retention of cargo, it seems to be involved in remodeling of the ERC and regulating exit of cargo from the compartment. Possible mechanisms for the recruitment of EHD1 to the ERC and a role for its remodeling function are suggested.

Chapter 1:

Introduction

1.1 Endocytosis and Recycling Regulate Receptor Display at the Plasma Membrane

The cell utilizes membrane receptors to interact with the external environment. These membrane receptors are synthesized by the cell in the endoplasmic reticulum, travel through the Golgi to reach the plasma membrane. When these receptors interact with their cognate substrate, they are internalized by the cell in a process termed as *receptor-mediated endocytosis* (RME). This process involves invagination of the plasma membrane and pinching off a vesicle containing the receptor pool, including cellular membrane proteins. The various mechanisms of receptor-mediated endocytosis can be broadly classified as *clathrin-mediated endocytosis* (CME) or *clathrin-independent endocytosis* (CIE). In CME, various adapter proteins are recruited to the plasma membrane, which in turn recruit clathrin. Clathrin triskelia form a cage around the budding vesicle (McMahon and Boucrot, 2011). Dynamin, a large GTPase, is a membrane fission catalyst that is recruited to necks of budding clathrin-coated vesicles (Hinshaw and Schmid, 1995; Sundborger and Hinshaw, 2014). It forms scaffolds around the necks of these vesicle and facilitates pinching off of these vesicles. Alternatively, receptors may be internalized by clathrin-independent endocytic machinery. A characteristic feature of CIE is the formation of uncoated transport carriers at the plasma membrane (Ferreira and Boucrot, 2017; Sandvig et al., 2008). Carriers may be mostly vesicular or partly tubular and have been known to form even without dynamin indicating involvement of as yet unknown fission catalyst(s). Receptors internalized by CIE usually lack cytoplasmic sequences and hence cannot be recognized by adapter proteins involved in CME (Maldonado-Báez et al., 2013). Diverse cargo, like GPI-anchored proteins, *major histocompatibility complex* proteins (MHC II and I), Glut1, and even GPCRs like the B2-adrenergic receptor are internalized primarily by CIE. After internalization, carriers generated via CME or CIE fuse to the sorting endosome (SE), an organelle which behaves as the sorting station of the cell (Hsu et al., 2012). Here receptors are sorted to different compartments as per the signals present in the receptors. Some receptors are directed to the trans-Golgi network (TGN). Receptors that need to be degraded are sorted into multivesicular bodies and are sorted to the lysosome. However, cells reuse a subset of the endocytosed receptors. For these receptors, the bound ligand is uncoupled and they are returned back to the plasma membrane for subsequent rounds of endocytosis. This process is called recycling and is essential for balancing the process of endocytosis (Maxfield and McGraw, 2004). Experiments in fibroblasts suggest that within an hour, cells are capable of internalizing equivalent to 50% of their surface area and 5-10% of the cell volume (Steinman et al., 1983). Despite this high rate cells are able to maintain their overall shape and volume

due to recycling processes that replenish membrane lipids and receptors at the plasma membrane. Recycling is also essential for varied processes such as cell migration, cell polarity and even cytokinesis (van Ijzendoorn and Ijzendoorn, 2006).

1.2 Recycling and the Endocytic Recycling Compartment

Recycling processes require the generation of tubular or vesicular carriers and can be broadly classified as “fast” and “slow”. The former refers to recycling of receptors directly from the SE with a $t_{1/2}$ of approximately 2 minutes (Maxfield and McGraw, 2004). However, many cells contain a specialized compartment called the endocytic recycling compartment (ERC), which is perinuclear in location and is organized near the microtubule organizing center (MTOC) (Grant and Donaldson, 2009) and may allow the cell to regulate the recycling of receptors. The ERC is a long-lived compartment, consisting of a network of vesicle and tubules and displays recycling rates of a $t_{1/2}$ of about 10 minutes. While numerous proteins have been shown to be necessary for slow recycling, mechanisms by which transport carriers are generated from this compartment remains unclear.

1.3 Identification of Proteins Involved in Recycling

Depletion of certain proteins severely affects the recycling process. Genetic screens carried out in *C. elegans* (Grant and Hirsh, 1999) were the first to show that deletion of a gene, called *receptor-mediated endocytosis 1 (rme-1)*, results in the expansion of recycling endosomes on the basolateral surface in intestinal cells of worms (Grant et al., 2001). The *rme-1* depleted worm was shown to be capable of uptake of basolateral fluid phase markers such as dextran, BSA or GFP, indicating that *rme-1* was in fact not required for receptor-mediated endocytosis. However, these markers accumulated in the enlarged endosomes observed in the intestinal cells (Grant et al., 2001). Moreover, the protein coded by this gene, RME-1, was found to be decorated on the surface of these endosomes. Together these results indicated that RME-1 is required for the exit of cargo from recycling endosomes.

In mammals, RME-1 displays high sequence identity to a family of C-terminal EPS15 homology domain (EHD)-containing proteins identified earlier (Grant et al., 2001; Mintz et al., 1999; Pohl et al., 2000). The EHD family consists of four paralogs in mammals, called EHD1-4. These proteins share high sequence identity (Grant and Caplan, 2008). While mammals have four paralogs, only EHD1 displays ubiquitous expression across all tissues (Blume et al., 2007). In cells, these paralogs are distributed to different sub-cellular compartments. EHD1 and EHD3

are associated with the ERC (Caplan et al., 2002; Galperin et al., 2002). EHD2 is localized to the plasma membrane (Daumke et al., 2007; Moren et al., 2012), while EHD4 has been shown to localize to a subset of early endosomes (Sharma et al., 2008).

Amongst the four EHDs, RME-1 displays the highest (67%) sequence identity to EHD1 (Grant and Caplan, 2008). The domain architecture is conserved across EHDs (Naslavsky and Caplan, 2011). They have a G-domain present at the N-terminus which is capable of binding and hydrolyzing ATP (Lee et al., 2005). The G-domain is flanked by helical domains on either side. Membrane binding stretch is located at the tips and loops formed by two helical domains, allowing recruitment of EHDs to membranes (Daumke et al., 2007). At the C-terminus, there is an EPS 15 homology domain (EH-domain). Structural studies have shown that EHD1's EH-domain specifically recognizes the Asparagine-Proline-Phenylalanine (NPF)-motif, present in interacting partners (Kieken et al., 2009). While highest binding is seen with proteins containing NPF, other XPF motifs (DPF, GPF and KPF) have also been shown to bind EH-domain, with lower affinity. Known binding partners of EHDs include Rab11FIP2 (Kieken et al., 2009, Naslavsky et al., 2006), Rabenosyn-5 (Naslavsky et al., 2005), MICAL-L1 (Kieken et al., 2010), EHBP-1 (Guilherme et al., 2004), Syndapin 1, Syndapin 2-s and Syndapin 2-l (Braun et al., 2005). These proteins contain NPF- motif flanked by acidic amino acid residues, which display a negative charge. EHD1-EH domain (EH-1) has an overall positive charge. Structural studies using NPF containing short peptide from Rab11FIP2 added to EH-1 suggest that the phenylalanine residue of the NPF motif dips into the EH-domain fold. This conformation is further stabilized by the electrostatic interaction of NPF-flanking negatively charged residues to positively charged surface of EH-1 (Henry et al., 2010; Kieken et al., 2010). Mutation of NPF to NPV or mutation of the flanking negatively charged amino acids to positive or neutral charged amino acids abrogates binding (Braun et al., 2005; Henry et al., 2010; Kieken et al., 2010; Zhang et al., 2012).

1.4 Role of EHD1 in Recycling

Endogenous EHD1 displays a punctate distribution in cells. Mutations in the G-domain that adversely affect ATP binding, or in the helical domain that affect membrane binding, result in the distribution becoming cytosolic (Blume et al., 2007; Caplan et al., 2002; Daumke et al., 2007; Lee et al., 2005). Overexpressed EHD1 in cells displays punctate and tubular distribution (Caplan et al., 2002; Sharma et al., 2010). Staining these cells with antibodies against MICAL-L1 (molecules interacting with *CasL*-Like protein 1), a protein that marks tubular recycling

endosomes, displays co-localization of EHD1 and MICAL-L1 distribution (Sharma et al., 2009, 2010), indicating that it may have a role in recycling.

To understand the role of EHD1 in recycling, one can follow the path of a membrane receptor in the cell. The pathway for recycling of transferrin receptor (TfnR) has been elucidated in cells (Bleil and Bretscher, 1982; Mayle et al., 2012). Incubating cells with a pulse of fluorescently-labeled transferrin followed by incubation with unlabeled transferrin allows for tracing the route of the receptor through the cell. In control cells, it is seen that, as the transferrin receptor is recycled, these cells lose that fluorescent-transferrin signal. However, EHD1 siRNA treated cells display a longer retention of the transferrin signal inside cells (George et al., 2007). Thus, EHD1 is required for the exit of the receptors from the ERC. EHD1 partner proteins are also required for efficient recycling. Overexpression of expression of the Syndapin-2-l-NFP motif results in a block in transferrin recycling (Braun et al., 2005), indicating that normal binding of EHD1 to its adapter is necessary for receptor recycling. Expression of mitochondrial surface-targeted EH-1 also displays recruitment of Syndapin-1 to mitochondria (Braun et al., 2005), showing that EHD1 physically interacts with these partner proteins in mammalian cells. This suggests a possible mechanism for targeting of EHD1 to the membrane compartment. A block in receptor recycling is also seen on knockdown of EHD1 binding partners (Braun et al. 2005; Naslavsky et al. 2004).

EHD1 is required for the recycling of a variety of receptors including major histocompatibility complex I (MHC1) (Caplan et al., 2002), calcium-dependent cytosolic phospholipase A2-alpha (cPLA2 α) (Cai et al., 2012), CD59 (Cai et al., 2011), β 1 integrin (Jovic et al., 2007), and L1/NgCAM (Lisiecka et al., 2010; Yap et al., 2010). Hence, EHD1 depletion affects a variety of processes like the complement pathway, cell migration (Jones et al., 2006) and axonal growth (Wu et al., 2016; Yap et al., 2010). Depletion of EHD1 affects recycling cargo internalized via both CME and CIE. Cells knocked down for EHD1 display defects in the recycling of transferrin receptor, a classical CME cargo, and CD59 and β 1 integrin which are internalized via CIE. A common feature across this different cargo is that on depletion of EHD1 they are retained within a perinuclear compartment in the cell, indicating that EHD1 is crucial for the exit of these receptors from the recycling compartment. Moreover, mock-treated cells stained for endogenous TfnR display a bright perinuclear distribution, marking the recycling compartment. In comparison, EHD1 depleted cells displayed long TfnR positive tubules extending from the recycling compartment (Lee et al., 2015). Extensive tubulation of

the recycling compartment in the background of EHD1 knockdown suggests that EHD1 is crucial for the scission of these tubules and exit of TfnR from this compartment.

The evidence for the role of EHD1 in receptor recycling is abundant. Yet there is still a need to elucidate the function of EHD1 in this process.

1.5 Reconstitution of EHD Function

Work from another EHD paralog, EHD2, has indicated that these protein are capable of binding and tubulating liposomes (Daumke et al., 2007). This ability to deform liposomes is conserved across species (Jakobsson et al., 2011; Pant et al., 2009). Cryo-EM of mouse EHD2 added to liposomes has revealed scaffolds of the protein on tubulated liposomes (Shah et al., 2014). Despite these advances, mechanistic insights into how EHD proteins remodel lipid membranes is still lacking. The work presented here aims to address this problem. It focuses on understanding the membrane-active properties of EHD1. Thus using a biochemical reconstitution approach in conjunction with fluorescence microscopy, this work analyzes and provides insights into the EHD1-membrane reaction.

Chapter 2: Materials **and Methods**

2.1 Material and Methods

2.1.1 Cloning, Expression, and Purification of Constructs

Human EHD1 (Open Biosystems, cat. no. MHS1010-98052880), LactC2 (a gift from Sergio Grinstein, Addgene plasmid # 22852) and Human EHD2 (Open Biosystems plasmid cat no. MHS1011-76680) were cloned into pET15b vector between 6xHis tag at the N-terminus and a Strep II tag at the C terminus. To generate EHD1-EGFP, mEGFP was cloned between EHD1 and Strep II sequence. N-terminal deletion mutants, EHD1^{Δ 2-9}, EHD1^{T94A} and EHD1^{T94A}-EGFP were made by site-directed mutagenesis using PCR. Sequences for all constructs were confirmed by DNA sequencing.

EHD1 is a soluble membrane-interacting protein and is therefore amenable to recombinant expression and purification. For expression, *E. coli* BL21(DE3) cells were transformed with the plasmid and protein expressed with auto-induction medium (Formedium, UK). Cells were grown for 36-48 hours, spun down at 6000g for 10 minutes. Cell pellets were washed by re-suspending in 1x PBS pH 7.4, spun down at 6000g for 10 minutes and stored at -40°C for future use. Prior to purification, cell pellets were thawed at RT for 5 min. Pellets were re-suspended in buffer (20mM HEPES pH 7.4, 300mM KCl supplemented with 1mM PMSF) and lysed by sonication. The lysate was spun down at 18000g for 10 minutes and the supernatant was incubated with 1 ml of HisPur Cobalt resin (Thermo) pre-equilibrated with buffer. The supernatant was incubated with resin for 1hr at 4°C, with constant shaking. The resin was collected in a PD-10 column (Biorad) and washed with 50mL of buffer followed by elution with 20mM HEPES pH 7.4, 300mM KCl, and 150mM Imidazole. The eluate was applied on a 5ml StrepTrap HP column (G.E Lifesciences) and washed extensively with 20mM HEPES pH 7.4, 150mM KCl buffer. The bound protein was eluted with 20mM HEPES pH 7.4, 150mM KCl buffer with 2.5mM desthiobiotin (Sigma). Samples prepared for each step of the purification were run on at 10% SDS PAGE. Fractions containing the highest amount of protein were pooled and used for experiments. Prior to the experiment, proteins were spun down at 100,000g for 20 min at 4°C to remove aggregates, and concentration of protein in the supernatant was estimated using absorbance at OD 280nm.

