The Membrane Traffic from the Endoplasmic Reticulum to the Plasma Membrane in *Entamoeba histoolytica* : The Analysis of Rab8A GTPase

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Abstract

Proteins translated by ribosomes in the rough endoplasmic reticulum (ER) are transported to the organelles or the plasma membrane via the Golgi apparatus. Further, some organisms uptake the nutrients from the extracellular environment by endocytosis or phagocytosis and utilize for its energy production. This sequential protein transport system, called membrane trafficking, is regulated by Rab small GTPases as a molecular switch. Rab small GTPases are the member of the Ras superfamily, and promote membrane fusion in the membrane trafficking.

Entamoeba histolytica, an intestinal protozoan parasite, engulfs bacteria and host cells. *E. histolytica* displays two distinct life stages: the dormant cyst and proliferative trophozoite stages. Infection initiates by oral ingestion of cysts. Cysts are differentiated to trophozoites (excystation) in the small intestinal lumen, and trophozoites colonize host intestinal tissues via the galactose and N-acetyl-D-galactosamine (Gal/GalNAc)–specific lectin. After the colonization, *E. histolytica* secretes protease, cysteine protease, and invades into the host intestinal tissues. In these events, Rab GTPases play pivotal roles in membrane fusion and regulated transport.

Interestingly, *E. histolytica* codes more than 100 Rab genes in their genome, whereas *Homo sapiens* possesses around 60 Rab genes despite of their multicellularity. Biological functions of most of Rab GTPases are still poorly understood in *E. histolytica.* In the previous phagosomal proteome analysis, colleagues of my lab identified 14 Rab proteins from the amoebic phagosomes. Among them, a homologue of Rab8, which plays a role in trafficking from the trans-Golgi network to the plasma membrane in *Homo sapiens* Rab8A or *Saccharomyces cerevisiae* ortholog, Sec3p, was identified. There is no report that Rab8 regulates phagocytosis directly in these organisms, while I here demonstrated that down-regulation of EhRab8A by small antisense RNA-mediated transcriptional gene silencing remarkably reduced adherence and phagocytosis of erythrocytes, bacteria, and carboxylated latex beads.

Further, surface biotinylation followed by SDS-PAGE analysis revealed that the surface expression of several proteins presumably involved in target recognition was reduced in the *EhRab8A* gene silenced strain. However the transport pathway and cargo proteins were unveiled. Here, I aimed to clarify the transport pathway and the machinery regulated by EhRab8A.

First, to confirm whether Rab8A functions for the phagocytosis by regulating the surface proteins, I established Rab8A overexpressed strain and dominant-negative/active strain and access the phagocytosis. Overexpression of wild-type EhRab8A augmented phagocytosis whereas expression of the dominant-negative form of EhRab8A resulted in reduced phagocytosis. These results indicated that EhRab8A regulates transport of surface receptor(s) for the prey from the ER to the plasma membrane. Next, I performed the localization analysis to determine the transport pathway of EhRab8A. I referred for the human Rab8 localization; Rab8 carries the traffic from the TGN to the plasma membrane with Rab11. The localization of EhRab11B was already reported, which is localized to the TGN as well as in other eukaryotes, however the localization of EhRab8A was completely differed from that of EhRab11B. EhRab8A was localized to the ER with EhBiP, which is an ER-residing chaperon protein, but the different compartment from EhSec13, which is one of a component of the COPII vesicle. According to these results, it was clarified that EhRab8A regulate the surface protein transport from the ER to the plasma membrane.

Second, to determine the further function or cargo protein of EhRab8A, the investigation of the binding protein of EhRab8A was accomplished. The Blue-Native Polyacrylamide gel Electrophoresis (BN-PAGE) following immunoprecipitation was performed to find out the binding proteins. In the results, 87 kDa complex was confirmed by the BN-PAGE and 35 and 40 kDa proteins were identified by immunoprecipitation. From the band of 35 kDa, the Cdc50 homolog, a non-catalytic subunit of lipid flippase, was identified as an EhRab8A binding protein. Indirect

immunofluorescence assay showed that endogenous EhCdc50 localized on the surface, but overexpression of EhCdc50 impaired its translocation to the plasma membrane and caused its accumulation in the ER. According to the previous report in other organisms, overexpression and accumulation of Cdc50 in the ER likely inhibited surface transport and function of the plasma membrane lipid flippase P4-ATPase. Further, overexpression strain of EhCdc50 gained resistance to miltefosine, which is consistent with the prediction that EhCdc50 overexpression caused its accumulation in the ER and mislocalization of the unidentified lipid flippase. EhRab8A gene silenced strain also showed increased resistance to miltefosine, supporting EhRab8A-dependent transport of EhCdc50. This is the first report that EhRab8A mediates the transport of EhCdc50 and lipid flippase P4-ATPase from the ER to the plasma membrane.

According to these results, Cdc50-P4ATPase complex, which carries the flip of phosphatidylcholines, is transported to the plasma membrane under the EhRab8A regulation. In general, proteins translated on the ribosome are taken into the COPII vesicles and transported to the Golgi, followed by other organelles. However, I showed that EhRab8A is responsible for the independent pathway. Additionally, it was determined that EhRab8A is localized on the ER, which is distinct from EhRab11B localization, suggesting that there is a EhRab8A-dependent transport machinery from the ER to the plasma membrane in *E. histolytica*. I also demonstrated that EhCdc50 is transported from the ER to the plasma membrane under EhRab8A regulation. In summary, I clarified the new transport pathway regulated by EhRab8A from the ER to the plasma membrane, which specifically transports the surface receptor or flippase. It may contribute to the elucidation of one of the diversified membrane trafficking mechanisms in *E. histolytica*.

Chapter 1. General Introduction

1-1 Membrane trafficking and Rab GTPases in Eukaryotes

Eukaryote is comprised of a wide variety of organisms; unicellular and multicellular. The former includes protozoan parasites such as *Plasmodium falciparum*, *Giardia lamblia*, *Entamoeba histolytica*, and a unicellular fungus *Saccharomyces cerevisiae*. The latter includes *Homo sapiens* and *Arabidopsis thaliana* [1]. In all eukaryotic cells, organelles carry out specialized functions that are ultimately controlled by proteins, which must be correctly targeted to the respective organelles. Thus, protein transport system, so-called membrane trafficking, is highly essential for the function of the cell [2].

Membrane trafficking is ubiquitous and well-conserved in eukaryotic cells. Small GTPases are main regulators, which are also conserved in all eukaryotes, in membrane trafficking [2,3]. In general, proteins newly translated by ribosomes in the rough endoplasmic reticulum (ER) or the cytoplasm enter into the ER lumen. After the proteins are modified by glycosylation or cleavage of the signal peptide, the proteins are packed into vesicles and then transported to the Golgi apparatus [4]. In this step, Sar1 family of small GTPases regulates vesicle formation. Sar1 GTPase takes part in the generation of COPII vesicles on the ER membrane, and then COPII vesicles, released from the ER, are uncoated and then fused to the Golgi apparatus [5]. At the trans-Golgi network (TGN), the cargo proteins are further packed into clathrin-coated vesicles by the regulation of the Arf family of small GTPases [6]. Finally, the cargo proteins included in the clathrin-coated vesicles are transported to the endosomes, lysosomes, or plasma membrane, where membrane fusion is regulated by Rab GTPase family [2]. These Small GTPases, Sar1, Arf and Rab GTPases, regulate membrane trafficking as molecular switches, undergoing recycling of GTP-bound active form and GDP-bound inactive form. These GTPases are activated and inactivated by guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP), respectively. GEF stimulates the release of guanosine diphosphate

(GDP) to allow binding of guanosine triphosphate (GTP). On the other hand, GAP stimulates the intrinsic GTPase activity of small GTPases and then converts GTP form to GDP form [7].

The function of Rab GTPases in vesicle trafficking differs with each isotype. In general, Rab GTPases are involved in vesicle tethering and membrane fusion together with their effector proteins [3]. Rab GTPases mediate vesicle tethering by recruiting tethering factors and SNARE proteins, which stimulates membrane fusion between donor and acceptor membranes. After membrane fusion, the GTP hydrolysis of Rab GTPase occurs, followed by the conversion of Rab GTPase to its inactive GDP-bound form, which resides in the cytoplasm [6,8]. In addition to membrane fusion, other Rab isotypes mediate vesicle transport along the cytoskeleton [10,11], such as actin filaments and microtubules, together with motor proteins. Rab GTPases also regulate the sorting of receptors together with adaptor proteins into the clathrin-coated vesicles from the plasma membrane during the initiation/start of endocytosis [6, 9].

Multicellular eukaryotes generally possess a large repertoire of Rab isotypes rather than that of unicellular eukaryotes [12]. For instance, *H. sapiens* and *A. thaliana* encode 60 and 29 Rab genes in their genome, respectively. Meanwhile, *S. cerevisiae*, *G. lamblia*, and *P. falciparum* possess 11, 8, and 11 Rab genes, respectively [13]. In contrast, the protozoan parasite *E. histolytica* has 102 Rab genes [14,15], suggesting that membrane trafficking in *E. histolytica* has a dynamic and specific role in membrane dynamics, which may be caused by the evolution of Rab GTPases.

1-2 Specific organelles involved in membrane trafficking in *E. histolytica*

E. histolytica is a protozoan parasite belonging to the large taxonomic group Amoebozoa, and is the causative agent of amebiasis in humans, accounting for an estimated 100,000 annual deaths according to the report from the World Health Organization (WHO, 1997). *E. histolytica* displays two distinct life stages: the dormant cyst and proliferative trophozoite stages. Infection is established by oral

ingestion of cysts. Cysts are differentiated to trophozoites (excystation) in the small intestinal lumen. Trophozoites colonize host intestinal tissues via the galactose and N-acetyl-D-galactosamine (Gal/GalNAc)–specific lectin [16,17]. To survive in the anaerobic environment within the colon, *E. histolytica* has adapted their organelles and its functions.

The absence of many typical organelles found in other eukaryotic cells such as the mitochondria, the stacked Golgi apparatus, rough ER, and a microtubule cytoskeleton has been reported in *E. histolytica* [18]. However, it is postulated that this parasite carries out functions such as post-translational modifications and protein targeting to subcellular compartments that are performed by the Golgi apparatus in usual eukaryotes. It is suggested that those functions take place in Golgi-like structures. In the case of mitochondria, *E. histolytica* possesses highly degenerate mitochondrion-related organelles (MROs) called mitosomes, which lack many features associated with canonical aerobic mitochondria [19]. *E. histolytica* mitosomes have minimal and mostly divergent transport systems such as protein import systems that lack a canonical targeting signal. According to these reports, the protein transport machinery might be diversified in *E. histolytica.*

1-3 The Rab GTPases in *E. histolytica*

Trophozoites of *E. histolytica* engulf human intestinal tissue cells and bacteria in the large intestine by phagocytosis. Phagocytosis is a ubiquitous mechanism that is evolutionarily conserved in a wide range of eukaryotes, and comprises several key steps: receptor-mediated recognition of the particle (prey), signal transduction, rearrangement of the cytoskeleton, and remodeling of the membrane and contents [20,21]. The surface receptor for a variety of targets activates a number of signaling cascades leading to target-dependent responses [22]. The subsequent maturation of phagosomes requires interactions between the phagosomes and various intracellular compartments, such as endosomes, lysosomes, the ER, and the Golgi apparatus

[23-25]. Membrane trafficking during phagocytosis, like receptor transport to the cell surface and membrane fusion between phagosomes and other intracellular compartments, is regulated by specific Rab GTPases localized to each organelle.

Previously, *Eh*RabB, *Eh*Rab5, EhRab7A, and *Eh*Rab7B have been shown to be sequentially involved in phagocytosis in *E. histolytica*. First, *Eh*RabB is recruited, along with phosphatidylinositol-3-phosphate, to the phagocytic cup. Simultaneously, a unique compartment called prephagosomal vacuole (PPV) is formed by the sequential action involving *Eh*Rab5 and EhRab7A. Finally, EhRab7A-positive PPVs and EhRab7B-positive lysosomes/late endosomes fuse with the phagosome to allow its full maturation [26-29]. Besides the above-mentioned EhRab proteins, localization and/or functions of only 6 amebic Rab proteins have been previously shown. It has been reported that EhRab11A is involved in encystation [30], whereas EhRab11B is involved in the transport and secretion of cysteine protease [31]. It was also shown that EhRabA and EhRab8A are localized to the leading edge of the cell and the plasma membrane, respectively [32-34].

To reveal the pathway regulated by respective Rab GTPase, it is necessary to find their corresponding effector proteins. However, effectors and binding proteins have not been identified for the previously reported EhRab proteins except for EhRab7A. Binding of the retromer complex, more specifically one of its components Vps26 [27], to Rab7 was first reported in *E. histolytica*, and subsequently observed in mammalian cells, indicating that Rab7 is involved in cargo recognition and sorting in the endosome of a wide range of eukaryotes.

It is supposed that Rab GTPases and their effector proteins have diverse functions compared with other eukaryotes. However, the roles of other Rab GTPases have not been elucidated. The realization of their unveiled function will contribute to the understanding of membrane trafficking not only in *E. histolytica* but also in other eukaryotes.

Chapter 2. The membrane trafficking pathway regulated by EhRab8A

2-1 Abstract

In *E. histolytica* Rab8A was reported to be involved in the transport regulation of a surface receptor, which is important for the target recognition in phagocytosis. Here, I showed that EhRab8A, which generally regulates the trafficking from the trans-Golgi network to the plasma membrane in other organisms, primarily resides in the ER and participates in phagocytosis. Overexpression of wild-type EhRab8A augmented phagocytosis whereas expression of the dominant-negative form of EhRab8A resulted in reduced phagocytosis. Further, EhRab8A was localized on the ER membrane, close to an ER-residing chaperon protein, EhBiP, but the different compartment from EhSec13, which is one of a component of the COPII vesicle. These results indicated that EhRab8A regulates transport of surface receptor(s) for the prey from the ER to the plasma membrane by the COPII-independent pathway. To the best of my knowledge, this is the first study to show that the ER-resident Rab8A is involved in phagocytosis through the transport of plasma membrane proteins.

2-2 Introduction

In the previous report, in order to clarify molecules and mechanisms involved in phagocytosis of *E. histolytica*, the proteomic analysis was performed to identify proteins associated with the phagosomes. 14 Rab GTPases, among 85 total proteins, were identified by liquid chromatography and mass spectrometry from the isolated phagosomes of *E. histolytica* [35-37]. Eh Rab8A, among 14 Rab GTPases from the phagosome proteomic analysis, was examined for the following analysis. Down-regulation of EhRab8A by small antisense RNA-mediated transcriptional gene silencing, remarkably reduced adherence and phagocytosis of erythrocytes, bacteria, and carboxylated latex beads. Surface biotinylation followed by SDS-PAGE analysis revealed that the surface expression of several proteins presumably involved in target recognition was reduced in the *EhRab8A* gene silenced strain. According to these reports, EhRab8A is involved in phagocytosis through the regulation of trafficking of a surface receptor to the plasma membrane.

Generally, in the eukaryotes secretory pathway, Rab11 functions upstream of Rab8. The GTP-bound active form of Rab11 binds its effector molecule, Rabin8, which is a GEF of Rab8 [38]. Consistently, partial colocalization of Rab8 and Rab11 in the TGN has been reported in mammalian cells [39,40]. This Rab cascade, comprising Ypt32p and Sec2p, homologues of Rab11 and Rabin8, respectively, is also conserved in the budding yeast [41]. In *E. histolytica,* it has previously reported that overexpression of EhRab11B resulted in massive mis-secretion of cysteine proteases, suggesting that EhRab11B is the primary regulator of cysteine protease transport from the TGN to the plasma membrane in *E. histolytica* [31]. On the other hand, the transport pathway regulated by EhRab8A was unveiled in *E. histolytica*.

2-3 Materials and Methods

2-3-1 Culture

Trophozoites of the *E. histolytica s*train HM-1: IMSS cl-6 were cultured axenically at

35°C in 13 × 100 mm screw-capped Pyrex glass tubes or plastic culture flasks in BI-S-33 medium, as previously described [42,43].

2-3-2 Construction and establishment of a myc-tagged EhRab8-expressing strain, and strains expressing dominant-active and -negative EhRab8A mutants

A plasmid constructed as follows for the creation of a transformant wherein EhRab8A with myc-tagged at the amino terminus, was expressed. A 600-bp DNA fragment containing the *EhRab8A*-coding sequence was amplified by PCR from cDNA with the following oligonucleotides: 5' -TCA GAA GAG GAT CTT ATG TCG GAG AAG GAT TCA ACA-3' and 5' -GTT CAA CTC GAG TTA ACA TCC AGT TGA TTC AGT-3' (the XhoI restriction sites are underlined). The amplified fragment was cloned into XhoI-digested pKT-3M [26] with an Infusion cloning kit (Clontech). Plasmids that expressed EhRab8A S21N (GDP-bound dominant-negative) and Q66L (GTP-bound dominant-active) mutants were constructed by PCR-mediated mutagenesis [44] using a PrimeSTAR Mutagenesis Basal Kit (Takara). The EhRab8A myc-tagged EhRab8-expressing strain, and strains expressing dominant-active and -negative EhRab8A mutants were established by transfection of the HM-1: IMSS cl-6 strain by liposome-mediated transfection, as previously described [26]; stable transformants were cultured in the medium containing 10 μg/ml Geneticin (Life Tech Oriental). Moreover, I established a control mock transformant by transfection of the HM-1: IMSS cl-6 strain with pKT-3M.

