Requirement of EHD Family of Endocytic Recycling Regulators for T-Cell Functions

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REQUIREMENT OF EHD FAMILY OF ENDOCYTIC RECYCLING REGULATORS FOR T-CELL FUNCTIONS

By

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**REQUIREMENT OF EHD FAMILY OF ENDOCYTIC RECYCLING REGULATORS FOR T-CELL FUNCTIONS**

Fany M. Iseka, Ph.D.

University of Nebraska, 2017

**Supervisor: Hamid Band, M.D, Ph.D.**

T-cells use the endocytic pathway for key cell biological functions, including receptor turn-over and maintenance of the immunological synapse. Some of the established players include the Rab GTPases, SNARE complex proteins, and others which in non-T-cell systems function together with Eps15 Homology Domain-containing (EHD) proteins. To date, the role of the EHD protein family in T-cell function remains unexplored. We generated conditional EHD1/3/4 knockout mice using CD4-Cre and crossed these with mice bearing a myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgene. We found that CD4\(^+\) T-cells from these mice exhibited a reduced antigen-driven cell proliferation and IL-2 secretion in vitro. In vivo, these mice exhibited reduced severity of experimental autoimmune encephalomyelitis. Further analyses showed that recycling of the TCR-CD3 complex was impaired, leading to increased lysosomal targeting and reduced surface levels on CD4\(^+\) T-cells of EHD1/3/4 knockout mice. Our studies reveal a novel role of the EHD family of endocytic recycling regulatory proteins in TCR-mediated T-cell functions.
To Pathy, Dinovic, Stephane, and Charlotte
ACKNOWLEDGEMENTS:

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in me and your love for me is inexpressible. Thank you for being the best parents I can ever ask for. Words cannot express my gratitude to you. I love and thank you both from the bottom of my heart.

To my immediate family, my husband, my son and new coming baby, I say thank you. To my husband, you have been more than what I could ask for. You are a very supportive, caring, unselfish, and loving man I have ever known. You have been my best friend, the best husband and the best father for our son. You started taking care of Dinovic since he was only 7 weeks and you still do. I cannot even count the number of times you had to leave work to come home and take care of him just so I can get ready for a presentation, or fellowship application, or whatever I needed help with. You are just awesome! And Dinovic, you have been the best gift from God to us. You have always been a healthy baby, very kind, very smart and just amazing. Thank you for understanding and being patient with me for all the times someone else had to watch and care for you while I was at school or studying. You both have been the best! I love you both so much and with all my heart I say thank you for making it possible for me to be able to finish my degree. I love you both!
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<thead>
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<th>Abbreviation</th>
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<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>Arf</td>
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<td>ATP</td>
<td>Adenosine-5'-Triphosphate</td>
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<td>ATPase</td>
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<td>BSA</td>
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<td>Clathrin Assembly Lymphoid Myeloid Leukemia</td>
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<td>Double Negative</td>
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<td>Experimental Autoimmune Encephalomyelitis</td>
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<td>Early Endosomes</td>
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<td>Endosomal Sorting Complexes Required for Transport</td>
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<td>FBS</td>
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<td>Glucose Transporter 4</td>
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<td>GPCRs</td>
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<td>GTPase</td>
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<td>HBV</td>
<td>Hepatitis B Virus</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HSC70</td>
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<td>ICAM-I</td>
<td>Intercellular Adhesion Molecule 1</td>
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<tr>
<td>IFT</td>
<td>Essential Intraflagellar Transport</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IS</td>
<td>Immunological Synapse</td>
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<td>KCa2.3</td>
<td>Small Conductance Ca²⁺-Activated K⁺ Channel Protein</td>
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<td>Lymphocyte Function-Associated 1</td>
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<td>MEFs</td>
<td>Mouse Embryonic Fibroblasts</td>
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<td>MHC-II</td>
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<td>MICAL-L1</td>
<td>Molecules Interacting with CasL-Like1</td>
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<td>MOG</td>
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<td>MVB</td>
<td>Multi-Vesicular Bodies</td>
</tr>
<tr>
<td>NCX</td>
<td>Na/Ca Exchanger</td>
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<tr>
<td>NgCAM</td>
<td>L1/Neuron-Glia Cell Adhesion Molecule</td>
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<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NPF</td>
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<td>4-OHT</td>
<td>4-Hydroxytamoxifen</td>
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<td>PA</td>
<td>Phosphatidic Acid</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PCDH 15</td>
<td>Protocadherin 15</td>
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<tr>
<td>PI (4, 5) P2</td>
<td>Phosphatidylinositol-4, 5-Bisphosphate</td>
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<tr>
<td>PIKfyve kinase</td>
<td>Phosphoinositide kinase, Five-type Zinc Finger Containing</td>
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<td>PLCY</td>
<td>Phospholipase C-y</td>
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<td>Full Form</td>
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<tr>
<td>pSMAC</td>
<td>Peripheral SMAC</td>
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<td>Ras-Like in Rat Brain</td>
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<td>Ribonucleic Acid</td>
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<td>RTKs</td>
<td>Receptor Tyrosine Kinases</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
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<td>SH3 domain</td>
<td>Src Homology 3 Domain</td>
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<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
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<td>SNAP</td>
<td>Synaptosomal-Associated Protein</td>
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<td>SNARE</td>
<td>Soluble N-Ethylmaleimide-Sensitive Fusion Factor Attachment Protein Receptors</td>
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<td>Single Positive</td>
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<td>TAMs</td>
<td>Immunoreceptor Tyrosine-Activation Motifs</td>
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<td>TGN</td>
<td>Trans-Golgi Network</td>
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<td>TSG</td>
<td>Tumor susceptibility gene</td>
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<tr>
<td>TMA</td>
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<td>Virological Synapses</td>
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<td>Western Blot</td>
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<td>Wild-Type</td>
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CHAPTER 1: INTRODUCTION
1. Endocytic Trafficking

1.1 Overview

Endocytic trafficking is an essential cellular process that has been evolutionarily conserved, from yeast to humans, with minor changes and specifications depending on the species. The endocytic trafficking is a fundamentally complex system extensively used in physiologically crucial processes such as cell migration (Maritzen, Schachtner, & Legler, 2015; Paul, Jacquemet, & Caswell, 2015), receptor turnover (Irannejad, Tsvetanova, Lobingier, & von Zastrow, 2015), cell signaling (Barbieri, Di Fiore, & Sigismund, 2016; Irannejad et al., 2015; Platta & Stenmark, 2011; Villasenor, Kalaidzidis, & Zerial, 2016), and cell-to-cell communication (Onnis, Finetti, & Baldari, 2016). It is also used in other pathways such as pathogen invasion (Kulpa et al., 2013; Pirooz et al., 2014; Takeuchi, Furuta, & Amano, 2011), cell division (Chesneau et al., 2012; Schweitzer, Sedwick, & D'Souza-Schorey, 2011), and membrane remodeling (Matsubayashi, Coulson-Gilmer, & Millard, 2015). It is important to mention that mis-regulation of endocytic trafficking is linked to different diseases including chronic hepatitis B virus (HBV) infections (Macovei, Petrareanu, Lazar, Florian, & Branza-Nichita, 2013; Watanabe et al., 2007), cancer (Mellman & Yarden, 2013; Mosesson, Mills, & Yarden, 2008), HIV infection (Kulpa et al., 2013), and autoimmune diseases (Iglesias-Bartolome et al., 2009). Therefore, understanding how this complex system works is of great interest in basic and clinical immunology.

Directed cell migration is an important phenomenon in embryonic development (Keller, 2002; Petrie, Doyle, & Yamada, 2009; Wallingford, Fraser, & Harland, 2002), during metastasis (Etienne-Manneville, 2008; Petrie et al., 2009) and during immune responses to pathogens or transformed cells (Petrie et al., 2009). For instance, immune cells are known to circulate in the blood and through chemotactic gradients released during inflammation to migrate to the site of infection. This very specific extravasation is achieved with the help of chemokine receptors,
growth factor receptors, and adhesive integrins (Maritzen et al., 2015). Integrins are one of the most important molecules required for, and are in the center of, cell migration.

Recent studies have shown that endocytic trafficking can regulate integrin functions. Integrins are known to continually and rapidly internalize and recycle back to the plasma membrane. For instance, some integrins were shown to internalize almost all their surface pool in just 30 minutes (Paul et al., 2015) and with a low degradation rate, most recycled back to the cell surface. This is important because as cells migrate, they form a rear and a front part, causing integrins to act as “feet” that allow the cells to move forward. It has been suggested that migrating cells use the process of endocytosis to move a pool of integrin “feet” from the rear of the cells to re-distribute them through exocytosis or by recycling them to the front part of cells to cause the forward movement (Paul et al., 2015). Other work has shown that this directional trafficking of integrins actually happens in three different ways, in which they can be trafficked from the front to the front, from the front to the rear of the cells, and from the perinuclear region to the rear of the cells (Paul et al., 2015). Endocytosis of integrin does not only promote the disassembly of adhesion, but it also contributes to the formation of adhesion reassembly by segregating a pool of integrins that can be recycled where new adhesions have to be made (site of action) (Paul et al., 2015). Lymphocytes use LFA-1 as their main integrin. The interaction between LFA-1 and its ligand, ICAM, allows T-cells to migrate into peripheral lymph nodes and to interact with antigen presenting cells (APCs) for T-cell activation (Evans et al., 2009). However, beside integrin, T-cells also need other receptors (such as co-stimulatory and co-inhibitory receptors and antigen specific receptors) for proper immune response. All of these receptors are strictly regulated by endocytic trafficking.

Immune cells have to maintain a proper balance between receptors on their cell surface and in the intracellular pool to function properly. Hence, they have to regulate and balance the rate of internalization, degradation, recycling and synthesis of new receptors. In most cases, the fate of
most receptors is determined by the status of their signaling. Receptor signaling begins at the plasma membrane; however, cells have found a way not only to sustain it, but also to terminate it through the process of endocytic trafficking (Irannejad et al., 2015). After ligand binding, receptors get activated mainly through phosphorylation of their cytoplasmic domain(s), which leads to activation of signaling cascades that eventually leads to different outcomes such as cell proliferation, survival, etc. Ligand binding induces internalization of receptors. These receptors go from the plasma membrane to early endosomes (EE) where they are sorted to either go to the lysosomes for signal termination or go back to the cell surface for re-stimulation. It is well-established that after internalization, a percentage of signaling receptors go from the EE to the multi-vesicular bodies (MVB) where they get sorted to go to the lysosome for either receptor-ligand degradation or for ligand degradation (Villasenor et al., 2016). Research also shows that another percentage of signaling receptors go from the EE to the cell surface using the fast route or the slow recycling route in which receptors go from the EE to recycling endosomes (RE) first and then back to the cell surface. All these steps are strictly regulated by families of endocytic trafficking regulators such as Rabs and EHD protein families that will be discussed later.

Endocytosis is important for segregating a pool of signaling receptors from the plasma membrane to the intracellular milieu to make space for new receptors and to allow signaling termination. Endocytosis also creates endosomes with pools of receptors that are ready to go back to the cell surface to sustain signaling at the cell surface and those that can act as a platform of intracellular signaling (Barbieri et al., 2016). Research has shown that receptors are able to continue signaling even inside endosomes in the intracellular milieu. In T-cells, internalized TCRs have been shown to continue signaling in endosomes, therefore expanding the overall time of sustained signaling (Yudushkin & Vale, 2010).

The endocytic trafficking is important in cell-to-cell communication especially among immune cells. Research has shown that during contact between a T-cell and an antigen
presenting cell (APC) or a target cell (which can be a transformed cell), they form a contact site called the immunological synapse (IS).

The IS has been shown to be not only a site of intense internalization and recycling, but also of directed secretions of cytokines, granzymes, microvesicles, and exosomes (Gutierrez-Vazquez, Villarroya-Beltri, Mittelbrunn, & Sanchez-Madrid, 2013). Exosomes in particular, have been shown to be bidirectional. Various studies have shown exosomes from APCs endocytosed in T-cells and vice versa. Other studies have also suggested that when engaged T-cell antigen receptors (TCRs) are internalized, some take with them the antigenic peptide or the pMHC inside the endosomes and when they are recycled, some of those are re-expressed on the cell surface of the T-cells. Re-expressed antigenic peptides or pMHC can act as APCs in those regions and activate other T-cells (a process called cross-dressing) (Campana, De Pasquale, Carrega, Ferlazzo, & Bonaccorsi, 2015; Game, Rogers, & Lechler, 2005; Tatari-Calderone, Semnani, Nutman, Schlom, & Sabzevari, 2002). Other exosomes carry with them other important molecules including small RNAs that were shown to change T-cell profiles (Gutierrez-Vazquez et al., 2013).

In addition, cell-to-cell communication is also exploited by certain pathogens. HIV for example uses the virological synapse (VS), which is very similar to the IS, as a way to replicate itself. Infected CD4+ T-cells form VS with other immune cells, including healthy CD4+ T-cells; vesicles containing HIV have been shown to be secreted into the VS and endocytosed by target cells. This newly infected cell can then form other VS with other healthy cells and spread the virus (Alvarez, Barria, & Chen, 2014).

Regardless of which way the cell chooses to utilize the endocytic trafficking pathway, they all involve the internalization of materials through the invagination of the plasma membrane into the cytoplasmic milieu. This mainly involves the pinching of the plasma membrane, and then the
release of the endosome containing internalized materials into the cytoplasmic milieu. Once in the cytoplasm, depending on their fate, the materials are sorted into specific endosomes. In the case of receptors, they either can go to the lysosomes for degradation or can recycle back to the cell surface for other rounds of activation.

1.2 Routes of Endocytosis

Cells use endocytic trafficking for many functions, including the internalization of receptors and uptake of nutrients, ligands, viruses and molecules. Cells use different routes to endocytose materials that include the clathrin-dependent pathway and the clathrin-independent pathway. The clathrin-independent pathway is further classified into caveolin-dependent, and clathrin-and caveolin-independent, ARF6 endocytosis (Fig 1.1). Regardless of the pathway, the internalized receptors enter the early endosome (EE) where they are sorted for either traffic into late endosome/MVB and lysosome or for recycling back to the cell surface. The latter proceeds either through the fast recycling route (directly to the plasma membrane) or through the slow recycling route via the endocytic recycling compartment (also called recycling endosome) (Gould & Lippincott-Schwartz, 2009; Jovic, Sharma, Rahajeng, & Caplan, 2010) (Fig 1.1).

Clathrin-mediated endocytosis (CME) is the most studied form of entry into the cytoplasm and it is used by all eukaryotic cells. This pathway has been extensively studied for the traffic of receptor tyrosine kinases (RTKs) such as EGFR and for G protein-coupled receptor (GPCRs). CME is defined by uptake of materials from the plasma membrane into the cytoplasm of cells through clathrin-coated vesicles. Five steps lead to the formation of a clathrin-coated vesicle (CCV). They include initiation (nucleation), cargo selection, coat assembly, scission, and uncoating (McMahon & Boucrot, 2011). Nucleation is the first step in CCV formation, in which a particular site in the plasma membrane is marked for invagination; it also involves the recruitment of clathrin machinery to the site. The membrane-sculpting F-BAR domain-
containing FCHo1 and 2 (FCHo1/2) proteins, together with other proteins, function as nucleator of clathrin-coated pits (CCP). FCHo1/2 bind to the plasma membrane to mark the site of CCV formation and recruit the scaffolding epidermal growth factor receptor substrate 15 (eps15) and intersectin (Henne et al., 2007; McMahon & Boucrot, 2011; Pechstein et al., 2010) which in turn recruit AP-2. This complex is able to form membrane curvature even before the recruitment of clathrin (Henne et al., 2007; Henne et al., 2010; McMahon & Boucrot, 2011). Other proteins such as SGIP1 (Henne et al., 2010) and other adaptors besides AP-2 are also involved in this step, and include AP180 and clathrin assembly lymphoid myeloid leukemia (CALM) (Daumke, Roux, & Haucke, 2014). All of these initiation proteins associate at specific sites in the plasma membrane that are enriched in phosphatidylinositol-4, 5-bisphosphate [PI (4, 5) P2] (Di Paolo & De Camilli, 2006) and mark those areas for clathrin recruitment and assembly.

The next step is the cargo selection in which cargos with specific sequence are recognized and bound to AP-2 and other cargo-specific adaptor proteins. CME is the only endocytic pathway with very well-characterized cargos. It has been shown that the destination of individual receptors and other membrane proteins depend on their intrinsic sequence-based signal motif located in the cytoplasmic domain (Traub, 2003). These internalization signals are required for transmembrane protein fast endocytosis (Traub, 2003). Transmembrane proteins are known to bind directly to the μ2 subunit of AP-2 (4 subunits: α and β2 adaptins, μ2 and the σ2) (Ohno et al., 1995). AP-2 recognizes and interacts with cargos containing sequences such as the YXXØ (where X is any amino acid and Ø is a bulky hydrophobic residue) and [DE]XXXL[LI] dileucine signals (Traub, 2003). Some cargos may also contain motifs such as the Asn-Pro-X-Tyr sequence (NPXY) that are recognized by other clathrin adaptor proteins (Traub, 2003).

After the selection of the cargos that are to be internalized, clathrin triskelia are then recruited from the cytosol of the cell to the site of the CCP on the plasma membrane by AP-2 and other accessory adaptor proteins to help form the CCV. Clathrin triskelia concentrate in those
CCP forming clathrin lattice; their polymerization stabilizes the curvature, cargo-specific adaptors and curvature effectors such as eps15 and intersectin. After their maturation, the CCV pinch off from the plasma membrane through the help of dynamin, recruited by BAR domain-containing proteins. These proteins have a preference in forming the curvature of necks for the forming vesicles, and are suggested to help in the process of neck formation of the vesicles that dynamin pinches off. These proteins include amphiphysin, endophilin, and sorting nexin 9 (SNX9) (McMahon & Boucrot, 2011; Meinecke et al., 2013).

After the scission by dynamin, the newly formed CCV is found in the cytosol, but before it can fuse with the early endosomes, they have to be stripped of their coats. CCVs go through a process of coat disassembly, by which the clathrin lattices go from an arranged conformation to individual triskelia by the help of the ATPase heat shock cognate 70 (HSC70) and its cofactor auxilin (Krantz et al., 2013). The triskelia are recycled for other CCV formation. The uncoated internalized endosome can now fuse with the early endosome where the cargos are sorted according to their fate.
**Figure 1.1: Pathways of Endocytosis.** Different routes of endocytosis include the clathrin-dependent (CME), caveolin-dependent (CavME), and the clathrin- and caveolin-independent internalization. Regardless of the route of internalization, the budded vesicle fuses with the EE where they are sorted to either recycle back to the cell surface or go to the late endosome for degradation. [(Gould & Lippincott-Schwartz, 2009) with permission from the publisher]
In addition to CME, cells also use other pathways for entry of materials into the cytoplasm that are called clathrin-independent endocytosis (CIE). CIE includes mainly caveolae-dependent (clathrin-independent) endocytosis and both clathrin- and caveolae-independent endocytosis called the Arf6 endocytosis (Maldonado-Baez, Williamson, & Donaldson, 2013).