2.1.2 Liposome Preparation

All lipids were purchased from Avanti Polar Lipids and stored at -80°C or -40°C before use. To prepare liposomes, glass tubes were cleaned with 1% SDS, Millipore water, methanol and chloroform and dried. Using a clean Hamilton syringe (Hamilton), lipids were aliquoted to make a final concentration of 1mM lipid mix and dried under a stream of air. The lipids were further dried under vacuum for 30 min in a speed-vac at 50°C to get rid of the solvent. Lipids were hydrated in 20mM HEPES pH 7.4 150mM KCl buffer and kept for swelling for 1 hour at 50°C in a water bath. Finally, the mix was extruded through 100nm pore-size polycarbonate filters (Avanti Polar Lipids). Liposomes were either used immediately or stored up to 1-2 weeks at 4°C .

2.1.3 Liposome Co-sedimentation Assay

1 μM of the protein was incubated with 100 μM liposomes (100 fold excess concentration) in 20mM HEPES pH7.4 150mM KCl buffer and incubated for 30 minutes at room temperature. The mix was spun down at 100,000g for 30 minutes and supernatant (unbound) and pellet (liposome-bound) were separated (Fig. 2.1). The fractions of supernatant and pellet were run on 10% SDS gel and stained with Commassie Brilliant Blue (CBB) to visualize protein bands. Protein intensity was estimated by densitometric analysis (Lee et al., 2015).

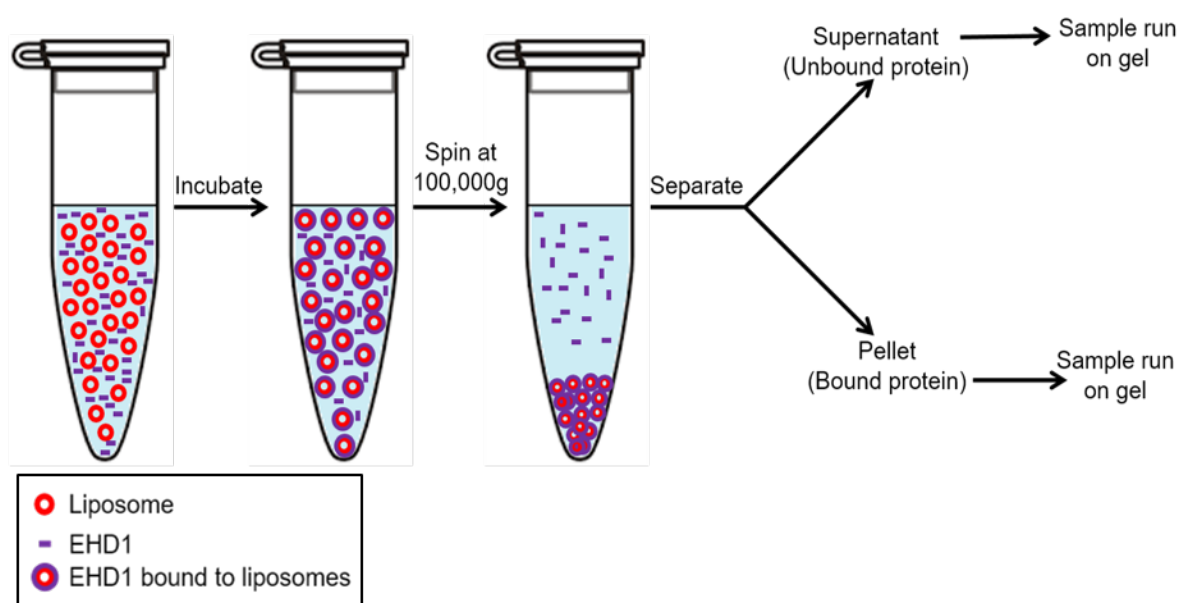


Figure 2.1 Schematic depiction of a liposome co-sedimentation assay

2.1.4 ATPase Assay

Prior to the experiment, proteins were spun down at 100,000g for 20 min to remove aggregates. Liposomes for the assay were prepared as mentioned before. To estimate ATPase activity, 1 μ M of the protein was incubated with 1mM ATP (Jena Biosciences, Germany) either in the absence or presence of liposomes in 20mM HEPES pH 7.4 150mM KCl, 1mM MgCl₂ buffer at 37°C. At intervals of 5 min, aliquots of the reaction were taken out and quenched with 0.5M EDTA. The inorganic phosphate released in the reaction was estimated colorimetrically using malachite green reagent (Baykov et al., 1988).

2.1.5 Expression in Cells and Microscopy

For expression in cells EHD1-EGFP, EHD1^{T72A}-EGFP and EHD1^{T94A}-EGFP were cloned into pcDNA3 mammalian expression vector, downstream to CMV-promoter. For transient transfection, PEI was used (polyethyleneimine, Sigma, cat. no. 408727) as described previously ((Ming Hsu and Uluda Ğ, 2012). In short, plasmid to be transfected was mixed with sterile PEI in the ratio of 2 μ g of plasmid: 5 μ l of 1mg/ml PEI in 450 μ l of OPTI-MEM medium (Gibco). The mix was incubated for 30-45 minutes at room temperature. The entire mix was added to cells growing in a 35mm dish growing with 1mL DMEM + 10 % FBS (Gibco) at about 40% confluent state. Cells were washed the next day and seeded on Labtek chambers (Fischer Scientific). For imaging, cells were fixed with 4% paraformaldehyde (PFA, Sigma) and washed with 0.1% triton, followed by PBS wash.

2.1.6 Supported Membrane Templates (SMrT)

To study the function of EHD1 a model membrane system developed in the lab, called supported *membrane templates* (SMrT) assay (Dar et al., 2015, 2017), was used. The lipid composition was chosen accordingly for optimal EHD1 activity and consisted of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; cat. no. 850375C), 1,2-dioleoyl-*sn*-glycero-3-phospho-l-serine (sodium salt; DOPS; cat. no. 840035C) and Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (p-Texas Red DHPE; Invitrogen, cat. no. T1395MP); prepared as a 1mM lipid stock in a molar ratio (59:40:1 mol %), reconstituted in chloroform (Sigma). SMrT templates were prepared according to the protocol as previously described (Dar et al., 2017) (Fig. 2.2). Briefly, 1-2 μ l of the lipid stock was coated evenly at one edge of a PEGylated glass coverslip and assembled in a flow cell (Bioptechs, PA) The PEG-coated surface provides a passive cushion for the lipids and prevents spilling of

membranes on the glass surface. Buffer (20mM HEPES pH 7.4 150mM KCl) is added to the chamber to hydrate the lipid mix, resulting in the swelling of membranes. Using a peristaltic pump, a laminar flow of buffer is set up inside the chamber. Due to the flow of buffer, swelled membranes are extruded out to form tubes that get laid out on the surface of the coverslip. The buffer was passed until tubes are evenly laid out. The tubes settle down and adhere to sites on the surface that have probably escaped passivation with PEG. Each SMrT preparation results in the formation of regions of supported lipid bilayers (SLBs) at the source and tube further downstream.

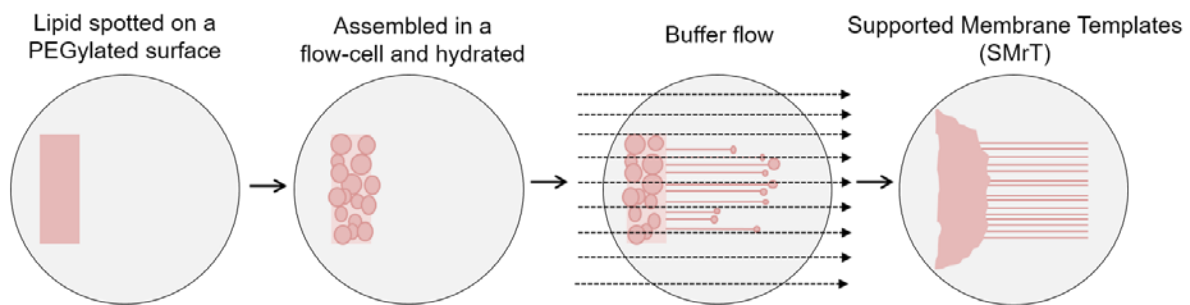


Figure 2.2 Schematic depicting preparation of SMrT

2.1.7 Microscopy and Analysis

Microscopy was carried out at 37°C using a temperature controller (Biopetechs, PA). Prior to each experiment, images of the supported lipid bilayer (SLB) at the source and tubes from different fields were acquired to determine the sizes of tube, as described previously (Pucadyil and Holkar, 2016). To estimate tube size, ROI (region of interest) of different areas were placed on background-subtracted images of SLB and the integrated fluorescence density (IntDen) was measured for each ROI. The IntDen is a measure of fluorescence intensity within the area of that ROI. Plotting a graph of IntDen vs the respective area for each ROI allows for estimation of fluorescence per unit area. Fitting the graph to a linear regression, the slope K_1 is obtained. Drawing ROI of fixed length (l), the same measurements were carried out for tubes to obtain fluorescence intensity for a tube ($\text{IntDen}_{\text{tube}}$). To estimate tube radii, following formula was used:

$$\text{Radius}(r) \text{ (nm)} = (\text{IntDen}_{\text{tube}} / (2 * \pi * l * K_1)) * 1000$$

Plotting the maximum fluorescence intensity for each tube vs the radius (r), and fitting the graph to a linear regression provides the slope K_2 . Thus, K_2 relates maximum pixel

intensities to tube radii. Dividing maximum pixel intensities by K_2 provides the radius of the tubes. Tube radius estimates made this way, describe the radius of the tube from the central lumen to the outer monolayer.

To study protein-membrane reactions, proteins were flowed in on SMrT templates in 20mM HEPES pH 7.4 150 mM KCl buffer. Nucleotides were added to the buffer and protein mix at a final concentration of 1mM, supplemented with 1mM $MgCl_2$. When acquiring movies, proteins were flowed in with buffer supplemented with an oxygen scavenger system, to reduce photobleaching by reactive oxygen species. Imaging was carried out on Olympus IX-71 inverted fluorescence microscope using 100X /1.4 N.A. objective lens for cells and SMrT assays. Images were acquired using an Evolve EMCCD camera (Photometrics). Confocal and STED microscopy was carried out on Leica TCS STED-3X Nanoscope using 100X /1.4 N.A. objective, using 660nm laser line for depletion in STED.

2.1.8 Image and Statistical Analysis

Image analysis was carried out using FIJI (Schindelin et al., 2012). Huygens Professional was used for deconvolution of STED images. Statistical analysis was done using GraphPad Prism (v. 6.0)

2.2 Results

Initial attempts for the EHD1 purification were with the single N-terminal tag only. Presence of the N-terminal histidine tag allowed purification of the protein by immobilized metal affinity chromatography and easy isolation of EHD1. However, since the construct was tagged at only the N-terminus and lacked any tag at the C-terminus, it was difficult to ensure that the protein was full length and not truncated at the C-terminus. Furthermore, buffer containing 300mM KCl was used to dissolve the pellet. Lysis and purification were carried out in this buffer, as the high salt concentration helps dissociating protein-protein interaction and hence reduces aggregation. However, the purified protein was dialyzed overnight in assay buffer (20mM HEPES pH 7.4, 150mM KCl), to get rid of high salt and imidazole used for elution. This protocol demanded the addition of additives such as 1mM DTT (to prevent oxidation of the protein and maintain a reducing environment) and 1mM EDTA (to inhibit proteolysis).

Using a dual tag tandem affinity purification overcomes many of these obstacles. Addition of a C-terminal Strep II tag (Fig. 2.3 a) and using both N- and C- terminal tags

sequentially for purification ensures that the construct obtained at the end of purification is full length. Using the C-terminal-tag, the protein is bound on the column and washed and eluted it in assay buffer (Fig. 2.3 b). This final step ensures that protein is purified in assay buffer, it can directly be used for experiments.

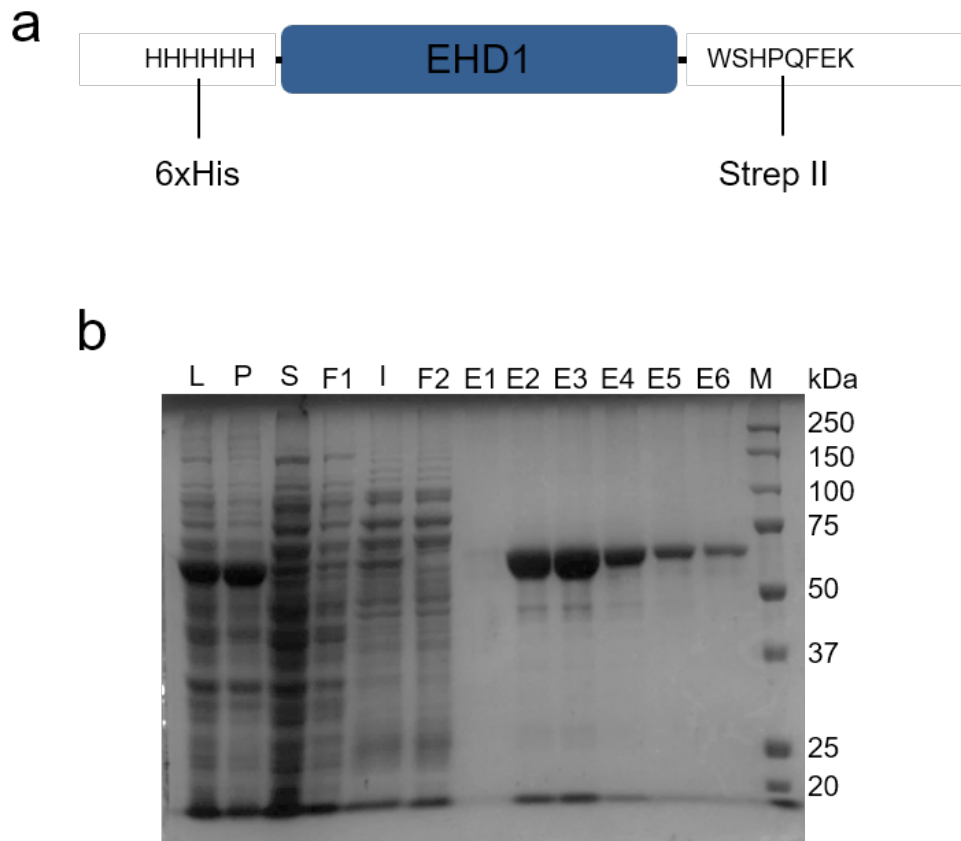


Figure 2.3 Purification of EHD1 a) EHD1 construct consisting of a 6xHis at the N-Terminus and a Strep II tag at the C terminus. b) EHD1 was purified and samples were run on a 10 % SDS gel . The samples are L=Lysate, P= pellet, S= supernatant after spin, FT1= Flowthrough from cobalt resin, I= Input obtained by elution of cobalt resin, FT2= Flowthrough from streptactin column, E1-E6= Elution fractions collected from the streptactin column, M=Protein marker.