2-3-3 Production of recombinant EhRab8A

In order to generate a plasmid expressing the maltose-binding protein (MBP)-fused EhRab8A-recombinant protein, the full-length EhRab8A open reading frame (ORF) was inserted into the pMAL-c2X vector (New England Biolabs, Ipswich, MA, USA). Recombinant protein was expressed using the *E. coli* strain BL21 (DE3) and purified

according to the manufacturer's instructions.

2-3-4 Antibodies

Antiserum was commercially raised against recombinant EhRab8A, EhBiP, EhSec13, or EhRab11B in rabbits (Eurofins Genomics, Japan). The EhRab8A IgG was further purified with HiTrap NHS-activated HP column (Amersham) coupled with purified recombinant MBP-conjugated-*Eh*Rab8A according to the manufacturer's instruction. The antiserum was purified using a HiTrap protein G kit (GE Healthcare).

2-3-5 Phagocytosis assay

Phagocytosis was assayed by microscopy. The myc-EhRab8A dominant-negative mutant-expressing cells were attached to slide glass and engulfed with PKH26-labeled (Sigma) erythrocytes for 20 min. The samples were fixed, stained with an anti-myc antibody, and visualized with an anti-mouse Alexa 488 antibody, as described in the following indirect immunofluorescence assay. The fluorescent intensity of the PKH26 signal in the trophozoites stained with the anti-myc antibody was captured on a Carl-Zeiss LSM780 confocal laser-scanning microscope, and the images were analyzed with ZEN software (Zeiss).

2-3-6 Subcellular fractionation

The accessibility by proteinase K assay was performed as reported previously with some modifications [28]. Approximately 3 x 10^5 amoeba cells were washed with cold PBS containing 2% glucose, resuspended in homogenization buffer (250 mM sucrose, 50 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mg/ml E-64), and homogenized on ice with 30 strokes by a Dounce homogenizer with a tight fitting pestle. After unbroken cells were removed by centrifugation at 400 x *g* for 2 min, the supernatant was centrifuged at 13,000 x *g* at 4°C for 10 min to obtain the pellet (p13) and supernatant (s13) fractions. The s13 fraction was further separated by centrifugation at 100,000 x *g* at 4°C for 1 h

to obtain soluble (s100) fractions. These fractions were subjected to immunoblot analyses with anti-EhRab8A, anti-CPBF1 [45], or anti-ICP1 antibodies [46].

2-3-7 Indirect immunofluorescence assay

An indirect immunofluorescence assay was conducted, essentially as previously described [26]. Trophozoites were transferred to 8-mm round Ills on a slide glass, fixed, permeabilized, and reacted with an anti-myc 9E10 monoclonal antibody (Santa Cruz Biotech) to detect myc-tagged *Eh*Rab8A wild-type and myc-tagged *Eh*Rab8A-GDP and GTP mutants, anti-EhRab11B (this study), anti-EhVps26 [27], anti-EhBiP (this study), anti-EhSec13 (this study) polyclonal rabbit sera.

2-4 Results

2-4-1 Establishment of a myc-tagged EhRab8-expressing strain

The transformant cells expressing EhRab8A with the amino-terminal myc-tag were established and subjected to immunoblots using anti-EhRab8A and anti-myc antibodies (Fig. 1A). Two bands were observed in the mock control using anti-EhRab8A antibody; the top (23.8 kDa, red asterisk) and bottom (22.8 kDa, black asterisk) bands likely corresponded to modified and non-modified endogenous EhRab8A. In myc-EhRab8A-expressing cells, four bands were observed; the two top bands (27.2 kDa; red cross, and 26.4; black cross) corresponded to the exogenously expressed myc-tagged EhRab8A with or without modification(s) whereas the bottom two bands correspond to the endogenous EhRab8A. The nature of the modification remains unknown. I examined the nature (membrane-bound or soluble) of two forms of EhRab8A by differential centrifugation. The 22.8- and 26.4-kDa bands were recovered in the pellet fraction after centrifugation at 13,000 × *g* together with a transmembrane protein CPBF1 [45], while the 23.8- and 27.2-kDa bands were found in the soluble fraction of centrifugation at 100,000 × *g*, together with a cytosolic soluble protein ICP1 [46] (Fig. 1B). The amount of exogenously expressed

myc-EhRab8A protein was 2.1 ± 0.64-fold higher than that of the endogenous protein (Fig. 1A).

2-4-2 Overexpression of wild-type Rab8A enhanced phagocytosis whereas a dominant-negative Rab8A mutant inhibited phagocytosis

The previous proteomic study of isolated phagosomes has revealed that 14 amebic Rab proteins are localized to phagosomes [35,36]. EhRab8A was analyzed as an important factor for the phagocytosis, and it was involved in the trafficking of proteins to the ER. To confirm if Rab8A functions for the phagocytosis by regulating the surface proteins, I established Rab8A overexpressed strain and dominant-negative/active strain and examine the phagocytosis efficient.

Nucleotide-fixed state mutations can be engineered to alter the activity of Rab proteins [2]. Two mutants of myc-EhRab8A, a GTP-fixed Q66L mutant and a GDP-fixed S21N mutant, were generated and introduced into the ameba trophozoites. One of the two mutants, in which Gln in the second GTP-binding motif was replaced with Leu, was designed to have reduced GTPase activity and regarded as a constitutively active (GTP-fixed) mutant (Q66L) [2,47]. The other mutant possessing a Ser-to-Asn mutation in the first GTP binding motif is assumed to possess a lower affinity for GTP than for GDP and function as a constitutively negative (GDP-fixed) form (S21N) [48]. I quantified the amount of fluorescein-labeled erythrocytes ingested by the trophozoites expressing either wild-type myc-EhRab8A or two mutant forms of myc-EhRab8A (Fig. 2A and B). The intensity of fluorescence reflected the amount of ingested erythrocytes significantly increased in wild-type myc-EhRab8A-expressing cells compared to the mock control (160% ± 17%; $p < 0.02$, Fig. 2B). In contrast, expression of the GTP-fixed mutant had a negative effect on erythrophagocytosis (68% ± 4.3% compared to the control; $p < 0.01$). Furthermore, the GTP-fixed mutant transformant exhibited growth retardation compared with mock control, the transformants expressing wild-type EhRab8A or GDP-fixed mutant (Fig. 2C) (mock;

18.3 ± 1.03 hr, wild-type EhRab8A, 17.8 ± 0.95 hr; GDP-fixed mutant, 17.6 ± 1.06 hr; GTP-fixed mutant; 22.4 ± 0.71 hr, $p < 0.03$ between GTP-fixed mutant and each of mock, GDP-fixed mutant transformant, or wild-type). Neither positive nor negative effect of the GDP-fixed mutation on erythrophagocytosis was observed.

2-4-3 EhRab8A localized to the ER, but neither to the trans-Golgi network nor lysosomes

EhRab8A is homologous to yeast Sec4p and human Rab8, with 49%–53% amino acid identity [14,32] (Fig. 3). Yeast Sec4p and human Rab8 are known to be involved in trafficking from the trans-Golgi network (TGN) to the PM [8,49]. Further, colocalization of Rab8 with Rab11, which is the upstream regulator of Rab8, has also been reported [50,51]. Thus, I examined whether EhRab8A was colocalized with EhRab11B, which was previously demonstrated to be on the TGN [31]. An indirect immunofluorescence assay of the myc-EhRab8A cells conducted using anti-EhRab8A and anti-myc antibodies revealed high colocalization (Pearson's correlation coefficient: $R = 0.83$) (Fig. 4A), indicating that endogenous EhRab8A and exogenously expressed myc-EhRab8A were localized to the same organelle(s)/compartments. EhRab8A did not colocalize with EhRab11B, which was visualized with an anti-EhRab11B antibody [31] ($R = 0.24$, Fig. 4B). Furthermore, EhRab8A did not colocalize with EhVps26, one of the retromer component that is involved in the recycling of receptors in the TGN and the endosomal compartment and well colocalized with the late endosomal marker EhRab7A [27,52,53] ($R = 0.05$, Fig. 5A). Localization of EhRab8A also clearly differed from that of EhRab7B, which is localized to lysosomes [28] (Fig. 5B). This observation was in contrast to other organisms [40,51].

Next, to find out the organelle compartment where EhRab8A is localized on, I accomplished the Percoll fractionation. It is possible to separate organelle compartments physically by two rounds of discontinuous Percoll gradient

ultracentrifugation[19], followed by immunoblot assay, in which the distribution pattern of EhRab8A was similar to that of Sec13 (EHI_001050, 38% amino-acid identity to human Sec13, E-value = 6e-46) [5], a component of the COPII vesicle (Fig. 6). Differently from this observation, EhRab8A was fractionated in a distinct fraction from Cpn60, the chaperonin family protein on the mitosome. It suggests that EhRab8A is localized on the ER. In mammals, Rab10, which shares 70% amino acid identity to Rab8 [13,54] (Fig. 3), is known to localize to endosomes (e.g., in Madin–Darby canine kidney epithelial cells) [55,56]. In addition, Rab10 is localized to the ER in *Xenopus laevis* egg, COS-7, and HeLa cells [57], which is consistent the result that EhRab8A localizes to the ER.

I next confirmed EhRab8A localization using antibodies raised against bacterial recombinant proteins of EhSec13 and the ER luminal chaperone EhBiP (EHI_199890, 64% amino acid identity to human BiP, E-value = 0) [58]. A subdomain of the ER, where COPII vesicle budding occurs, was named the ER exit site (ERES) [59], and it was visualized with the anti-EhSec13 antibody. EhSec13 did not colocalize with EhRab8A (R = 0.15, Fig. 7A). Next, an indirect immunofluorescence assay performed using the anti-EhBiP antibody revealed partial colocalization of myc-EhRab8A and EhBiP, suggesting that EhRab8A was localized to the ER or a compartment adjacent to the ER (R = 0.7, Fig. 7B).

Confocal fluorescence micrographs revealed that the localization of myc-EhRab8A Q66L mutant was similar to that of wild-type EhRab8A, and also well colocalized to EhBiP, whereas S21N mutant revealed dispersed cytosolic staining like the Rab8 SN mutant in other organisms [60,61] (Fig. 8). The localization of Q66L and S21N mutants is consistent with presumed characteristics of GTP- and GDP-fixed mutants. Expression of GTP- or GDP-fixed mutants did not affect the ER morphology, which is visualized with anti-BiP antibody.

2-5 Discussion

2-5-1 ER-localized EhRab8A is required for phagocytosis

In this study, I have shown using two genetic approaches that in *E. histolyitca* unique ER-localized EhRab8 is involved in phagocytosis. I have shown that overexpression of the wild-type or GTP-fixed mutant of EhRab8A enhanced or reduced phagocytosis, respectively (Fig. 2). Rab8 is a member of the highly conserved Rab subfamily in eukaryotes, including Metazoa (*Caenorhabditis elegans*, *Nematostella vectensis*), Choanozoa (*Monosiga brevicollis*), Fungi (*Saccharomyces cerevisiae*), Amoebozoa (*Dictyostelium discoideum*), Viridiplantae (*Arabidopsis thaliana*), Stramenopiles (*Phytophthora sojae*), Ciliophora (*Tetrahymena thermophila)*, and Chizopyrenida (*Naegleria gruberi*), but missing in Diplomonadida (*Giardia lamblia*) and Trichomonadida (*Trichomonas vaginalis*) [13,15,62]. In mammals and yeasts, Rab8 is involved in trafficking from the TGN to the PM [3]. In free-living *D. discoideum*, Rab8 is localized to a contractile vacuole, which is a specialized osmoregulatory organelle involved in water expulsion [63]. A line of evidence provided in this study suggests that EhRab8A plays a unique function in *Entamoeba*: EhRab8A is involved in the ameba's adherence to prey by mediating the transport of candidate receptor(s) to the plasma membrane. Until now, many ER proteins including Rab1 have been identified in proteome analyses of macrophage phagosomes [23,25]; the results suggested that the ER may supply membranes to the plasma membrane to engulf large foreign particles and to recruit MHC class I molecules to phagosomes [64,65]. Although it remains to be debated whether the ER is involved in phagocytosis, more specifically the ER provides membrane to newly-formed phagocytosis in *E. histolytica*, several ER proteins were identified from purified phagosomes by previous proteomic analyses in *E. histolytica*: calreticulin, BiP [66], and COPII components [67]. These data are consistent with the notion that the amoebic ER is involved in progression of phagocytosis. It has been previously shown that an ER luminal protein, calreticulin, is present on the amoebic cell surface

and involved in erythrophagocytosis, suggesting that the ER and the plasma membrane is dynamically interconnected in *E. histolytica* [68,69].

2-5-2 Lack of the Rab11-Rab8 cascade for transport from the TGN to the PM in *E. histolytica*

In the mammalian cells, Rab8 functions with Rab11 and Rabin8, which is a GEF of Rab8, as a Rab11-Rabin8-Rab8 cascade [3]. In contrast, in *E. histolytica*, EhRab8A and EhRab11B were not colocalized (Fig. 4), indicating that EhRab8A and EhRab11B regulate non-overlapping steps of the same pathway or independent pathways. It was reported that EhRab11B regulates cysteine protease transport from the TGN to the plasma membrane in *E. histolytica* [31]. These data suggest that the role and its regulation of Rab8 in *Entamoeba* are highly divergent from that in Opisthokonts. Identification and characterization of effector proteins (and upstream GEF) of EhRab8A, which is underway, may help elucidate the evolution of Rab8-mediated membrane trafficking in divergent eukaryotes.

2-5-3 EhRab8A may be involved in a non-conventional traffic pathway from the ER to the plasma membrane

The absence of EhRab8A at the ERES (Fig. 7) was noteworthy. In general, cargo proteins transported to the Golgi are recognized by p24 families of proteins and a component of COPII, Sec24 protein, and then packed into the COPII vesicles in the ERES [70,71]. During formation of the COPII vesicle, a member of the Arf family of GTPases called Sar1, together with the p24 families of proteins, plays a pivotal role in cargo sorting into COPII vesicles in the ER [72]. Thus, my data suggested that the cargo sorting in a pathway regulated by EhRab8A differed from the canonical mechanism for the COPII vesicle formation mediated by the p24 family and Sec24.

Human Rab10 is closely related to Rab8 and localized to the ER (70% amino acid identity, Fig. 3), and forms a specialized domain linked to phospholipid synthesis

involving phosphatidylinositol synthase (PIS) and ethanolamine phosphotransferase 1 (CEPT1) [57]. It has also been shown that expression of a mutant Rab10 or depletion of endogenous Rab10 reduces ER tubular extension, likely attributable to a disturbance of the Rab10-associated enzymes involved in phospholipid biosynthesis in the ER subdomain [57]. The *E. histolytica* genome encodes both PIS and CEPT (EHI_069630 for CDP-DAG inositol 3-phosphatidyltransferase; EHI_148580 and EHI_152340 for ethanolamine/choline kinase). Thus, it is plausible that EhRab8A regulates the transport of plasma membrane proteins via the lipid biosynthesis. My preliminary co-immunoprecipitation of EhRab8A identified several lipid metabolizing enzymes, which also supports a premise that EhRab8A is involved in lipid/phospholipid biosynthesis and transfer.

2-6 Figure Legends

Fig. 1. Subcellular fractionation of EhRab8A

A. Immunoblot analysis of endogenous (wild-type) and myc-EhRab8A detected by anti-EhRab8A (left) and anti-myc (right) antibodies. In mock cells, two bands corresponding to endogenous unmodified (a black asterisk) and modified EhRab8A (a red asterisk) were detected. In myc-EhRab8A transformant cells, unmodified (black crosses) and modified myc-tagged EhRab8A (red crosses) were detected. B. Immunoblot analysis of EhRab8A in fractionated cell lysates. Lysates from myc-EhRab8A transformant cells were fractionated by centrifugation into the low-speed pellet (p13, the pellet fraction of 13,000 x *g* centrifugation), the high-speed pellet (p100, the pellet fraction of 100,000 x *g* centrifugation), and the supernatant fractions (s100, the supernatant fraction of 100,000 x *g* centrifugation). These fractions were subjected to immunoblot analysis using anti-EhRab8A, anti-CPBF1 (a membrane protein), and anti-ICP1 (a cytosolic protein) antibodies. Note that two bands corresponding to endogenous unmodified (a black asterisk) and myc-tagged unmodified EhRab8A (a black cross) were fractionated to the membrane fractions

(p13 and p100), and the other two bands corresponding to endogenous modified (a red asterisk) and myc-tagged modified EhRab8A (a red cross) were partitioned to the cytosolic fraction (s100).