Caveolae-mediated endocytosis (CavME) is defined as flask plasma membrane invagination of 60-80 nm diameters that are abundant in most eukaryotic cells. Caveolae are associated with cholesterol-rich lipid-raft domains and some glycosphingolipids and sphingomyelins (Parton & Simons, 2007); they are used more extensively in some cells (such as adipocytes and endothelial cells) than others. Caveolae pits are made of proteins from the caveolin family, with Caveolin-1 (Cav1) being the most important. These proteins have been shown to work in association with the cavins family of proteins (Elkin, Lakoduk, & Schmid, 2016; Parton & Simons, 2007; Parton & del Pozo, 2013).

CavME is different from other CIE in a couple of ways: i) CavME do not show a defined coat structure (electron dense coat) as seen in CIE, nor show progression of neck formation during cargo internalization; and ii) unlike clathrin, caveolae are differentially expressed in some tissues and cells. Caveolae have been shown to make up about 50% of the plasma membrane surface in cells such as adipocytes and endothelial cells. Even within a particular cell, caveolae were preferentially shown to concentrate more in one area than another (Parton & del Pozo, 2013). For example, Cav1 homo-oligomers were shown to accumulate at the apical site while Cav1 and Cav2 hetero-oligomers were accumulated at the basolateral side of epithelial cells. In addition, Cav1 was shown to concentrate at the rear end of endothelial cells during planar movement (migration) and to co-localize with caveolae (Parat, Anand-Apte, & Fox, 2003).

It is believed that caveolin oligomers traffic to the plasma membrane. Cavin proteins are also recruited there and stabilize caveolin oligomers. What recruits cavin to the caveolae site is
not clear, but interactions with caveolae and CAV1 may play a significant role. CavME is less defined compared to the CME; however, recent studies have linked it to signal regulation, receptor localization and trafficking, and regulation of lipids trafficking and metabolism (Parton & del Pozo, 2013).

As more studies have emerged on understanding the entry of materials into the cytosol of cells, it has become clear that other alternative routes of entry exist besides CME and caveolae-dependent pathways. These include newly discovered clathrin- and caveolae-independent endocytosis with pits containing flotillins (e.g. uptake of cholera toxin and endocytosis of glycosylphosphatidylinositol (GPI)-linked proteins) (Glebov, Bright, & Nichols, 2006), pathways that involve small GTPases Rac1 and Cdc42, endophilin-dynamin- RhoA-dependent pathway (e.g. Interleukin-2 receptor endocytosis) (Lamaze et al., 2001), and the pathways that requires the small GTPase called Arf6 (e.g. MHC-I) (Elkin et al., 2016).

Most cells use both clathrin-mediated and clathrin-independent endocytosis at the same time depending on the need and the kind of receptor transported. Such communication was observed in the case of Rab35 which is transported by the CME, but is required for the transport of cargos through CIE (Dutta & Donaldson, 2015).

1.3 Endocytic Trafficking Endosomes

Regardless of the route of endocytosis, all vesicles containing internalized materials end up in the EE where they are sorted to go to the lysosomes or to go back to the cell surface through the fast route (directly to the cell surface) or through the slow route using the RE. The EE is a very dynamic structure with the capacity of undergoing homotypic fusion. The EE is composed of micro-domains of tubule extensions of approximately 60 nm in diameter and large vesicles of approximately 400 nm in diameter. The EEs are each specialized for the sorting of specific cargoes within minutes after their internalization, according to their fates, into sub-domains that
will sort them to the RE for recycling back to the plasma membrane or to the MVB for degradation. The acidification of vesicles from these two different sub-domains also varies with approximate pH ranging from 6.2 to 5.5 in the lumen of MVB and a pH of 6.5 in the tubular recycling endosomes (Jovic et al., 2010). In addition, the pH in the lumen of EE is approximately 6.3-6.8 and allows the dissociation of most ligands from their receptors. An example of this is seen in the case of the low density lipoprotein receptor that dissociates from its ligand (low density lipoprotein) in the EE after internalization, and the receptor is sorted into a tubular sub-domain for recycling while the ligand is sorted into the larger vesicle sub-domain for degradation. The opposite is seen in the case of ligand-bound EGFR that is sorted together with the ligand into the more circular or large vesicle sub-domain for degradation.

Internalized materials, including signaling receptors that are targeted to go back to the cell surface, are sorted into micro-domains in the EE that are constantly forming tubules. Those tubules containing cargo are labeled with Rab4 and Rab5 and, can either go back directly to the plasma membrane using the fast route or can fuse with or differentiate into recycling endosomes (RE) to go through the slow route (Jovic et al., 2010). When stained with Rab11, for example, REs showed perinuclear localization. A good example of a signaling receptor that goes through both recycling pathway is the transferrin receptor (TfR). Studies that measured the recycling of TfR confirmed the existence of a slow (t_{1/2}=15-30 minutes) and a fast route (t_{1/2}= 5 minutes) for recycling with different kinetics (Hopkins & Trowbridge, 1983; Swiatecka-Urban, 2013). This tubular compartment is usually marked by the presence of Rab11 and/or EHD1, which will be discussed in another sub-chapter.

The RE is not only important for the recycling of receptors, but it is crucial to sustain signaling at the plasma membrane. It is required for antigen presentation by APCs, for the maintenance of the immunological synapse necessary for proper immune responses, for the
maintenance of cell polarity in polarized cells, for cell division, for migration, and for neuronal synapse and neuron survival.

Receptors in the EE to be degraded have a sorting signal that allows them to be targeted to the lysosome. For signaling receptors including the epidermal growth factor receptor (EGFR), a mono-ubiquitination of one or more tyrosine residues in their cytoplasmic domain is required for their targeting to the lysosomes. It is believed that receptors targeted to the lysosome accumulate in areas in the EE that are rich in flat clathrin lattice. The hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in the EE binds ubiquitinated cargo, clathrin in the flat lattice and other proteins such as the tumor susceptibility gene (Tsg)101 subunit of endosomal sorting complexes required for transport (ESCRT-I), that in turn recruit ESCRT-II and ESCRT-III. Together they bud off the EE and form the multivesicular bodies (MVB), which are somewhat rounded and will then fuse to the lysosome for the degradation of the signaling receptor (Jovic et al., 2010).

Another pathway that bridges from the EE is the retrograde transport to the Golgi complex. Cargoes from the EE targeted to the trans-Golgi network (TGN) are sorted into tubular sub-domains similar to those used for the recycling. The differences between these two tubular sub-domains are that those used for the TGN are from more mature EE, are enriched with SNX1/sortilin and show the presence of the retromer machinery with a high concentration of phosphatidylinositol 3, 5-bisphosphate (PtdIns (3, 5) P2) generated by phosphoinositide kinase, five-type zinc finger containing (PIKfyve kinase) (Jovic et al., 2010; Mari et al., 2008; Rutherford et al., 2006). Caveolin-1 serves as a marker of endosomes in the transport of cargo from the plasma membrane to the Golgi complex (Nichols, 2002).

EEs are not just important in sorting and directing cargoes to specific endosomes for their fate, they are also important in signaling. Different GPCRs, such as thyroid-stimulating hormone
receptor, have been shown to regulate signaling of MAPK and PI3K signaling pathway and the production of secondary passengers such as cAMP while inside EE (Calebiro et al., 2009).

Recent studies have shown other kinds of recycling beside the typical slow and fast route of recycling, well-described with the traffic of TfR. It was shown that the RE that recycles TfR is different than the more tubular RE that are seen in the recycling of CIE cargoes such as MHC-I (Swiatecka-Urban, 2013). Although, in both cases, these endosomes are positive for some similar markers such as Rab11; they also have more specific markers such as Arf6 that mark them more specifically for one type of cargo than the other. Cells have to maintain a proper balance between the rates of internalization, degradation, recycling and synthesis for normal function. The endocytic trafficking pathways involves the uptake of various materials including receptors (which were described above), nutrients, pathogens, and more. Despite the most common and well-studied pathways of endocytic trafficking mentioned above, other pathways exist such as phagocytosis of large particles (used mainly by APCs), pinocytosis, and trogocytosis that are all strictly regulated and used by cells for various reasons.

2. Regulators of the Endocytic Trafficking

2.1. Rab GTPases.

Endocytic trafficking is a very complex process with defined steps that are strictly regulated by a small number of protein families including the Ras-associated binding (Rab) proteins. Rab proteins are evolutionarily conserved proteins that are present in organisms ranging from yeast to humans. For many decades, the Rab proteins have been shown to be the master regulators of different steps of the endocytic traffic including the transport to the cell surface, to the MVB then to the lysosomes for degradation, and transport to and from the TGN. Rabs are small (21-25 kDa) monomeric GTP-binding proteins that cycle between the active form (GTP-bound) and the inactive form (GDP-bound). In their GTP-bound/active state, Rab proteins
localize to intracellular membrane where they have been shown to recruit and interact with their effectors. It is through these effectors that Rab proteins functions. Upstream regulators called the guanine nucleotide exchange factors (GEFs) in the cytoplasmic surface of different membranes-bound organelles control the active or GTP-bound state of Rabs. GTPase activating proteins (GAPs) (Bhui & Roy, 2014; Jovic et al., 2010; Wandinger-Ness & Zerial, 2014) regulated the inactive or GDP-bound state of Rabs, causing the hydrolysis of the Rab-GTP complex, converting it into Rab-GDP, and causing its release from the target membrane to the cytosol.

Rab proteins that are known to localize in the EE include Rab5, Rab4, and most recently classified Rab35; all playing different functions in the EE. Rab5 regulate the entry of vesicles containing internalized cargoes from the plasma membrane to the EE. Rab5 is also important for the generation of phosphatidylinositol-3 phosphate (PtdIns (3) P) lipids, for homotypic fusion and for the movement of sub-domains of EE on actin and microtubules tracks. Some of Rab5 effectors include PtdIns (3) P-Kinase/h VPS34/p150 (VPS34), early endosomal antigen-1 (EEA1), rabenosyn-5, and APPL1 and APPL2 (Jovic et al., 2010).

Rab4 and Rab35 are regulators of the traffic pathway that localize in the EE. Both Rab4 and Rab35 regulate the transport or exit of constitutively and specialized recycling cargos from the EE directly back to the plasma membrane using the fast route. In addition, Rab4 is shown to regulate the exit of cargo from the EE to the RE for slow recycling. Key effectors of Rab4 include rabenosyn-5, rabaptin-4, rabaptin-5 and rabaptin-5β. Rab35 is more associated with the recycling of cargoes that were internalized through CIE. Cargoes trafficking in Arf6-positive endosomes are positive for Rab35, but not Rab4, indicating that Rab35 is more associated with the recycling of cargo from CIE (Chesneau et al., 2012; Etienne-Manneville, 2008; Walseng, Bakke, & Roche, 2008). Other Rabs (less studied) that also localize at the EE include Rab10, Rab14, Rab21 and Rab22 (Jovic et al., 2010).
Rab7 and Rab9 regulate the transport in the late endosome. Rab7 is now commonly used as a marker for late endosomes that mature into or fuse with the lysosomes. Rab9 has been shown to be more associated with the transport from late endosomes to the trans-Golgi. Another important group of Rabs is the Rab11 protein. Rab11, together with Rab4, and Rab35 mediate the recycling of cargo to the cell surface; however, Rab11 localizes in the RE and is more specific for cargoes going through the slow recycling. Rab11 regulate the recycling of cargoes from the EE and the transport of cargoes from the Golgi complex (Goldenring, 2015; Jovic et al., 2010). Rab11 effectors include rabphilin11, Rab11 binding protein (Rab11 BP), family interacting protein2 (FIP2), FIP3, FIP4, secretory protein 15 (Sec15), and Rab interacting protein 11 (RIP11).

Other regulators of the endocytic trafficking in addition to the Rab family of GTPases are within, the EHD-containing protein family (Zhang, Naslavsky, & Caplan, 2012).

### 2.2. EH Domain-Containing Protein Family

The EH domain was originally identified in 1993, as a three repeat sequence in the NH$_2$-terminal of eps15 (EGFR pathway substrate clone.15) (Fazioli, Minichiello, Matoskova, Wong, & Di Fiore, 1993), and later on the same repeats were identified in another EGFR substrate called eps15R (Coda et al., 1998). Since then, the number of proteins identified as containing this domain has increased, and is now estimated to be over 50 EH domain-containing proteins discovered, that include intersectin, Resps1, POB1, END3P and others (Fig 1.2). This domain is important for protein-protein interaction and has been observed in organisms ranging from yeast to humans. Most EH domain-containing proteins have more than one repeat of this domain in their NH$_2$ terminus, but neither the position of this domain nor the number of repeats appears to be a requirement for function (Fig 1.2). The EH domain has a preference of binding to proteins with asparagine-proline-phenylalanine (NPF) motifs such as Numb I. Studies have also shown that the positions of amino acids around this motif are important for this protein-protein interaction (Fazioli et al., 1993; Santolini, Salcini, Kay, Yamabhai, & Di Fiore, 1999).
Most of the well-studied EH domain-containing proteins such as eps15 and intersectin are mainly involved in the early steps of endocytosis and are shown to be critical regulators of endocytic traffic. In addition to the NH2-terminal EH domain-containing proteins, a family of proteins (as a sub-family of the EH domain proteins) was discovered that expresses only one repeat of the EH domain located in the COOH-terminus and, is also involved in the endocytic traffic, called the EHD protein family.
Figure 1.2: Architecture of EH Domain-Containing Proteins. There are now over 50 proteins in this family of EH domain-containing proteins. The structures of a few of them are shown here. Most of them contain more than one repeat of the EH-domain on their N-terminus. The EH-domain is for their interaction with other proteins that contain NPF motifs. [(Santolini et al., 1999) with permission from the publisher]
3. EHD Protein Family

3.1 EHD Protein Structure

EHDs are evolutionarily conserved proteins that include four members (EHD1-4) in mammals and one member in Caenorhabditis elegans, Drosophila melanogaster, plants such as Arabidopsis thaliana, and in eukaryotic parasites such as Plasmodium falciparum, Leishmania major and Entamoeba histolytica (Daumke et al., 2007). In mammals, EHD1 was the first member characterized, before the discovery of the other three members, EHD2, EHD3, and EHD4 (Mintz et al., 1999; Pohl et al., 2000). Based on nucleotide sequence alignment, EHD1 and EHD2 have about 74% homology, up to 82% homology between EHD1 and EHD3, and up to 76% homology between EHD1 and EHD4 (Pohl et al., 2000). This family of proteins also has very similar amino acid sequence and protein structure. These results suggest that EHD3 is the most similar to EHD1 compared to the other members. Genes encoding these proteins were mapped to different chromosomes with EHD1 to 11q13, EHD2 to 19q 13.3, EHD3 to 2q21 and EHD4 to 15q11.1 (Pohl et al., 2000).

EHD proteins are ubiquitously expressed in most mouse organs analyzed, but show differences in expression levels in various tissues. For example, EHD1 is highly expressed in lung, spleen and in testis, but low in the kidney and liver. EHD2 is high in lung, mammary glands, and fat tissues, but is either low or not expressed in kidney and in brain. EHD3 has a high expression in heart, but has a low expression in the lung and liver. EHD4 is highly expressed in the heart and pancreas, but has a low expression in the mammary glands and liver (Mintz et al., 1999; Pohl et al., 2000).

All EHDs have three domains including the EH domain, the helical domain and the P-loop domain (also called the ATP/GTP-binding site) (Fig 1.3A). The EH domain is used for protein-protein interaction and strongly binds to proteins with tripeptide asparagine-proline-
phenylalanine (NPF) motif followed by two acidic residue (Daumke et al., 2007; Kieken et al., 2010). These amino acids around the NPF motif have been shown to be critical for binding. A conserved positively charged amino acid, lysine 483, is important for the function of the EH domain. Mutation of this lysine to glutamic acid had no obvious defect on the structure of the EH domain, but it caused a mis-localization of mutant EHD1 from tubular membranes to more punctate structures. These results were similar to those seen when the entire EH domain was truncated, suggesting a critical role of the EH-phosphoinositide interaction in membrane tabulation (Naslavsky, Rahajeng, Chenavas, Sorgen, & Caplan, 2007). The coiled-coil domain (helical domain) is for oligomerization and the P-loop (G-domain) binds and hydrolyzes ATP instead of GTP. The P-loop is also required for dimerization and acts as the membrane-binding region. The structure of EHD proteins also shows a linker region that connects the domains that proceed the EH domain. The presence of an EH domain in EHDs suggested that they might have a role in receptor-mediated endocytosis as shown with other EH domain-containing proteins such as eps15 (Mintz et al., 1999).