Chapter 3: EHD1

Forms Membrane

Active Oligomers

3.1 Minimal Requirements for EHD1 Recruitment to Membranes

Prior work on the membrane-protein interactions of EHDs has suggested that EHD1 does not display binding to any specific lipid species (Blume et al., 2007). On the other hand, EHD1 can be recruited to liposomes by increasing the net negative surface charge (Lee et al., 2015). The membrane binding activity of EHD proteins resides in the helical domain. For EHD2, mutation in this domain (F322A, K324D, K327D, K328D, and K329D) results in cytoplasmic distribution upon expression in cells (Daumke et al., 2007) indicating that they are required for membrane localization. Overexpressed of helical domain mutant EHD1^{K324D} also displays a cytoplasmic distribution when compared to wild-type and shows reduced binding to liposomes (Lee et al., 2015). To determine the minimal requirements for membrane recruitment purified EHD1 was subjected to a liposome co-sedimentation assay. For this, 100nm-extruded liposomes containing varying amounts of DOPS (0, 20, 40, 60, 80, and 100 mol %) in the background of DOPC were prepared. PS has a single negative charge and increasing PS concentration in the membrane reflects an increase in net surface charge on the membrane. Moreover, depletion of PS in cells causes phenotypes similar to those seen in EHD1-depleted cells, suggesting that negative charge contributes to recruitment in cells (Lee et al., 2015). Liposome co-sedimentation was carried out as described previously (Fig. 2.1, Chapter 2). On centrifugation liposomes, being denser than soluble proteins, sediment to form a tight pellet, pulling down bound protein. Unbound protein is retained in the supernatant. Samples of bound and unbound protein are prepared and run on a 10 % SDS gel (Fig 3.1 a). EHD1 displays increased binding to liposomes with increasing net negative charge. The protein shows sigmoidal binding with an increase in net negative charge (Fig. 3.1 b). EHD1 liposome binding reaches a plateau with 80-100 mol% DOPS containing liposomes. At these concentrations a major fraction of the total protein added was bound to liposomes. On fitting the data to a one-site specific binding equation, an EC₅₀ of 40 mol% DOPS is obtained. Thus, the purified protein displays membrane recruitment with PS liposomes, similar to previous reports (Lee et al., 2015). Performing the same assay with EHD1^{F322A}, a mutant that affects membrane binding displays a rightward shift of the graph and requires up to 80 mol% PS for recruitment (Kushwah, 2016). This indicates that the binding seen is specifically via the membrane binding loop in the protein. It was also observed that on reducing the amount of PS while maintaining the same charge density by the inclusion of 5 mol% DOPI4P (Dioleoyl phosphatidylinositol-4-phosphate), an equivalent amount of EHD1 was recruited to liposomes (Kushwah, 2016). Thus negatively charged PS membranes represent the simplest system for recruitment of EHD1.

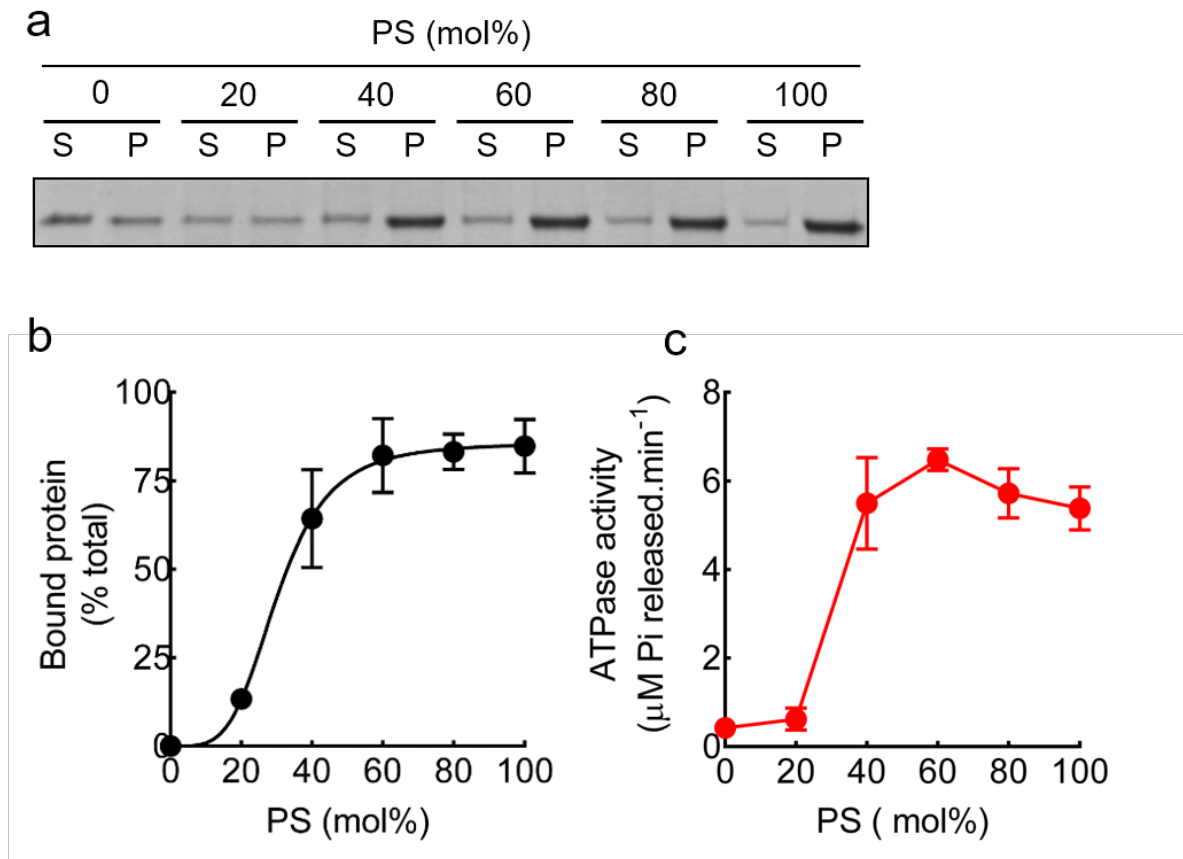


Figure 3.1 EHD1 can bind membranes and can hydrolyse ATP a) Sedimentation assay of EHD1 with liposomes of increasing DOPS content. Each sample was spun down and separated into supernatant (S) and pellet (P). b) Quantitation of sedimentation assay (mean $\pm$ s.d., $n = 3$) c) ATPase assay for EHD1 with liposomes with increasing DOPS content (mean $\pm$ s.d., $n = 3$)

3.2 EHD1 Displays Membrane Stimulated ATPase Activity

Small GTPase proteins usually cycle between an inactive GDP-bound state and an active GTP-bound state. Their activity is regulated by specific proteins, called GEFs (*guanine nucleotide exchange factors*), which facilitate nucleotide exchange, and GAPs (*GTPase-activating proteins*), which facilitate GTP hydrolysis (Bos et al., 2007). The dynamin superfamily comprises large GTPase proteins. A common feature displayed by the dynamin superfamily proteins is that they do not need GEFs and GAPs for regulation (Ferguson and De Camilli, 2012). Dynamin, for example, has an intrinsic low affinity for GTP and a low GTP hydrolysis rate which gets stimulated up to 100-fold on assembly induced by binding to membranes (Warnock and Schmid, 1996). In these reactions, the dynamin molecules themselves behave like GAPs and GEFs (Chappie et al., 2010). This property is shared by EHD proteins; mouse EHD2 has a basal ATPase rate of 0.7 hr^{-1} which is stimulated 8 fold (5.6 hr^{-1})

on incubation with Folch liposomes (Daumke et al., 2007). To test whether EHD1 also displays basal and stimulated ATPase activity, the purified protein was subjected to an ATPase assay using the same range of liposomes. Malachite green assay was used to test EHD1's ATPase activity (as described in Chapter 2). In the absence of liposomes, EHD1 displayed a basal ATPase activity of 0.6 min^{-1} . This activity is stimulated ~ 10 fold on binding membranes (Fig. 3.1 c) and was found to be ~ 40 -fold higher than that reported for EHD2 (Daumke et al., 2007).

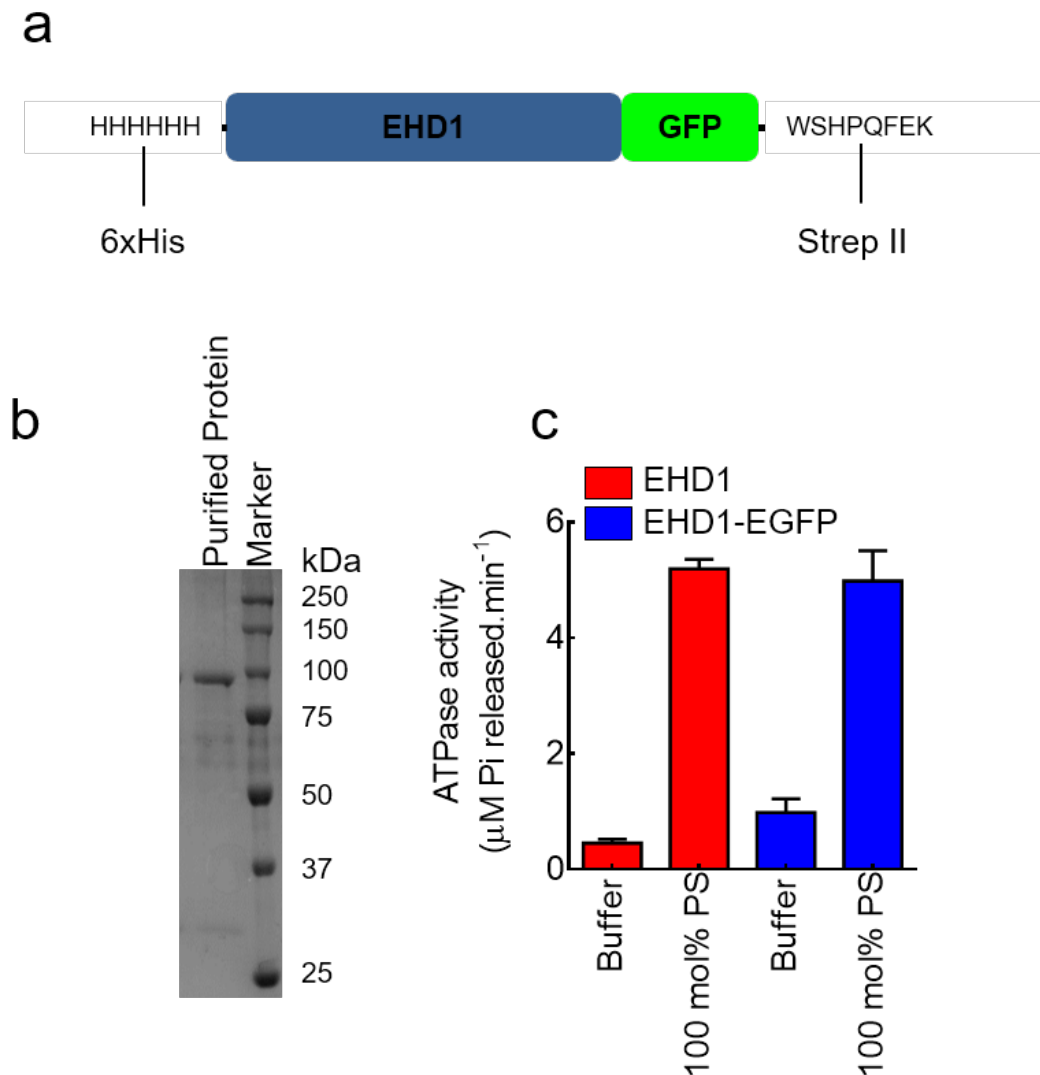


Figure 3.2 EHD1-EGFP is functional a) EHD1-EGFP construct b) EHD1-EGFP was purified and samples were run on a 10 % SDS gel. c) Comparison between ATPase activity for untagged EHD1 and **EHD1-EGFP** (mean $\pm$ s.d., $n = 3$ for EHD1 (untagged) and $n=2$ for EHD1-EGFP)

3.3 A Fluorescent-tagged EHD1 Construct

To understand the dynamics of protein assembly on membranes, visualizing protein distribution on membranes is crucial. Amongst the various techniques available two methods were tested, viz; labeling the purified protein with a fluorescent dye or alternatively, genetically encoding a fluorescent tag on the protein. The former relies on covalent conjugation of an organic dye directly to cysteine residues in the protein. EHD1 has a single cysteine residue present in its G-domain. While it is possible to label EHD1 with a fluorescent dye, labeling results in the loss of activity of the protein. Hence, instead of chemically conjugating a fluorescent dye, EGFP tagged versions of the protein were generated.

N-terminal GST- and EGFP have been used before to assess EHD cellular distribution and function (Cai et al., 2013; Daumke et al., 2007; Galperin et al., 2002; Lee et al., 2005; Shah et al., 2014). However, when tested for ATPase activity, the N-terminal EGFP tagged construct displayed higher basal ATPase activity and no stimulation of ATPase activity on incubation with PS liposomes. It was observed that addition of any bulky protein at the N-terminus, such as GST-tag or EGFP affected the ATPase activity of the protein. (Kushwah, 2016).