Fig. 2. Expression of EhRab8A GTP-bound mutant caused a defect in phagocytosis.

A. Immunofluorescence imaging of EhRab8A and erythrophagocytosis. Transformant trophozoites expressing myc-EhRab8A wild-type or myc-EhRab8A-Q66L mutant, and mock transformant cells were mixed with PKH-labeled erythrocytes for 20 min at 37°C and reacted with an anti-myc antibody in an immunofluorescence assay. B. Efficiency of erythrophagocytosis by the transformants expressing myc-EhRab8A wild-type, myc-EhRab8A-Q66L, or myc-EhRab8A-S21N mutant, and mock transformant. The total fluorescence intensities of the PKH26-labeled erythrocytes ingested by a single trophozoite were measured, and the average of the total fluorescence intensities per trophozoite for each strain are shown. Note that expression of myc-EhRab8A wild-type and the myc-EhRab8A–Q66L mutant enhanced and reduced erythrophagocytosis, respectively. C. The population doubling time of myc-EhRab8A wild-type, myc-EhRab8A-Q66L, myc-EhRab8A-S21N mutant, and mock transformant cells. The correlation coefficients

Fig. 3. Sequence alignment and identity

A. Sequence alignment of EhRab8A, EhRab11B, *Homo sapiens* Rab8A, Rab10, and *Saccharomyces cerevisiae* Sec4p. Amino acids sequences were aligned with the ClustalW (ver 2.1). The GTP-binding consensus region and switch I and II regions, which are involved in the interaction with effectors, are shown in the yellow box and red lines, respectively. B. Percentage identity of EhRab8A, EhRab11B, *Homo sapiens* Rab8A, Rab10, and *Saccharomyces cerevisiae* Sec4p. Amino acid percentage identity matrix was created by ClustalW.

Fig. 4. Lack of colocalization of exogenously expressed myc-EhRab8A and the trans-Golgi network marker

Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and endogenous EhRab8A, stained with anti-EhRab8A (A, red), anti-EhRab11B antibodies (B, red). Histograms of the green and red intensities along the red lines are shown at the bottom left of panels A and B. Major peaks of green, red, and merged signals are yellow. The lengths of the lines are also shown. R = Pearson's correlation coefficient. Bars, 5 μm.

Fig. 5. Lack of colocalization of exogenously expressed myc-EhRab8A and the endosome and lysosome markers

Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and the endosome, stained with anti-EhVps26 antibody (A, red), and the lysosome, stained with lysotracker (B, red). Histograms of the green and red intensities along the red lines are shown at the bottom left of panels A and B. Major peaks of green, red, and lysosome depicted by white arrows. ßThe lengths of the lines are also shown. R = Pearson's correlation coefficient. Bars, 5 μm.

Fig. 6. Percoll gradient fractionation of EhRab8A

Homogenate prepared from wild-type cells was fractionated by two series of Percoll gradient ultracentrifugation according to the previous report [19], and subjected to immunoblot using anti-Cpn60 (mitosome), anti-EhRab8A, and anti-Sec13 antibodies, respectively. First Percoll fractions which is positive for Cpn60 were subjected to the 2nd Percoll fractionation. Membrane-bound Sec13 was fractionated to J to L, and Cpn60 was detected from the L to N fractions. EhRab8A was co-fractionated with Sec13 positive fraction (J and K).

Fig. 7. Colocalization of exogenously expressed myc-EhRab8A and

endoplasmic reticulum markers

Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green), and the anti-EhSec13 (A, red) or anti-EhBiP (B, red) antibodies. Histograms of the green and red intensities along the lines indicated in the islets on the right bottom of each image are shown at the bottom left of panels A–B. Major peaks of green, red, and merged signals are depicted by arrows. The lengths of the lines are also shown. In the bottom right of panels A–B, scatter plots showing the results of the colocalization analyses are shown. R = Pearson's correlation coefficient. Bars, 5 μm.

Fig. 8. Subcellular localization of myc-EhRab8A wild-type and mutants.

The GTP-bound Q66L or GDP-bound S21N mutants were expressed under the regulation of the constitutive cysteine synthase promoter. Exogenous myc-EhRab8A was stained with the anti-myc 9E10 antibody. GTP-bound myc-EhRab8A Q66L was localized to the endoplasmic reticulum stained with the anti-BiP antibody, and the patter was similar to that of the wild-type myc-EhRab8A. GDP-bound myc-EhRab8A S21N showed cytosolic staining.

Figure 1

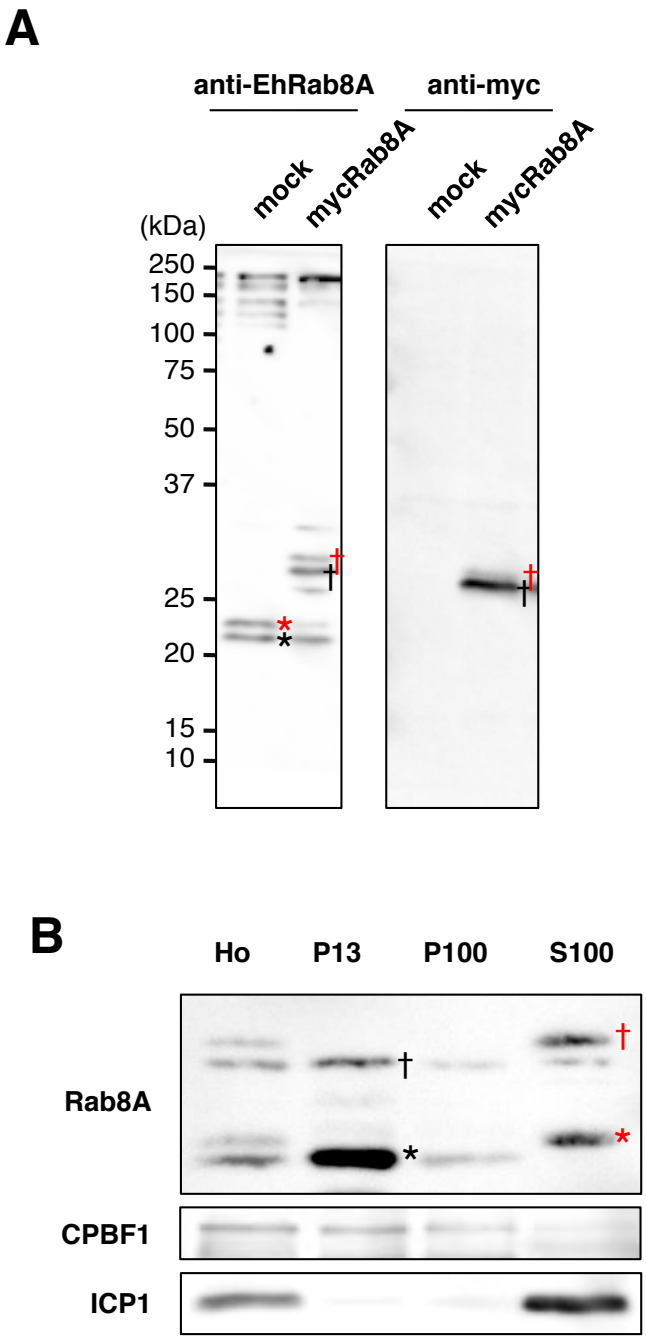
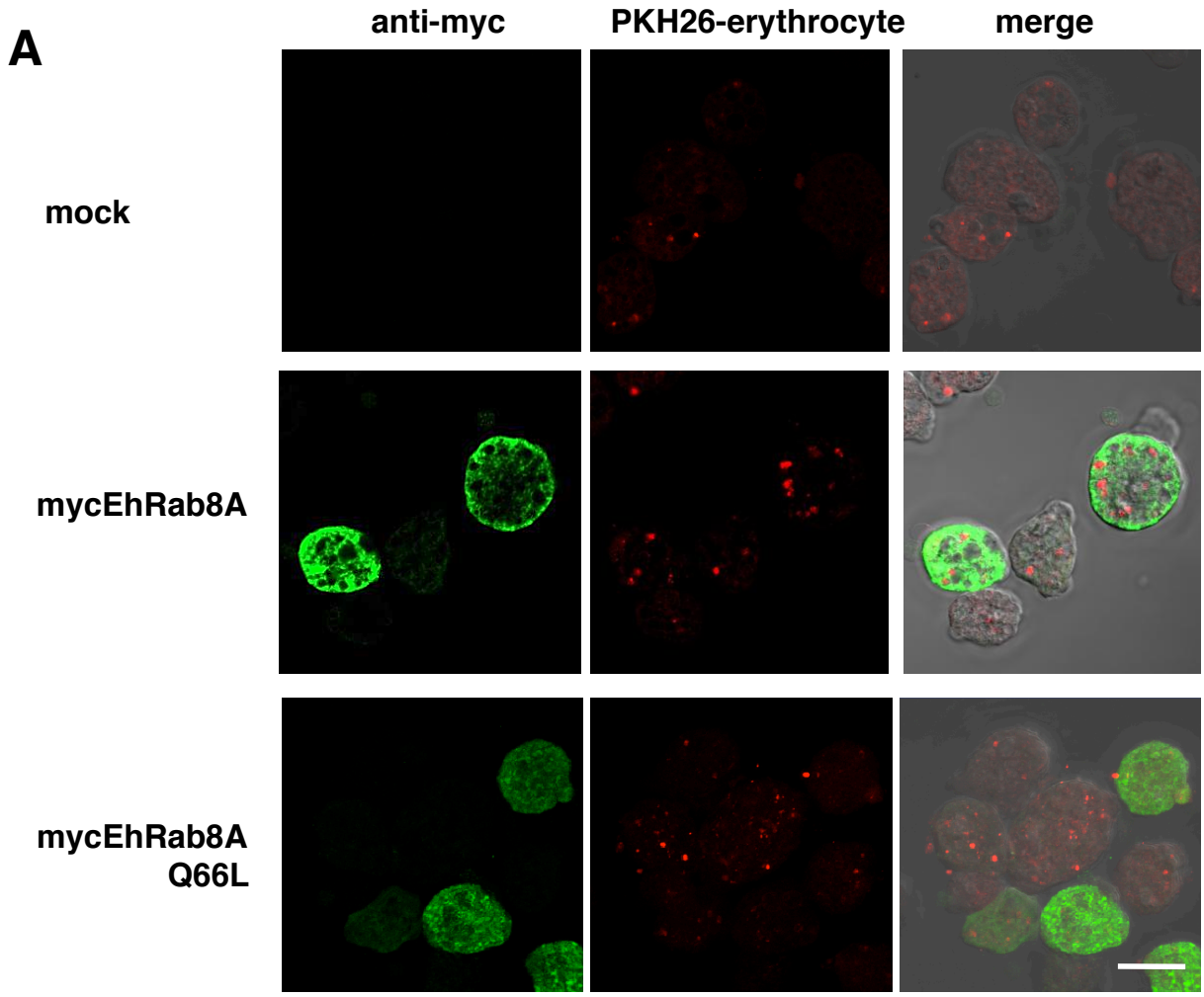
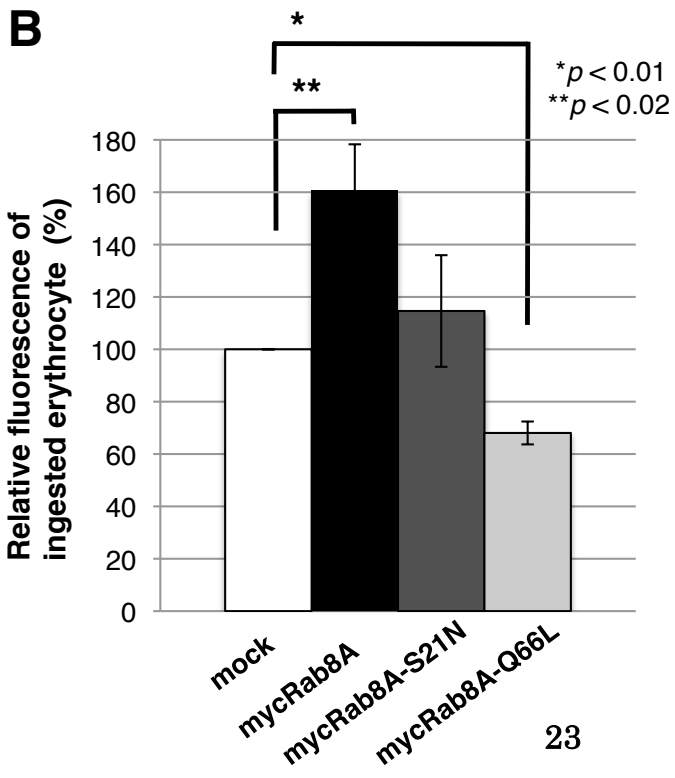

A
anti-EhRab8A
anti-myc
mock
mycRab8A
mock
mycRab8A
(kDa)
250
150
100
75
50
37
25
20
15
10
B
Ho
P13
P100
S100
Rab8A
CPBF1
ICP1

Figure 2


A
anti-myc
PKH26-erythrocyte
merge
mock
mycEhRab8A
mycEhRab8A Q66L


B
*
**
*p < 0.01
**p < 0.02
Relative fluorescence of ingested erythrocyte (%)
180
160
140
120
100
80
60
40
20
0
mock
mycRab8A
mycRab8A-S21N
mycRab8A-Q66L

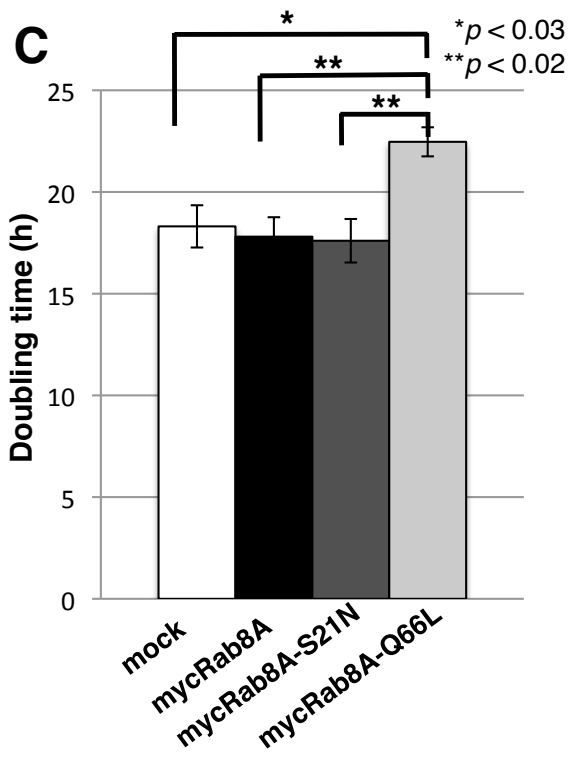
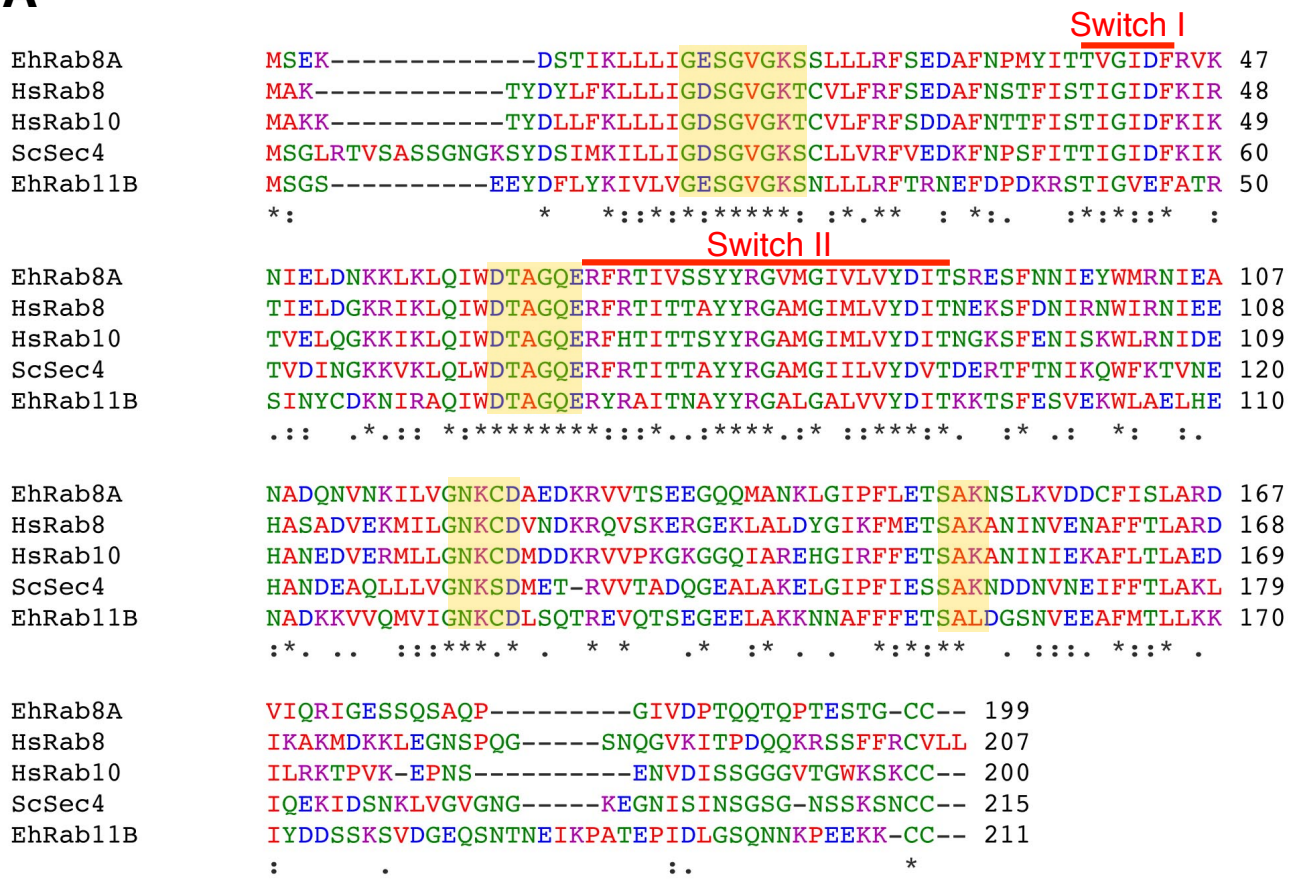