Out of the 4 members, EHD2 and EHD4 are the only ones with a three-dimensional protein structure resolved; however, the structure of the EH domain of EHD1 has also been resolved (Fig 1.3B & Fig 1.4A). Studies on the characterization of the EHD2 structure provided evidence that EHD proteins are involved in membrane remodeling in vivo and this task is ATP dependent (Daumke et al., 2007). The study showed that EHD proteins have many similarities with the dynamin superfamily including the ability to tabulate liposomes in vitro, to oligomerize around lipid tubules in ring-like structures, and to hydrolyze nucleotide in response to lipid binding (Daumke et al., 2007). Recent studies have characterized the structure of an N-terminally truncated, activated EHD4 dimer (Melo et al., 2017). This group also performed a side-by-side analysis comparing the non-activated/nonhydrolyzable ATP- bound EHD structure to the activated EHD structure (Fig 1.3 and Fig 1.4).
While in the cytosol, EHD proteins are in an auto-inhibited conformation, where the N-terminus is locked in the GTPase domain and the EH domains block assembly. However, once recruited into the membrane, different conformational changes allow their functions. In the first conformational change/rotation, the N-terminus of EHD proteins is released from the GTPase domain; this was suggested to cause the activation of the EHD (Melo et al., 2017). The other change is the rotation of the helical domains that adjust the position of the membrane-binding site. This rotation seemed to regulate membrane binding. At the same time, this also causes the linker to the EH domain to be freed from the GTPase domain. As a consequence, this then causes the EH domain to become free compared to their inactive form. This was suggested to resemble the interaction of an EH domain with an NPF motif binding protein such as MICAL-L1 (Melo et al., 2017). Other conformations permit the formation and stabilization of EHDs oligomerization at the membrane, and lead to membrane curvature ((Melo et al., 2017), Fig 1.4). EHD proteins form homo and hetero-oligomers with the exception of EHD2 that appears to form only homodimers. Other studies in mammalian cells have shown that EHD1 and EHD3 form homo and hetero-oligomers, and EHD1 has been shown to form heterodimers with EHD4 in vivo (Yap, Lasiecka, Caplan, & Winckler, 2010).
**Figure 1.3: Structure s of EHD Proteins.** A) Generic structure of EHD proteins showing the 3 domains including the helical domain, ATP-binding-G-domain, and the EH-domain. B) Dimers of EHD2 protein in the presence of a nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMPPNP). [(Daumke et al., 2007) with permission from the publisher]
Figure 1.4: Structure of Activated EHD4 Dimers. A) Structure of the ATPγS-bound EHD4ΔN. The purified N-terminally truncated EHD4 variant (amino acids 22–541). B) Activation model of EHDs. Numbers refer to release of the N-terminus into the membrane (1), rotation of the helical domain (2), release of the EH domains from the autoinhibitory site (3), insertion of the KPF-loop into the hydrophobic pocket of the GTPase domain (4), membrane binding and oligomerization (5), and membrane tubulation (6). [(Melo et al., 2017) with permission from the publisher]
3.2 Functions of EHD Proteins in vitro and in vivo:

Since the discovery of all four members of the EHD protein family, interest in studying their functions, both in vitro and in vivo, have increased. EHD1, compared to the other family members, is the most studied and well-characterized. EHD1 has been described to regulate the transport of various materials including signaling molecules from the early endosomes back to the plasma membrane through the recycling endosome (Naslavsky & Caplan, 2011). However, some studies associated the function of EHD1 to earlier steps of the endocytic trafficking (such as the internalization of receptors) (Naslavsky, Rahajeng, Rapaport, Horowitz, & Caplan, 2007). EHD1 was first identified in a genetic screen that was looking for endocytic genes in Caenorhabditis elegans. The investigators used a dominant-negative construct with a point mutation near the EH domain and showed mis-localization of the endocytic RE and slowing of transferrin receptor recycling (George et al., 2007; Lin, Grant, Hirsh, & Maxfield, 2001; Mintz et al., 1999).

EHD proteins are cytosolic proteins that are recruited to the endosomes through interactions with specific lipids such as phosphatidic acid (PA) that are enriched in the endosomal membrane. Recent in vitro studies (Giridharan, Cai, Vitale, Naslavsky, & Caplan, 2013) have revealed that PA recruit MICAL-L1 (Molecules Interacting with CasL-Like1) and the F-BAR protein Syndapin2 (Syn2, also called PACSIN2), to the RE and form a stable structure that is involved in the curvature of the endosomal membrane and tubule formation by Syn2. EHD1 is recruited to those tubules through a direct interaction with MICAL-L1. EHD1 also directly interacts with Syn2, so once recruited, EHD1 binds to both MICAL-L1 and Syn2, which then performs vesiculation or scission of the pre-formed tubules (Braun et al., 2005; Giridharan et al., 2013; Rahajeng, Giridharan, Cai, Naslavsky, & Caplan, 2012; Reinecke, Katafiasz, Naslavsky, & Caplan, 2014). EHD1 mainly marks the RE and primarily exhibits perinuclear localization where it has been shown to regulate the recycling of various molecules such as TfR, MHC-I, MHC-II, β1 Integrin, and GLUT4 ((Caplan et al., 2002; George et al., 2007; Guilherme, Soriano,
The *in vitro* studies were instrumental in defining the mechanism of EHD proteins functions in the endocytic trafficking pathway. *In vivo* studies of EHD proteins revealed that these proteins are essential in fundamentally and physiologically relevant processes such as eye development, embryonic development, muscle formation, and fertility. The EHD1 null mouse was engineered in our laboratory and was found to be embryonically lethal in mice having a predominantly B6 background (Bhattacharyya et al., 2016). Embryos from these mice were studied in detail and they showed a neural tube closure defect. Further mechanistic investigation showed short and stubby cilia with dysregulated ciliary SHH signaling due to a defective transport of smoothened (a SHH receptor that was shown to be regulated by EHD1) (Bhattacharyya et al., 2016). EHD1 null mice in a mixed 129:B6 background showed eye defects including anophthalmia, aphakia, microphthalmia and congenital cataracts (Arya et al., 2015).

Another study showed that the lamprey I-EHD (conserved EHD1/3 ortholog) was found to be enriched at the synaptic release site and to regulate the clathrin/dynamin-dependent synaptic vesicle budding and dynamin function (Jakobsson et al., 2011). EHD1 has also been studied in rat brains and found to negatively regulate synaptic vesicle exocytosis through binding to a protein associated with synaptic vesicle called snapin (Wei et al., 2010).

EHD proteins have an EH domain that binds target proteins with an NPF motif followed by an acidic residue. EHD1 has been shown to directly interact with proteins including, but not limited to, Rab11 effector Rab11-FIP2, syndapin 1 and syndapin 2, snapin, SNAP29, rabenosyn-5, and MICAL-L1. In addition to its well-known function in the RE, EHD1 has also been shown to be associated with Rabankyrin-5 and Vps26 to regulate the retromer pathways (Zhang et al., 2012).
Figure 1.5: Endocytic Trafficking of Signaling Receptors. This diagram has been modified to show where in the endocytic trafficking pathway the EHD proteins seem to function. These functions are based on various in vitro experiments from different groups and some confirmation from some in vivo models. EHD proteins have been shown to play similar and distinct roles in different steps of the endocytic trafficking pathway. [Modified from (Scita & Di Fiore, 2010) with permission from the publisher]
EHD3 is the closest to EHD1 in sequence identity. EHD3 has been studied in different systems in vitro. Those studies have shown that EHD3 regulates the exit of cargos from the EE to the RE (Naslavsky, Rahajeng, Sharma, Jovic, & Caplan, 2006) (Fig 1.5). Unlike EHD 1 that was shown to cause scission of pre-existing tubules, EHD3 is important in endosomal membrane tubulation (Henmi et al., 2016). EHD3 null mice do not have any overt phenotypes. However, another group found EHD3 expression level increased during heart failure in large animals such as dogs (Gudmundsson et al., 2012). EHD3 null mice also had cardiac abnormalities including arrhythmias and blunted response to adrenergic stimulation, and reduced expression of Na/Ca exchanger (NCX1) (Curran et al., 2014). In glioma cancer tissues, EHD3 was found to function as a tumor suppressor by controlling cell cycle arrest and apoptosis (Chukkapalli et al., 2014).

EHD4 is involved in trafficking of the EE and control the exit of cargo from both the RE and the late endosomes (Fig 1.5). To demonstrate the role of EHD4 in recycling, the Caplan laboratory studied the effect on recycling of TfR and MHC-class I, and used low-density lipoprotein to demonstrate its role in degradation (Sharma, Naslavsky, & Caplan, 2008). In neurons, the neurotrophin (NT) family of target-derived growth factors controls neurons’ survival in both the periphery and in the CNS by signaling through Trk receptor tyrosine kinase (which has ligands such as TrKA, etc.). EHD4, called Pincher in this study, was shown to regulate the internalization of TrKA and TrKB, to associate with TrK endosomes in the MVB, and to regulate their retrograde transport and signaling by associating with ErK5, a downstream effector of the retrograde survival signaling (Valdez et al., 2005). To demonstrate the role of EHD4 in vivo, our laboratory engineered EHD4 null mice. These mice showed that EHD4 was required for normal pre-pubertal testis size in the male. These EHD4 null males had reduced fertility (George et al., 2010). As seen with other EHD whole body knockout systems, EHD1 expression was high in adult mice and, we believe, compensated for the loss of EHD4 in some organs.
EHD proteins have been shown to have both shared and distinct roles in endocytic trafficking (George et al., 2007), and these have caused their genetic studies to be confounded due to their compensatory functions. While EHD3 null mice have no obvious phenotype, EHD3 and EHD4 double knockout mice had smaller, pale kidneys, died at very young age (between 3-24 weeks), and developed renal thrombotic microangiopathy (TMA)-like glomerular lesions (George et al., 2011). Cadherin-23 (CDH23) and protocadherin 15 (PCDH15), members of the cadherin superfamily, form some of the extracellular filaments that connect stereocilia and kinocilium of a hair cell into a bundle (Muller, 2008). Deletion of CDH23 and PCDH15 genes has been associated with deafness. A study showed that EHD4 interacts with CDH23 in hair cells; however, EHD4 null showed no defect in hearing suggesting a compensatory function for EHD1 as these mice showed a 2-fold increase in EHD1 expression (Sengupta et al., 2009).

EHD2 is the most distinct of all the other EHD proteins. EHD1, EHD3 and EHD4 are located on intracellular tubular/vesicular membranes, while EHD2 is located to the cytoplasmic interface of the plasma membrane. All EHDs form hetero-oligomers, except for EHD2 that only forms homo-oligomers. EHD1 protein localization to the membrane depends on interactions with MICAL-L1 and with phosphatidylinositol lipids, as the mutation in lysine 483 inhibits EHD1 localization to the tubular structures (Naslavsky et al., 2007). However, the localization of EHD2 to the plasma membrane is regulated by its interaction with phospholipid PIP2 and is independent of actin microfilaments (Simone, Caplan, & Naslavsky, 2013). Based on its localization on the cytoplasmic side of the plasma membrane and its association with phospholipids, studies were done to determine whether EHD2 has any role in the early steps of internalization such as vesicle formation. EHD2 was identified as a third structural element or component of the caveolae at the plasma membrane and is involved in controlling the stability and turnover of the caveolae. EHD2 was also shown to interact directly with cavin1 and pacsin2/Syn2, such that its deletion resulted in more dynamic and short-lived caveolae (Moren et al., 2012).
EHD2 localization in the plasma membrane suggested possible actin interaction with actin. One study connected EHD2 to the actin cytoskeleton through its interaction with EHB1. EHB1 has a CH domain similar to that of the type 1 CH domain of α-actin, which binds F-actin (Guilherme et al., 2004). Another study showed that EHD2 interacts with Nek3 and is associated with Vav1, a Nek3-regulated GEF for Rho GTPases. Since Vav1 regulates Rac1 activity, this study suggested that EHD2 regulates the traffic from the plasma membrane by controlling Rac1 activity (Benjamin et al., 2011). EHD2 is highly expressed in fat, and regulates the internalization of Glut4 in adipocytes (Guilherme et al., 2004). EHD2 is involved in other pathways besides the internalization of material from the plasma membrane (Fig 1.5). It directly binds to the second C2 domain of myoferlin and regulate myoblast fusion (Doherty et al., 2008) and, translocate to the nucleus as well as suppresses transcription (Pekar et al., 2012) and accumulates at the site of injury in human myotubes during membrane repair processes (Marg et al., 2012).

The EHD protein family is involved in various steps of endocytic trafficking including internalization, endosomal sorting (at the early endosome, late endosome, and recycling endosome), and retrograde transport. EHD proteins regulate the process as of cell migration, embryonic development, muscle size regulation, fertility, synaptic release in neurons, and receptor trafficking from the plasma membrane to their designated destination, and have more recently been associated with diseases such as cancer and neurological disorders such as Alzheimer’s (Buggia-Prevot et al., 2013; Chukkapalli et al., 2014). However, although studies have shown the expression of EHD proteins in lymphoid tissues such as the spleen, prior to the research described in this dissertation their role in T cell function had not been studied.
4. T-cell Functions

4.1 Overview of T-cell Development

T-cell development starts in the bone marrow from hematopoietic stem cells. Some of the stem cells give rise to the common lymphoid progenitors that differentiate into immature precursor T-cells. The immature precursors migrate to the thymus and proceed through thymic developmental stages. While in the thymus, they differentiate into a double negative stage (DN). DN T-cells express neither CD4 nor the CD8 co-receptor. Based on surface expression of CD44 and CD25, the DN T-cells are further classified into DN1, DN2, DN3 and DN4. The DN stage defines if the T-cell will express the αβ T-cell antigen receptor (TCR) or γδ TCR T cell lineage. In the case of a αβ TCR, the DN T-cell differentiates into the double positive stage (DP) where it expresses both the CD4 and CD8 co-receptors. The DP T-cells go through positive and negative selection to eliminate potential self-reactive T-cells. Both selections result in the generation of single positive (SP) CD4+ and CD8+ T-cells. The SP cells then migrate to secondary lymphoid tissues and interact with APCs that present antigenic peptide; this interaction leads to activation and confers a defense against pathogens (Ellmeier, Haust, & Tschismarov, 2013; Sawada, Scarborough, Killeen, & Littman, 1994). During T-cell activation, the contact site between the T-cells and APCs form a structural domain called the immunological synapse (IS) that is required for an effective T-cell activation and effector functions.

APCs (including macrophages, dendritic cells and B cells) surveille tissues looking for pathogens or dying cells. Loaded with antigenic peptides, they migrate from tissues to lymphoid organs and present the peptides to T-cells, specifically CD4+ T-cells. APCs internalize antigens through phagocytosis and/or receptor-mediated endocytosis. APCs then process those antigens to small peptides that can be loaded into the MHC molecules. This process is called antigen processing. The next step is the loading of those antigenic peptides into either MHC class I or
MHC class II depending on the source of the antigen which come from either the extracellular or the cytosolic environment for MHC class II or MHC class I, respectively. The peptides loaded into MHC class II, for example, are transported to the cell surface and will present peptides to CD4+ T-cells. On the other hand, T-cells also proceed through different steps and check points to be ready to elicit a specific response to the particular antigenic peptide recognized (Neefjes, Jongsma, Paul, & Bakke, 2011).

Naïve cells (unstimulated T-cells) are primarily stimulated by dendritic cells; this causes the T-cell to proliferate and differentiate into effector cells. Priming of naïve cells is critical for initiating T-cell responses against the pathogen; however, re-stimulation of effector T-cells through a second exposure to the same antigen causes a faster and more robust response against the pathogen. T-cell activation involves more than antigen recognition by antigen-specific TCRs and co-receptors; requiring regulation by a variety of molecular interactions with costimulatory and co-inhibitory molecules, cytokine receptors, adhesion molecules, and intracellular signaling cascades.

4.2 T cell Activation and the Immunological Synapse (IS)

The TCR/CD3 complex is comprised of a TCR αβ heterodimer that is required and responsible for antigen recognition, and the non-polymorphic CD3 proteins that are responsible for initiating the intracellular signaling cascade. The CD3 protein is made of the heterodimers γδ and γε and the homodimer ζ-ζ (Portoles & Rojo, 2009; Shores et al., 1994; Smith-Garvin, Koretzky, & Jordan, 2009; Yuseff, Lankar, & Lennon-Dumenil, 2009). After ligand recognition, Lck (a Src family tyrosine kinase) becomes activated and phosphorylates immunoreceptor tyrosine-activation motifs (TAMs) within the cytoplasmic tail of the CD3 complex. This leads to calcium signaling and recruitment of other tyrosine kinases such as Zap-70, that bind to the phosphorylated ITAMs. Zap-70 in turn acts as a scaffolding protein and recruits signaling
adaptors and effector molecules such as LAT, SLP-76, Grb2/Gads, and phospholipase C-γ (PLCY). Zap-70 also activates other signaling molecules such as protein kinase C-θ (PKCθ), inducing cytokine production and controlling the activation of genes involved in T-cell survival and effector functions (Hashimoto-Tane et al., 2010; Yuseff et al., 2009). T-cell activation in the absence of co-stimulatory molecules causes the T-cells to become anergic (a non-responsive state) which leads to failure of T-cells to respond to further re-stimulations. Interaction of co-stimulatory molecules such as CD28 with their ligands CD80 and CD86 provides additional signaling required for avoidance of anergy and for productive T-cell activation (Smith-Garvin et al., 2009).

The IS represents the site of contact between T-cells and APCs or target cells (e.g., tumor cells). The IS is initiated early during T-cell activation and results in the accumulation of TCRs in apposition with peptide-MHC on the APC. Signaling begins at the periphery of the IS with the formation of microclusters that typically contain phosphorylated TCRs, associated signaling molecules (e.g. Zap70, SLP-76, etc.), and co-stimulatory molecules (e.g. CD28) (Hashimoto-Tane et al., 2010) (Fig 1.6). These microclusters are transported to the center of the IS and form the central supra-molecular activation complex (cSMAC) (Fig 1.6). Other key signaling proteins that localize to the cSMAC include Lck and LAT. The cSMAC is surrounded by an accumulation of cell adhesion molecules which pair with counter-receptors on APC, such as LFA1-ICAM1, forming a sealing zone called peripheral SMAC (pSMAC) (Griffiths, Tsun, & Stinchcombe, 2010; Hashimoto-Tane et al., 2010; Huppa, Gleimer, Sumen, & Davis, 2003; Jo et al., 2010), Fig 1.6). cSMAC is a site of both strong receptor activation and increased receptor degradation (Lee et al., 2003).
4.3. Endocytic Trafficking in T cells

Recent studies (Cemerski & Shaw, 2006; Finetti et al., 2009) have demonstrated that clustering of TCRs at the IS is a combination of lateral diffusion, cytoskeleton-derived movement, and polarized recycling. TCR/CD3 association starts at the ER and is completed at the Golgi. From the Golgi, TCR/CD3 is transported through endosomal vesicles to the cell surface. Several studies have shown that once the TCRs are on the surface, they undergo several rounds through the constitutive recycling pathway before being targeted for degradation (Das et al., 2004; Finetti et al., 2009; Lee et al., 2003). The TCR/CD3 complexes go through the CME for internalization. Once internalized, they co-localize with TfR in the RE and in the IS, suggesting that internalized TCRs traffic through the recycling endosome back to the cell surface (Das et al., 2004).