Since the N-terminal EGFP tagged construct displayed non-optimal ATPase activity, EGFP was instead attached to the C-terminus of the protein (Fig. 3.2 a). The C-terminal tagged EHD1 was purified (Fig 3.2 b) and tested for ATPase activity. EHD1-EGFP displays basal and assembly stimulated ATPase activity, similar to the untagged construct (Fig 3.2 c). Thus, EHD1-EGFP is a functional equivalent construct to wild type (untagged) and provides an easy means for visualization of the protein with fluorescence microscopy.

3.4 Model Membranes to Visualize Protein-Membrane Dynamics

EHD proteins are capable of binding and remodeling membranes. EHD2 added to liposomes with ATP induces tubulation of liposomes (Daumke et al., 2007; Shah et al., 2014). Addition of EHD1 to permeabilized cells displayed remodeling of membrane tubes stained by MICAL L-1 (Cai et al., 2013). While these experiments provide insights into the functions of the protein, the mechanism by which these proteins facilitate membrane remodeling still remains elusive. To fully comprehend the membrane-active functions of EHD1 a minimal membrane reconstitution system, amenable to observation of protein-membrane dynamics, is necessary.

To visualize the membrane remodeling activity of EHD1 using fluorescence microscopy, SMrT (Supported Membrane Templates) assay (Dar et al., 2017) was used (Fig. 2.2, Chapter 2). Briefly, SMrT is made by spotting and drying a lipid mix on a PEG (polyethylene glycol)-conjugated glass coverslip. Subsequent hydration of lipids by assembly in a flow cell with buffer results in swelling of the membrane to form vesicles. The PEG-coated surface provides a passive cushion for the lipids and prevents spilling of membranes on the glass surface. The unidirectional flow of buffer in the flow cell induces extrusion of vesicles to form membrane tubes that get laid out over the surface of the glass coverslip. Tubes formed are sturdy and stable, in the presence of buffer. By using *p*Texas Red-DHPE, an environment- and membrane curvature-insensitive fluorescent lipid added to the lipid mix, these templates can be visualized under the microscope.

The SMrT assay allows sampling two membrane regions, displaying different curvature. At the region where the lipid mix is spotted, hydration and successive washes with buffer result in the formation of planar supported lipid bilayers (SLBs). These are membrane sheets that have no curvature. Connected to these SLBs is an array of membrane tubes, formed due to extrusion of vesicles (Fig. 3.3 a). Membrane tubes are highly curved templates and span a wide range of sizes (Fig. 3.3 b). The range of sizes obtained correlates well with the gradients of curvatures observed for tubular recycling endosomes (TRE) in cells (Xie et al., 2016).

SMrT can be prepared using practically any lipid mix. For experiments with EHD1, templates are made using a lipid mix containing DOPC: DOPS: *p*Texas Red-DHPE (59:40:1 mol %). As a control, EGFP tagged LactC2 domain (EGFP-LactC2), which specifically binds PS (Yeung et al., 2008), is added to the templates. EGFP-LactC2 displays uniform distribution on SLBs and tubes (Kamerkar and Pucadyil, unpublished results) (Fig. 3.3 c) confirming that PS is uniformly distributed in the membrane templates.

3.5 Nucleotide Binding Determines EHD1 Recruitment to Membranes

To the membrane templates, EHD1-EGFP was added and incubated for 10 minutes at 37°C. The system was subsequently washed with buffer and images were acquired in appropriate channels. It was seen that adding EHD1-EGFP with AMP-PNP, a non-hydrolysable ATP analog, induces recruitment of EHD1 to membranes. On SLBs EHD1 displays a non-uniform distribution and appears clustered (Fig. 3.4 a). This distribution is markedly different from that seen for LactC2 (Fig. 3.3 c). EHD1 clusters may represent oligomers of the protein formed on the membrane. In stark contrast, in the absence of any

nucleotide (Apo), EHD1-EGFP fluorescence was absent on the membrane. When added with ADP, minimal recruitment to SLBs is observed. Thus in the ATP-bound state (EHD1-EGFP with AMP-PNP), maximal recruitment to lipid membranes was observed. Taken together, these experiments suggest that ATP binding is required for recruitment of the protein to membranes.

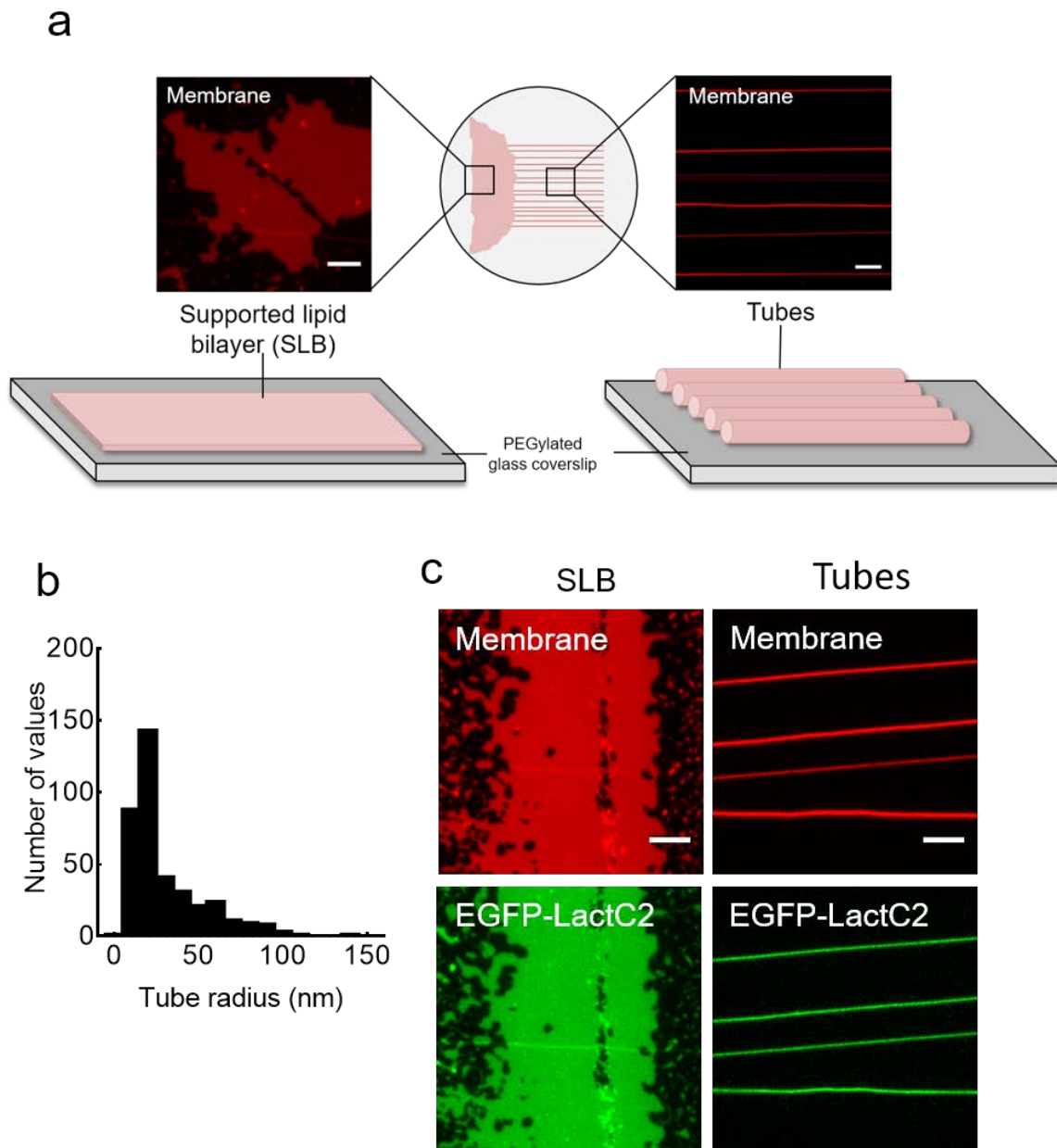


Figure 3.3 Two regions on membrane templates a) Representation of two membrane regions produced in a typical SMrT assay b) Distribution of tube radii obtain on in SMrT assays $N_{\text{SMrT}} = 5$ c) Distribution of EGFP-LactC2 on SMrT template. Scale bar = $5\mu\text{m}$

On membrane tubes also, EHD1-EGFP added with AMP-PNP appears clustered (Fig. 3.4 b). When compared to recruitment on SLBs, recruitment on membrane tubes was enhanced.

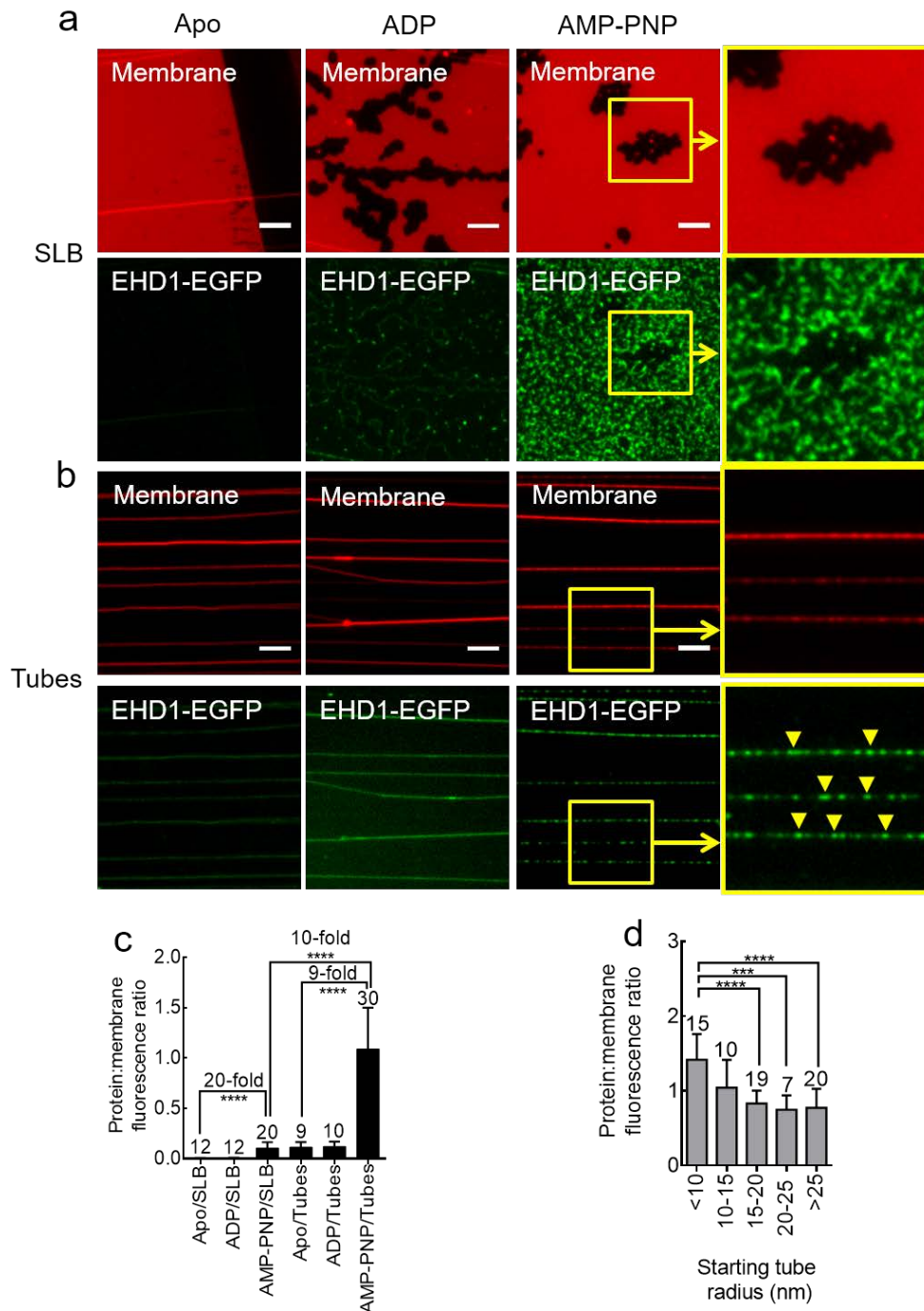


Figure 3.4 EHD1-EGFP binding with different nucleotide analogs Binding of EHD1-EGFP to SLBs (a) and tubes (b) in different nucleotide bound states. Yellow arrowheads indicate clusters of EHD1-EGFP. Scale bars = 5 μ m c) Comparison of protein: membrane fluorescence ratio for SLBs and tubes in different nucleotide bound states. Data represents mean $\pm$ SD for n (SLB and tubes) as indicated. Statistical significance assessed using Mann-Whitney test. **** denotes $P < 0.0001$. d) Protein: membrane fluorescence ration for AMPPNP-bound EHD1-EGFP compared to tubes of varying starting tube radii. Data represents mean $\pm$ SD of n (tubes) as indicated. Statistical significance assessed using Mann-Whitney test. *** denotes $P = 0.0002$, **** denotes $P < 0.0001$.

EHD1-EGFP displayed 10-fold higher binding to curved membrane templates over SLBs (Fig. 3.4 c). Even when the protein was added without any nucleotide or with ADP, minimal recruitment of the protein to membrane tubes is seen. Compared to SLBs, even in the APO state or with ADP, more protein is recruited on membrane tubes. Unlike the distribution seen with AMP-PNP, EHD1-EGFP displays uniform distribution under these conditions. This suggests that EHD1 displays an intrinsic ability to sense membrane curvature and prefers binding to curved membrane templates (Fig. 3.4 d).

EHD1-EGFP displays clusters when added with AMP-PNP. However, when EHD1-EGFP is added to templates with ATP γ S, a slow-hydrolyzing ATP analog, a dramatic increase in recruitment of the protein is observed. With ATP γ S, EHD1-EGFP appears as long filaments on the SLBs and covers more area on membrane tubes (Fig. 3.5 a). Interestingly when EHD1-EGFP is assembled on membrane templates with ATP γ S and incubated with ATP, rapid dissociation of EHD1 from SLBs is observed (Fig. 3.5 b). In contrast, on tubes, more protein is retained (Fig. 3.5 c) indicating that in the presence of ATP hydrolysis, intrinsic curvature sensitivity of the protein allows it to resist dissociation.