C
*
**
**
*p < 0.03
**p < 0.02
Doubling time (h)
25
20
15
10
5
0
mock
mycRab8A
mycRab8A-S21N
mycRab8A-Q66L

Figure 3

A



B

	EhRab8A	HsRab8	HsRab10	ScSec4	EhRab11B
EhRab8A	100				
HsRab8	53	100			
HsRab10	52	70	100		
ScSec4	49	52	55	100	
EhRab11B	40	41	42	40	100

Figure 4

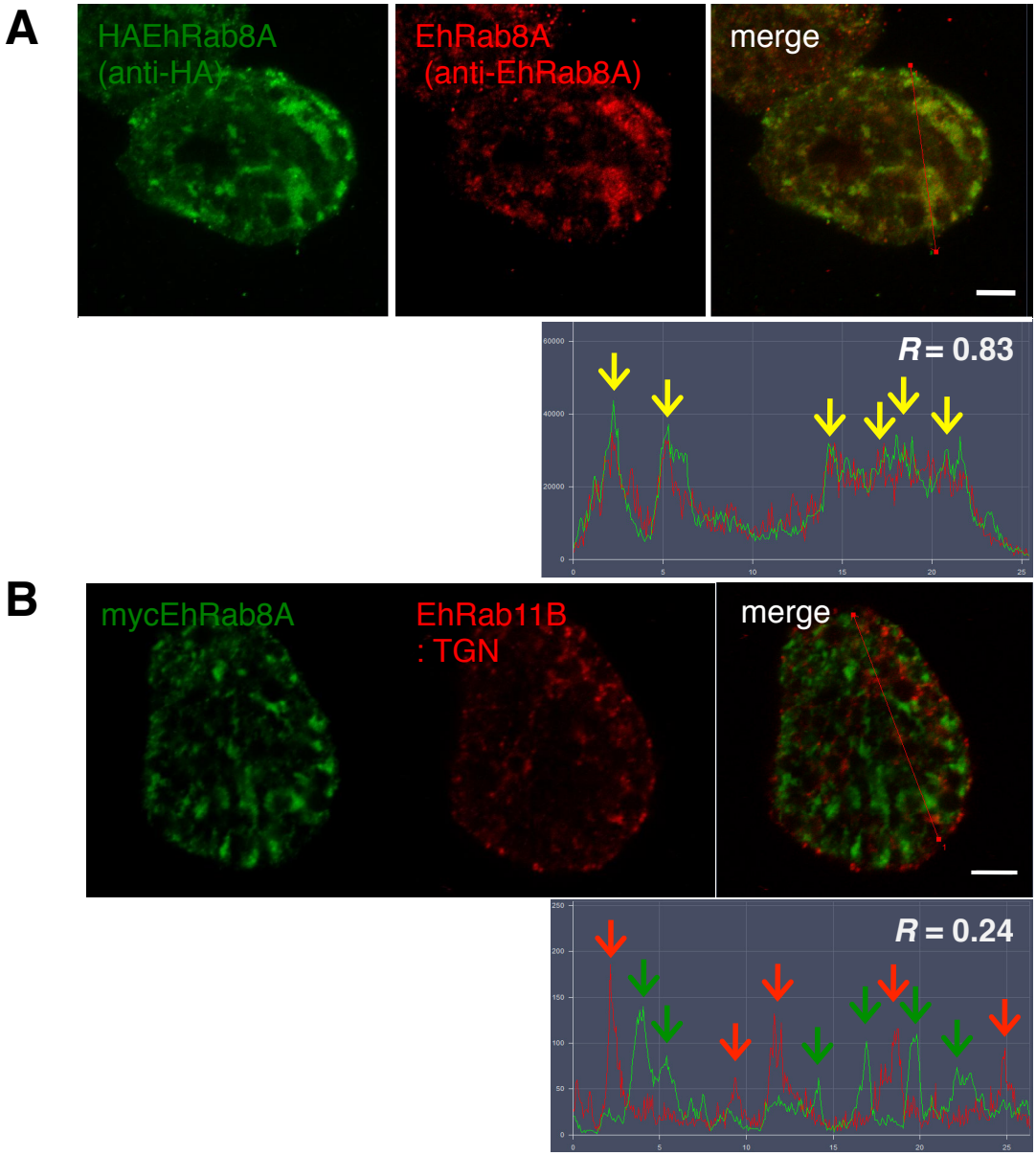

A
HAEhRab8A
(anti-HA)
EhRab8A
(anti-EhRab8A)
merge
R = 0.83
B
mycEhRab8A
EhRab11B
: TGN
merge
R = 0.24

Figure 5

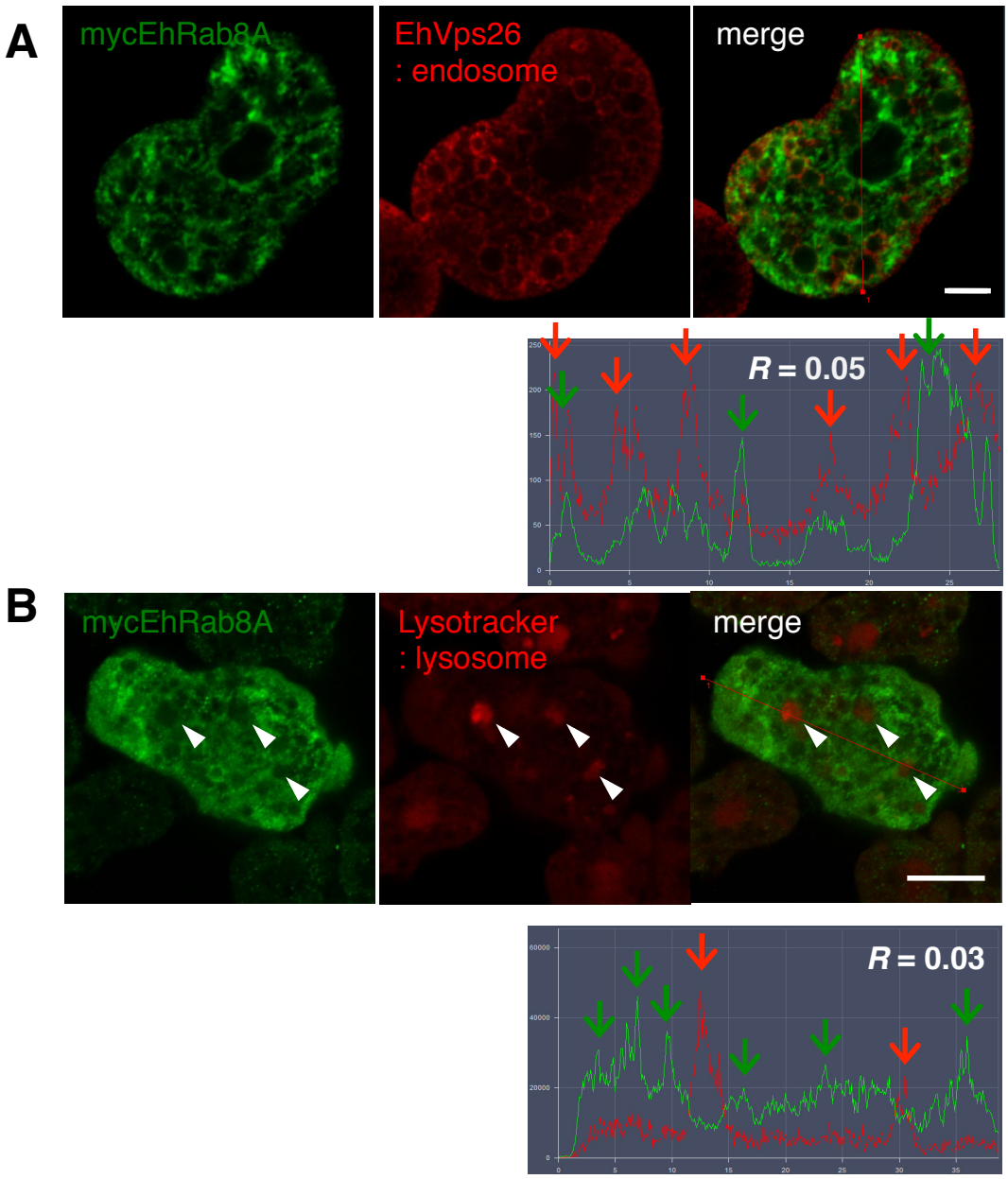
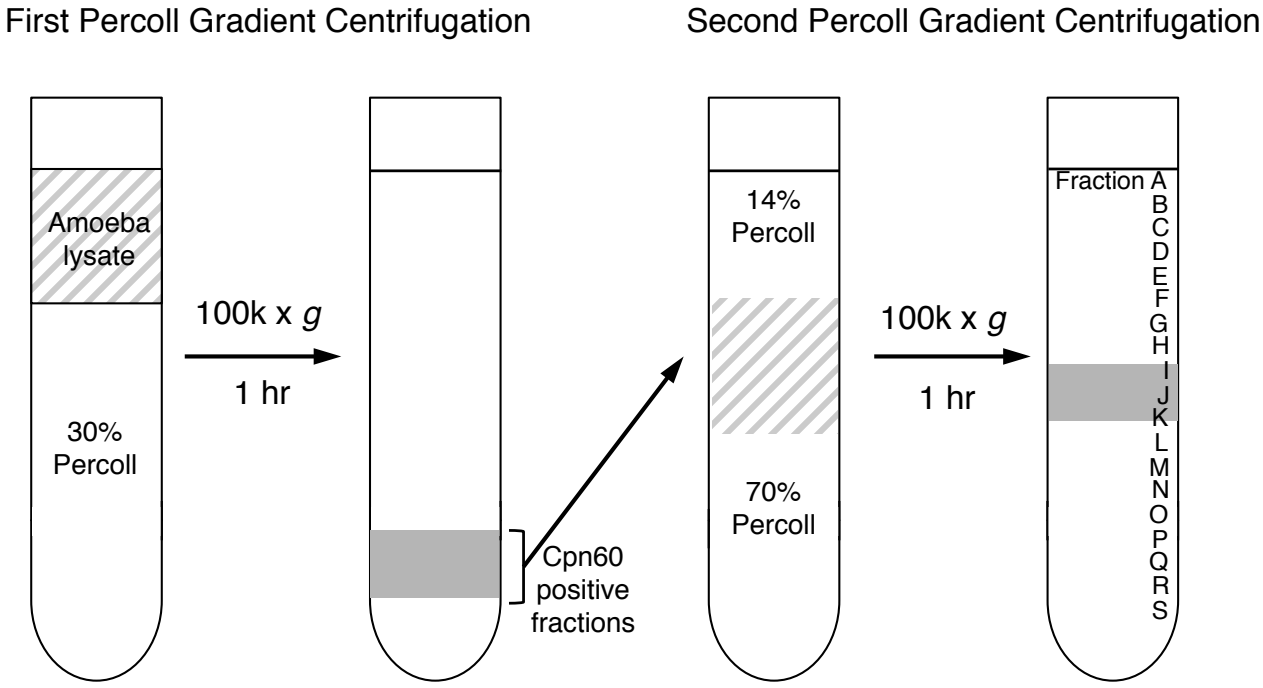

A
mycEhRab8A
EhVps26
: endosome
merge
R = 0.05
B
mycEhRab8A
Lysotracker
: lysosome
merge
R = 0.03