It is well documented that T-cells become polarized during ligand activation, leading to the rearrangement of intracellular organelles such as the recycling endosomes, the Golgi, and the centrosome or MTOC directly underneath the IS (Yuseff et al., 2009). The main purpose of MTOC/Golgi rearrangement is to direct secretion of Golgi-derived lymphokines from T-cells to the bound APC (Poo, Conrad, & Janeway, 1988), and to allow trafficking of newly synthesized TCRs from the Golgi to the surface. In addition, MTOC/Golgi rearrangement has also been required for the delivery of granules to the IS, hence controlling the killer function of cytotoxic T lymphocytes (CTLs) (Kloc, Kubiak, Li, & Ghobrial, 2014; Stinchcombe, Majorovits, Bossi, Fuller, & Griffiths, 2006). Polarization of recycling endosomes is important for effective accumulation of TCRs at the IS. Studies have shown that constitutive internalization and recycling of TCRs are necessary for their accumulation at the IS (Das et al., 2004; Yuseff et al., 2009). In some studies, internalization was seen as early as 6 min after T-cell-APC contact and up to 90% of surface TCRs were internalized within 30-60 min after the contact (Huppa et al., 2003).
Figure 1.6: Later Stages of the IS in T-cells: Vesicle Trafficking and Changes in Polarization and Membrane Morphology Determines Effector Functions. Membrane rearrangement leads to the formation of the cSMAC where immune-engaged receptors are clustered and the pSMAC that contains adhesion molecules forms a seal that keeps the T-cell and the APCs in contact during activation. [(Yuseff et al., 2009) with permission from the publisher]
T-cell activation leads to phosphorylation of the ITAM in the cytoplasmic tail of CD3 proteins. Stimulation by a strong agonist triggers TCR internalization and degradation from the cSMAC, which leads to the down-modulation of the TCR signaling (Lee et al., 2003). Importantly, signal attenuation can also be caused by the function of co-inhibitory molecules such as CTLA-4, which competes with CD28 for binding to CD80 (or B7-1) and CD86 (B7-2) and disrupts tyrosine phosphorylation events through recruitment of intracellular SH-2 phosphatase (Huppa et al., 2003). However, while degradation through the lysosome is still considered one of the main ways of signal attenuation, some studies have shown that partially or slightly phosphorylated TCRs recycle back to the cell surface after internalization, while fully phosphorylated TCRs traffic to the lysosomes for degradation (Lee et al., 2003). These studies, together with other studies demonstrated that a small portion of surface TCRs are recycled back to the surface during activation, suggesting that the recycling endosome is a critical compartment controlling the traffic of recycling TCRs and newly synthesized TCRs to the surface. Therefore, the RE not only provides T-cells with TCRs that are ready for stimulation, but also at the same time sustains signaling by controlling the formation and maintenance of the IS.

It has become clear and well accepted that the IS is a focal point of strong endocytic trafficking (Griffiths et al., 2010) (Fig 1.6). Most of the molecules that are required for the formation and maintenance of the IS such as TCRs (Huppa et al., 2003), CD28 (Cefai et al., 1998), LFA-1 (Fabbri et al., 2005; Stanley, Tooze, & Hogg, 2012) on T cells and ICAM-I (Jo et al., 2010), MHC class I (Caplan et al., 2002) and MHC class II (Walseng et al., 2008) on APCs have been shown to undergo internalization and recycling back to the T-cell-APC interface during activation. Besides receptors, signaling molecules such as LAT and Lck also traffic to the IS (Soares et al., 2013); yet, the mechanisms of trafficking for most of these proteins are not well understood.
Endocytic trafficking in T-cells has been well studied in the context of T-cell activation by either a ligand or antibodies (such as anti-CD3/CD28), but process of endocytic trafficking in unstimulated (naïve or resting) cells has received little focus. A study showed that activation of PKC induces internalization of TCR with subsequent sorting to a non-degradative compartment from which the TCR recycles back to the cell surface in a functional state (Dietrich et al., 1998). They also demonstrated that PKC-mediated internalization requires the phosphorylation of CD3\(^\gamma\)S126. The same group showed that de-phosphorylation of CD3\(^\gamma\) following PKC-mediated internalization was required for TCR recycling and it was dependent neither on microtubules and actin polymerization nor on CD45 or Src kinases. Their study was among the first to suggest that PKC-dependent and ligand-dependent TCR sorting are two different pathways of receptor sorting. Their data also suggested that PKC-mediated TCR sorting via the RE is mainly used in unstimulated T-cells (before ligand binding), which are known to constitutively internalize and recycle their receptor with very little degradation as opposed to activated T-cells. During ligand binding, both the PKC and tyrosine kinases are activated, but leads towards lysosomal sorting of TCR; therefore, suggesting that ligand-dependent sorting is more dominant than PKC-dependent sorting during activation (Dietrich et al., 1998).

4.4. Similarities Between IS and other Synapses

The IS exhibits various similarities with the neuronal synapse, and neuromuscular Junction (Boulanger, Huh, & Shatz, 2001; Dustin & Colman, 2002; Tarakanov & Goncharova, 2009), both shown to be regulated by EHDs. EHD proteins were found to localize to and regulate neural synapses (Ioannou & Marat, 2012) and were implicated in various steps of endosomal trafficking in neuronal synapses (Ioannou & Marat, 2012; Lasiecka, Yap, Caplan, & Winckler, 2010; Valdez et al., 2005; Yap et al., 2010). EHD1, in particular, was found to negatively regulate exocytosis in the vesicle release site by affecting the binding of the SNARE complex protein, SNAP-25 to snapin, preventing the association of synaptotagmin-1 to the SNARE complex (Wei
et al., 2010). SNAP-25 is also a t-SNARE expressed in T cells as SNAP-23 and shown to cluster at the IS (Das et al., 2004). Another study showed that the lamprey I-EHD (conserved EHD1/3 orthologue) was found to be enriched at the synaptic release site and regulated the clathrin/dynamin-dependent synaptic vesicle budding and dynamin function (Jakobsson et al., 2011). Collaborative studies between our laboratory and the Hoffman group at George Washington University demonstrated that endogenous EHD1 localizes at the neuromuscular junction in vivo (Mate et al., 2012). The fact that the EHDs play important roles in other synapses that have shown similarities with the IS suggest that they could also regulate the IS.

Several studies have suggested a link between the process of ciliogenesis and IS formation (Griffiths et al., 2010). Our lab has found that the embryonic lethality observed in EHD1 null mice is associated with open neural tubes, loss of primary cilia on neuroepithelial cells, and defected ciliary SHH signaling due to a dysregulated transport of smoothened, a SHH receptor shown to be regulated by EHD1 (Bhattacharyya et al., 2016). Importantly, SHH signaling has been shown to control T-cell killing at the IS (de la Roche et al., 2013). In vitro studies of EHD1-null MEFs confirmed the role of EHD1 in primary ciliogenesis (Bhattacharyya et al., 2016). In addition, although hematopoietic cells lack primary cilia, an essential intraflagellar transport (IFT) component, IFT20, was found to be expressed by T-cells and is required for the recycling of the TCR/CD3 complex and other receptors that traffic to the IS back to the surface (Finetti et al., 2009; Finetti et al., 2014). Furthermore, knockdown of IFT20 resulted in impaired recruitment and signaling at the IS.

4.5 Potential Role of EHD Protein Family in T cell Function and in the IS

EHD proteins regulate the recycling of a number of receptors that traffic through the clathrin-dependent and clathrin-independent pathway, including TfR, MHC class I, MHC class II, etc. (Caplan et al., 2002; Lin et al., 2001; Naslavsky & Caplan, 2011; Walseng et al., 2008).
Furthermore, uncoordinated 119 protein (unc119) which regulate the activation of Rab11, controls the exit of Lck, (a Src kinase that phosphorylates the ITAM of the TCR-CD3 complex and is recruited to the IS) from Rab11 endosomes to the plasma membrane (Gorska, Liang, Karim, & Alam, 2009), while the function of Rab11 is regulated by EHD proteins (George et al., 2007; Naslavsky et al., 2006; Naslavsky & Caplan, 2011). Another study showed that the recycling of LFA-1 is dependent on the traffic of ICAM-1 to the IS (Jo et al., 2010). Furthermore, the same group demonstrated that ICAM-1 co-localized with MHC class II in intracellular endosomes and at the APC side of the IS during ligand stimulation (Jo et al., 2010). These results suggested that ICAM-1 uses the same traffic pathway as MHC class II, which has been shown to be regulated by EHD1 (Walseng et al., 2008). These data suggest the possibility that EHD1 could be responsible for the transport of both MHC class II and ICAM-1 to the IS on APCs during ligand stimulation.

Based on the importance of the endocytic traffic in T-cell function and IS formation and maintenance, we wanted to investigate the role of the EHD proteins in both T-cell function and in the IS. Given the importance of the endocytic trafficking in T-cell function and in IS during activation and the role of the EHD proteins in the neuronal synapse, in the neuromuscular junction, and in ciliogenesis, we hypothesized that EHD protein will regulate the endocytic recycling of receptors and the stability of IS/APC contact formation/maintenance in unstimulated and activated T-cells, respectively.
CHAPTER 2: MATERIALS AND METHODS
2.5. Materials and Methods for Chapter 3

5.1 Reagents & Antibodies

**Reagents:** BSA (cat. # A7906-100G), paraformaldehyde (cat. # 158127-500G), Triton X-100 (cat. # 93418), EGTA (cat. # E8145-50G), sodium orthovanadate (Na$_3$VO$_4$, cat. # S6508-50G), sodium deoxycholate (cat. # D6750-100G), 4-hydroxytamoxifen (cat. # T176-10MG), and Brefeldin A (BFA, cat. # B7651) were from Sigma-Aldrich (St. Louis, MO). Propidium iodide staining solution (cat. # 00-6990-42) was from eBiosciences. $^3$H-thymidine (cat. # 2407001, 2.0 Ci/mmol) was from MP Biomedical. Bafilomycin A1 (cat. # BML-CM110-0100) was from Enzo Life Sciences. MOG peptide (amino acids 35-55) (cat. # BP001328-PRO-371) was from Syd Labs. OVA peptide (amino acid 323-336) (sequence: ISQAVHAAHAEINE) was synthesized by Tufts University Core facility (Medford, MA, 02155). CFA (cat. # 231131) and heat-inactivated Mycobacterium tuberculosis were from DIFCO Laboratories. Sodium fluoride (NaF, cat. # S299-500), sodium chloride (NaCl, cat. # S271-10), and Tris (cat. BP152-5) were from Fisher Chemicals. Pertussis toxin was from ENZO Life Sciences. ECL development reagent (cat. # 32106), BSA for bicinchoninic acid assay (cat. # 23209), and PMSF (cat. # 36978) were from Thermo-Scientific. CFSE (cat. # C34554), CellTrace Violet stain (cat. # C34557), RPMI-1640 (cat. # SH30027.02), Penicillin/streptomycin (cat. # 15140-122) and FBS (cat. # 10427-028; lot # 1662765A120-01) were from Life Technologies. Live/dead fixable blue dead cell stain kit was from Molecular Probes. IL-2 ELISA kit (cat. # M2000) was from R&D Systems, Inc.

**Antibodies:** Anti-CD28 (cat. # 122007) and anti-LFA-1 (cat. # 141011) was from Biolegend. Anti-CD3ε (referred to as anti-CD3) (cat. # 553064, and 553057), anti-CD25 (cat. # 558642), anti-CD4 (cat. # 553051, and cat # 553047), anti-CD8α (cat. #560469), anti-CD28 (cat. # 553294) and anti-CD44 (cat. # 553135) was from BD Bioscience. Anti-B220 (cat. # 25-0452-81), and anti-CD3ε (referred to as anti-CD3) (cat. # 56-0032-80) were from eBioscience. Anti-HSC70
Anti-EHD1 (cat. # ab109311) was from Abcam. Anti-β-Actin (cat. # A5316) was from Sigma-Aldrich. APC-conjugated anti-Annexin V (cat. # 17-8007-72) was from eBiosciences. Polyclonal rabbit antibodies recognizing EHD1 plus EHD4, EHD2 or EHD3 have been described previously (45). Secondary fluorochrome-conjugated antibodies were from Life Technologies.

5.2 Mice Generation & Genotyping

Whole-body knockout mice (Ehd1-null) derived from Ehd1flox/flox mice have been described previously (Rainey et al., 2010). Ehd1-null mice were maintained on mixed 129, B6 background. $EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice in a predominantly C57BL/6 background were crossed with B6. Cg-Tg(Cd4-cre)1Cwi/BfluJ to generate Cd4-cre; $EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice. These mice were further crossed with C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J, 2D2 Tcr or MOG Tcr to generate Cd4-cre; 2D2Tcr; $EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice. $EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice in a predominantly C57BL/6 background were also crossed with tamoxifen-inducible CreERT2-expressing mice from Jackson Laboratories (Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$; strain 008463) to generate Cre$^{ERT2}; EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice. These mice were further crossed with C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J, 2D2 Tcr or MOG Tcr) to generate Cre$^{ERT2}; 2D2Tcr; EHD1^{0/0}; EHD3^{0/0}; EHD4^{0/0}$ mice. Genotypes were confirmed by subjecting tail clip DNA to PCR analysis using the KAPA mouse genotyping kit (KAPA Biosystems). Mice were treated humanely according to the National Institutes of Health (NIH) and University of Nebraska Medical Center guidelines. Animal studies were pre-approved by the Institutional Animal Care and Use Committee (#14-067).
### Table 2.5.3. Primers Used for Tail PCR Genotyping (include modification codes if applicable)

<table>
<thead>
<tr>
<th>Oligo Names</th>
<th>Sequences 5’ to 3’</th>
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<tr>
<td>Ehd1-1</td>
<td>(AAGTCAGAAGACAACCTTCTGGAGTTCCCT)</td>
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<tr>
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</tr>
<tr>
<td>CreR</td>
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5.4 Western blotting

Lymphoid tissues or isolated cells were lysed in ice-cold Triton X-100 lysis buffer (0.5% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM VO4) or with RIPA lysis buffer (same as Triton X-100 lysis buffer with an increase of Triton X-100 to 1% and an addition of 5 mM EDTA, 1 mM EGTA, 1% SDS, and 0.5% sodium deoxycholate). Lysates were vortexed, centrifuged at 13,000 rpm for 30 minutes at 4°C either immediately or after overnight rocking in the cold room, and supernatants were collected. Protein lysates were quantified using the bicinchoninic acid (BCA) assay. 40 μg aliquots of lysate protein per sample were resolved by SDS/PAGE and transferred to PVDF membranes (from Immobilon-P, cat # IPVH00010). In certain experiments, lysates from equal numbers of cells were resolved by SDS/PAGE. The membranes were blocked in TBS/5% BSA, incubated with the appropriate primary antibodies diluted in TBS-0.1% Tween 20 for 1 hr, washed in TBS-0.1% Tween (3x for 5 minutes each) followed by a 45-min incubation with HRP-conjugated secondary antibody in the same buffer. The membrane was then washed in TBS-0.1% Tween (3x for 5 minutes each) and ECL-based detection was performed.

5.5 CD4+ T-cell isolation

To isolate primary CD4+ T-cells, a negative selection protocol was performed as described (Kamala, 2008) using magnetic beads (Invitrogen Biotin binder kit cat. # 11533D) and biotinylated antibodies (Biolegend), and purity was established to be 91-95% based on flow cytometry.

5.6 Fluorescence Activated Cell Sorting (FACS)

T-cells were incubated on ice in the dark for 15 to 30 min (depending on the experiment) with appropriate conjugated antibodies at the manufacturer’s recommended dilution in FACS buffer (0.1% BSA in PBS). Cells were pelleted, washed twice, and suspended in 400 μl of cold FACS
buffer. In other cases, cells were fixed with 4% cold PFA for 15 min at room temperature after staining; then washed and suspended in 400 µl of cold FACS buffer. Cells were protected from light until analyses using either the LSR II Green or LSR II cytometer (BD Bioscience). FACS data were analyzed using DIVA (BD FACSDIVA TM Software), FlowJo (FLOWJO, LLC Data Analysis Software, Ashland, OR) and ModFit LT software (Verity Software House, Topsham, ME).

5.7 CFSE and CellTrace Violet-T-cell proliferation dye dilution assay

Spleen cells (5x10^6 cells/ml) from CD4-Cre; 2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl mice and control (2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl) mice were stained with CFSE or CellTrace Violet according to the manufacturer’s instructions. Cells were treated with 50 µg/ml of MOG35-55 peptide for 72 hrs. On the indicated day, cells were stained with FITC-CD4 before analysis. Dilution of CFSE or CellTrace Violet fluorescence as an indicator of cell division was assessed via FACS analysis. Data were analyzed using FlowJo and ModFit LT software. ModFit LT software was also used to determine the Proliferation Index (PI). In some instances, the cells were not stained with the proliferation dye, but were stained with anti-AnnexinV and Propidium Iodide staining solution after 72 hrs of stimulation and analyzed by FACS for cell death analysis.

5.8 ^3^H-thymidine incorporation assay and T-cell expansion

Spleen cells (5x10^5 cells/well) from CD4-Cre; 2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl or control (2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl) mice were seeded in 96-well U-bottom plates in 100 µl medium in the presence of varying concentrations of MOG35-55 peptide for 72 hrs. Cells were pulsed with 1 µCi of ^3^H-thymidine per well for the last 6 hrs of incubation, harvested onto filter disks and the radioactivity (counts per minute) counted using a scintillation counter (Packard). For T-cell expansion, spleen cells or LN cells from CD4-Cre; 2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl mice and control (2D2Tcr; EHD1^fl/fl; EHD3^fl/fl; EHD4^fl/fl) mice were stimulated with 10-
20 µg/ml of MOG<sub>35-55</sub> peptide and T-cells were expanded in the presence of 30 U/ml IL-2 for 7-8 more days (in fresh IL-2-containing media changed every two days after three days of stimulation).

5.9 <em>In vitro</em> deletion of EHD proteins (EHD1, 3 and 4)

Spleen cells or LN cells from <em>EHD<sub>1</sub>fl/fl</em>; <em>EHD<sub>3</sub>fl/fl</em>; <em>EHD<sub>4</sub>fl/fl</em>; <em>Cre<sup>ERT2</sup></em>; 2D2Tcr mice were pre-stimulated with either the MOG peptide or with anti-CD3 and anti-CD28 for three to four days in the presence (+) or absence (-) of 4-hydroxytamoxifen (4-OHT) (200 nM-300 nM) for deletion of EHD 1, 3 and 4. Cells were washed in PBS, stained with CFSE, and were re-stimulated for proliferation as previously described.

5.10 ELISA

Isolated spleen cells (5x10<sup>5</sup> cells/well) were cultured in 96-well U-bottom plates in the presence of 10 µg/ml of MOG<sub>35-55</sub> peptide at 37°C for indicated time points. IL-2 secretion was measured in culture supernatants using an ELISA kit according to the manufacturer’s instructions.