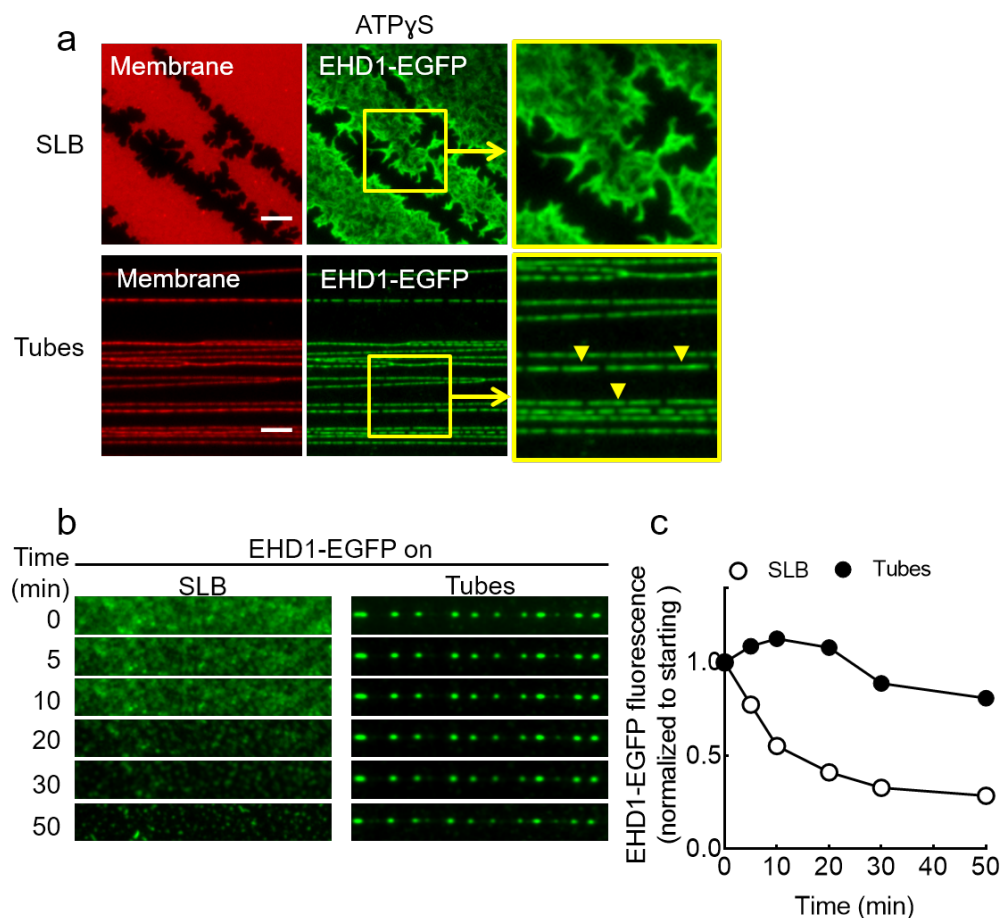


Figure 3.5 EHD1 distribution with ATPyS a) Binding of EHD1-EGFP with ATPyS to SLBs (*top*) and tubes (bottom). Yellow arrowheads indicate clusters of EHD1-EGFP on tubes. Scale bar = 5µm b) Representative images of SLBs and tubes demonstrating the effect of addition of ATP to EHD1-EGFP assembled with ATPyS. c) Quantification of EHD1-EGFP fluorescence on SLB and tubes. Data represents mean $\pm$ SD for three regions of interest on SLBs and tubes.

3.6 Mutations in G-domain Affect Protein Distribution in Cells

Reconstitution experiments with EHD1-EGFP clearly show that ATP-binding determines the distribution of the protein. To emulate this in a cell system, mutants that disrupt ATP-EHD1 interactions were generated and expressed in cells.

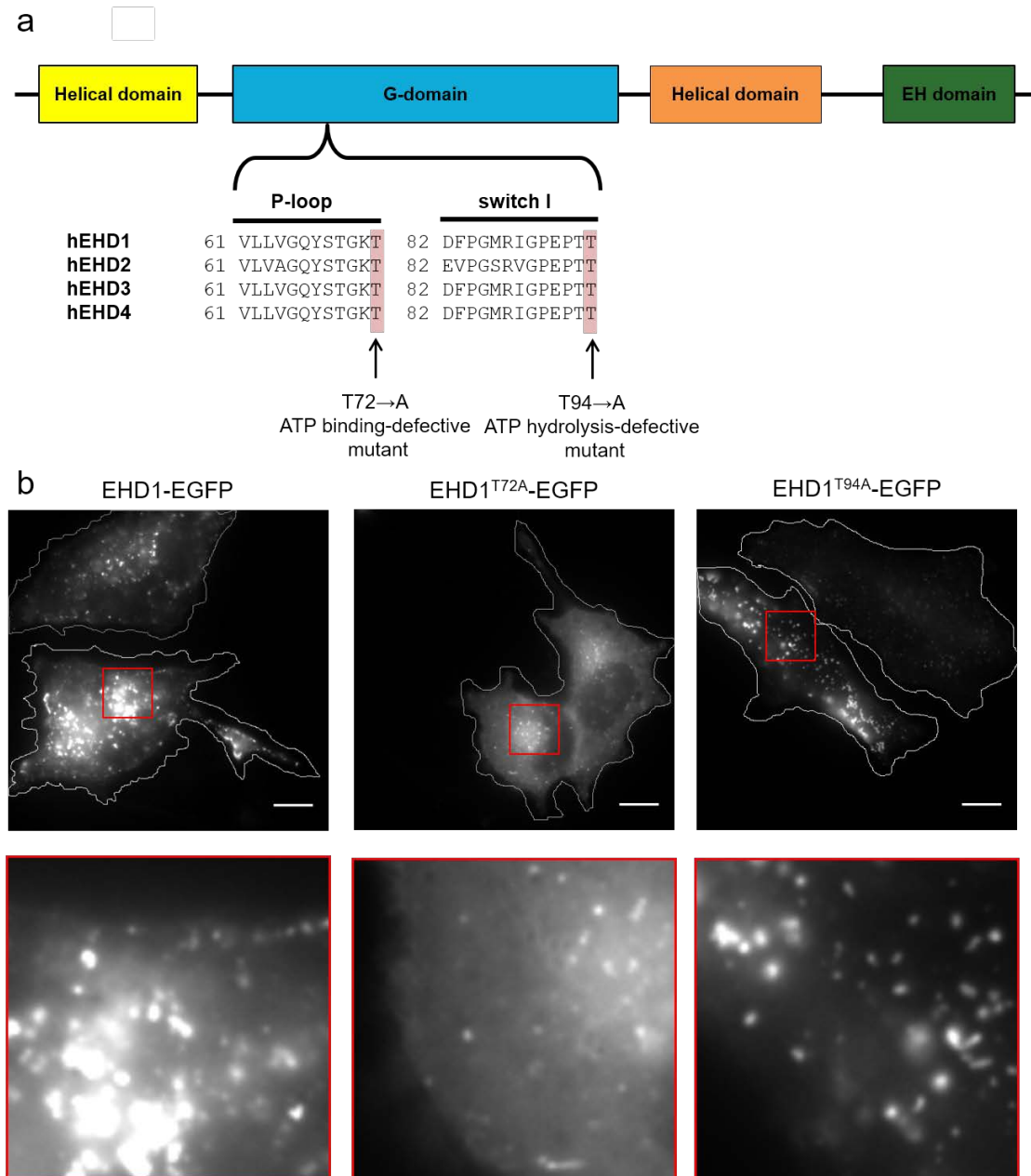


Figure 3.6 G-domain mutations affect EHD1 distribution a) Schematic showing domain organization of EHD1 and location of conserved threonine residues b) (*Top*) HeLa cells expressing EHD1-EGFP or EHD1^{T72A}-EGFP or EHD1^{T94A}-EGFP. Enlarged images displaying perinuclear region in cells (*bottom*). Scale bar = 10µm

G-domain mutants (Fig. 3.6 a), previously described in literature (Blume et al., 2007; Daumke et al., 2007), alter EHD-ATP interaction. Mutating the threonine residue at 72 to alanine (T72A) abrogates EHD1's ATP binding mimicking the Apo state of the protein. On the other hand, mutating the threonine residue at 94 to alanine (T94A) renders the protein defective in ATP hydrolysis, mimicking the ATP bound state.

On expressing EHD1-EGFP in HeLa cells it was seen that at steady state EHD1 displays both punctate and cytosolic distribution. Large clusters are seen in the perinuclear region. Mutant proteins display a different distribution from wild type protein. EHD1^{T72A}-EGFP displays a more cytosolic distribution with lesser puncta, while EHD1^{T94A}-EGFP displays a more punctate distribution (Fig. 3.6 b). Thus ATP binding and hydrolysis regulates the distribution of EHD1 in cells.

3.7 EHD1 Oligomers are Membrane Active

Oligomers of EHD1-EGFP with AMP-PNP on tubes are membrane-active and remodel the underlying membrane tube. In the membrane channel, tubes display coincident remodeling, with bright fluorescent puncta that coincide with clusters of EHD1. Protein scaffolds appear well separated on narrow tubes as compared to wide tubes (Fig. 3.7 a) which is highlighted by comparing COV (coefficient of variation) of EGFP fluorescence to starting tube radius (Fig. 3.7 b).

Compared to the distribution of EHD1 on membrane tubes, the distribution of Alexa-488 labeled dynamin 1 (A488-dyn1) is very different (Dar and Pucadyil, unpublished results). Dynamin also forms membrane active scaffolds. Unlike EHD1, dynamin scaffolds assemble on membrane tubes even in the absence of any nucleotide. However, dynamin scaffolds constrict the underlying membrane tube, causing the expulsion of the underlying membrane on either side of the scaffolds. This is reflected in the anti-correlated line profile comparison of membrane and Alexa-488 labeled dynamin fluorescence (Fig. 3.7 c). It is easy to see that this profile is very different from that seen with EHD1 (Fig. 3.7 a). EHD1-EGFP with AMP-PNP displays a positive correlation with membrane fluorescence while A488-dyn1 is negatively

correlated. As tubes are diffraction limited, the brightness of the tube is directly proportional to the net membrane area occupied within that focal volume. Thus the bright puncta induced by EHD1 oligomers reflect “bulging” of the underlying membrane tube. Interestingly, the bulged tube radius (R_b) seems to depend on the initial radius of the tube (Fig. 3.7 d). Moreover, EHD1-induced bulging also resulted in concomitant thinning of intervening membrane regions.

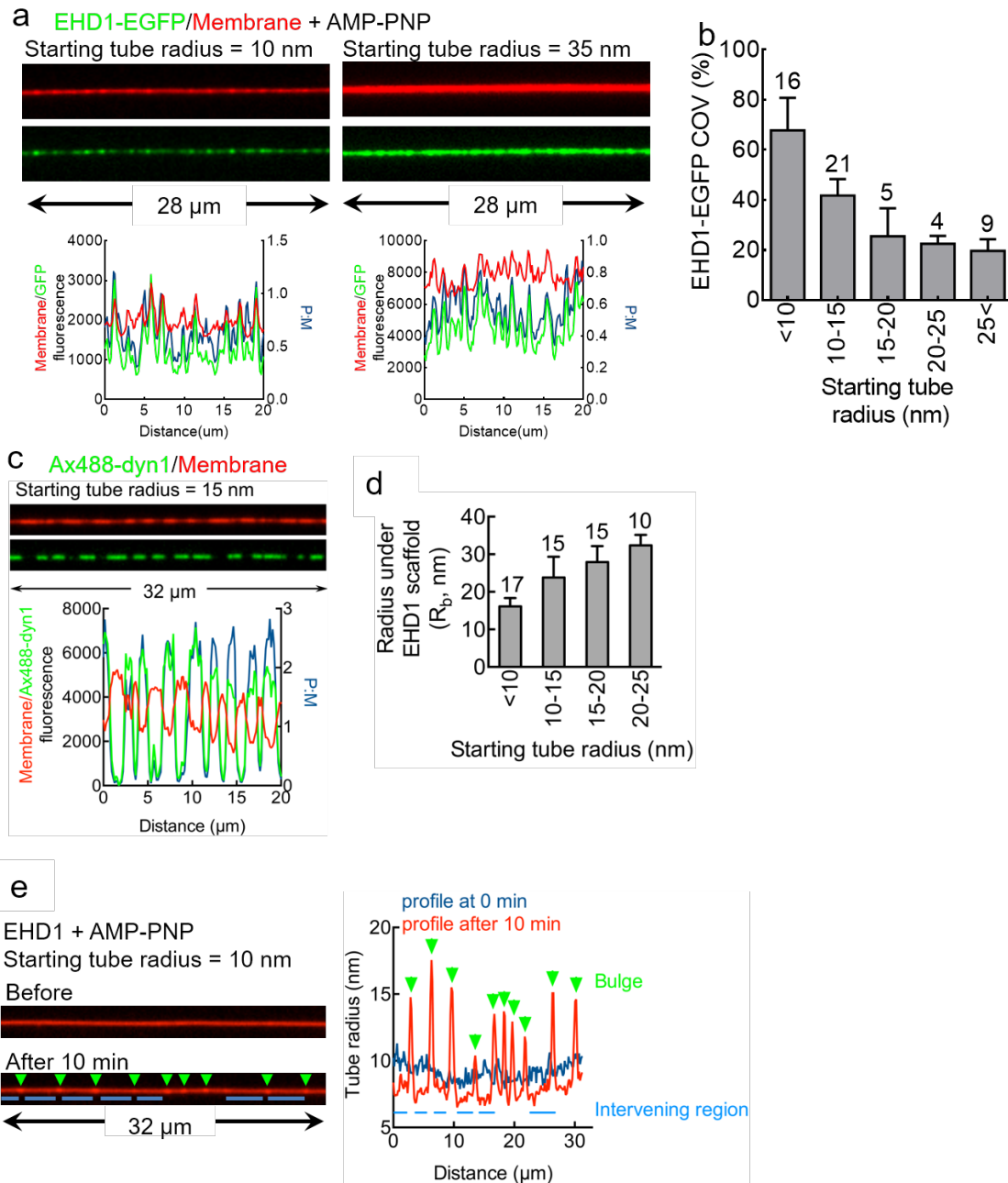


Figure 3.7 Nucleotide bound EHD1 remodels membranes a) Representative images of distribution of EHD1-EGFP added with AMP-PNP on tubes. Line profile indicate fluorescence ratio of protein to membrane (P: M) b) Plot represents coefficient of variation (COV) of EHD1-EGFP fluorescence as a function of starting tube radius. Data represents mean $\pm$ SD for n_{tubes} as indicated c) Representative images of distribution of Ax488 labelled dynamin1 on tubes. Line profile indicate fluorescence ratio of protein to membrane (P: M) d) Plot showing dependence of scaffold size on starting tube radius. Data represents mean $\pm$ SD of n_{tubes} as indicated e) Representative images (and associated line profile) of a tube displaying remodelling on addition of EHD1 with AMP-PNP acquired in the membrane channel at different time points, as indicated. Remodelled tube displays bulging (green arrowheads) and thinning of intervening membrane (blue lines)

Thus, on a tube of 10nm starting tube radius, bulging caused thinning of intervening regions, which reached estimates of up to $\sim 7\text{nm}$ in radius (Fig 3.7 e). This effect is brought about by compensatory mechanisms working to conserve tube volume in response changes in the shape of the membrane. The opposite can be seen with dynamin induced constriction, which displays an expansion of the membrane on either side of the scaffold. Thus EHD1 induced membrane remodeling is very different from that seen with dynamin.