Figure 6



2nd Percoll

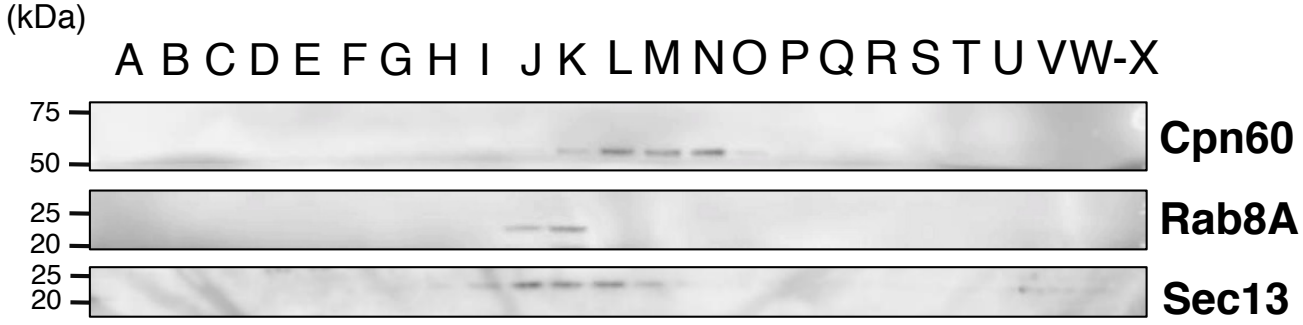


Figure 7

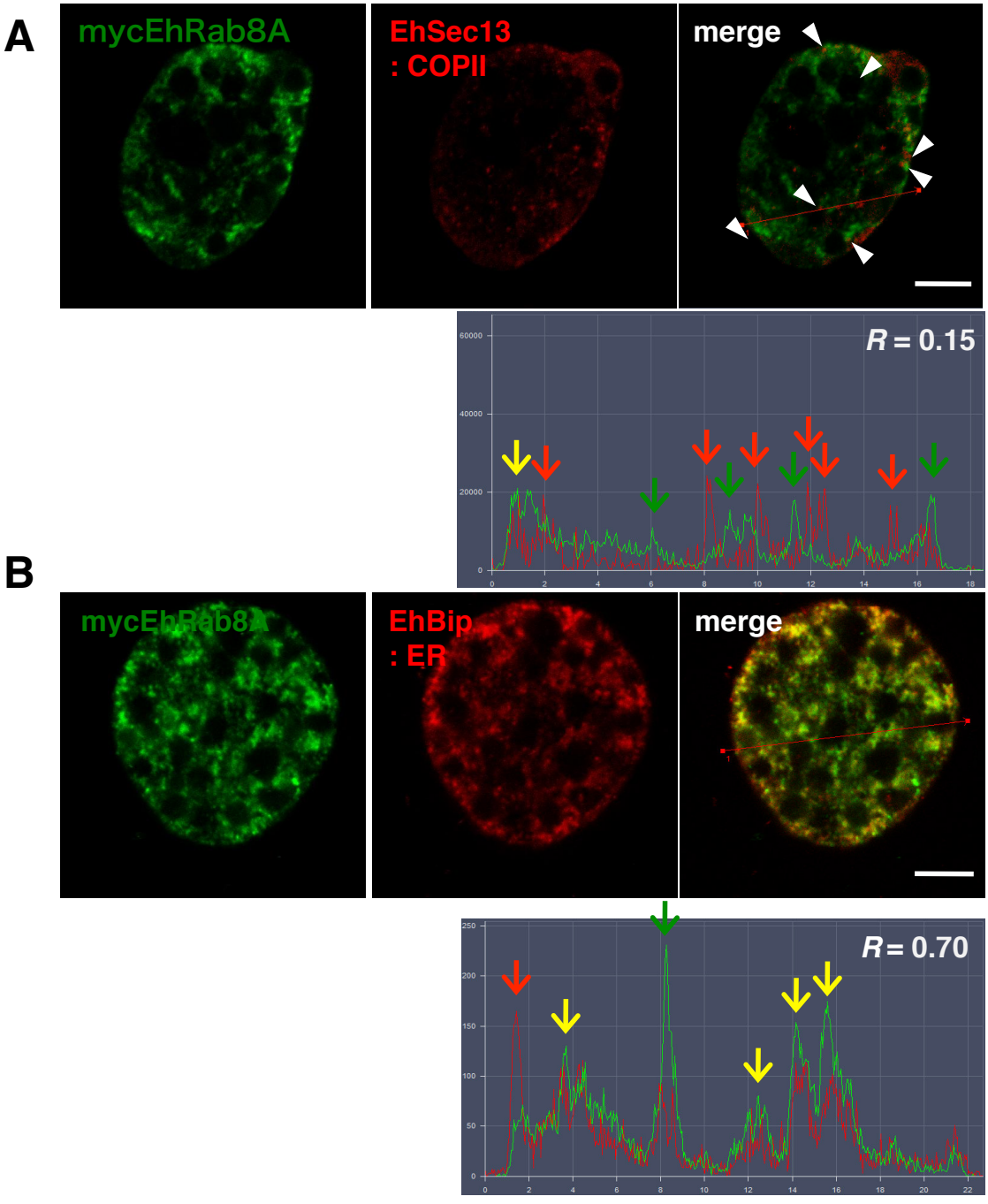
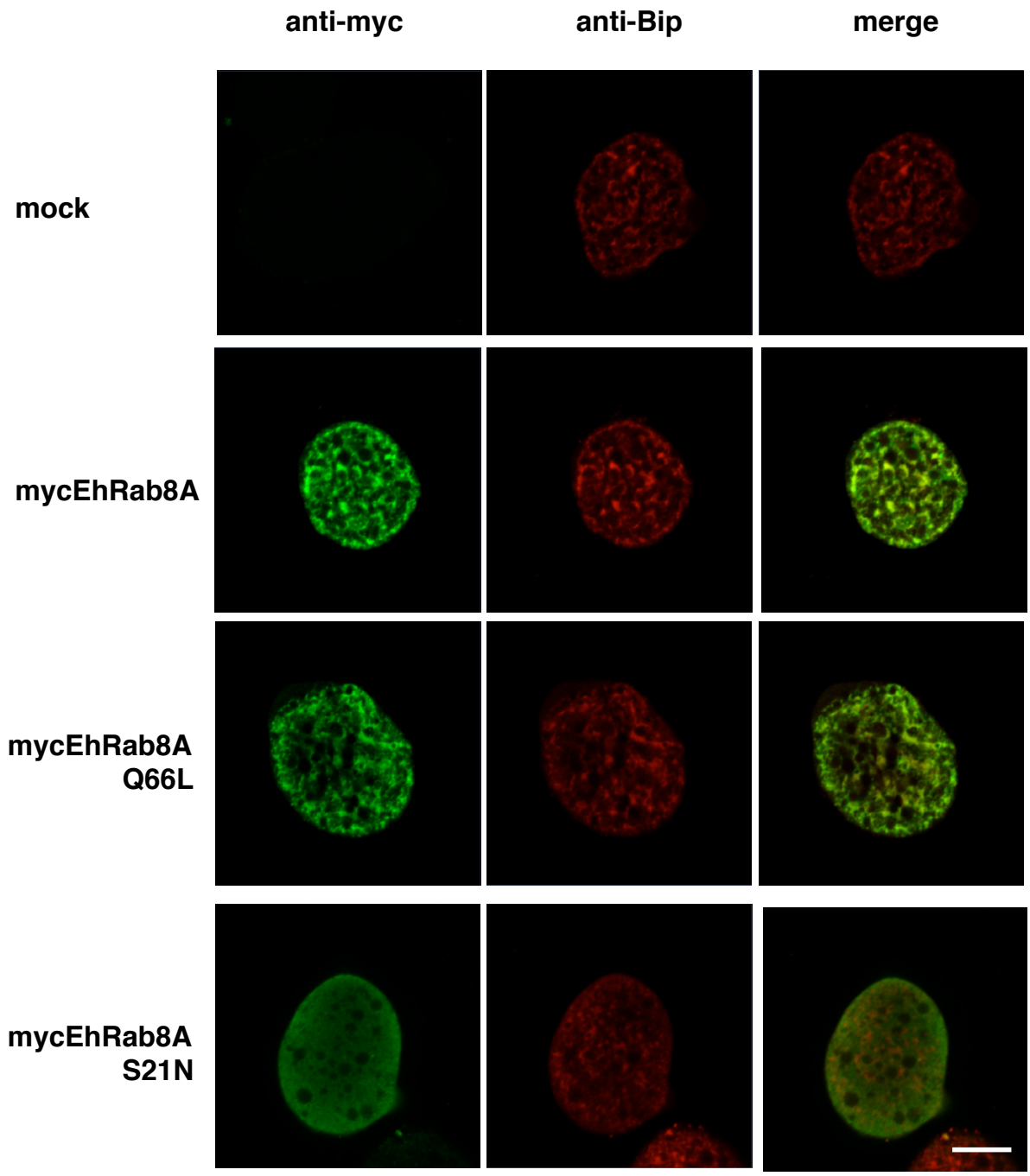

A
mycEhRab8A
EhSec13
: COPII
merge
R = 0.15
B
mycEhRab8A
EhBip
: ER
merge
R = 0.70

Figure 8


anti-myc
anti-Bip
merge
mock
mycEhRab8A
mycEhRab8A Q66L
mycEhRab8A S21N

Chapter 3. Identification of EhCdc50 as binding protein of EhRab8A

3-1 Abstract

Membrane traffic plays a pivotal role in virulence in the enteric protozoan parasite *Entamoeba histolytica*. EhRab8A small GTPase is a key regulator of membrane traffic at the endoplasmic reticulum (ER) of this protist and is involved in the transport of plasma membrane proteins. Here I identified the binding proteins of EhRab8A. The Cdc50 homolog, a non-catalytic subunit of lipid flippase, was identified as an EhRab8A binding protein candidate by affinity coimmunoprecipitation. Binding of EhRab8A to EhCdc50 was also confirmed by reciprocal immunoprecipitation and blue-native polyacrylamide gel electrophoresis, the latter of which revealed an 87 kDa complex. Indirect immunofluorescence imaging with and without Triton X100 showed that endogenous EhCdc50 localized on the surface in the absence of permeabilizing agent but was observed on the intracellular structures and overlapped with the ER marker Bip when Triton X100 was used. Overexpression of N-terminal HA-tagged EhCdc50 impaired its translocation to the plasma membrane and caused its accumulation in the ER. As reported previously in other organisms, overexpression and accumulation of Cdc50 in the ER likely inhibited surface transport and function of the plasma membrane lipid flippase P4-ATPase. Interestingly, HA-EhCdc50-expressing trophozoites gained resistance to miltefosine, which is consistent with the prediction that HA-EhCdc50 overexpression caused its accumulation in the ER and mislocalization of the unidentified lipid flippase. Similarly, EhRab8A gene silenced trophozoites showed increased resistance to miltefosine, supporting EhRab8A-dependent transport of EhCdc50. This study demonstrated for the first time that EhRab8A mediates the transport of EhCdc50 and lipid flippase P4-ATPase from the ER to the plasma membrane.

3-2 Introduction

Identification of binding proteins of Rab GTPases often provides necessary clues to the mechanisms of trafficking pathways [73]. A plethora of binding proteins and effectors for most Rab GTPases have been studied to elucidate the molecular mechanism of the Rab-mediated trafficking in human and major model organisms [3]. For example, Rab11 and Rab8 coordinately control trafficking from the trans-Golgi to the plasma membrane in mammalian cells together with their effector proteins, Rab11-family interacting proteins (FIPs) and Rabin8 (Rab8-guanine nucleotide exchange factor). Neurite outgrowth requires a cascade of Rab11, Rabin8 and Rab8 activities in the recycling endosomes [74]. In ciliary membrane trafficking, Rab11 and Rab8 sort rhodopsin at the trans-Golgi membrane and transport rhodopsin to the periciliary membrane of photoreceptor cells followed by the Golgi recruitment of FIP3 and Rabin8 [75]. In the social amoeba *Dictyostelium discoideum*, intracellular osmoregulation is controlled by contractile vacuole fusion with the plasma membrane under hypotonic conditions; and this process is regulated by the serial recruitment of Rab11, Rab11 effector, Rab8 and Rab8-GTPase activating protein on the contractile vacuole membrane [76]. Thus, the Rab11 and Rab8 cascade, which is mediated by Rab8 effector proteins, is conserved in many tissues and organisms. Other effector protein is also reported in yeast Rab8 homolog, Sec4p. GTP-bound active form of Sec4p interacts with Sro7, a member of the structurally conserved tomosyn/Sro7 family. Sro7 functions as a Sec4 effector that helps to tether post-Golgi vesicles to the plasma membrane together with SNARE complex, followed by membrane fusion [77]. Transport pathway of EhRab8A was identified, however its function or cargo proteins have not been clarified. It is crucial to find out the binding protein of EhRab8A to elucidate the function.

Cdc50, which is a non-catalytic subunit and form heteromeric complexes with P4ATPase, is conserved protein in eukaryotes. Members of the P4 subfamily of P-type

ATPases catalyze phospholipid transport and create membrane lipid asymmetry [78]. The Cdc50-P4ATPase complex is transported from the ER to the TGN, then delivered to the late secretory and endocytic compartments, and the plasma membrane. According to the previous report in eukaryotes, the trafficking pathway of the Cdc50-P4ATPase complex is COPII dependent as well as other proteins, and there is no report that Cdc50 and P4ATPase interact with Rab GTPases directly.

3-3 Materials and Methods

3-3-1 Antibodies

The anti-EhBip, anti-EhRab8A, anti-EhVps26 [27] antibodies used were described previously. The anti-HA antibody clone 16B12 and anti-Myc clone 9E10 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-EhCdc50 antibody was produced as described below.

3-3-2 Creation of *E. histolytica* transformant lines

A plasmid expressing N-terminal 3HA-tagged EhCdc50 (HA-EhCdc50) was constructed as follows. A 966-base pair DNA fragment containing the EhCDC50-coding sequence was amplified by PCR from *E. histolytica* genomic DNA using the following oligonucleotides: 5′-CCCGGGATGTCAGAGAAAGTAAAGGGACTTG-3′ and 5′-CTCGAGTTACCATCGAAGAAATCTC-3′ (the SmaI and XhoI restriction sites are underlined). The amplified fragment was cloned into pCR™-Blunt II-TOPO™ with a Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). This plasmid was digested with SmaI and XhoI and the insert was cloned into SmaI- and XhoI-digested pKT-3HA. The plasmid was introduced into trophozoites as described previously [79]. Construction of the 3Myc-EhRab8A (Myc-EhRab8A)-expressing line from HM-1 and EhRab8A gene-silenced line from G3 strain were described in chapter2.

3-3-3 Blue-Native Polyacrylamide gel Electrophoresis (BN-PAGE)

Approximately 1 × 10^6 cells were harvested at the late logarithmic growth phase, washed with cold phosphate-buffered saline (PBS) and homogenized in 0.5 mL homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM E-64, pH 7.5) with 30 strokes of a Dounce homogenizer. Samples were centrifuged at 13,000 g at 4 °C for 10 min to obtain the pellet fraction (p13). The p13 fraction was solubilized in homogenization buffer containing 2% digitonin at 4 °C for 30 min. Solubilized samples were collected by centrifugation (13,000 g, 4°C, 10 min) and subjected to BN-PAGE using the NativePAGE™ NovexH Bis-Tris Gel System (Invitrogen) according to the manufacturer's protocol. The resolved proteins were transferred to a polyvinylidene fluoride membrane and the Myc-EhRab8A protein complex was detected using anti-Myc antibody.

3-3-4 Coimmunoprecipitation

Approximately 4 × 10^6 Myc-EhRab8A-expressing or mock control trophozoites were harvested in the logarithmic growth phase, washed with 2% glucose/PBS and resuspended in 1 mL of PBS. The cell suspension was incubated with 2 mM 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP) (Sigma-Aldrich, St. Louis, MO, USA) for cross-linking for 30 min at room temperature according to the manufacturer's protocol. The samples were mixed with 1 mL homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl2, 1 mM E-64, pH 7.5) and homogenized on ice with 30 strokes of a Dounce homogenizer. After unbroken cells were removed by centrifugation at 400 g for 2 min, the supernatant was centrifuged to obtain the p13 fraction which was primarily enriched with EhRab8A. The p13 fraction was resuspended in approximately 0.5 mL of homogenization buffer containing 2% digitonin at a 2 mg/mL protein concentration and then incubated with Protein G Sepharose (Sigma-Aldrich) at 4 °C for 60 min to reduce non-specific binding during coimmunoprecipitation. The Myc-EhRab8A protein complex was

immunoprecipitated with anti-Myc-antibody-conjugated agarose (Sigma-Aldrich) at 4 °C for 3 h and then washed with homogenization buffer containing 0.5% digitonin. The Myc-EhRab8A protein complex was eluted with homogenization buffer containing 2% digitonin and 0.4 mM Myc peptide (Sigma-Aldrich).

3-3-5 Liquid Chromatography-Tandem Mass Spectrometric Analysis (LC-MS/MS)

In-gel trypsin digestion of protein bands of interest and LC-MS/MS mass spectrometric analysis (Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) were performed as previously described [80,81].

3-3-6 Tunicamycin treatment assay

The tunicamycin treatment assay was performed as previously described [27]. HA-EhCdc50 expressing cells were treated with tunicamycin at 1, 3, 10 μg/mL, and incubated for 24 h at 35.5 °C. After incubation, cells were lysed and analyzed by immunoblotting with anti-HA, anti-EhCP-A5 and anti-EhVps26 antibodies.

3-3-7 Production of EhCdc50 antibody

The exoplasmic region of EhCdc50, corresponding to amino acid residues 108-271, was inserted into the pCold-GST vector (Takara Bio, Shiga, Japan). EhCdc50108-271 recombinant protein was expressed in the *Escherichia coli* BL21 (DE3) strain (Thermo Fisher Scientific) and purified according to the manufacturer's instructions. Anti- EhCdc50 antiserum was commercially raised against recombinant $EhCdc50_{108\text{-}271}$ in rabbits (Eurofins Genomics, Val Fleuri, Luxembourg).

3-3-8 Indirect immunofluorescence assay

An indirect immunofluorescence assay was conducted essentially as previously described [26]. Trophozoites were seeded into 8-mm round wells on a glass slide, fixed

with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100 and reacted with antibodies. Alexa 488- or Alexa 568-conjugated anti-mouse or rabbit IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Images were acquired using an LSM780 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Images were analyzed using Zeiss ZEN software.

3-3-9 Miltefosine sensitivity assay

Trophozoites were seeded into 96-well plates at 0.5×10^4 cells/well in 280 μL of BI-S-33 medium containing 5–80 μM Miltefosine. After 18 h of culture, the medium was removed and the viability of attached trophozoites was estimated by measuring of the absorbance at 450 nm using a DTX880 Multimode Detector (Beckman Coulter, Brea, CA, USA) after incubating of the trophozoites with 100 μL of Opti-MEM medium containing 10% WST-1 reagent (Roche, Basel, Switzerland) for 20 min at 37 °C [82]. Experiments were repeated three times with triplicate samples evaluated in each experiment.

3-3-10 Phagocytosis assay

Approximately 1×10^5 trophozoites of HA-EhCdc50 expressing and mock transformants were seeded on an 8-mm round wells slide glass. After removal of unattached trophozoites, 30 μL of PKH26 red fluorescent-labeled (Sigma) erythrocytes at 10^7 cells/mL were added to the trophozoites to allow phagocytosis for 30 min. The samples were fixed and stained with HA-antibody followed by visualization with anti-mouse Alexa 488 antibody. The fluoresce intensity of PKH26 incorporated by the HA-EhCdc50 expressing and mock tranformant trophozoites was measured on an LSM780 confocal microscope (Carl Zeiss) and analyzed with ZEN software (Carl Zeiss).

3-4 Results

3-4-1 Identification of EhCdc50 as an EhRab8A interacting protein in *E. histolytica*

Previously I demonstrated that EhRab8A is localized to the ER in the steady state, which differs from the Rab8 ortholog in other organisms [38]. In mammals, Rab8 is localized to the trans-Golgi or endocytic compartments and involved in transport to the plasma membrane [38]. However, the function of the Golgi apparatus and the trans-Golgi in *Entamoeba* is highly diverse in protein glycosylation and organelle function [18]. Curiously, EhRab8A gene silencing demonstrated that EhRab8A is involved in the trafficking of at least three surface proteins with molecular masses of 200, 60 and 30 kDa. In general, the protein sorting of secretory proteins in the ER is known to be regulated by the Sar1 GTPase but not by Rab GTPases [83]. To clarify the mechanisms of the EhRab8A-dependent trafficking across the ER, I attempted to identify EhRab8A interacting proteins by coimmunoprecipitation of the epitope-tagged EhRab8A.