5.11 Cell surface TCR internalization assay

Internalization of cell surface pool of TCR-CD3 was assessed as previously described (Carrasco, Navarro, & Toribio, 2003; H. Liu, Rhodes, Wiest, & Vignali, 2000) with modifications. Briefly, freshly isolated LN cells at 5x10<sup>6</sup> cells/ml in 96-well U-bottom plates (in triplicates) were incubated in the presence of 10 µg/ml BFA at 37°C for the indicated times to allow the surface TCR to internalize in the absence of newly synthesized TCR transport to the cell surface. At each time point, cells were transferred into ice and subsequently stained with PE-conjugated anti-CD3 followed by FACS analysis to quantify the surface TCR levels remaining at each time point. The extent of the TCR remaining at the cell surface was calculated by expressing the median
fluorescent intensity (MFI) of PE-anti-CD3 staining at each time point relative to time 0, which was set as 100%.

5.12 Analysis of TCR recycling

The recycling of pre-existing intracellular pools of TCR-CD3 was carried out by adapting a previously described protocol (Dietrich et al., 2002). Briefly, the accessibility of the cell surface CD3 on freshly isolated LN cells (at 5x10⁶ cells/ml in 96-well U-bottom plates in triplicates) was blocked by staining with a predetermined saturating concentration of unconjugated anti-CD3 at 4°C for 30 min. The cells were extensively washed and incubated at 37°C. At the indicated times, the cells were transferred to 4°C and stained with a PE-conjugated version of the same anti-CD3 antibody that was used to block the staining of the initial cell surface cohort of CD3. The MFI of PE-anti-CD3 cell surface FACS staining was used as a measure of the recycling of intracellular TCR-CD3. Recycling of cell surface TCR-CD3 pool following internalization was assayed by a modified protocol (Osborne, Piotrowski, Dick, Zhang, & Billadeau, 2015; Sullivan & Coscoy, 2008). Freshly isolated LN cells were first stained with PE-conjugated anti-CD3 for 30 min on ice. After extensive washes, the cells were incubated at 37°C for 30 min (in complete RPMI) to allow the antibody-labelled TCR-CD3 to undergo internalization. The cells were then subjected to an acid wash to strip any remaining antibody bound to CD3 on T-cell surface. The cells were washed in cold FACS buffer and re-suspended in complete RPMI followed by incubation at 37°C for the indicated times to allow recycling of the labelled internalized pool of TCR-CD3 back to the cell surface. The cells were spun down, acid washed, and re-suspended in FACS buffer for FACS analysis to determine the remaining intracellular fraction of the internalized TCR-CD3. The percentage of TCR-CD3 recycling was calculated from the equation \(((T_0-T_i)/T_0) \times 100\), where \(T_0\) is the MFI of cells incubated for 0 min before the second acid wash, while \(T_i\) is the MFI of cells subjected to a second acid wash after incubation for the indicated times.
5.13 Confocal Microscopy

Lymph node T-cells were expanded as described under T-cell expansion. A total of 5 x10^6 expanded T-cells in complete RPMI medium were plated onto Poly-L-lysine-coated coverslips placed in 24-well plates and allowed to attach at 37°C for 10 min. Cells on coverslips were fixed in 4% ice-cold PFA for 20 min and then incubated with 0.1M glycine (in PBB) for 3 min at room temperature. After washing, the cells were incubated with the blocking plus permeabilization buffer (2% BSA +0.1% Triton in PBS) for 30 min at room temperature. Cells were stained with anti-Lamp-1 and anti-CD3 primary antibodies overnight a 4°C. After three washes, the cells were stained with secondary anti-rat (for Lamp-1) and anti-Armenian hamster antibodies for 45 min at room temperature. After washes, the cells were mounted with VECTASHIELD Hard Set mounting medium with DAPI (Vector laboratories, Cat # H-1500). Images were acquired at room temperature using a ZEISS ELYRA S.1 and superresolution structured illumination (SR-SIM). The sCMOS camera mounted on side port was used. Objective lenses: Plan-APOCHROMAT 63x/1.40 Oil DIC. For resolution: lateral resolution (XY): 120 nm, axial resolution (Z): 300 nm (typical experimental FWHM values with objective lens Plan-APOCHROMAT 63x/1.40 Oil DIC, subresolution beads of 40 nm diameter and excitation at 488 nm). Merged fluorescence pictures were generated and analyzed using ZEN ®2012 software from Carl Zeiss.

5.14 Induction and monitoring of Experimental Autoimmune Encephalomyelitis (EAE)

Six to eight week old female CD4-Cre; 2D2Tcr; EHD1<sup>fl/fl</sup>; EHD3<sup>fl/fl</sup>; EHD4<sup>fl/fl</sup> or control (2D2Tcr; EHD1<sup>fl/fl</sup>; EHD3<sup>fl/fl</sup>; EHD4<sup>fl/fl</sup>) mice were subcutaneously immunized in the flank with 200 µg of MOG<sub>35-55</sub> peptide emulsified (1:1) in complete Freund’s adjuvant (CFA) containing 0.5 mg heat-killed Mycobacterium tuberculosis H37RA in a total of 200 µl emulsion per mouse, together with an intraperitoneal injection of pertussis toxin (200 ng/mouse) in 200 µl PBS followed by a second dose of pertussis toxin two days later. Mice were monitored daily and scored for clinical signs of
EAE on a scale of 0-5 whereby 0 = no disease; 1 = decreased tail tone; 2 = hind limb weakness or partial paralysis; 3 = complete hind limb paralysis; 4 = front and hind limb paralysis; and 5 = moribund state or dead (Bettelli et al., 2003). Composite scores of each cohort were used to determine statistical significance.

5.15 Statistics

An unpaired student’s t test was used to calculate the p-values. Data are presented as mean ± SEM and p<0.05 served as the threshold for statistical significance.
CHAPTER 3: REQUIREMENT OF EHD PROTEIN FAMILY FOR T CELL FUNCTION
3.6. Introductions

Understanding of the mechanisms that underlie proper T-cell function is of great interest in basic immunology and immunopathology. T-cell activation requires recognition of an antigenic peptide bound to MHC proteins on antigen presenting cells (APC) by the T-cell antigen receptor (TCR) and downstream signaling mediated by cytoplasmic regions of the TCR-CD3 complex. Effective generation of an immune response requires concurrent engagement of and signaling through co-stimulatory proteins (e.g., CD28) on T cells by their cognate ligands on the APC surface (Huppa et al., 2003). Therefore, the mechanisms that ensure optimal levels of the TCR and accessory receptors on the T-cell surface prior to antigen stimulation are fundamentally critical to generating an effective immune response. However, mechanisms that regulate the surface pool of TCR and its accessory receptors have been primarily investigated in the context of T-cell activation. During T-cell activation, TCR signaling elicited by the APC-presented antigen, together with co-stimulatory signals, leads to spatial reorganization of the TCR and accessory receptors, such as CD28 and lymphocyte function-associated 1 (LFA-1), to form an area of intimate contact with APCs, the immunological synapse (IS) (Hashimoto-Tane et al., 2010; Jo et al., 2010). The endocytic pathways supply the IS with newly synthesized and recycled receptors to replenish those that are targeted to the lysosome for degradation (Das et al., 2004; Lee et al., 2003; Valitutti, Muller, Salio, & Lanzavecchia, 1997).

Cells use clathrin-dependent as well as clathrin-independent pathways to internalize surface receptors into early (EE)/sorting endosome compartments, from where they traffic to late endosome/multi-vesicular bodies and lysosomes for degradation, or recycle back to the cell surface. To go back to the cell surface, receptors either through a Rab4⁺ fast recycling endosome (RE) (directly to the plasma membrane) or through a slow recycling route via the Rab11⁺ RE (Gould & Lippincott-Schwartz, 2009; Naslavsky & Caplan, 2011). This sorting process is known to be regulated by Rab family of small GTPases and their interacting partners (Walseng et al.,
T-cell receptors such as TCR-CD3 (Huppa et al., 2003), CD28 (Cefai et al., 1998) and LFA-1 (Fabbri et al., 2005; Stanley et al., 2012) are known to be internalized and recycled to the IS during T-cell activation. Internalized TCR-CD3 traverses the Rab5+ EE, followed by transport through Rab4+ and Rab11+ endosomes, indicating the involvement of both fast and slow recycling pathways (Kumar, Kremer, Dominguez, Tadi, & Hedin, 2011; H. Liu et al., 2000). Naïve and resting T-cells constitutively internalize and recycle their surface receptors and the balance of these processes dictates their pre-stimulation surface levels and hence the levels of T-cell activation (H. Liu et al., 2000). Yet, the mechanisms of constitutive basal traffic of TCR-CD3 are not fully understood.

Recently, a new family of endocytic regulators, the EPS15 Homology Domain-containing proteins (EHD1-4), has been identified. These proteins exhibit a conserved domain architecture with helical regions near the N-terminus and the middle of the protein surrounding an ATP-binding G-domain that can hydrolyze ATP slowly, and a C-terminal EH domain that binds to proteins with asparagine-proline-phenylalanine (NPF)-containing or related tripeptide motifs as a major mechanism of protein-protein interactions (Mintz et al., 1999; Pohl et al., 2000). Structural studies of EHD2 reveal that the helical regions form a curved membrane lipid-binding interface, and that the ATPase domain folds similar to the GTPase domain of dynamin and mediates the formation of stable dimers (Daumke et al., 2007). Other structural findings support a model that dimeric EHDs organize into oligomeric rings around curved membrane structures and function in membrane tubulation and vesiculation (Blume, Halbach, Behrendt, Paulsson, & Plomann, 2007; Daumke et al., 2007; Ioannou & Marat, 2012; Jakobsson et al., 2011; Shah et al., 2014). Consistent with these biochemical observations, cell biological studies have shown that EHD proteins regulate recycling of several surface receptors that traffic through clathrin-dependent or clathrin-independent pathways, including transferrin receptor, MHC class I, β1 integrin, AMPA receptors, insulin-like growth factor 1 receptor (IGF1R), insulin-responsive glucose transporter 4 (GLUT4), and others (Caplan et al., 2002; George et al., 2007; Grant et al., 2001; Guilherme et
al., 2004; Jovic et al., 2007; Lasiecka et al., 2010; Lin et al., 2001; Naslavsky & Caplan, 2011; Rapaport et al., 2006). However, the physiological relevance of the many in vitro assigned roles of EHD proteins remains to be defined.

We used gene targeting in mice to reveal the in vivo functional roles of EHD proteins and cell surface receptors whose traffic is regulated by EHDs (Curran et al., 2014; Cypher et al., 2016; Demonbreun et al., 2015; Doherty et al., 2008; Gudmundsson et al., 2010; Posey et al., 2011; Posey et al., 2014). For example, EHD1 KO mice exhibit strain-dependent phenotypes varying degrees of embryonic lethality, male infertility, ocular developmental defects, and neural tube closure defects due to impairment of ciliogenesis and SHH signaling (Arya et al., 2015; Bhattacharyya et al., 2016).

To date, any roles of EHD-family proteins in TCR traffic or T-cell function are unknown. Given their importance in the regulation of a variety of other cell surface receptors and the consequences of deleting their genes, singly or in combination, on organ/cell function in vivo, we hypothesized that EHD proteins could play an important functional role in T cells by regulating receptor traffic. Consistent with such a hypothesis, EHD proteins have been shown to interact with Rab effectors, such as Rabenosyn-5, a dual Rab4 and Rab5 effector in the early endosome (Naslavsky, Boehm, Backlund, & Caplan, 2004), and Rab11 effector, Rab11-FP2, which regulates the exit of vesicles from the recycling endosome back to the plasma membrane (Naslavsky et al., 2006). Moreover, EHD proteins (EHD1, EHD3, and EHD4) were shown to interact with SNARE complex proteins such as SNAP-25, SNAP29 (Lu et al., 2015; Rotem-Yehudar, Galperin, & Horowitz, 2001), Syndapin I and II (Braun et al., 2005; Giridharan et al., 2013; Naslavsky & Caplan, 2011), and other proteins in non-T-cell systems. EHD proteins have been shown to localize in endosomes positive for Rab5, Rab4, Rab8, Rab11 or Rab35 (Caplan et al., 2002; Lasiecka et al., 2010; Sharma et al., 2008; Walseng et al., 2008), compartments that are implicated in TCR traffic (Finetti et al., 2014; Finetti et al., 2015; Patino-Lopez et al., 2008). Thus, we posited that EHD proteins could play an important functional role in T cells.
We found that EHD 1, 3 and 4 are expressed in lymphoid tissues and in purified CD4+ T cells. Accordingly, we generated myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgene (2D2-TCR Tg)-bearing mice in which floxed EHD 1, 3 and 4 were deleted using a CD4-Cre transgene. *In vitro*, CD4+ T-cells of EHD1fl/fl; EHD3fl/fl; EHD4fl/fl; Cd4-cre; 2D2Tcr mice (EHD1/3/4 knockout) exhibit reduced antigen-driven cell proliferation and IL-2 secretion. *In vivo*, these mice exhibit a reduced severity of experimental autoimmune encephalomyelitis (EAE) elicited by immunization with the MOG peptide. We show that surface levels of TCR, CD28, and LFA-1 are reduced and TCR recycling is impaired in CD4+ T cells of mice with a CD4-Cre-mediated EHD1/3/4 knockout. Therefore, our studies reveal a novel role of the EHD family of endocytic recycling proteins in the surface display of TCR in unstimulated T cells that contributes to their positive functional role in antigen-induced T-cell activation and immunopathology.

3.7. Results:

7.1 Multiple EHD Family Members are Expressed in Lymphoid Organs and Purified CD4+ T-cells.

Our previous Western blotting studies of EHD protein expression in the mouse thymus and spleen (George et al., 2010; George et al., 2011; Rainey et al., 2010), which we extended here to lymph nodes (LN) (Fig. 1A), showed that multiple EHD family members are expressed, albeit unequally, in lymphoid organs. This suggested that multiple EHD family members are likely to be expressed in individual immune cell types, consistent with our recent findings that bone marrow-derived macrophages (BMDMs) express both EHD1 and EHD4 (Cypher et al., 2016). With the focus of this study on functional analyses in the context of CD4+ T-cells, our additional analyses showed that EHD 1, 3 and 4 are expressed in primary murine CD4+ T-cells (Fig. 1B) while EHD2 protein was undetectable to barely detectable. Analyses of mRNA expression databases (ImmGen and BioGPS) further support this expression pattern. Notably, western
blotting of purified LN T-cells at various time points of anti-CD3/CD28 stimulation showed that levels of EHD proteins vary as a function of CD4+ T-cell activation, with a time-dependent increase in EHD 1 and 4 levels and a reduction in EHD3 levels (Fig. 2). These findings suggested that EHD proteins may play important roles in T-cell function.

Our previous knockout studies have revealed relatively unique as well as redundant functional roles of EHD proteins, with the knockout of EHD1 alone producing by far the most obvious developmental and adult organ functional aberrations, depending on the strain background (Arya et al., 2015; Bhattacharyya et al., 2016; Rainey et al., 2010; Rapaport et al., 2006). We assessed lymphoid organs of EHD1−/− mice for any immune defects and we saw that globally, their lymphoid tissues maintained normal structural architecture compare to those from the Wt mice (Fig. 3A). Therefore, we assessed whether EHD1 deficiency had any impact on T-cell activation. Notably, the initial analysis of EHD1−/− splenic T-cells did not reveal any detectable abnormality in anti-CD3/CD28 induced cell proliferation (Fig. 3B). These findings supported a redundant functional role of EHD proteins, at least in the context of T-cell proliferation. Consistent with this, an increase in EHD4 levels was noted in LN from EHD1−/− mice (Fig. 3C). In addition, we also noted an increase in the levels of both EHD1 and EHD4 in spleen and lymph nodes of EHD3−/− mice (Fig. 3D). Initial analysis of EHD4−/− T-cells shows that they proliferate less than CD4+ T cells from control mice (Fig. 3E, 3F). We also noted that the deletion of EHD4 in lymph node cells did not cause an increase in the level of EHD1 and EHD3. Given the likelihood of redundant EHD function, we used a combinatorial knockout strategy to further explore their role in T-cells.
7.2 CD4-Cre-Mediated Conditional Deletion of EHD1/3/4 in T-cells Leads to a Reduced Proportion of CD4+ T-cells in Secondary Lymphoid Tissues.

Given the likelihood of redundant EHD function, we generated mice with a CD4-Cre-depenpednt, T-cell directed, conditional deletion of floxed Ehd1, Ehd3, and Ehd4 genes. To examine the impact of EHD 1/3/4 deletions in T-cells in the context of antigen-specific responses, we also incorporated the MOG peptide-specific 2D2-Tcr transgene (Bettelli et al., 2003) into our conditional knockout model (referred to as CD4-Cre+ mice) (Fig. 4). Littermates without a CD4-Cre transgene were used as controls (referred to as CD4-Cre- mice). Western blot analysis of CD4+ T-cells isolated from the 2D2-Tcr transgenic mice showed that the presence of the transgenic TCR by itself did not have any detectable impact on the levels of EHD proteins (Fig. 5A). CD4-Cre+ mice were developmentally indistinguishable from CD4-Cre- mice and no gross phenotypic changes were seen in adult mice. We check the specificity of the transgenic receptor to the antigen and showed that they are specific to the MOG peptide (Fig. 5B, 5C, & 5D). Tail PCR and Western blot analysis confirmed the CD4-dependent deletion of EHD1/3/4 (Fig. 5E and Fig. 6A).

Next, to examine whether EHD1/3/4 deletion impacted T-cell development, we isolated single cells from lymphoid organs of 4-week-old mice and analyzed these for changes in T-cell numbers using cell counting combined with FACS. We found that total numbers of thymocytes, splenocytes, and LN cells were comparable in mice from both groups (Fig. 6B). Similarly, comparable cell numbers were observed when spleens of older (6-8 weeks old) mice of the two genotypes were analyzed (Fig. 6C). We carried out FACS analyses on the live/B220+ T-cell population, and assessed the proportion of cells at various developmental stages based on staining with antibodies against CD4, CD8, CD25 and CD44 (Dashtsoodol, Watarai, Sakata, & Taniguchi, 2008; G. Liu et al., 2014; Wang et al., 2009). Single positive thymocytes in 2D2-Tcr Tg mice are known to be skewed toward the CD4+ T-cell compartment; this is also seen in spleen and LN
CD4-Cre-mediated deletion of EHD 1/3/4 did not affect the relative percentages or total cell numbers within various thymic developmental stages when analyzed in 4-week-old mice (Fig. 6D and 6E).

In contrast, CD4-Cre-mediated EHD1/3/4 deletion led to a decrease in the percentage of T-cells in the periphery. This phenotype was seen in both the spleen and LN (Fig. 6F, 6G). However, the absolute number of CD4+ T-cells in these organs did not differ significantly between CD4-Cre positive and control mice. In addition, an apparent increase in peripheral B cell numbers and their percentages were seen (Fig. 6F, 6G); the basis of this phenotype is unknown but is likely to reflect altered T-cell- B-cell interactions due to T-cell deficiency of EHD1/3/4. We also saw a decrease in the percentage of CD8+ T-cells in spleens of CD4-Cre positive mice, but this did not reflect an increase in CD8+ T-cell numbers in these same organs. From these results, we conclude that expression of EHD proteins is dispensable during thymic T-cell development, but may play a role in peripheral T-cell function.