Chapter 4: ATPase–
dependent Self-
assembly of EHD1
Promotes Membrane
Fission

4.1 ATP Hydrolysis by EHD1 Causes Membrane Fission

4.1.1 Membrane Remodeling with ATP

Passing EHD1 with ATP on SMrT induces rapid remodeling of membrane tubes. Extensive bulging and thinning of membrane tubes occurs, and many tubes undergo fission (Fig. 4.1 a). Analysis of tubes of different dimensions revealed that membrane remodeling is apparent on tubes with radius below ~ 25 nm.

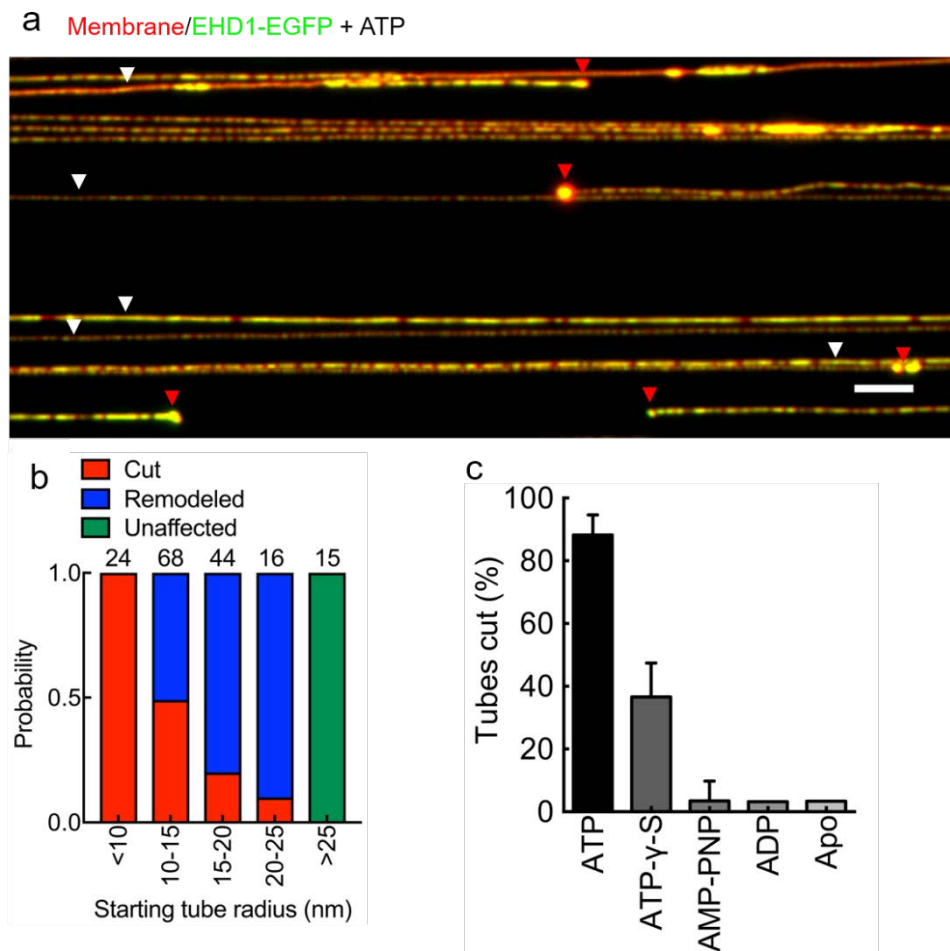


Figure 4.1 EHD1 with ATP remodels membrane and causes fission a) Representative images displaying effect of addition of EHD1-EGFP with ATP. Red arrowheads mark tubes that are cut, white arrowheads mark uncut tubes. Scale bar = $5\mu\text{m}$ b) probability of remodelling or fission, as a function of starting tube radius. Numbers represent n (tubes) as indicated c) Comparison of percent tubes cut using ATP or different ATP-analogs. Data represents mean $\pm$ SD for $n = 3$ experiments.

Narrower tubes display remodeling and fission. Below 25nm , a distribution of remodeled and cut tubes is obtained suggesting that membrane remodeling and fission is

dependent on the starting tube radius (Fig. 4.1 b). Freestanding tubes also undergo scission, indicating that this reaction is due to an intrinsic property of the protein and not due to any background sticking. However, since these tubes are not pinned to the surface, they are often out-of-focus and fission results in the rapid retraction of the tube from the field of view, compounding difficulty in analysis. Membrane fission is also dependent on ATP hydrolysis (Fig. 4.1 c). Analysis of tubes undergoing fission when EHD1 is added with different nucleotide analogs reveals that maximum fission is seen with ATP. Using ATP γ S, the slow hydrolyzing analog, displays lesser fission while flowing in protein with the non-hydrolysable analog AMP-PNP, ADP, or in the absence of any nucleotide (Apo) displays the least amount of cut tubes.

4.1.2 Rate of ATP Hydrolysis Governs Fission

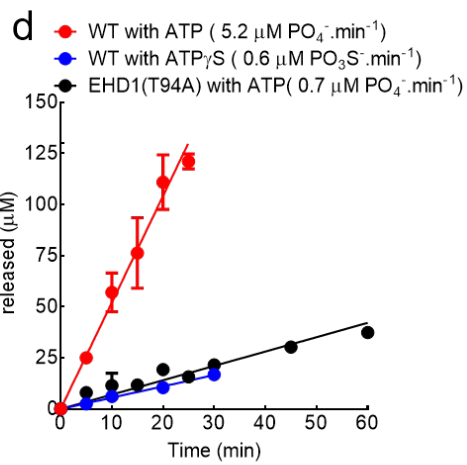
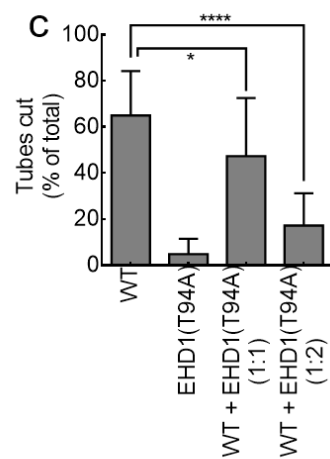
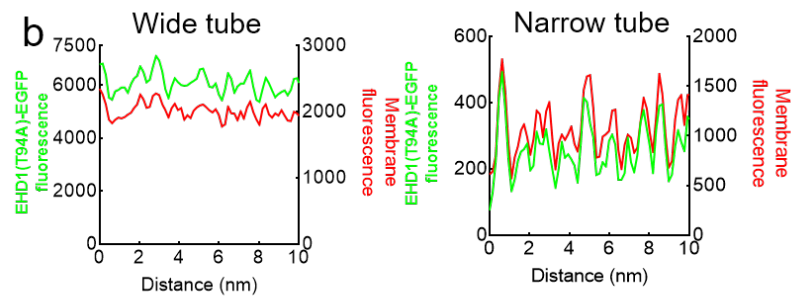
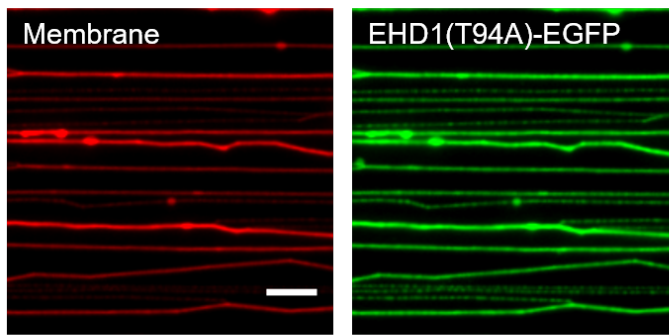
That hydrolysis requires membrane fission can be tested by using a mutant defective in ATP hydrolysis. On purifying and flowing in EHD1^{T94A}-EGFP with ATP on SMrT, the mutant protein forms oligomers on membrane tubes, which are also capable of bulging the underlying membrane (Fig 4.2 a, b). Interestingly, in comparison with WT, EHD1^{T94A} displays reduced fission. Furthermore, mixing WT and the mutant protein displays a reduction in fission of membrane tubes (Fig 4.2 c). Increasing the amount of EHD1^{T94A} in the mix further reduces the total number of tubes cut, suggesting that EHD1^{T94A} displays a dominant negative phenotype.

Comparing WT and EHD1^{T94A} ATPase activity in the presence of 100 mol% PS liposomes reveals that the mutant is indeed defective in ATP hydrolysis. EHD1^{T94A} hydrolyzes ATP at a slower rate than WT protein (Fig 4.2 d). The rate of hydrolysis of ATP by the mutant is comparable to that seen with WT hydrolyzing ATP γ S. This may explain why the mutant protein is capable of bulging membranes but displays lesser fission. Consistent with this, incubation of EHD1^{T94A} with ATP for longer time points, displays a steady increase in the percentage of tubes cut (Fig. 4.2 e). Thus ATP hydrolysis rate determines the fission reaction.

4.1.3 ATP Hydrolysis Induces with Growth of EHD1 Scaffolds

Since EHD1 hydrolyzes ATP γ S slowly, it offers a method to slow down the reaction. EHD1-EGFP was flowed with ATP γ S on SMrT templates and the system was imaged using STED microscopy. Interestingly, EHD1-EGFP scaffolds imaged on membrane tubes display an ordered assembly. These scaffolds displayed a symmetric helical-looking structure, assembled on bulges (Fig. 4.3 a).

a EHD1(T94A)-EGFP + ATP



e EHD1(T94A) + ATP

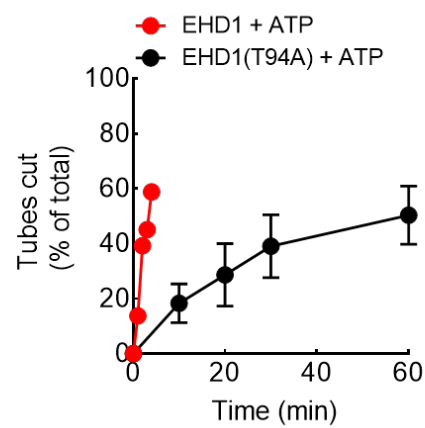
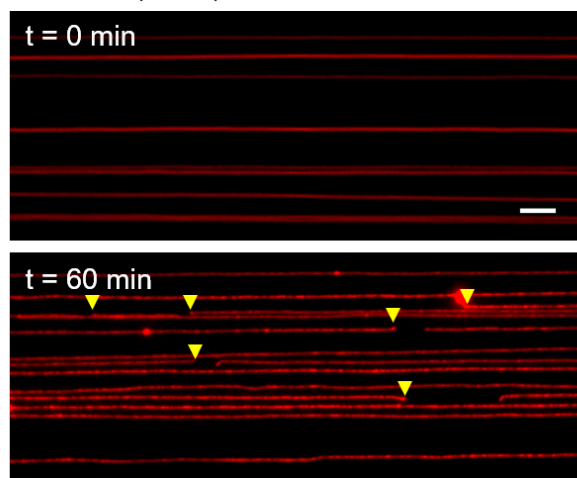


Figure 4.2 Rate of ATP hydrolysis governs fission a) Representative fluorescence images of EHD1^{T94A}-EGFP added with ATP to templates b) Plots showing comparison of distribution of EHD1^{T94A}-EGFP on narrow and wide tubes c) Plots describing tubes cut (percent of total tubes) for each case, as indicated. Data represent mean $\pm$ SD (n $\sim$ 350 tubes). Statistical significance assessed using Mann-Whitney test. * denotes $P = 0.419$ while **** denotes $P < 0.0001$ respectively d) Comparison of kinetics of nucleotide hydrolysis by EHD1 and EHD1^{T94A} e) Representative fluorescence images of addition of EHD1^{T94A} with ATP to templates captured at the indicated time points. Yellow arrowhead mark cut tubes. (Right) Plot describes the tubes cut (as a percentage of total tubes) with proteins as indicated (mean $\pm$ SD)

To understand how EHD1 scaffolds may induce membrane fission, EHD1-EGFP was flowed at 10 fold lower concentration, to reduce background fluorescence, with ATP on membrane tubes and the reaction was monitored. Remarkably, with ATP EHD1-EGFP displays nucleation at different regions on the membrane tube followed by rapid self-assembly induced growth of the scaffold, bi-directionally along the length of the tube (Fig. 4.3 b).