To see whether EhRab8A forms a stable complex with other proteins and, if so, to estimate its apparent molecular mass, I conducted BN-PAGE following immunoprecipitation (Fig. 9A). Immunoblotting with the anti-Myc antibody revealed an 87-kDa band in the immunoprecipitated sample from lysates of N-terminal Myc-tagged EhRab8A-expressing cells (Fig. 9A, arrowhead), suggestive of possible interacting protein(s). The putative EhRab8A interacting protein(s) that were coimmunoprecipitated with Myc-EhRab8A using an anti-Myc antibody were detected as two specific bands of approximately 35 and 40 kDa (Fig. 9B, arrowheads). The two bands were excised from the gel and analyzed by liquid chromatography and time-of-flight tandem mass spectrometry (LC-ToF MS/MS) (Table 1, 2). From the 35 kDa band, two candidate proteins, EHI_142740 and EHI_118780, were detected. EHI_142740 was detected exclusively from Myc-EhRab8A expressing cells but not from mock control cells. This protein showed homology with human CDC50 (33%

identity, e-value 1.3×10^{-33}) and *Leishmania* Cdc50 homologue, Los3 (25% identity, e-value 3.0×10^{-25}), which are known as a non-catalytic subunit of lipid flippase P4-ATPase. Thus, I designated hereinafter EHI_142740 as EhCdc50 (GenBank Accession number, LC389589). The other candidate detected in the 35 kDa band, EHI_118780, showed homology to a nuclear pore protein. From the 40 kDa band, six candidate proteins involved in lipid metabolism were identified: sphingomyelin phosphodiesterase (EHI_100080), glycerophosphodiester phosphodiesterase (EHI_068320), cytosolic tldc domain-containing protein (EHI_134660), C2 domain containing protein (EHI_069950), lysosomal vacuolar ATP synthase subunit (EHI_106350) and mitosome luminal protein sulfate adenylyltransferase (EHI_197160). Among these candidates, only glycerophosphodiester phosphodiesterase (EHI_068320) is known to be localized to the ER membrane in other organisms and thus considered to be a candidate of EhRab8A binding protein. However, the localization and function of the 40 kDa band as a potential EhRab8A interacting protein and the binding of these proteins to EhRab8A remain elusive, because I failed to establish amoeba transformants expressing EHI_068320 despite my repeated attempts.

3-4-2 Confirmation of binding of EhCdc50 and EhRab8A

The interaction of EhCdc50 and EhRab8A was confirmed by reciprocal coimmunoprecipitation of EhRab8A from N-terminal HA-tagged-EhCdc50-expressing cells (Fig. 9C). The immunoprecipitated HA-EhCdc50 was recognized as a 42.5 kDa band using an anti-HA antibody; the size was slightly larger than the calculated molecular mass of HA-EhCdc50 of 38.9 kDa. This is consistent with the prediction that Cdc50 is a glycosylated transmembrane protein (see below). EhRab8A was detected as a 23 kDa band with anti-EhRab8A antiserum in the coimmunoprecipitated sample, while the negative control marker for endosomal

protein EhVps26 [27], which was previously shown to be differentially localized from EhRab8A, was not detected in the sample. These data clearly validated the physical interaction between EhRab8A and EhCdc50.

3-4-3 EhCdc50 is N-glycosylated protein

The Cdc50 proteins are structurally well conserved at the primary sequence levels throughout eukaryotes and contain two transmembrane domains separated by a large exoplasmic loop (Fig. 11A, see below), which contains two to four N-glycosylation sites [Asn-Xaa-(Ser/Thr)] in *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and human [84-86]. In human Cdc50A and *S. cerevisiae* Cdc50p, it has been demonstrated that post-translational modifications at the four N-glycosylation sites are necessary for the stable expression of lipid flippase P4-ATPase, complex formation and trafficking from the ER [85,86]. In *E. histolytica*, sequence-based prediction suggested that three of four N-glycosylation sites are present in the ectodomain (Asn141, Asn162 and Asn244) (Fig. 12A). Inhibition of N-glycosylation by tunicamycin treatment decreased the apparent molecular weight of HA-EhCdc50 from 42.5 kDa to 37.8 kDa, as shown by immunoblot analysis using an anti-HA antibody (Fig. 10), indicating that EhCdc50 is modified with N-linked oligosaccharides.

3-4-4 Localization of EhCdc50 and the inhibition of its transport to the plasma membrane by overexpression

Cdc50 is known as a non-catalytic subunit of lipid flippase P4-ATPase and the complex localizes to the endosomes and plasma membrane in the steady state [87]. Gene silencing of EhCdc50 was unsuccessful despite my repeated transfections, indicating EhCdc50 is essential for the growth. To investigate the localization of endogenous and exogenously overexpressed EhCdc50, I established a native antibody against recombinant EhCdc50$_{108\text{-}271}$ (Fig. 11A). EhCdc50 was detected on the cell surface in the mock transformant with this anti-EhCdc50 antibody (similar results

were observed in wild type cells; data not shown) when trophozoites were not treated with Triton X-100, suggesting that endogenous EhCdc50 was uniformly distributed on the entire plasma membrane (Fig. 11B). The surface labeling by anti-EhCdc50 antibody was significantly reduced when recombinant EhCdc50$_{108\text{-}271}$ protein was used for competition; the relative intensity of the peripheral staining by anti-EhCdc50 antibody was reduced to 19 ± 12% of the level in the absence of recombinant EhCdc50$_{108\text{-}271}$ protein by the preincubation of the anti-EhCdc50 antibody with recombinant EhCdc50$_{108\text{-}271}$ protein (Fig. 11B, C), validating specificity of this antibody against EhCdc50. I next examined the localization of EhCdc50 in HA-EhCdc50-expressing cells using an anti-EhCdc50 antibody. In HA-EhCdc50-expressing cells, EhCdc50 was not detected on the cell surface of the nonpermeabilized trophozoites with ani-EhCdc50 antibody (Fig. 11B, right panel), suggesting that transport of both HA-EhCdc50 and native EhCdc50 to the plasma membrane was prevented. When HA-EhCdc50 cells were perforated with Triton X-100, HA-EhCdc50 appeared to be associated with the ER-like network structures (Fig. 4A, upper panel), which was confirmed by co-staining with an anti-EhBip (ER luminal chaperone) antibody (Fig. 13A). HA-EhCdc50 showed strong colocalization with EhBip (Pearson's correlation coefficient: R = 0.65). HA-EhCdc50 also showed mild colocalization with EhRab8A, as visualized using anti-HA and anti-EhRab8A antibodies (R = 0.47) (Fig. 13B). Colocalization of EhRab8A and EhBip was previously demonstrated with a Pearson's correlation coefficient of 0.7. These results indicate that overexpressed HA-EhCdc50 is mainly localized to the ER and partially colocalized with EhRab8A. Similar observations suggesting that overexpressed Cdc50 was accumulated in the ER were reported in other organisms including human and yeast [78,88].

3-4-5 Overexpression of EhCdc50 or gene silencing of EhRab8A showed decreased miltefosine susceptibility

Many of phospholipid flippase P4-ATPases require interactions with the Cdc50 family for their exit from the ER [88]. In *Arabidopsis*, *S. cerevisiae* and mammalian cells, either overexpression or knockout of Cdc50 caused accumulation of Cdc50 together with P4-ATPase to the ER, which led to defects in the internalization of phospholipids [78,88]. I hypothesized that EhCdc50 and a catalytic subunit of phospholipid flippase P4-ATPase on the plasma membrane are involved in translocation of phospholipids from the exoplasmic to the cytoplasmic face in *E. histolytica*. The effect on phospholipid translocation in EhCdc50-overexpressing cells was evaluated using the phosphocholine analogue miltefosine, which is toxic and causes amebic cell growth inhibition. Miltefosine is an alkylated phosphocholine, originally developed as an anticancer drug and is effective against six different *Leishmania* species, including *L. donovani, L. aethippoca, L. tropica, L. mexicana, L. panamensis* and *L. major*. It has been shown in *Leishmania* that the deletion of the Cdc50 homologue, LdRos3, confers resistance to miltefosine [89]. I examined the susceptibility of an HA-EhCdc50-expressing strain against miltefosine. After 18-h treatment with miltefosine, the cell viability of the HA-EhCdc50 expressing strain was higher than that of the control mock transformant (The IC_{50} values of mock and HA-EhCdc50-expressing strains against miltefosine, 22.2 ± 3.2 and 33.4 ± 4.4 μM, respectively; p value <0.05) (Fig. 14A). Similarly, the EhRab8A gene-silenced strain showed partial resistance to miltefosine (The IC_{50} values of G3 parental and EhRab8A gene silenced strains were 21.8 ± 3.6 and 29.2 ± 2.8 μM, respectively; p value <0.05) (Fig. 14B).

3-5 Discussion

3-5-1 Identification of EhCdc50 as EhRab8A binding protein

Cdc50 is an essential accessory protein for the exit of lipid flippase P4-ATPase from

the ER in yeast, *Leishmania*, human and plant [90]. In this study, I identified the non-catalytic subunit, EhCdc50, of lipid flippase as a binding protein of EhRab8A small GTPase. The interaction between EhRab8A and EhCdc50 was confirmed by reciprocal immunoprecipitation (Fig. 9) and immunofluorescence assay (Fig. 13) in the present study and appears to be a common feature conserved among eukaryotes. Rab8 appears to be highly conserved in eukaryotes including Opistokonta (Metazoa and fungi), Viridiplantae (*A. thaliana*) and Amoebozoa (*E. histolytica* and *Dictyostelium discoideum*) [62,13] but not in Trypanosomatidae (*Leishmania* and *Trypanosoma*) and some protozoan parasites that belong to the Alveolata (*Plasmodium* and *Toxoplasma*) [91,92]. However, EhRab8A is unique in that it resides in the ER, while in human macrophages and *Dictyostelium*, Rab8 homologues localize to phagosomes or endocytic compartments [75,76]. In *E. histolytica*, EhRab8A is involved in the transport of surface proteins involved in the cellular attachment and phagocytosis. Thus, identification of Cdc50 as a binding protein of Rab small GTPase is plausible, as the flippase and thus its non-catalytic subunit, must be specifically transported via an EhRab8A-dependent secretory pathway, although this has never been demonstrated in other organisms.

EhRab8A gene-silencing decreased amebic adhesion to host cells and reduced the presentation of major three surface proteins with apparent molecular weights of 200, 60 and 30 kDa. As intrinsic EhCdc50 has a calculated molecular mass of 35 kDa, it does not seem to correspond to one of the three previously identified major surface proteins. This explains why the HA-EhCdc50 expressing strain, in which HA-EhCdc50 is accumulated in the ER (Fig. 13), did not show a defect in phagocytosis of erythrocytes (Fig. 14). Taken together, EhRab8A is likely involved in the transport of multiple proteins including EhCdc50 as well as three previously reported major surface proteins from the ER to the cell surface.

3-5-2 N-linked oligosaccharide modification and possible role of EhCdc50 in the exit from the ER

It has been shown that in mammalian and yeast cells Cdc50 has an apparent molecular mass of 50–60 kDa, is glycosylated in the Golgi and presented on the cell surface [90]. In *Entamoeba*, EhCdc50 was detected as a band with an approximate molecular weight of 35 kDa by SDS-PAGE (Fig. 10), which is consistent with the estimated molecular mass of the polypeptide (35.7 kDa), suggesting that EhCdc50 is not glycosylated [93]. However, as EhCdc50 possesses two hydrophobic transmembrane domains, which are likely responsible for the aberrant mobility on SDS-PAGE and offsetting possible glycosylations. Indeed, tunicamycin treatment reduced the molecular mass of HA-EhCdc50 by approximately 4.7 kDa (Fig. 10), indicating that some of the three potential N-glycosylation sites in EhCdc50 (Fig. 12) may be glycosylated. This predicted addition of a relatively short sugar chain is likely explained by the fact that *E. histolytica* forms an unusual simple 1.8 kDa N-glycan precursor of $Man_5GlcNAc_2$ in the ER rather than the common 2.5 kDa $Glc_3Man_9GlcNAc_2$, which is present in most animals, plants and fungi [93].

N-glycosylation of Cdc50 is required for stability and activity of the Cdc50/ATPase complex in mammalian cells [86]. It is currently unclear whether N-glycosylation of EhCdc50 is necessary for the interaction with EhRab8A. The results of BN-PAGE and immunoblotting showed that EhRab8A formed an 87 kDa complex (Fig. 9A), suggesting that the ~110 kDa amoebic EhP4-ATPase, which is present as a family of 11 proteins encoded in the genome (see below), is not part of the EhRab8A/EhCdc50 complex. After EhRab8A recognizes EhCdc50 and completes its sorting in the ER, EhRab8A is presumably replaced by EhP4-ATPase and then the N-glycosylated EhCdc50/EhP4-ATPase complex can be transported to the plasma membrane.

3-5-3 A possible novel Rab8A-dependent and COPII-independent pathway for Cdc50 traffic

One of the assumed roles of Cdc50 is trafficking of P4-ATPase from the ER to the cell surface. As suggested by a previous proteome analysis of the COPII coat component in yeast, P4-ATPase Nep1p physically interacts with the COPII coat components Sec13p, Sec23p and Sec24p [94]. It has been also shown that yeast P4-ATPase Drs2p interacts with Sec23p [94]. Analogously, EhCdc50 appears to be transported in a similar manner as Sar1p GTPase and COPII. However, EhRab8A does not colocalize with the COPII component on the ER, indicating the presence of an unconventional COPII-independent and EhRab8A-dependent ER exit pathway in *Entamoeba*.

It has been reported in many organisms that both repression and overexpression of Cdc50 and/or P4-ATPase prevents correct targeting of the P4-ATPase/Cdc50 complex and results in accumulation of the complex in the ER, concomitant with the loss of the plasma membrane-associated lipid flippase activity [78,88,95]. With a few exceptions, the class 2 P4-ATPases, yeast P4-ATPase Neo1p and mammalian ATP9A and ATP9B, do not require the Cdc50 family. Yeast Neo1p forms a complex with the scaffolding protein Ysl2p [96] and mammalian ATP9A and ATP9B exit the ER to localize with the respective cell membranes in the absence of Cdc50 [97]. I observed the plasma membrane localization of EhCdc50 in the steady state (Fig. 11). In contrast to the single gene copy of EhCdc50, I found 11 potential P4-ATPase genes in the *E. histolytica* genome: EHI_141350, EHI_096620, EHI_135220, EHI_188210, EHI_174280, EHI_049640, EHI_197300, EHI_168260, EHI_024120, EHI_140130 and EHI_009460. Three of them, EHI_049640, EHI_168260 and EHI_197300, were classified as class 2 P4-ATPases, suggesting that the EhCdc50 and P4-ATPase in the amoebic genome and several P4-ATPases exit the ER via an EhCdc50-independent mechanism.

3-5-4 EhRab8A and EhCdc50 are involved in miltefosine sensitivity in *Entamoeba*

Miltefosine is known to inhibit choline-phosphate cytidylyltransferase involved in the phosphatidylcholine biosynthesis in mammalian cells, [98] and homologous proteins are present in *Leishmania* [99]. However, the leishmanicidal mode of action on miltefosine is not completely understood and most information regarding its properties was obtained from miltefosine-resistant parasites generated in vitro and clinical isolates. Miltefosine resistance in *Leishmania* occurs mainly through the reduction in drug incorporation associated with the introduction of mutations into the P4-ATPase miltefosine transporter, MT and Cdc50 homologue, LdLos3 (Perez-Victoria *et al.*, 2006). The resistance mechanism is likely conserved among *Leishmania* species causing either cutaneous (*L. amazonensis* and *L. major*) or visceral (*L. donovani) leishmaniasis* [100,101]. Here I showed that overexpression of HA-EhCdc50 caused defects in EhCdc50/P4-ATPase trafficking and a concomitant increase in the tolerance to miltefosine (Fig. 14), suggesting that EhCdc50 is involved in the transport of phospholipids and miltefosine by P4-ATPase. Additionally, down-regulation of EhRab8A similarly caused tolerance to miltefosine (Fig. 14B), supporting the prediction that EhRab8A is necessary for the transport of EhCdc50/P4-ATPase. My result indicates that any mutations in EhRab8A that affect the affinity with EhCdc50 or an increase in the expression of EhCdc50 can confer resistance to miltefosine in *Entamoeba*. Thus, EhRab8A and EhCdc50 are of interest as potential mechanisms of drug resistance against miltefosine in case it is used in clinical cases in future. In the human fungal pathogen *Cryptococcus*, it is reported that Cdc50 is involved in its virulence as well as in drug resistance [102].

The mechanisms of EhCdc50/P4-ATPase in phospholipid transport and the EhRab8A-mediated regulation of EhCdc50/P4-ATPase trafficking remain unclear in *E. histolytica*. *E. histolytica* possesses full capacity of de novo biosynthesis of phospholipids including phosphatidylcholine and phosphatidylethanolamine and the

ability to scavenge components from the environment under in vitro cultivation conditions [42]. Furthermore, *Entamoeba* possesses a list of lipid binding proteins in their genome [103,104]; one of such lipid transfer proteins, EhPCTP-L, was shown to bind phosphatidylserine and phosphatidic acid and localizes to the cell surface [105]. The catalytic P4-ATPase subunit of the phospholipid flipase responsible for miltefosine transport should be identified to better understand the molecular mechanisms of phospholipid transport on the plasma membrane and its involvement in drug resistant and pathogenesis in *Entamoeba*.