7.3 Expression of EHD Proteins is Required for Optimal Antigen-Induced T-cell Activation in vitro and for EAE in vivo.

Incorporation of a MOG antigen-specific TC transgene in our EHD1/3/4 deletion models allowed analyses of CD4+ T-cell response to a specific antigen, both in vitro and in vivo, the latter in the context of the development of EAE. We asked whether the mature T-cells in these mice exhibit any functional deficits. Initial experiments, using spleen cells from the parental 2D2-TCR transgenic and control mice with MOG or ovalbumin peptides, established the optimal concentrations of MOG peptide to induce T-cell proliferation (not shown). Analysis of CD4+ T-cells from spleen using the CellTrace Violet stain and ³H-thymidine incorporation showed a significant decrease in proliferation of T-cells from CD4-Cre+ mice compared to control CD4-Cre- (Fig. 7A, 7B). Visualization of progressive cell divisions using ModFit LT software, showed that while CD4-Cre+ and CD4-Cre- CD4+ T-cells progress through a comparable number of
divisions, EHD1/3/4-null T-cells exhibit a significant underrepresentation at late divisions and an increased proportion of cells at earlier divisions (Fig. 7A). The EHD1/3/4-null T-cells also exhibited a significantly lower overall proliferation index (Fig. 7A), further supporting the conclusion that these cells proliferate slower than the control T-cells. To exclude the possibility that the apparent decrease in the number of EHD1/3/4-null T-cells at late stages of proliferation might be due to their apoptosis, we stimulated the CD4+ T-cells as previously described and stained these with PI and anti-Annexin V followed by FACS analysis. We observed no increase in the percentage of apoptotic cells in antigen-stimulated CD4+ T-cells isolated from CD4-Cre+ compared to CD4-Cre− (control) mice (Fig. 7C). Thus, the mature peripheral CD4+ T-cells of CD4-Cre− exhibit a marked defect in the specific antigen-induced cell proliferation in vitro.

To assess whether the impact of EHD1/3/4 protein deficiency on CD4+ T-cell proliferation reflects their abnormal development or an actual importance in the events associated with T-cell activation, we used T-cells from 2D2-TCR Tg-bearing mice carrying a tamoxifen-inducible ERT-Cre that allows in vitro deletion of EHD1/3/4. EHD1/3/4 deletions in vitro led to a significant decrease in antigen-induced proliferation compared to their control T-cells (Fig. 7D, 7E, 7F). These results support a T-cell intrinsic positive role of EHD proteins in sustaining antigen-driven T-cell responses in mature peripheral T-cells.

Among the cytokines that initiate antigen-activated T-cells into proliferation is the activation-induced secretion of IL-2 and the induced expression of the corresponding receptor on the T-cell surface (Boyman & Sprent, 2012; Malek, Yu, Scibelli, Lichtenheld, & Codias, 2001). Therefore, we assessed the impact of EHD1/3/4 deletions on IL-2 secretion. We observed a significantly lower level of antigen-induced IL-2 secretion by EHD1/3/4-null CD4+ T-cells (Fig. 7G) with a difference detectable at the earliest time point (12 hrs) analyzed, making it unlikely that the reduction is due to a smaller pool of T-cells caused by reduced proliferation. The
significant reduction in IL-2 secretion is likely to represent one mechanism for the defective T-cell proliferation upon deletion of EHD1/3/4.

The impact of EHD1/3/4 deficiencies was not restricted to in vitro T-cell responses alone. Using immunization with the MOG35-55, we were able to test the impact of EHD1/3/4 deficiencies on the development of EAE in vivo. We immunized cohorts of control (CD4-Cre−) vs. experimental (CD4-Cre+) mice with MOG35-55 and assessed onset and severity of EAE over time. Interestingly, while no significant difference in the disease onset was seen, EHD1/3/4–null group showed a decrease in the disease severity compared to control littermates (Fig. 7H). These results further support our conclusion that the expression of EHD proteins is important for full CD4+ T-cell responses to antigen. Altogether, these in vitro and in vivo analyses established, for the first time, that EHD proteins play an important positive role in antigen-driven T-cell activation and immune response.

7.4 EHD Proteins Regulate the Traffic of the TCR-CD3 Complex to the Cell Surface.

At a cellular level, EHD proteins have been characterized as regulators of the endocytic traffic of cell surface receptors as mentioned in the introduction. Endocytic traffic of these receptors is key to their targeting to the immunological synapse (Andres et al., 2004; Cefai et al., 1998; Fabbri et al., 2005; Huppa et al., 2003; H. Liu et al., 2000; Qureshi et al., 2012; Stanley et al., 2012; Valk, Rudd, & Schneider, 2008; von Essen et al., 2004). To assess whether EHD proteins may indeed regulate the traffic of TCR-CD3 or the accessory receptors, we first examined the cell surface levels of TCR-CD3, CD28, and LFA-1 by FACS analysis of unstimulated T cells. As assessed by comparing the MFI values, T-cells from CD4-Cre+ mice expressed significantly lower levels of TCR-CD3 on the cell surface compared to T-cells from control mice (Fig. 8A). These analyses also showed that in vitro activated CD4+ T-cells from
CD4-Cre+ mice express lower levels of TCR-CD3, CD28, CD25, CTLA-4 and LFA-1 compared to those from control mice (Fig. 8A).

Since the TCR-CD3 complex is the primary determinant of antigen-specific T-cell activation and subsequent responses, we focused on the TCR for further analyses. We examined the kinetics of TCR-CD3 internalization and recycling in unstimulated CD4+ T-cells. Analysis of TCR-CD3 internalization using FACS showed that there was not significant difference in internalization of TCR in EHD1/3/4 -null T-cells compared to control T-cells (Fig. 8B). Next, we analyzed recycling to the cell surface of the pre-existing intracellular pool of TCR/CD3. For this purpose, anti-CD3ε antibody recognition epitope on the pre-existing surface TCR-CD3 was blocked with an un-conjugated antibody and the appearance of intracellular TCR-CD3 on the cell surface at various time points was monitored by staining with a conjugated anti-CD3ε. These analyses showed that EHD1/3/4 -null CD4+ T-cells exhibit a significantly reduced recycling of pre-existing intracellular TCR-CD3 to the cell surface at all time points analyzed compared to control T-cells (Fig. 8C). Next, we assessed the recycling of the pre-existing cell surface pool of TCR-CD3 by pre-labelling it with a PE-conjugated anti-CD3 and allowing it to internalize. After acid wash (to strip any remaining antibody bound to TCR-CD3 on T-cell surface), the internalized PE-labeled TCR-CD3 was allowed to reappear at the cell surface and re-stripped to remove the labeled antibody. Even under these assay conditions, we observed a significant decrease in the recycling of TCR-CD3 in EHD1/3/4 -null CD4+ T-cells compared to control cells (Fig. 8D). Overall, these results show a key requirement for EHD 1, 3 and 4 in basal recycling of TCR-CD3 from intracellular endocytic recycling compartments back to the cell surface.
7.5 Deletion of EHD1, 3 and 4 Promotes the Lysosomal Degradation of TCR-CD3.

Given the impairment of the endocytic recycling together with the reduced steady-state surface TCR-CD3 levels in freshly isolated EHD1/3/4-null T cells (Fig. 8A, 8C, 8D), we considered the possibility that the pool of TCR-CD3 that is retained in intracellular compartments may be aberrantly targeted for lysosomal degradation. To address this, EHD1/3/4-null T cells (from CD4-Cre-mediated and 4OHT-induced ERT-Cre deletion) were examined by western blotting for total levels of CD3ɛ. In both cases, we found that deletion of EHD proteins led to a significant decrease in the total levels of CD3ɛ (Fig. 9A, 9B). Given our findings with the CSF-1 receptor in macrophages (Cypher et al., 2016), we hypothesized that this reduction was likely due to lysosomal targeting and degradation. To test this hypothesis, we treated the EHD1/3/4-null T-cells with or without bafilomycin-A1, an inhibitor of lysosomal degradation, for 4 h and analyzed the impact on total CD3ɛ levels using western blotting and confocal (Fig. 10). Western blotting of cells treated with Bafilomycin A1 for 4 h showed a recovery of the CD3ɛ protein level in EHD1/3/4-null T cells with levels in treated cells comparable to those in control cells (Fig. 10A, 10B). We performed confocal imaging of control and EHD1/3/4-null T cells incubated with or without bafilomycin-A1. Some co-localization was seen in Bafilomycin A1-treated control T cells (Fig. 10C, upper panel). Notably, significantly more lysosome-localized CD3ɛ was seen in EHD1/3/4-null cells treated with bafilomycin-A1 (Fig. 10C, lower panel and Fig 10D right). However, relatively low CD3ɛ staining was co-localized with the lysosomal marker LAMP-1 in untreated cells (Fig. 10D left). These results support the conclusion that expression of EHD1/3/4 is required for efficient transport of TCR-CD3 from endosomal compartments back to the cell surface and to prevent its aberrant traffic into lysosomes.
3.8. Discussions

Cellular studies have identified members of the EHD protein family as key new regulatory elements of endocytic trafficking of several cell surface receptors, but their physiological functions remain relatively unexplored and their roles in the immune system unknown. Here, we investigate the role of EHD proteins in the context of antigen-specific CD4+ T-cells in vitro and in vivo. Our genetic studies, ablating genes encoding three members of the EHD protein family (EHD 1, 3 and 4) by CD4+ T-cells in the context of MOG transgenic TCR, demonstrate for the first time, an important, positive role of EHD proteins in antigen-specific T-cell activation in vitro and an autoimmune response in vivo. Mechanistically, we show that EHD proteins are required for basal recycling of TCR-CD3 from intracellular endocytic pools to the cell surface, thereby determining the subsequent level of T-cell activation. Combined with our recent study demonstrating a key positive role of EHD1 in the transport of newly synthesized CSF-1R from the Golgi to the cell surface of BMDMs (Cypher et al., 2016) and previous cell biological findings that EHD1 facilitates MHC class I recycling from the endocytic recycling compartment to the cell surface in a cell line system (Caplan et al., 2002), our results strongly support the likelihood that EHD proteins play critical and potentially diverse roles in the immune system.

Analysis of thymic T-cell development did not reveal any significant alterations in total cellularity, T-cell subsets or relative proportions of T-cells at different stages in mice with a CD4-Cre mediated deletion of EHD1/3/4, indicating that EHD proteins are largely dispensable for thymic development beyond the CD4/8 double-positive stage. Whether this might reflect any role of the remaining EHD family member, EHD2, remains a possibility that will require future studies. It is also possible that EHD proteins are important at earlier stages of thymic development and future use of other Cre elements active at these stages will be needed to address this possibility. Also, since our analyses, of which the primary purpose was not to probe thymic
development, were carried out in the context of a pre-rearranged TCR transgene, this could have masked a potential developmental role of EHD proteins in the context of a normally diverse TCR repertoire.

In contrast to the relatively unperturbed thymic development, CD4-Cre-mediated EHD1/3/4 deletion led to a decrease in the percentage of T cells in the periphery. This phenotype was seen in secondary lymphoid tissues, including the lymph nodes and the spleen. These results suggested that EHD proteins may play a more important role in mature peripheral T cells. The precise basis for this phenotype remains unclear but lower functional responses to TCR engagement, as seen in ex vivo analyses (discussed below), could play a role since TCR signals are important in peripheral T-cell maintenance (Cantrell, 1996; Daniels & Teixeiro, 2015; Surh & Sprent, 2008). In addition, an apparent increase in the peripheral B cell numbers and their percentages was seen; the basis of this phenotype is unknown but is likely to reflect altered T-cell- B-cell interactions due to T-cell deficiency of EHD1/3/4.

The in vitro analyses of T cells from the CD4-Cre deletion model demonstrated significantly defective antigen-elicited CD4⁺ T-cell proliferation and IL-2 cytokine secretion responses upon EHD1/3/4 deletion. Similar results were observed using CD4⁺ T cells derived from an alternate model where in vitro deletion of floxed EHD1, 3 and 4 genes was induced with 4-OHT to bypass any in vivo developmental abnormalities. These results support a T-cell intrinsic positive role of EHD proteins in sustaining antigen-driven T-cell responses in mature peripheral T cells, a role independent of any potential involvement of EHD proteins in events associated with thymic T-cell development.

Coordination of multiple EAE-inducing T-cell functions, including their early activation, expansion, migration to the brain across the blood/brain barrier, and effector functions to mediate demyelination in the CNS are required for full disease induction (Furtado et al., 2008; O'Connor
et al., 2008). Therefore, we analyzed the impact of EHD1/3/4 deficiencies on the onset and progression of EAE. Since *in vitro* studies showed the T-cell proliferation and cytokine (IL-2) secretion to be impaired upon EHD1/3/4 deficiencies, we expected to see a delayed onset and reduced severity of EAE. While we saw no difference in the onset of disease, EHD1/3/4-null mice showed a decrease in the disease severity compared to control littermates. Therefore, further *in vitro* and *in vivo* studies using models developed here should help to comprehensively reveal other aspects of T-cell function that also rely on EHD protein expression.

Given the reduced TCR-CD3 levels in the CD4+ T-cells of our EHD1/3/4 -null mouse model prior to deliberate T-cell activation, our focus here has been on the role of EHD proteins in basal (prior to antigenic stimulation) TCR-CD3 traffic, which has not been mechanistically dissected in much detail. Therefore, further studies to identify partner proteins through which EHDs regulate TCR traffic will be of great interest. Notably, endocytic trafficking pathways play key roles during T-cell activation by orchestrating the polarized transport of TCR-CD3 and signaling molecules such as Lck to maintain an active immunologic synapse, and Rab4- and Rab11-positive endosomes are implicated in these processes (Soares et al., 2013).

Cell biological studies have linked EHD proteins to the regulation of traffic in Rab11+ recycling endosomes as well as compartments regulated by other Rabs. Thus, further studies to examine the potential importance of EHD proteins in regulating endocytic traffic into and out of the IS during T-cell activation will be of great interest. In this regard, we and others have recently identified a key role of EHD proteins in primary cilia biogenesis (Bhattacharyya et al., 2016; Lu et al., 2015) and recent studies have shown that the maintenance of IS in cilia-less T-cells involves ciliogenesis-related proteins such as IFT20 which localize in the IS and are required for recycling of TCR-CD3 (Finetti et al., 2009; Finetti et al., 2014; Finetti et al., 2015). Although IFT20 KO under CD4-Cre led to only minor defects in T-cell development and collagen- induced arthritis (an experimental model of rheumatoid arthritis), earlier deletion using Lck-Cre led to
defective thymic development and T-cell function in vivo (Yuan, Garrett-Sinha, Sarkar, & Yang, 2014); the latter are partially similar to the phenotype of our EHD1/3/4 KO mice. Interestingly, EHD1 and EHD3 were found to be important for the recruitment of transition zone proteins and IFT20 during ciliary vesicle formation (Lu et al., 2015). Though our study has been done mainly on freshly isolated and rested cells, it seems plausible that EHD proteins may also regulate the traffic of TCR-CD3 in the IS to promote T-cell activation and subsequent responses.

Notably, the CD4-Cre mediated Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) deletion led to T-cell defects strongly resembling the phenotype of EHD1/3/4 deficiency with minimal thymic developmental defects, reduced proliferation and cytokine production, a decrease in the surface TCR-CD3 levels, increase in lysosomal degradation of TCR-CD3, and reduced severity of EAE (Piotrowski, Gomez, Schoon, Mangalam, & Billadeau, 2013). WASH is an Arp2/3 activator that localizes on distinct endosomal subdomains and functions in endosomal trafficking (Jia et al., 2010). Given these phenotypic similarities of the two KO models and the interaction of the WASH protein with retromer complex and the involvement of EHD proteins in retromer-dependent transport (Gautreau, Oguievetskaia, & Ungermann, 2014; Seaman, 2012), further exploration of a link between EHD proteins and WASH complex in regulating TCR-CD3 traffic will be of great interest.

Overall, studies presented here using genetic models and functional analyses, in vivo and in vitro, reveal an important albeit redundant positive role of the newly described EHD family of endocytic recycling regulatory proteins in T-cell function. Mechanistically, we show that EHD proteins are required to facilitate basal recycling of TCR-CD3 from intracellular endocytic compartments back to the cell surface to ensure the surface expression of optimal TCR-CD3 levels for subsequent antigen-driven T-cell activation. Future studies using the unique models described here should add to our understanding of the importance of endocytic traffic in the
control of TCR-CD3 and other receptors that regulate T-cell functions, as well as the roles of these proteins in other cells in the innate and adaptive arms of the immune system.

3.9. Figures

Figure 1: Expression of EHDs in Lymphoid Tissues and Isolated CD4⁺ T-cells. A) Lymphoid tissues express all EHD proteins. One membrane (top) was probed with an antibody recognizing both EHD1 and EHD4, and a second one (bottom) probed for EHD3 followed by re-probing for EHD2. β-Actin was used as loading control. B) EHDs expression in T-cells. 40 µg of cell lysates from freshly isolated CD4⁺ T-cells (from the spleen of a WT mouse) and Jurkat cells were loaded on a 10% SDS PAGE gel; cell lysate from thymocytes was used as positive control. HSC70 was used as loading control. Membranes were probed for EHD 1&4 (one antibody that detects both EHD 1and 4), and for EHD2. The EHD2 membrane was stripped and re-probed for EHD3. (M=marker).
<table>
<thead>
<tr>
<th>M</th>
<th>CD4+ T Cell</th>
<th>Jurkat</th>
<th>Thymus</th>
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<td>HSC70</td>
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Figure 2: EHD Proteins Show Differential Expression during T-cell Stimulation. A. CD4+ T-cells were freshly isolated from C57Bl/6 mice and were incubated with (+) or without (-) ant-mouse CD3ε/CD28 for 0, 24, and 48hrs for T-cell stimulation. Lysate of CD4+ T-cells from these time points and from the spleen and thymus of the same mice were run on 10% SDS PAGE gel. The membrane was probed for EHD1, EHD3, EHD4, and for β-Actin (loading control). B. Quantification of the western blots. Levels of EHD proteins were normalized to β-Actin. This is a representative figure of two different experiments.
**A**

<table>
<thead>
<tr>
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**B**

Bar graph showing the fold change of EHD1 normalized to β-actin over time.