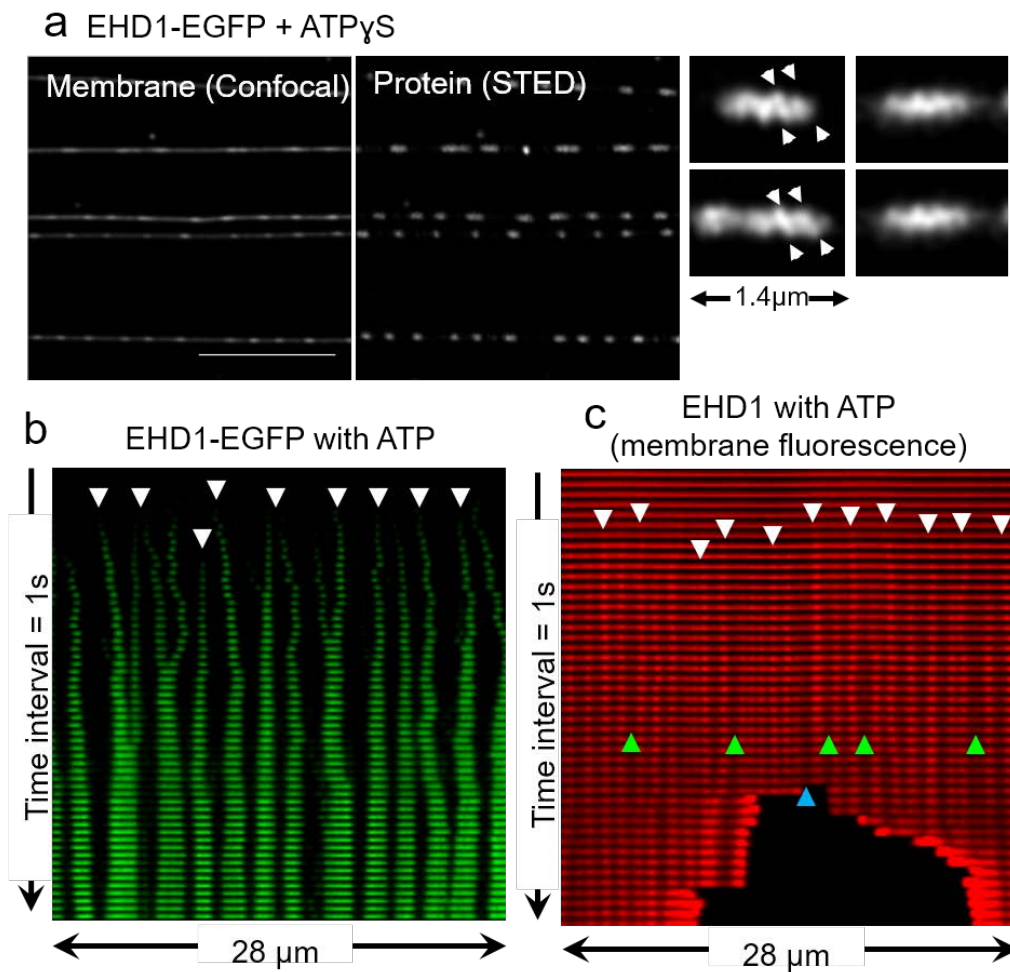


Figure 4.3 Growing EHD1 scaffolds induce membrane fission a) Representative Confocal and STED images of the EHD1-EGFP added to tubes with ATP γ S as indicated. (*Right*) panels displays magnified STED images of EHD1-EGFP scaffolds. White arrowheads display possible orientation of helical rungs. Scale bar = 5 μ m b) Representative montage of tube displaying nucleation of EHD1-EGFP (white arrowheads) and growth along the length of the tube c) Representative montage tubes showing bulging (white arrowheads) and thinning of intervening membrane (green arrowheads) till fission (blue arrowhead)

The expanding EHD1 oligomers quickly cover a significant area on the membrane tube. This results in rapid expansion of the bulged region under the scaffold and leads to thinning of the intervening membrane. As ATP reaction proceeds, expansion of bulges along with continued thinning of membrane tubes leads to scission. Tube scission appears to occur at the thinned region between bulges (Fig 4.3 c). Thus EHD1, via a unique mechanism of membrane bulging combined with ATP hydrolysis-induced self-assembly of the polymer grows along the tube, causes thinning of the intervening membrane, and leading to fission.

4.2 Role of the N-terminus in EHD1 Function

4.2.1. The N-terminus Harbors a Second Conserved Membrane Binding Site

The N-terminal residues are highly conserved across EHDs, suggesting a conserved function. A previous X-ray crystal structure for mouse EHD2 failed to obtain coherent electron density for the N-terminus, suggesting that it was flexible (Daumke et al., 2007). Using site-directed spin-labeling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy, the N-terminal residues 2-9 of mouse EHD2 were studied to determine the conformation of this region. (Shah et al., 2014). These experiments suggested that the N-terminus was flexible and adopted two conformations. Due to the presence of hydrophobic residues, the N-terminus was shown to be capable of shallow insertion into membranes, suggesting that it was a different membrane binding site from the helical domain (Daumke et al., 2007). Furthermore, for the accurate determination of the location of the N-terminus in EHD2, Leu5 was mutated to methionine and a selenomethionine (SeMet)-derivatized crystal of EHD2 was obtained. Electron density for SeMet was mapped to a hydrophobic pocket present in the G domain, where it was spatially localized adjacent to a KPFxxxNPF loop. This motif directly interacts with the EH domain in other proteins. Thus it was hypothesized that the N-terminus bridges the contact between the KPFxxxNPF loop and the G domain and thus regulates protein function. Indeed, mutation of the KPFxxxNPF loop of EHD2 results in

cytosolic localization of the protein in cells (Bahl et al., 2015). However, it is difficult to comment on the role N- terminus via these experiments.

Recently, a new structure was derived for the N-terminal truncated mouse EHD4, which raised further questions about the regulatory role of the N-terminus. EHD4 is one of the EHD paralogs found in the cell and has been shown to be predominantly associated with early endosomes (Sharma et al., 2008). N-terminal truncated mouse EHD4 (amino acids 22-514; EHD4^{ΔN}) was purified, crystallized and analyzed by X-ray diffraction (Melo et al., 2017). EHD4^{ΔN} displays membrane binding and oligomerization inducing liposome tubulation. Interestingly, it displayed a 200-fold stimulation in ATPase activity in presence of Folch liposomes. This was seven-fold higher than that previously reported for EHD2 (Daumke et al., 2007). The most striking feature of the structure was the reorientation of the helical domain by ~50° as compared to EHD2. The crystal packing placed monomers in a linear arrangement, with helical domains in each dimer facing outwards. This was claimed to be the “open structure” for EHD proteins while the old EHD2 structure was suggested to be “auto-inhibited”. They suggested that the deletion of the N-terminus was responsible for the change in structure and increased ATPase activity.

The above experiments point towards a role of the N-terminus in regulating EHD function. Hence it is important to understand the role of the N-terminus in the context of the EHD1-membrane interaction.

4.2.2 EHD1^{Δ2-9} Membrane Binding and ATPase Activity is Equivalent to Wild-type

To understand the contribution of the N-terminal residues to EHD1 function a construct was engineered in which the membrane inserting residues 2-9 (Shah et al., 2014) were deleted. This construct referred to as EHD1^{Δ2-9} was recombinantly expressed and purified (See Chapter 2, Section 2.1) and tested for its ability to bind membranes and catalyze ATP hydrolysis. EHD1^{Δ2-9} was found to bind to negatively charged membranes with affinities similar to wild-type, displaying half-maximal binding at 40 mol% DOPS concentration (Fig. 4.4 a). N-terminus deletion did not affect recruitment of the protein to liposomes.

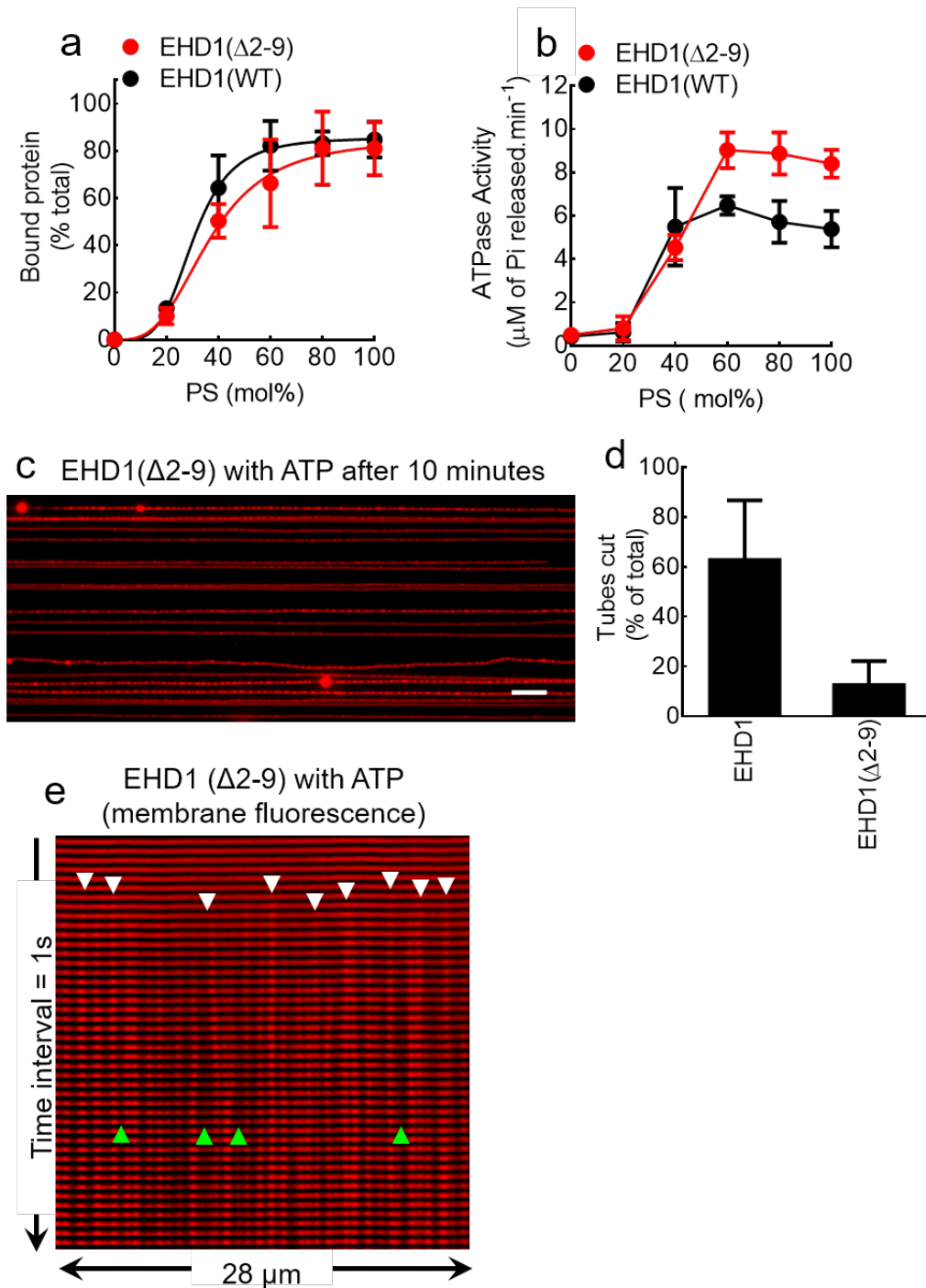


Figure 4.4 N-terminus of EHD1 is required for fission a) Comparison of liposome co-sedimentation assay for proteins as indicated. Data represents mean $\pm$ SD, n = 3 b) Comparison of ATPase activity for proteins as indicated. Data represents mean $\pm$ SD, n = 3 c) Representative fluorescence image of addition of EHD1 $^{\Delta$ 2-9 Added to template with ATP. Scale bar = 5 μ m d) Plot showing number of tubes cut as a percentage of total tubes analyzed (mean $\pm$ SD) e) Representative montage of a tube acquired in the membrane channel showing bulging(white arrowheads) an thinning of membrane tube (green arrowheads)

EHD1^{Δ2-9} displayed basal ATPase rate of 0.5 min⁻¹ in the absence of any DOPS which was stimulated to ~8.5 min⁻¹ when maximally bound to membranes (Fig. 4.4 b). Though the N-terminus has been implicated in playing an auto-inhibitory function for other EHD proteins (Hoernke et al., 2017; Melo et al., 2017; Shah et al., 2014), deletion of the N-terminus in EHD1 did not seem to relinquish the dependence on membrane binding to stimulate ATPase activity.

4.2.3 EHD1^{Δ2-9} Cannot Catalyze Membrane Fission

While EHD1^{Δ2-9} showed no significant difference in membrane binding or ATPase activity as compared to WT, the differences become apparent on comparing the protein on a SMrT assay. WT protein added to membrane tubes resulted in the bulging and subsequent fission of these tubes. EHD1^{Δ2-9} is also capable of bulging membrane tubes (Fig. 4.4 c). However, when added to membranes tubes, EHD1^{Δ2-9} is unable to cause scission (Fig. 4.4 c,d). Instead, the tubes get arrested at a “pre-fission” bulged state. As mentioned before, the bulges formed by the WT protein expand bi-directionally and facilitate membrane fission. However, EHD1^{Δ2-9} induced bulges seem to be defective in expansion and dissipate over time (Fig. 4.4 e). Thus the N-terminus is crucial for membrane fission.