3-6 Figure Legends

Fig. 9. Identification and confirmation of EhCdc50 as an EhRab8A binding protein

A. Immunoblot analysis of Myc-EhRab8A with anti-Myc antibody following BN-PAGE from representative image of three independent experiments. B. SDS-PAGE analysis of Myc-EhRab8A-binding proteins. Myc-EhRab8A-binding proteins coimmunoprecipitated with anti-Myc antibody were separated on SDS-PAGE and detected with silver staining. Representative data of three independent experiments was shown. C. Reciprocal coimmunoprecipitation of EhRab8A via interaction with EhCdc50. EhCdc50 binding protein was immunoprecipitated from lysates of HA-EhCdc50-expressing cells with anti-HA antibody, followed by immunoblotting with anti-HA, anti-EhRab8A and anti-EhVps26 antibodies. Anti-EhVps26 antibody was used as a negative control.

Fig. 10. Demonstration of N-linked glycosylation on EhCdc50

HA-EhCdc50 expressing cells were treated with tunicamycin at 1, 3, 10 μg/mL for 24 h. Cells were lysed and analyzed by immunoblotting with anti-HA, anti-EhCP-A5 and anti-EhVps26 antibodies. The apparent molecular weight of HA-EhCdc50 was decreased by the tunicamycin treatment. EhCP-A5 and EhVps26 are the glycosylated

and non-glycosylated proteins, respectively.

Fig. 11. Surface staining of endogenous EhCdc50 in wild-type and overexpressed HA-tagged EhCdc50 cells

A. Schematic diagram of the domain organization of EhCdc50. TMD, transmembrane domain. A part of the exoplasmic domain (a.a. 108–271) that was used to produce *E. coli* recombinant protein to raise antiserum is indicated with a dotted arrow. B. Indirect immunofluorescence assay of endogenous EhCdc50 in mock control and HA-tagged EhCdc50-expressing cells without permeabilization with Triton X100. Pretreatment of the EhCdc50 antiserum with the recombinant protein (middle panel) abolished the surface labeling. Scale bar, 5 μm. C. Quantification of surface labeling with anti-EhCdc50 antibody shown in B. Peripheral signal intensity of 30 independent trophozoites was captured by Zeiss ZEN software. Bar graph shows the means and standard deviations of the relative peripheral fluorescence intensity of EhCdc50 with or without preincubation of the anti-EhCdc50 antibody with recombinant $EhCdc50_{108\text{-}271}$ protein at a molar ratio of 1:100 in the mock and HA-EhCdc50 expressing cells, of three independent experiments.

Fig. 12. Amino acid sequence alignment of Cdc50 homologues

A. Sequences were aligned using ClustalW2 server (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Accession numbers were: *E. histolytica* EhCdc50 (EHI_142740), human CDC50A (Q9NV96), *S. cerevisiae* Cdc50p (YCR094W), *L. donovani* LdRos3 (ABB05176) and *A. thaliana* ALIS5 (Q8L8W0). N-glycosylation sites of EhCdc50 were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and depicted with black arrowheads. N-glycosylation sites of Cdc50 homologues reported in humans, *S. cerevisiae* and *A. thaliana*, are indicated with small black crosses. Two transmembrane regions of EhCdc50 were predicted with the TMHMM Server v.2.0

(http://www.cbs.dtu.dk/services/TMHMM/) and depicted with red lines and "TMD." B. Amino acid percentage identity matrix was created using the ClustalW2 server.

Fig. 13. Immunofluorescence assay showing ER localization of overexpressed HA-EhCdc50

HA-EhCdc50 expressing trophozoites were stained with anti-Bip (A, red), anti-EhRab8A (B, red) and anti-HA (green) antibodies (top panels) after permeabilization with Triton X100. Histograms of the green and red signal intensities along the line indicated in the merged images are shown in the bottom left panels. R = Pearson's correlation coefficient. Bars, 5 μm.

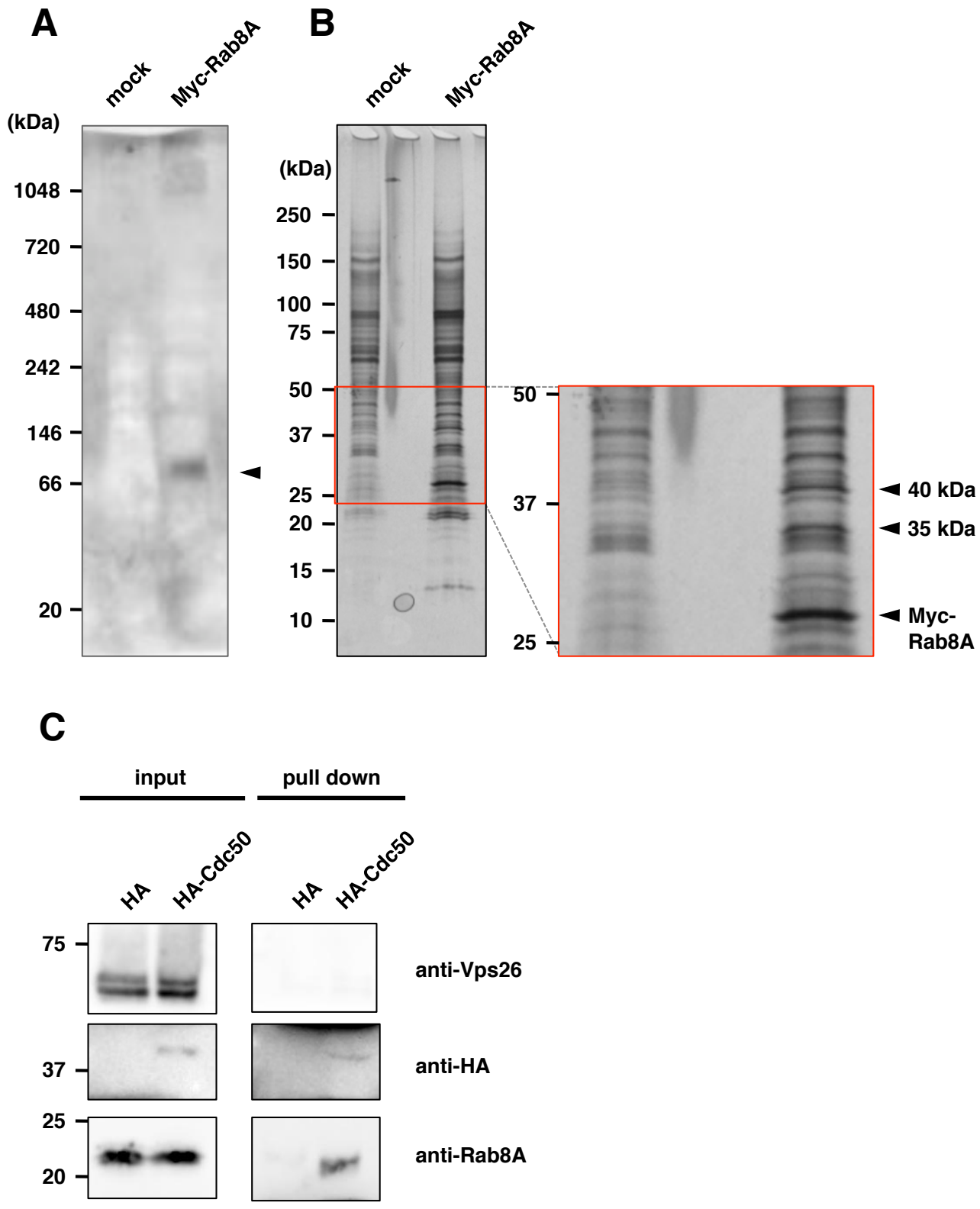
Fig. 14. Miltefosine sensitivity of EhCdc50 overexpressing and EhRab8A gene silencing cells

A. Miltefosine sensitivity of EhCdc50 overexpressing cell. Percentage survival of HA-EhCdc50 expressing line and its corresponding mock transfected controls of HM-1 after treatment with indicated concentrations of miltefosine for 18 h. B. Miltefosine sensitivity of EhRab8A gene silencing cell. Percentage survival of EhRab8A gene silenced line and its corresponding mock transfected controls of G3 strains. Calculated IC_{50} values using GraphPad Prism ver.6 software are also shown in the right panel. Bar graph shows the means and standard deviations of three independent experiments. The correlation coefficients were calculated using Student's *t*-test.

Fig. 15. Effect of HA-EhCdc50 overexpression on erythrophagocytosis

Efficiency of erythrophagocytosis by the transformants expressing HA-EhCdc50, and mock transformant. The total fluorescence intensities of the PKH26-labeled erythrocytes ingested by a single trophozoite were measured, and the average of the total fluorescence intensities per trophozoite for each strain are shown.

Figure 9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(kDa)
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720
480
242
146
66
20
B
mock
Myc-Rab8A
(kDa)
250
150
100
75
50
37
25
20
15
10
50
37
25
40 kDa
35 kDa
Myc-
Rab8A
C
input
pull down
HA
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HA-Cdc50
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25
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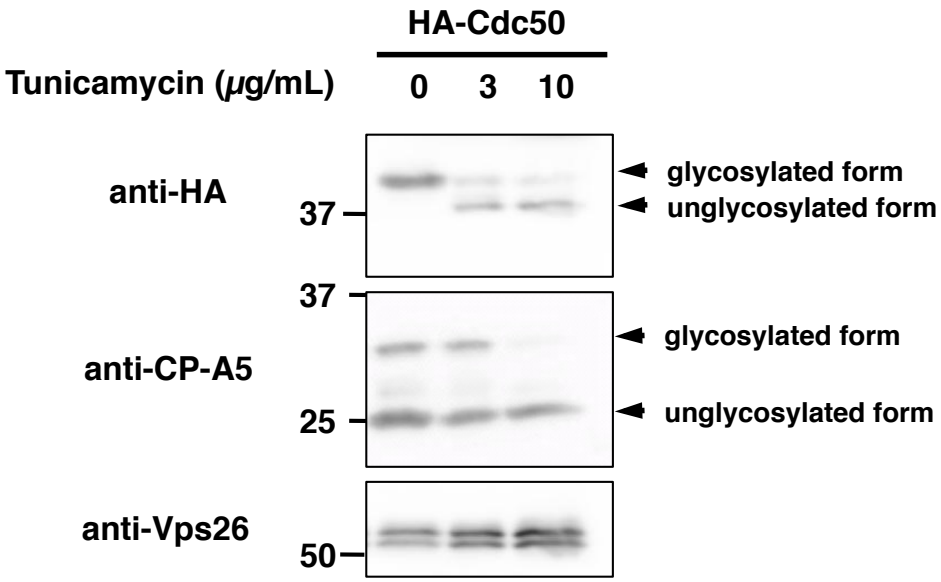
anti-Vps26
anti-HA
anti-Rab8A

Figure 10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Tunicamycin (μg/mL)
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3
10
anti-HA
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glycosylated form
unglycosylated form
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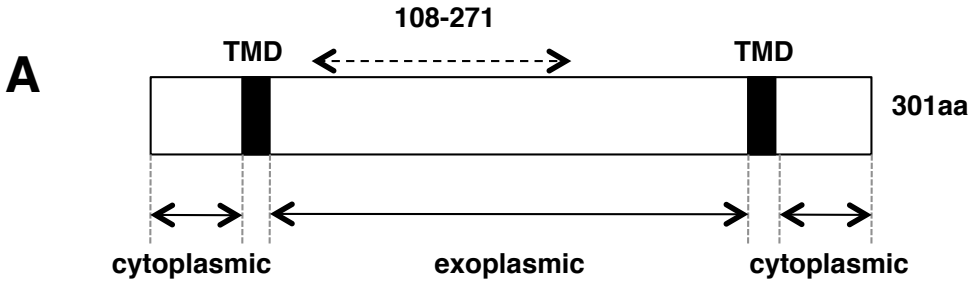
anti-CP-A5
glycosylated form
25
unglycosylated form
anti-Vps26
50

Figure 11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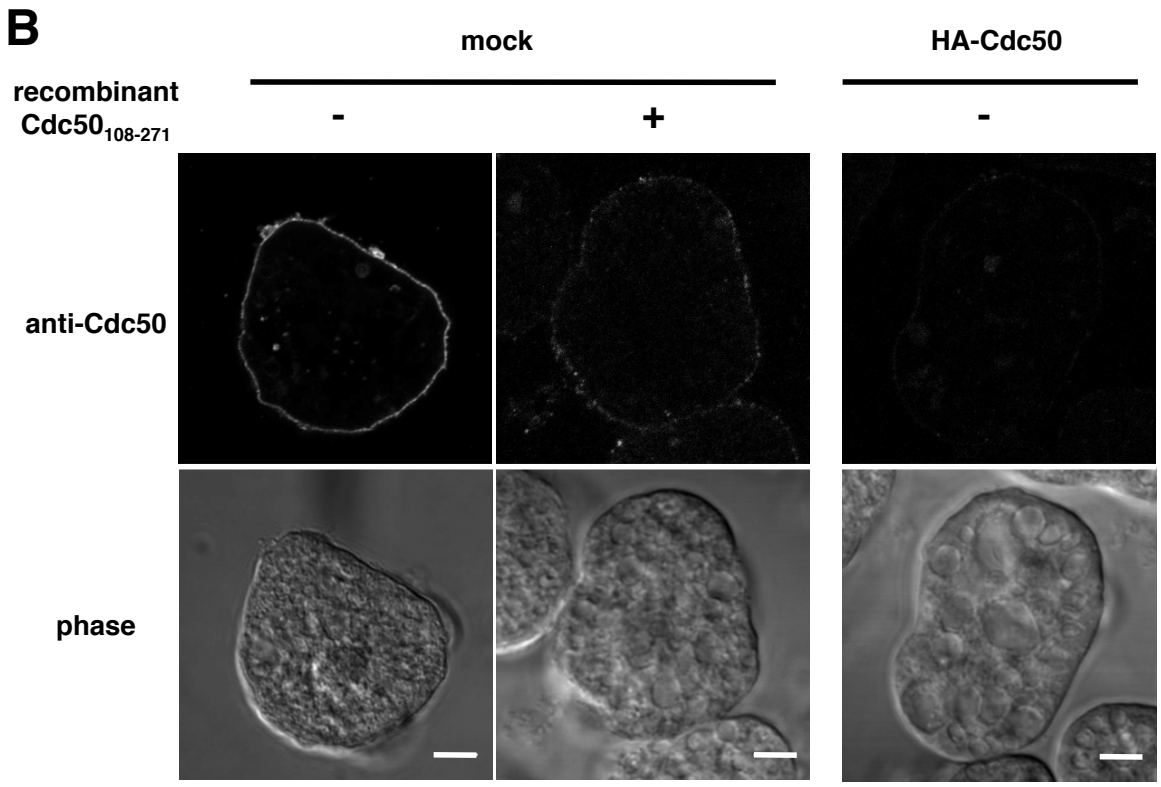
exoplasmic
cytoplasmic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HA-Cdc50
recombinant
Cdc50108-271
-
+
-
anti-Cdc50
phase