- **unstimulated**
- **Stimulated**

Time (hrs): 0, 24, 48
(Fig. 2B) Continued,

Fold Change of EHD4 Normalized to β-actin

Fold Change of EHD3 Normalized to β-actin

Unstimulated
Stimulated

Time (hrs)

0 24 48

0 24 48

0 0.5 1 1.5 2 2.5

0 0.5 1 1.5 2 2.5 3

Unstimulated
Stimulated
Figure 3: Potential Redundant Functions of EHD Protein Family. A) EHD1 staining of the Lymph node and spleen from EHD1\textsuperscript{−/−} and Wt mice showing deletion of EHD1. B) Freshly isolated CD4\textsuperscript{+} T-cells from EHD1\textsuperscript{−/−} and Wt littermate mice were stimulated with anti-CD3\(\varepsilon\) and anti CD28 for 72hrs and were subject to thymidine incorporation for the last 6 hrs of incubation. A representative figure of 3 independent experiments is shown. C) EHD1, EHD3 and EHD4 protein expression in lymphoid organs from Wt and EHD1\textsuperscript{−/−} mice. Tissue lysate from these organs were loaded on a 10\% SDS PAGE gel. D) Western blotting showing upregulation of EHD1 and EHD4 in the spleen and lymph nodes of EHD3\textsuperscript{−/−} compared to tissues from Wt mice and upregulation of EHD4 in spleen and lymph nodes of EHD1\textsuperscript{−/−} compared to control tissues. Blots were probed for EHD1 and EHD4. E) Lymph nodes cells from Wt mice and EHD4\textsuperscript{−/−} mice were stained with CFSE then stimulated with various concentration of anti-CD3 (1 \(\mu\)g/ml and 2.5 \(\mu\)g/ml) for 3 days. Cells were stained with anti-CD4 to look at the proliferation profile of CD4\textsuperscript{+} T-cells. FACS analysis of data modelled using ModFit software is shown. PI = proliferation index ± SEM of N = 3. F) Western blotting of lymph nodes from Wt and EHD4\textsuperscript{−/−} mice showing deletion of EHD4.
A

**Lymph Node**

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<tr>
<th></th>
<th>WT</th>
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**Spleen**

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B

![Graph showing thymidine incorporation](image)

C

<table>
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<tr>
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<td>EHD1</td>
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<tr>
<td>β-actin</td>
<td>β-actin</td>
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<td>WT</td>
<td>WT</td>
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<tr>
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(Fig. 3C) continued,

![Graphs showing fold change of EHD proteins in Thymus and LN](image)

**D**

![Image showing protein expression in Thymus, Spleen, and Lymph Nodes](image)
E

CD4+ T cell from Wt

CD4+ T cell from EHD4-/-

Anti-CD3ε (1µg/ml)

Pl = 4.4 ± 1.0

Pl = 3.5 ± 0.8

Anti-CD3ε (2.5 µg/ml)

Pl = 5.4 ± 0.9

Pl = 4.2 ± 0.9

F

Lymph Nodes

WT WT EHD4-/- EHD4-/- WT WT EHD4-/- EHD4-/-

EHD4 EHD1

β-Actin

EHD3

β-Actin
Deletion of EHD 1,3,4 Specifically in CD4⁺ T cell using CD4-Cre

Figure 4: Generation of the TgTCR; CD4-Cre; EHD (1, 3, 4) flox/flox. Young mice (3-4 weeks old) were used for the FACs profile of the thymic development and older mice (6-8 weeks old) we used for rest of the experiments. Littermates that were CD4-Cre negative were used as control mice.
Figure 5: The CD4 Transgenic TCR Mice (2D2) Express Normal Levels of EHD Proteins and Respond to MOG Peptide, but not the OVA Control Peptide. (A) Western blot of tissue lysates from the thymus and spleen of 2D2 mice (T = Tg TCR), Wt TCR littermate (W = non transgenic), and EHD1 null mice (N = EHD1 null). N & W are used as control mice, M = marker. EHD3 membrane was stripped and re-probed for EHD2. 100 µg of tissue lysates were loaded in a 10% SDS PAGE gel. (B) Spleen cells from littermate with WT TCR, the WT C57BL/6J, and the Tg TCR mice were cultured with either the OVA peptide or the MOG peptide. Cells were pulsed with thymidine for the last 6 hrs of the 72 hrs incubation. Cells were harvested and radioactivity was counted. C) CFSE labeling was also used to check the specificity of the TgTCR. BMDC were pulsed with either the MOG peptide or OVA control peptide then were co-cultured together with CFSE labelled freshly purified CD4+T cells (92%) from the TgTCR for 3 days. FACs analysis of day 0 and day 3 are shown. D) Purity level of CD4+ T-cells used in this experiment. E) PCR of a mouse litter genotyped with DNA extracted from tails showing an example of 4 different offspring (#1, 2, 3, & 4) with or without CD4-Cre; TgTCR, and carrying EHD (1,3,4) flox/flox for the conditional deletion of EHD1, 3, & 4 in T-cells. F) FACS profile of cells from different lymphoid organs of TgTCR and control mice. Thymus showing % of CD4+ and CD8+ T-cells. Spleen showing % of CD4+ and CD8+ T-cells and % of T-cells that were positive for Vα3.2 and Vβ11 (expressed only on T-cells with TgTCR).
A

Thymus | Spleen
---|---
N | W | T

EHD3

EHD4

EHD1

HSC 70

EHD2

HSC 70

Re-probe

B

[Graph showing thymidine incorporation vs. peptide concentration]

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<tr>
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<tbody>
<tr>
<td>Wt TCR MOG</td>
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<tr>
<td>Wt TCR OVA</td>
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<tr>
<td>C57BL/6 OVA</td>
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<tr>
<td>C57BL/6 MOG</td>
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<tr>
<td>Tg TCR OVA</td>
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<td>Tg TCR MOG</td>
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Concentration (µg/ml) of peptide

[H^3] Thymidine incorporated (x10^4)
C  

MOG\textsuperscript{35-55} peptide stimulation  

OVA control peptide stimulation  

Day 0  

Day 3  

D
FACS Profile of the Thymus from Different Mice
(Fig. 5F) Continued,

FACS Profile of the Spleen From Different Mice

WT
C57BL/6J

WT TCR
(litter mate)

TgTCR
/2D2

CD4

CD8

Vα3.2

Vβ11
Figure 6: CD4-Cre Mediated Conditional Deletion of EHD 1, 3 and 4 does not Impact Thymic T-cell Development but Leads to a Relative Decrease in CD4+ T-cells in Secondary Lymphoid Tissues. 

A) Western Blot verification of EHD1/3/4 deletions is shown. Lysates of freshly isolated splenocytes from the same mice before T-cell expansion were used as controls. A representative blot of N = 6 is shown. 

B & C) There was no change in the absolute number of cells in the different lymphoid tissues of CD4-Cre+ mice. In B) cell numbers were counted in single cell preparations of the indicated lymphoid organs of 4-week-old CD4-Cre+ and CD4-Cre- (control) mice; N = 5. In C, the total spleen cell numbers from adult (6-8 weeks old) mice are shown; N = 5. 

D & E) Analysis of thymic developmental stages in CD4-Cre+ and CD4-Cre- mice are shown. D) Top and middle panels show representative FACS plots with gating strategies used for analysis of cell subsets and developmental stages in thymocytes of 4-week-old mice. The numbers in quadrants represent the mean percentage of total thymocytes. Bottom shows quantification of middle panel. 

E) Top shows FACS plots with gating strategies and bottom shows quantification of relative percentages of cell subsets or cell numbers as in D), N = 4. 

F & G) Reduced percentages of T-cells in the spleen and increased percentages of B cells in the spleen and LN are shown. Spleen (F) and LN cells (G) from 4-week-old CD4-Cre+ and CD4-Cre- mice were analyzed by FACS for markers of B cells (B220+), CD8+ T-cells and CD4+ T-cells (N=4). Total cell numbers were calculated by multiplying the total live cell counts with the percentage of each subset. All values plotted as the mean ± SEM for indicated sample size. *, p<0.05; **, p<0.01.
A

CD4-Cre:

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<tr>
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<th>CD4+T cells</th>
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<tr>
<td>+</td>
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</table>

EHD4

EHD1

EHD3

β-Actin

72 kDa

55 kDa

72 kDa

B

4 Weeks old

CD4-Cre Negative

CD4-Cre Positive

NS

C

6-8 Weeks old mice

CD4-Cre Negative

CD4-Cre Positive

NS
Figure 7: Conditional Deletion of EHD 1, 3 and 4 in T-cells Leads to Reduced Cell Proliferation and IL-2 Secretion. A) A reduced proliferation by Cell-Trace Violet dye dilution assay is shown. Successive cell divisions were modeled with ModFit LT software and are shown as peaks of different colors. The figure is a representative of N = 3. The mean percentages of cells at successive divisions are shown on the right as a histogram. The proliferation index (PI) shown on top is the mean ± SEM of N = 3. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ******, p<0.00001. B) Reduced proliferation seen by \(^3\)H-thymidine incorporation assay is shown. Shown is a representative figure of N = 2. C) Cells were stimulated as previously mentioned for the proliferation assays and stained with propidium iodide (PI) and anti-Annexin V antibody followed by FACS analysis for relative proportions of PI\(^+\)/Annexin V\(^+\) apoptotic cells. Values represent mean ± SEM of N = 2 (NS, not significant). D) Spleen cells from EHD\(^{fl/fl}\), EHD\(^{fl/fl}\), EHD\(^{fl/fl}\); Cre\(^{ERT2}\); 2D2Tcr mice were stimulated with anti-CD3/CD28 in the presence or absence of 4-OH-tamoxifen (4-OHT) for four days to derive in vitro EHD 1/3/4-deleted T-cells vs. their WT controls. Cells were washed, loaded with CFSE dye and re-stimulated with anti-CD3/CD28 for 72 hrs. Shown is a representative figure of N = 2. E) Western blot used to show depletion of EHD 1/3/4 in tamoxifen-treated T-cells. F) Quantification of the percentage of proliferating cells from the data shown in D). G) Reduced IL-2 secretion is shown. Values represent mean ± SEM, N = 4, * p<0.05, **p<0.01. H) There was a reduced severity of EAE in CD4-Cre\(^+\) mice. The plotted values are the composite mean scores ± SEM, N = 5 in each group, *, p<0.05. Clinical scores were recorded as described in Methods.
A

Relative Cell number

Cell Trace Violet, Fluorescence Intensity

CD4-Cre negative

PI = 5.6 ± 0.2

CD4-Cre positive

PI = 3.6 ± 0.3

% of cells per generation

Generations

CD4-Cre negative

CD4-Cre positive

****
*****
**
***
**

parent G2 G3 G4 G5 G6

% of cells per generation

Generations

CD4-Cre negative

CD4-Cre positive

****
*****
**
***
**

parent G2 G3 G4 G5 G6
B

[\text{[^3}H\text{]} \text{Thymidine Incorporated in CPM (x10^3)}]

MOG^{35-55} \text{Concentration (ug/mL)}

\text{CD4-Cre negative}

\text{CD4-Cre positive}

**

**

*

C

\text{% of Apoptotic Cells}

\text{CD4-Cre Negative}

\text{CD4-Cre Positive}

NS

PI and AV
**D**

![Graph showing CFSE, Fluorescence Intensity Counts for Unstimulated, No Treatment, 4-OHT treated samples.](image)

**E**

![Images showing expression levels of EHD4, EHD1, EHD3, and β-Actin under 4-OHT treated and non-treated conditions.](image)

**F**

![Bar graph showing the percentage of proliferating CD4+T cells under No Treatment and 4-OHT conditions.](image)
**Figures G and H**

**G**
- **IL-2 (pg/ml) (10^2)**
- **CD4-Cre negative**
- **CD4-Cre positive**

**12h 24h**

**H**
- **EAE Clinical Scores**
- **Days after Immunization**
- **CD4-Cre Negative**
- **CD4-Cre Positive**
Figure 8: Conditional Deletion of EHD 1, 3 and 4 Leads to Impairment of Basal Recycling of TCR-CD3 from Endocytic Compartments to the Cell Surface. **A**) The surface level of TCR-CD3, CD28 and LFA1 at steady state is shown. MFI values plotted are mean ± S. E. of N = 4 (*, p<0.05). **B**) A relatively intact basal TCR-CD3 internalization is shown. N = 3; NS, not significant. **C**) Reduced recycling of pre-existing endosomal TCR-CD3 is shown. The data represent mean ± SEM. of N = 4 (*p<0.05). **D**) The recycling of the labelled cell surface TCR-CD3 is shown. Cells were analyzed by FACS and MFI values used to quantify the proportion of internalized receptors that were still intracellular (acid wash-resistant fraction) and the percent of recycling was calculated as described in Methods. Values plotted are mean ± SEM of N = 4. *, p<0.05, **, p<0.01.
A

**MFI of Different Receptors (unstimulated) \((x10^2)\)**

- **CD3**
- **CD28**
- **LFA-1**
- **CD4**

**CD4-Cre Negative**

**CD4-Cre Positive**


**MFI of Different Receptors 24h after T cell Activation \((x10^2)\)**

- **CD3e**
- **CD25**
- **CD28**
- **CTLA-4**

**CD4-Cre Negative**

**CD4-Cre Positive**
(Fig. 8A) Continued,
C

Constitutive Recycling of TCR-CD3

![Graph showing the percentage of CD3 recycling to the cell surface over time for CD4-Cre negative and positive cells.](image)

D

Recycling of surface-labelled TCR-CD3

![Graph showing the percentage of CD3 recycling to the cell surface over time for CD4-Cre negative and positive cells.](image)
Figure 9: Conditional Deletion of EHD 1, 3 and 4 Leads to a Reduced Level of the CD3ε Component of TCR-CD3. A) Expanded T-cells from LN of CD4-Cre− or CD4-Cre+ mice probed for CD3ε and either EHD1/4 or EHD3 proteins. B) Quantification of CD3ε signals. Values plotted are mean ± SEM. of N = 5. C) LN cells from EHD1fl/fl; EHD3fl/fl; EHD4fl/fl; CreER(T2; 2D2-Tcr or EHD1fl/fl; EHD3fl/fl; EHD4fl/fl; 2D2Tcr (control) mice were stimulated with anti-CD3/CD28 for four days in the presence (+) or absence (−) of 4-OHT. Lysate from equal numbers of cells were loaded in each lane. Western blot was carried out as in B. Shown is a representative blot run in duplicate. D) Quantification of CD3ε signals for blot shown in C). E) Quantification of CD3ε signals for Cre-ERT+ T-cells. Values plotted are mean ± SEM of N = 3 *p<0.05, **P<0.01.
D

Quantification of Western Blot Normalized to β-actin

No Treatment 4-OHT

Cre-ERT - Cre-ERT +

E

Fold Change of CD3ε Normalized to B-actin

No Treatment 4-OHT

CD3ε
Figure 10: Conditional Deletion of EHD 1, 3 and 4 Leads to Lysosomal Degradation of TCR-CD3. 

A) T-cells expanded from LN of CD4-Cre⁻ or CD4-Cre⁺ mice (7-8 days) were washed and treated with (+) or without (-) 100 nM Bafilomycin-A1 for 4 h at 37°C. Lysate from equal numbers of cells probed for CD3Ɛ and EHD1/4 and re-probed for EHD3, with β-actin as a loading control. A representative blot is shown. 

B) Quantification from data exhibited in A are shown here. Values plotted are mean ± SEM of N = 3. 

C) Expanded T-cells from LN of CD4-Cre⁻ or CD4-Cre⁺ mice were treated with Bafilomycin-A1 as in A and stained with anti-CD3Ɛ (Red) and anti-LAMP1 (Green) antibodies followed by confocal imaging. Shown is a representative image of N=3. Scale bar = 2 µm. 

D) Right, cells not treated with Bafilomycin-A, but stained as those in C). The red boxes indicate that the intracellular pools of CD3Ɛ were used to determine Pearson correlation coefficients from analyses of >50 cells per group as an indication of the extent of CD3Ɛ/LAMP1 co-localization, which is shown in D, Left. Values are mean ± SEM, *p<0.05.
**A**

CD4+ T cells

<table>
<thead>
<tr>
<th></th>
<th>No incubation</th>
<th>After 4hrs in 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bafilomycin A1:</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CD4-Cre:</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- CD3ε
- EHD4
- EHD1
- EHD3
- β-Actin

**B**

Fold Change of CD3ε Normalized to β-Actin

- CD4-Cre negative
- CD4-Cre positive

* NS
Pearson Correlation

CD4-Cre negative
CD4-Cre positive

TCR-CD3 ZoomedLamp-1 Merge

D

CD4-Cre negative
CD4-Cre positive

Lamp-1/TCR-CD3e

0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Pearson Correlation

Lamp-1/TCR-CD3e

*
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS
10. **Summary of all the Studies**

The endocytic trafficking pathways is a complex pathway used by all vertebrates. This pathway is extensively used in critical processes such as cell migration (Maritzen et al., 2015; Paul et al., 2015), receptor turn over (Irannejad et al., 2015), cell signaling (Barbieri et al., 2016; Irannejad et al., 2015; Platta & Stenmark, 2011; Villasenor et al., 2016), cell-to-cell communication (Onnis et al., 2016), and in other pathways such as pathogen invasion (Kulpa et al., 2013; Pirooz et al., 2014; Takeuchi et al., 2011), cell division (Chesneau et al., 2012; Schweitzer et al., 2011), and membrane remodeling (Matsubayashi et al., 2015). It is well documented that mis-regulation of the endocytic trafficking is linked to different diseases including chronic hepatitis B virus (HBV) infections (Macovei et al., 2013; Watanabe et al., 2007), cancer (Mellman & Yarden, 2013; Mosesson et al., 2008), HIV infection (Kulpa et al., 2013), autoimmune diseases (Iglesias-Bartolome et al., 2009), etc. Therefore, understanding how regulators of this complex pathway function is of great interest in basic and clinical immunology.

On a cellular level, cells use this pathway for various reasons including the internalization of receptors and uptake of nutrients, ligands, other molecules and viruses. Cells use different routes to endocytose materials that include the clathrin-dependent pathway and the clathrin-independent pathway. However, regardless of the pathway, the internalized receptors enter the early endosome (EE) where they are sorted for either traffic into late endosome/MVB and lysosome or for recycling back to the cell surface. This pathway is regulated by a few families of regulators such as Rab family of GTPases and more recently a growing number of researchers have demonstrated that the EHD protein family is among key regulators of this complex pathway.