Chapter 5: Insights **into Membrane-** **active Properties of** **EHD2**

5.1 EHD2 Displays High Sequence Identity to EHD1

The EHD-family of proteins consists of four different paralogs named EHD1-4 respectively. These proteins are differentially localized in the cell (Blume et al., 2007) and display high sequence identity (George et al., 2007; Naslavsky and Caplan, 2005). Much of what is known about the structure of EHD proteins comes from the X-ray crystal structure described for mouse EHD2 (Daumke et al., 2007). Through crystal structure analysis it was determined that EHD2 has a dynamin-like G-domain flanked on either side by helical domains with a C-terminal EH-domain. The helical domains form loops containing hydrophobic residues flanked by positively charged lysine residues at the tip, this region forms the membrane-binding patch. The G-domain of EHD2 is capable of binding and hydrolyzing ATP while the EH-domain has been implicated in the binding of partner proteins (Henry et al., 2010; Wong et al., 1995). In cells, EHD2 localizes to the plasma membrane and has been suggested to be associated with the necks of caveolae (Moren et al., 2012) and actin (Marg et al., 2012; Simone et al., 2014). *In vitro*, EHD2 has been shown to be capable of binding membranes and in the presence of ATP causes tubulation of liposomes (Shah et al., 2014).

Despite the enormous information available about EHD2, there is little evidence for the mechanism by which EHD2 remodels membranes. Negative stained EM and Cryo-EM micrographs of EHD2 added to liposomes with ATP display tubulated liposomes with a protein coat of EHD2 surrounding the liposomes (Daumke et al., 2007; Shah et al., 2014). However, these images do not display extensive remodeling, as seen with EHD1. To address whether these differences (between EHD2 and EHD1) are due to the differences in assay systems (liposome as opposed to membrane tubes), it is necessary to study both proteins under the same conditions. Thus, EHD2's membrane binding, ATPase activity, and membrane remodeling activity was analyzed and compared with EHD1.

5.2 EHD2 Binds Membranes with Higher Affinity than EHD1

Liposome co-sedimentation assay with EHD2 revealed that it is capable of binding to PS liposomes with higher affinity than EHD1. EHD2 membrane binding reached saturation with liposomes containing 40 mol % DOPS, with half-maximal binding seen at 10 mol % DOPS (Fig. 4.5 a). This was very different from EHD1, which displayed half-maximal binding at about 40 mol % DOPS. Thus EHD2 displays a greater affinity for negative charge than EHD1.

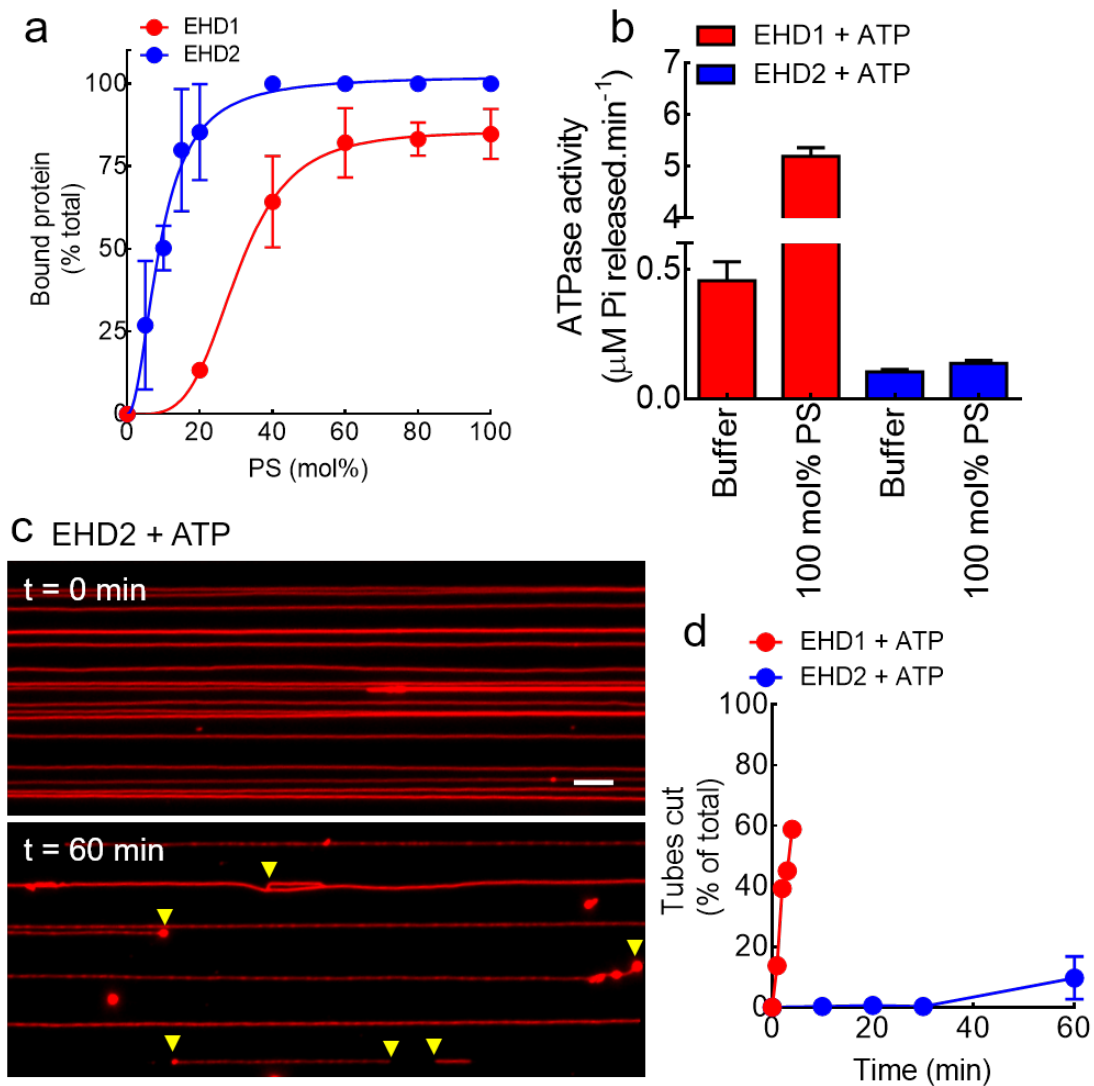


Figure 5.1 EHD2 is different from EHD1 a) Comparison of liposome co-sedimentation assay for EHD1 and EHD2 b) Comparison of EHD1 and EHD2 ATPase activity in buffer or with 100% DOPS liposomes c) Representative fluorescence images of SMrT assay for EHD2 with ATP acquired at different time points as indicated. Yellow arrowheads indicate cut tubes. Scale bar = 5 μm d) Plot of number of tubes cut (percentage to total tubes) for each protein as indicated. Data represents mean $\pm$ SD.

5.4 EHD2 Displays Lower ATPase Activity than EHD1

Despite its higher binding to membranes, EHD2 displays significantly low ATPase activity. In the presence of 100 mol% DOPS liposomes, EHD2 ATPase activity was around 0.13 min^{-1} which was ~ 40 fold lower than that seen with EHD1 (Fig 4.5 b). Thus, under the same conditions, EHD2 displays lower ATPase activity than that seen with EHD1.

5.5 EHD2 Catalyzes Membrane Fission Slower than EHD1

Consistent with its lower ATPase activity, EHD2 displays slower membrane remodeling as compared to EHD1. Upon addition of EHD2 with ATP on membrane templates, bulging of membrane tubes is observed only after a long incubation time (Fig. 4.5 c). Additionally, EHD2 displays reduced membrane fission capability (Fig. 4.6). Thus, when tested under identical experimental conditions, the differences between these paralogs can be clearly elucidated.

Discussion

This work with EHDs has identified a unique mechanism by which a protein can bring about remodeling of membranes (Fig. 6.1). Our experiments highlight that EHD1 requires ATP for membrane remodeling. In the ATP-bound state EHD1 binds membranes, causing them to bulge outwards. ATP hydrolysis drives extensive polymerization of the protein by self-assembly. As protein scaffolds grow bi-directionally, they extend the underlying bulge, consequently thinning the membrane regions between scaffolds. Uncontrolled growth and extensive remodeling by EHD1 leads to further thinning of the membrane, leading to scission.

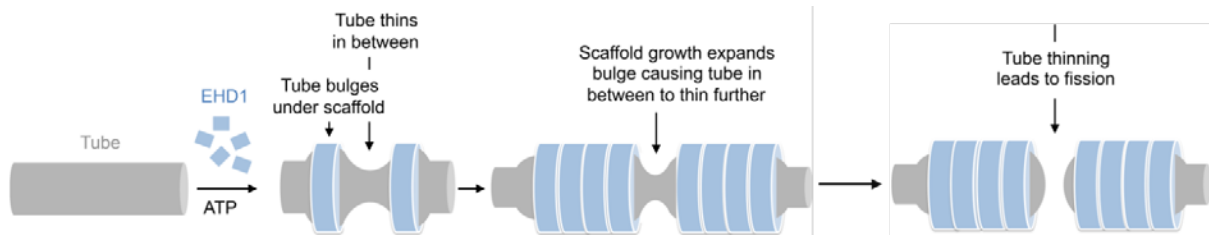


Figure 6.1 Schematic representation of remodelling of membrane tube by EHD1

Ample experiments have described the mechanism of action of other membrane scaffolding proteins like those belonging to the BAR domain family and dynamin. These proteins are capable of binding, oligomerizing and constricting the underlying membrane surface (Daumke et al., 2014; Jarsch et al., 2016; McMahon and Gallop, 2005). EHD scaffolds, on the other hand, promote bulging of the underlying membrane. X-ray crystal structures of EHDs suggest that they form crisscross dimers (Daumke et al., 2007; Melo et al., 2017; Shah et al., 2014). These scissor-shaped dimers have been shown to form a curved membrane-binding surface (Daumke et al., 2007; Melo et al., 2017). It is possible that membrane bulging observed is due to the adaptation of the negatively charged membrane to a positively charged curved surface formed by EHD oligomers and further work is necessary to understand the details of bulge formation. Mutations in the oligomerization interface could possibly help dissect these functions (Hoernke et al., 2017).

ATP-binding promotes recruitment of the protein to membranes, though EHD1 displays higher recruitment to curved membrane templates over planar membranes. Long incubation times display a reduction in EHD1 density on the membrane. This may be due to the exhaustion of ATP, which in turn may trigger disassembly of the protein scaffold as a consequence of reduced number EHD-membrane interactions in the scaffold. However, the protein's affinity for curved membrane templates counteracts this disassembly process, and more protein is found on tubes over SLBs. As a consequence of these factors, at steady-state

condition, EHD1 may be less abundant on relatively planar membranes and instead may cycle between a cytosolic dimer form and a population of protein assembled on membrane tubes.

In the absence of nucleotide, EHD1 displays minimal recruitment to membrane tubes. Dynamin does not display this, and self-assembles on membrane tubes. Addition of GTP to dynamin scaffolds assembled on membrane tubes triggers a further constriction event, causing scission (Dar et al., 2015; Sweitzer and Hinshaw, 1998). However, for EHD1, ATP hydrolysis induces self-assembly of the protein along the tube, probably through exposure of an oligomerization interface in the transition state. Such a mechanism of self-assembly via dimerized G-domain interfaces has been reported for dynamin (Carr and Hinshaw, 1997; Chappie et al., 2010). Growing EHD1 scaffolds cause concomitant growth of the bulge. The N-terminal residues also harbor a membrane binding site and may be responsible for anchoring the protein scaffold in the membrane. Without the N-terminus, EHD1 is capable of bulging the underlying membrane but is defective in the expansion of the bulges, and hence also defective in scission. Compared to EHD1, EHD2 binds membranes with higher affinity. Also, EHD2 displays a 40-fold lower ATPase activity, compared to EHD1. This suggests that rather than actively remodeling the underlying membrane by self-assembling into discrete scaffolds, EHD2 may be capable of uniformly coating the entire membrane surface. Consistent with this, EHD2 depletion causes increase mobility of caveolae on the membrane and does not contribute to fission (Stoeber et al., 2012). Since EHDs proteins hetero-oligomerize, EHD2 may recruit other EHDs which could then induce membrane remodeling (Yeow et al., 2017). The dominant negative mutant EHD1^{T94A} also shows reduced ATP hydrolysis rate and slower membrane remodeling. These results also highlight the importance of the rate of ATP hydrolysis in EHD1 induced membrane-remodeling. Despite sharing significant sequence identity, one must be careful when comparing features across these paralogs.

Growing EHD1 scaffolds expand the underlying membrane, and to conserve total membrane volume, bulging results in concomitant thinning of membrane tubes. Thinning of the membrane tube works to bring opposing bilayers closer together, probably leading to the formation of a hemifission intermediate that resolves into fission (Frolov et al., 2015). This suggests a novel mechanism of bulging induced membrane fission. While it is easy to understand these features using a simple *in vitro* setup it would be interesting to see EHD1 is similarly capable of remodeling more complex membranes, like the ERC. Previous studies have shown that depletion of EHD1 causes expansion of the recycling compartment (Lee et al., 2015) and accumulation of cargo at the ERC (Cai et al., 2011, 2012; Jovic et al., 2007),

suggesting that it plays a role in membrane remodeling and fission at the ERC. How would EHD1 be recruited to the ERC? Recent work has suggested that the kinesin motor KIF13A is required for generation of tubules from sorting endosomes (Delevoye et al., 2014). Since curvature determines recruitment and further remodeling, these tubules could form the substrate for EHD1 activity. Modulating the PS content or other mechanisms to increase the negative charge displayed on the compartment surface could also regulate EHD1 recruitment (Lee et al., 2015). Once bound, EHD1 could induce bulging of membrane tubules inducing concomitant thinning and scission of the membrane, facilitating the release of membrane-bound cargo. However, this mechanism of “bulging-induced fission” is indirect when compared to dynamin, where GTP-hydrolysis is translated into conformational changes in the scaffold, which generates the force to constrict and cut membranes (Antonny et al., 2016; Ferguson and De Camilli, 2012; Roux et al., 2010; Sweitzer and Hinshaw, 1998; Warnock and Schmid, 1996). It is also possible that thinning induced by bulging could facilitate the recruitment of another as yet unidentified protein(s) which in turn could catalyze fission. In that case, EHD1 may play a role in promoting extensive remodeling on membrane tubes. Here, EHD1-binding proteins may help regulate the growth, self-assembly, and disassembly of EHD1. It would be interesting to see whether these proteins (such as MICAL L-1) or other ERC resident proteins could influence the recruitment and distribution EHD1.

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