Our group and others have studied the role of EHD proteins both *in vitro* and *in vivo*. They regulate the transport of various receptors from different steps of the endocytic trafficking pathway. They have been shown to regulate the recycling of receptors from the EE to the RE, the
EE to the LE, and the retrograde transport through the Golgi. In non-immune cells systems, EHD proteins have been shown to regulate the transport of some key receptors such as MHC-I, TfR, and MHC-II that have been demonstrated in immune cells, to accumulate at the immunological synapse and be essential for the formation and maintenance of the IS (Caplan et al., 2002; Lin et al., 2001; Naslavsky & Caplan, 2011; Walseng et al., 2008). In addition, EHD proteins have been shown to regulate the neuronal synapse (Das et al., 2004; Ioannou & Marat, 2012; Ioannou & Marat, 2012; Jakobsson et al., 2011; Lasiecka et al., 2010; Valdez et al., 2005; Wei et al., 2010; Yap et al., 2010), the neuromuscular junction (Mate et al., 2012), and the process of ciliogenesis (Bhattacharyya et al., 2016). All of these processes have been demonstrated to exhibit various similarities with the immunological synapse (Bezakova & Ruegg, 2003; Boulanger et al., 2001; Dustin & Colman, 2002; Finetti et al., 2009; Finetti et al., 2014; Griffiths et al., 2010; Tarakanov & Goncharova, 2009). However, though there is growing number of researchers interested in studying this family of proteins, their roles in immune cell functions were never investigated.

Immune surveillance and rapid response by the immune system is essential to eliminate invading infectious agents and endogenous oncogenically-transformed cells. T-cells are major regulators and executioners of these functions. Effective generation of an immune response requires concurrent engagement of and signaling through co-stimulatory proteins (e.g., CD28) on T-cells by their cognate ligands on the APC surface (Huppa et al., 2003). Therefore, the mechanisms that ensure optimal levels of the TCR and accessory receptors on the T-cell surface prior to antigen stimulation are fundamentally critical to generating an effective immune response. However, mechanisms that regulate the surface pool of TCR and its accessory receptors have been primarily investigated in the context of T-cell activation.

During T-cell activation, TCR signaling elicited by the APC-presented antigen, together with co-stimulatory signals, leads to spatial reorganization of the TCR and accessory receptors, such as CD28 and LFA-1, to form an area of intimate contact with APCs, the IS (Hashimoto-
Tane et al., 2010; Jo et al., 2010). The endocytic pathways supply the IS with newly-synthesized and recycled receptors to replenish those that are targeted to the lysosome for degradation (Das et al., 2004; Lee et al., 2003; Valitutti et al., 1997). T-cell receptors such as TCR-CD3 (Huppa et al., 2003), CD28 (Cefai et al., 1998) and LFA-1 (Fabbri et al., 2005; Stanley et al., 2012) are known to be internalized and recycled to the IS during T-cell activation. Internalized TCR-CD3 traverses the Rab5+ EE, followed by transport through Rab4+ and Rab11+ endosomes, indicating the involvement of both fast and slow recycling pathways (Kumar et al., 2011; H. Liu et al., 2000). Naïve and resting T-cells constitutively internalize and recycle their surface receptors and the balance of these processes dictates their pre-stimulation surface levels and hence the levels of T-cell activation (H. Liu et al., 2000). Yet, the mechanisms of constitutive basal traffic of TCR-CD3 are not fully understood.

Based on the importance of the endocytic traffic in T-cell function and IS formation and maintenance, we wanted to investigate the role of the EHD proteins in both T-cell function and in the IS. Given the importance of the endocytic trafficking in T-cell function and in IS during activation and the role the EHD proteins in the neuronal synapse, in neuromuscular junction, and in ciliogenesis, we hypothesized that EHD protein will regulate the endocytic recycling of receptors in unstimulated and activated T cells respectively.

Our group and others have shown that EHD proteins are expressed in spleen, so to expand on this, we checked the expression of EHD proteins in the spleen, thymus and in lymph nodes using western blotting and immunofluorescence staining. Our data confirmed previous data and also showed in other lymphoid organs that were not published before that all members of the EHD proteins family are expressed in all lymphoid organs tested (Fig. 1). We then check their expression levels in primary T cells and cell lines. We show for the first time that multiple EHD proteins family members are expressed both in primary CD4+T-cells and in Jurkat cell line. This was shown both through western blotting and IF (Fig. 1 and not shown).
To determine if EHD protein were important during T-cell activation, we stimulated freshly isolated CD4+ T cells with or without anti-CD3/CD28 for 2 days and check the levels of EHD proteins at different time points. In our surprise, we saw that EHD1 and EHD4 levels went up over time with stimulation, while the level of EHD3 went down (Fig. 2). These results suggested that EHD proteins are important in T cell functions.

We have used gene targeting in mice to reveal the in vivo functional roles of EHD proteins and cell surface receptors whose traffic is regulated by EHDs (Curran et al., 2014; Cypher et al., 2016; Demonbreun et al., 2015; Doherty et al., 2008; Gudmundsson et al., 2010; Posey et al., 2011; Posey et al., 2014) in other systems. We have previously generated single and combination knockout of EHD protein family. For example, EHD1 KO mice exhibit strain-dependent phenotypes varying degrees of embryonic lethality, male infertility, ocular developmental defects, and neural tube closure defects due to impairment of ciliogenesis and SHH signaling (Arya et al., 2015; Bhattacharyya et al., 2016). EHD3 null mice have cardiac abnormalities including arrhythmias and blunted response to adrenergic stimulation, and reduced expression of Na/Ca exchanger (NCX1) (Curran et al., 2014). EHD4 null mice showed that EHD 4 was required for normal pre-pubertal testis size in male and these males had reduced fertility. Whenever these mice were analyzed, they did not show any obvious immune defect phenotypes. A closer look at the CD4+ T-cells from EHD1 and EHD4 null mice showed that deletion of EHD1 protein individually did not cause a significant impact on T cell activation; however, deletion of EHD4 individually showed a decrease in T cell proliferation.

Given the likelihood of redundant EHD function, we generated mice with a CD4-Cre-depependent, T-cell directed, conditional deletion of floxed Ehd1, Ehd3, and Ehd4 genes. To examine the impact of EHD 1/3/4 deletions in T-cells in the context of antigen-specific responses, we also incorporated the MOG peptide-specific 2D2-Tcr transgene (Bettelli et al., 2003) into our conditional knockout model. Analysis of thymic T-cell development did not reveal any
significant alterations in total cellularity, T-cell subsets or relative proportions of T-cells at different stages in mice with a CD4-Cre mediated deletion of EHD1/3/4, indicating that EHD proteins are largely dispensable for thymic development beyond the CD4/8 double-positive stage. One limitation of this model is the fact that there is still one member present in these T-cells, so whether this might reflect any role of the remaining EHD family member, EHD2, remains a possibility that will require future studies. The second limitation of our model is that we are using CD4-cre in which deletion take place at the CD4/8 double positive. It is still possible that EHD proteins are important at earlier stages of thymic development and future use of other Cre elements active at those stages will be needed to address this possibility. The third limitation of our models is the fact that our analyses were carried out in the context of a pre-rearranged TCR transgene; this could have masked a potential developmental role of EHD proteins in the context of a normally diverse TCR repertoire. More analysis of the thymic development is being conducted at this point to check if EHD proteins have any role during thymic development beyond the double positive stage of T-cell development.

In contrast to the relatively unperturbed thymic development, CD4-Cre mediated EHD1/3/4 deletion led to a decrease in the percentage of T-cells in the periphery. This phenotype was seen in secondary lymphoid tissues, including the lymph nodes and the spleen. These results suggested that EHD proteins might play a more important role in mature peripheral T-cells. In addition, an apparent increase in the peripheral B cell numbers and their percentages was seen. The basis of this phenotype is unknown but is likely to reflect altered T-cell- B-cell interactions due to T-cell deficiency of EHD1/3/4.

The in vitro analyses of T-cells from the CD4-Cre deletion model demonstrated significantly defective antigen-elicited CD4+ T-cell proliferation and IL-2 cytokine secretion responses upon EHD1/3/4 deletion. Similar results were observed using CD4+ T-cells derived from an alternate model where in vitro deletion of floxed EHD1, 3 and 4 genes was induced with
4-OHT to bypass any *in vivo* developmental abnormalities. These results support a T-cell intrinsic positive role of EHD proteins in sustaining antigen-driven T-cell responses in mature peripheral T-cells, a role independent of any potential involvement of EHD proteins in events associated with thymic T-cell development.

Research has shown that self-reactive T-cells from patients with multiple sclerosis and type 1 diabetes form impaired IS. Their T-cells had TCRs signaling in the periphery with little or no accumulation of pMHC or TCR-pMHC complex transport to the cSMAC (Schubert et al., 2012). Our antigen specific model, the 2D2 mouse model, is a well-established and accepted animal model of EAE which recapitulates many aspects of MS and has led to the development of different therapies approved for treatment of MS (Steinman & Zamvil, 2006). Understanding the trafficking mechanisms that ensure the polarized display of cell surface receptors in the IS will enhance our understanding of a key process in immune responses and could provide new approaches of immune intervention in highly prevalent diseases such as cancer and autoimmunity (Derniame, Vignaud, Faure, & Bene, 2008; Nicolaou et al., 2007).

Coordination of multiple EAE-inducing T-cell functions, including their early activation, expansion, migration to the brain across the blood/brain barrier, and effector functions to mediate demyelination in the CNS are required for full disease induction (Furtado et al., 2008; O’Connor et al., 2008). It is important to note that about 4% of the 2D2 mice develop spontaneous EAE by 3 to 5 months of age; however, all the mice used for these studies were 6-8 weeks old and the disease was induced by immunization with the MOG peptide. We analyzed the impact of EHD1/3/4 deficiencies on the onset and progression of EAE. Since *in vitro* studies showed the T-cell proliferation and cytokine (IL-2) secretion to be impaired upon EHD1/3/4 deficiencies, we expected to see a delayed onset and reduced severity of EAE. While we saw no difference in the onset of disease, EHD1/3/4-null mice showed a decrease in the disease severity compared to control littermates.
Our studies on the importance of EHD proteins in IS formation and maintenance showed that EHD1 and EHD3 accumulate at the IS (T cell-APCs contact site) during T-cell activation (data not shown). Although our analysis never showed if the deletion of EHD proteins in T-cells could lead to impaired IS formation, the accumulation at the IS that we observed in our WT T-cells still suggest that EHD could be required for the traffic of key receptors and integrin such as TCRs, CD28, CD25, and LFA-1 in the IS. Again, these data suggest that EHD could have an important role in the IS as those CD4⁺ T-cells have to be activated first, then migrate to the brain to cause demyelination of the CNS. Therefore, these results demonstrate that EHD proteins play a functional role in T-cells in vivo. Further in vitro and in vivo studies using models developed here should help to comprehensively reveal other aspects of T-cell function that also rely on EHD protein expression.

Given the reduced TCR-CD3 levels in the CD4⁺T-cells of our EHD1/3/4 -null mouse model prior to deliberate T-cell activation, our focus was on the role of EHD proteins in basal (prior to antigenic stimulation) TCR-CD3 traffic, which has not been mechanistically dissected in much detail. Therefore, further studies to identify partner proteins through which EHDs regulate TCR traffic will be of great interest. Notably, endocytic trafficking pathways play key roles during T-cell activation by orchestrating the polarized transport of TCR-CD3 and signaling molecules such as Lck to maintain an active immunologic synapse, and Rab4- and Rab11-positive endosomes are implicated in these processes (Soares et al., 2013).

To assess whether EHD proteins regulate the traffic of TCR-CD3 or the accessory receptors, we first examined the cell surface levels of TCR-CD3, CD28, and LFA-1 by FACS analysis of unstimulated T-cells. T-cells from CD4-Cre⁺ mice expressed significantly lower levels of TCR-CD3 on the cell surface compared to T-cells from control mice (Fig 8A). When we analyzed in vitro activated CD4⁺ T-cells from CD4-Cre⁺ mice, we saw that they expressed lower
levels of TCR-CD3, CD28, CD25, CTLA-4 and LFA-1 compared to those from control mice. We also checked the levels of CD4 both in non-activated and activated and it did not change.

Since EHD proteins are regulators of the endocytic trafficking pathway, we examined the kinetics of TCR-CD3 internalization and recycling in unstimulated CD4+ T-cells. Analysis of TCR-CD3 internalization using FACS showed that there was not significant difference in internalization of TCR in EHD1/3/4 -null T-cells compared to control T-cells (Fig. 8B). However, these T-cells exhibited a significantly reduced recycling of both the pre-existing intracellular pool of TCR-CD3 and the PE-labeled surface TCR-CD3 (back) to the cell surface at all-time points analyzed compared to control T-cells. Overall, these results show a key requirement of EHD 1, 3 and 4 in basal recycling of TCR-CD3 from intracellular endocytic recycling compartments back to the cell surface.

We were also interested in checking the total level of TCR-CD3 in these EHD1/3/4-null T-cells (from CD4-Cre-mediated and 4OHT-induced ERT-Cre deletion). We examined the total levels of CD3ε by western blotting and we found that deletion of EHD proteins led to a significant decrease in the total levels of CD3ε (Fig. 9). Given our findings with the CSF-1 receptor in macrophages (Cypher et al., 2016), we hypothesized that this reduction was likely due to lysosomal targeting and degradation. To test this hypothesis, we treated the EHD1/3/4-null T-cells with or without bafilomycin-A1, an inhibitor of lysosomal degradation, for 4 h and analyzed the impact on total CD3ε levels using western blotting and confocal. Western blot of cells treated with Bafilomycin A1 for 4 h showed a recovery of the CD3ε protein level in EHD1/3/4 -null T-cells, with levels in treated cells comparable to those in control cells. Confocal imaging of control and EHD1/3/4 -null T-cells incubated with or without bafilomycin-A1 showed that relatively low CD3ε staining was co-localized with the lysosomal marker LAMP-1 in untreated cells, but some co-localization was also seen in Bafilomycin A1-treated control T-cells. However, we saw significantly more lysosome-localized CD3ε in EHD1/3/4-null cells treated with
bafilomycin-A1. These data suggested that EHD proteins promote the recycling of the TCR-CD3 while preventing their lysosomal degradation.

11. Conclusions

Overall, studies presented here using genetic models and functional analyses in vivo and in vitro, reveal an important albeit redundant positive role of the newly-described EHD family of endocytic recycling regulatory proteins in T-cell function. Mechanistically, we show that EHD proteins are required to facilitate basal recycling of TCR-CD3 from intracellular endocytic compartments back to the cell surface to ensure the surface expression of optimal TCR-CD3 levels for subsequent antigen-driven T-cell activation. Our founding support the conclusion that expression of EHD1/3/4 is required for efficient transport of TCR-CD3 from endosomal compartments back to the cell surface and to prevent its aberrant traffic into lysosomes.

We also show that EHD1 and EHD3 accumulate at the IS (data not shown). These data suggest that EHD proteins could regulate the transport of important receptors and signaling molecule at the IS during T-cell activation. Altogether, our studies support the role of EHD proteins both before and during T-cell activation. Future studies using the unique models described here should add to our understanding of the importance of endocytic traffic in the control of TCR-CD3 and other receptors that regulate T-cell functions as well as the roles of these proteins in other cells in the innate and adaptive arms of the immune system.

12. Future Directions

Although our studies have reveal novel role of EHD proteins in T-cell function, there are still many questions that are left unanswered. Our model system used the deletion of EHD1, EHD3, and EHD4, while leaving EHD2 untouched. Although CD4+ T-cells express a low level of EHD2, it still does not rule out the possibility that EHD2 could have some role in T-cell function.
It will therefore be important to check that function of EHD2 either using the EHD2 null mice or using the inducible system established in our laboratory. Another question that is left unanswered is the actual role of each of the EHD proteins deleted in our system. Although initial studies were done using the EHD1null mice showed no significant difference compared to WT mice, initial studies using EHD4 null mice showed that their T-cells have a decreased proliferation compared to control T-cells. It will be interesting to check if T-cells from EHD3 null mice will have no difference in proliferation compared to control T cells since EHD3 seems to be used differently than other EHDs during activation.

Our analysis of young mice showed that the percentage and absolute number of B cells increased in both the spleen and lymph nodes. The basis of this phenotype is not known. It will be great interest to investigate which sub-type of B cells caused this increase and to also check if their functions were affected. We also saw a slight change in the percentage of CD8+T-cells; however, since our model (2D2 mice) is known to be skewed toward the CD4+T-cell compartment, it makes it difficult to know what role EHD proteins have in CD8+T-cells. The use of a non-transgenic model to study thymic development and different T-cell sub-sets in the periphery is needed.

We have analyzed some T-cell surface receptors such as CD28, LFA-1, CD25, and CTLA-4 and saw that deletion of EHD proteins led to their decrease from the cell surface. It will be interesting to study the role of EHD proteins in the traffic of CTLA-4, since they localize in endosomes in unstimulated T-cells and must be transported to the cell surface during T-cell activation.

The mechanism by which EHD proteins regulate the traffic of TCR-CD3 is not known. Since either the TCR or the CD3ε themselves do not contain NPF motifs, it suggest that there is another protein that links or associate them to endosomes positive for EHD proteins to regulate...
their recycling back to the cell surface. It will be interesting to investigate which proteins link EHD proteins to TCR-CD3 for their transport.

Our confocal analysis showed EHD protein expression in APCs (data not shown). Since we have an in vitro inducible system, we can study the role of EHD proteins in APCs. Also further studies to examine the potential importance of EHD proteins in regulating endocytic traffic into and out of the IS during T-cell activation will be of great interest. In this regard, our group and others have recently identified a key role of EHD proteins in primary cilia biogenesis (Bhattacharyya et al., 2016; Lu et al., 2015). Recent studies have shown that the maintenance of IS in cilia-less T-cells involves ciliogenesis-related proteins such as IFT20 which localize in the IS and are required for recycling of TCR-CD3 (Finetti et al., 2009; Finetti et al., 2014; Finetti et al., 2015). Although IFT20 KO under CD4-Cre led to only minor defects in T-cell development and collagen- induced arthritis (an experimental model of rheumatoid arthritis), earlier deletion using Lck-Cre led to defective thymic development and T-cells function in vivo (Yuan et al., 2014); the latter are partially similar to the phenotype of our EHD1/3/4 KO mice. Interestingly, the requirement of EHD1 and EHD3 were found to be important for the recruitment of transition zone proteins and IFT20 during ciliary vesicle formation (Lu et al., 2015). Though our study has been done mainly on freshly isolated and rested cells, it seems plausible that EHD proteins may also regulate the traffic of TCR-CD3 in the IS to promote T-cell activation and subsequent responses.
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