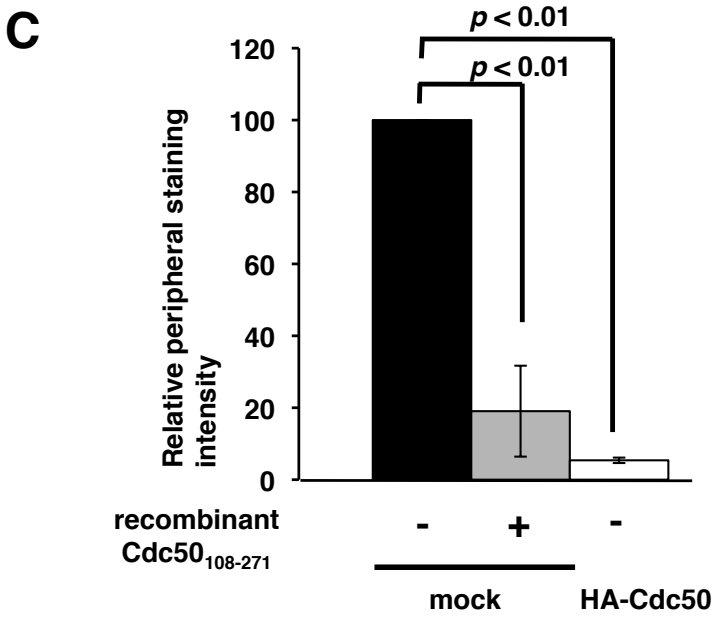
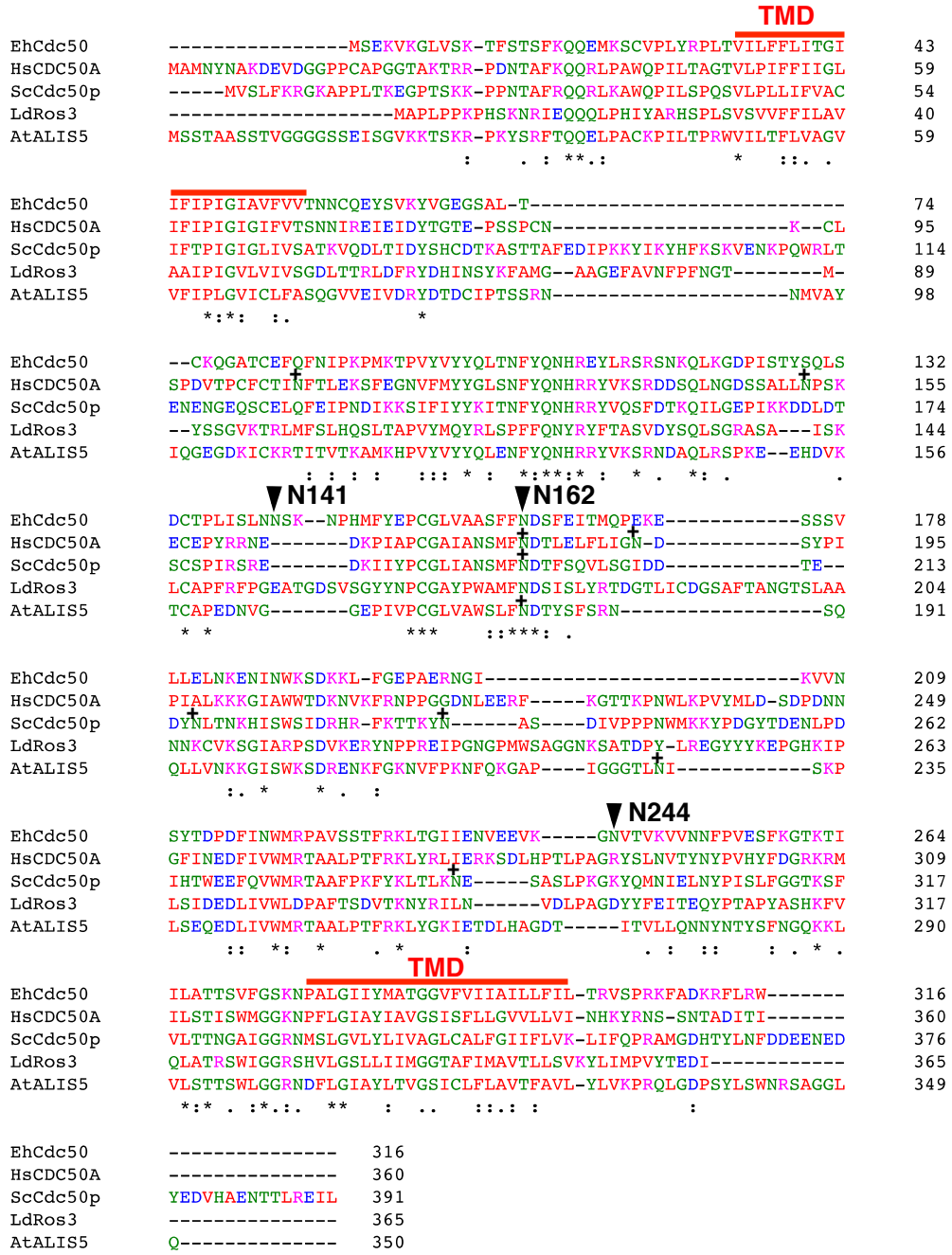

C
p < 0.01
p < 0.01
120
100
80
60
40
20
0
Relative peripheral staining intensity
recombinant
Cdc50108-271
-
+
-
mock
HA-Cdc50

Figure 12

A



B

	EhCdc50	HsCdc50A	ScCdc50p	LdRos3	AtALIS5
EhCdc50	100	33	30	25	36
HsCdc50A		100	35	28	37
ScCdc50p			100	22	30
LdRos3				100	26
AtALIS5					100

Figure 13

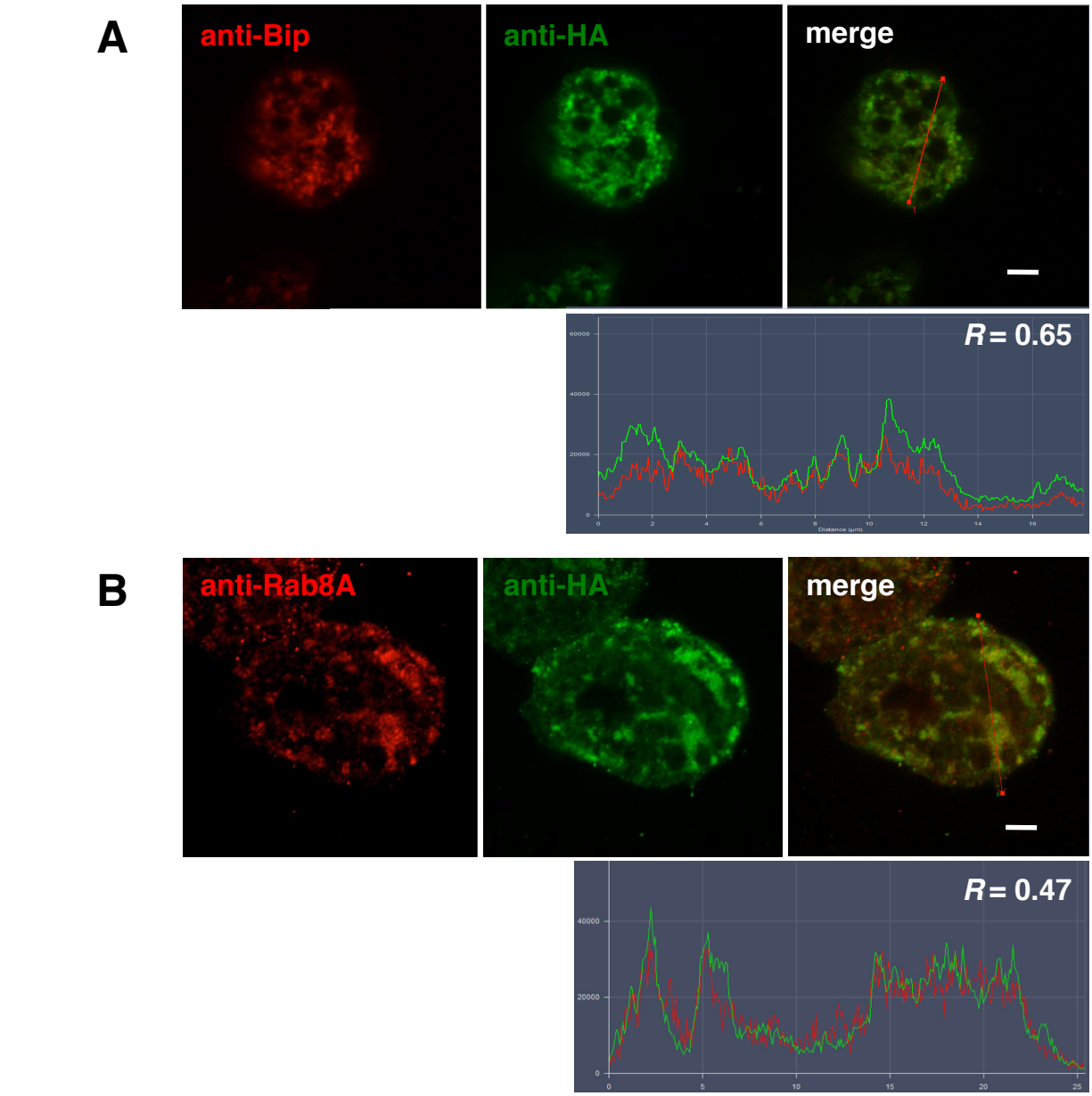
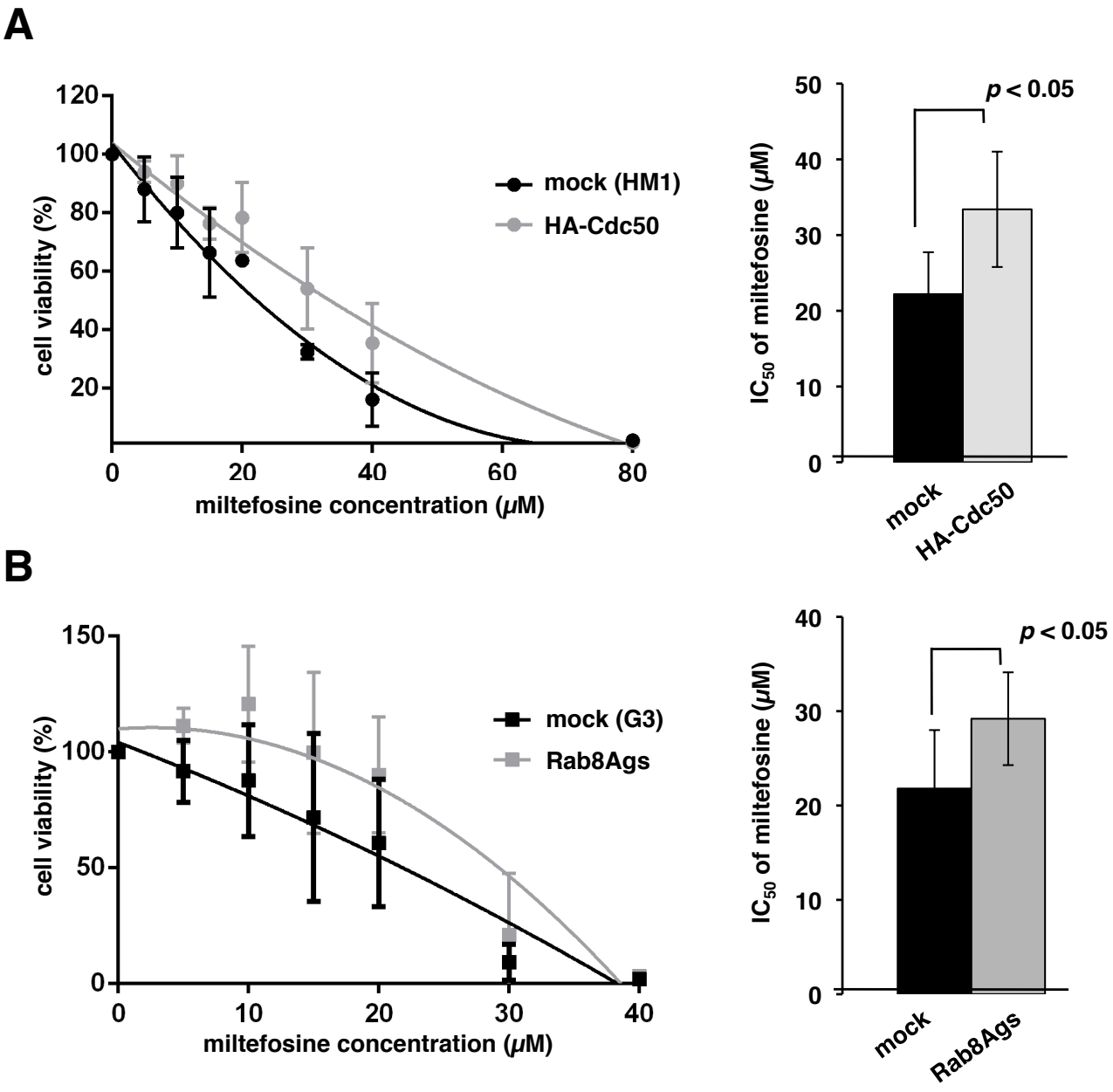


Figure 14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120
100
80
60
40
20
cell viability (%)
0
20
40
60
80
miltefosine concentration (μM)
mock (HM1)
HA-Cdc50
50
40
30
20
10
0
IC50 of miltefosine (μM)
p < 0.05
mock
HA-Cdc50
B
150
100
50
0
cell viability (%)
0
10
20
30
40
miltefosine concentration (μM)
mock (G3)
Rab8Ags
40
30
20
10
0
IC50 of miltefosine (μM)
p < 0.05
mock
Rab8Ags

Figure 15

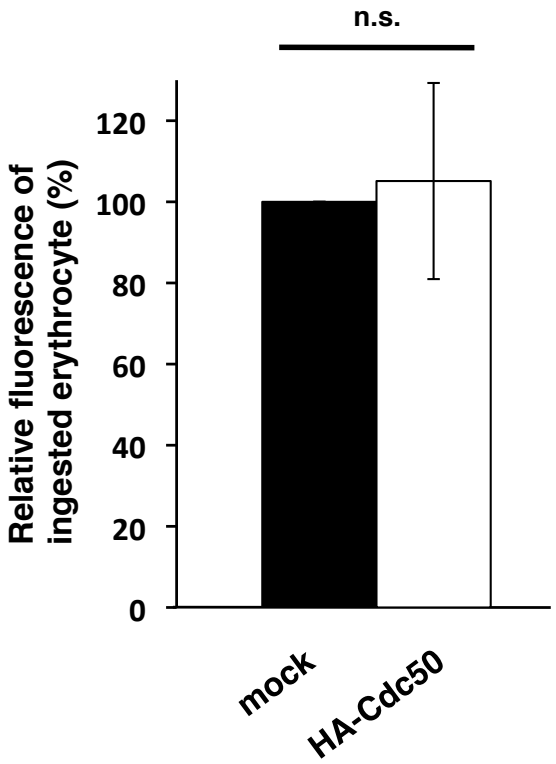
n.s.
Relative fluorescence of ingested erythrocyte (%)
120
100
80
60
40
20
0
mock
HA-Cdc50

Table 1. Thirty-five kDa proteins coimmunoprecipitated with Myc-EhRab8A.

Annotation	Gene ID	Molecular Weight (kDa)	Normalized Relative Ratio against Common Peptides, mock/mycRab8A	Subcellular Localization in Other Organisms	E-Value (Species)
Cdc50	EHI_142740	36	0/2.68	trans-Golgi/endosome/PM/ER	1.3×10^{-43} (*Arabidopsis thaliana*)
Nuclear pore protein	EHI_118780	38	2.80/9.36	nucleus	5.8×10^{-18} *(Chaetomiu thermopholum)*

Table 2. Forty kDa proteins coimmunoprecipitated with Myc-EhRab8A.

Annotation	Gene ID	Molecular Weight (kDa)	Normalized Relative Ratio against Common Peptides, mock/mycRab8A	Subcellular Localization in Other Organisms	E-Value (Species)
Sphingomyelinase phosphodiesterase	EHI_100080	46	0/1.02	Acid organella	1.4×10^{-39} (*Dictyostelium discoideum*)
tldc domain-containing protein	EHI_134660	42	0/1.01	cytosol	4×10^{-7} *(Heterostelium album*)
Vacuolar ATP synthase subunit δ	EHI_106350	40	0/1.01	lysosome membrane	1.3×10^{-100} *(Dictyostelium discoideum)*
Sulfate adenylyltransferase	EHI_197160	48	0/1.01	amoebic mitosome lumen	8.8×10^{-141} *(Desulfovibrio desulfuricans)*
Glycerophosphodiester phosphodiesterase	EHI_068320	45	0.84/2.02	ER	4.6×10^{-25} (*Bacillus subtilis*)
C2 domain containing protein	EHI_069950	37	4.19/9.09	cytosol	5.1×10^{-7} *(Arabidopsis thaliana)*

Chapter 4. General Discussion

In this study, I revealed that EhRab8A regulates protein transport on the ER membrane to the plasma membrane. This is the first report that the Rab GTPase, which is localized on the ER, regulates protein sorting at the ER. In general, protein sorting into the COPII vesicles from the ER is regulated by the Sar1 GTPase, During the formation of COPII-coated vesicles on the ER, Sar1 is activated by the ER integral membrane protein Sec12, which functions as Sar1 GEF. Membrane-bound Sar1-GTP recruits Sec23-Sec24 heterodimer, and Sec23/24-Sar1 complex selects transmembrane and cargo proteins, that are packed into the COPII vesicles [94]. Subsequently, Sec23-Sec24 heterodimer recruits Sec13-Sec31 heterodimer on to the prebudding complex, executing of the outer layer of the coat [106]. After completion of COPII coat assembly, Sec23 GAP activated GTP-hydrolysis of Sar1, and then vesicle uncoating occurs. The uncoated vesicle is finally fused with the cis-Golgi membrane by the regulation of Rab1 GTPase. Sar1 and all the component of COPII coat is conserved in *Entamoeba,* and I successfully detected with the ER budding site using anti-Sec13 antibody. My result that the localization of EhRab8A was differed from COPII component Sec13 indicated that EhRab8A was localized to different subcompartment from COPII budding sites, implicating the presence of functionally distinct cargo sorting site by EhRab8A from EhSar1 in *E. histolytica*. Rab mainly controls membrane fusion with the target membrane, but only a few cases of Rab involved in cargo selection and sorting: human Rab5 sorts transferrin receptors into the clathrin-coated pits at the plasma membrane during the clathrin-mediated endocytosis [107]. This observation suggests that the EhRab8A is also involved in the cargo selection in the ER membrane independent from the Sar1 and COPII vesicles.

In addition, I discovered that the EhRab8A pathway is independent from EhRab11B regulated pathway in *E. histolytica*: EhRab8A localization was differed from EhRab11B. Rab cascade between Rab11 and Rab8, that is directed to the plasma membrane transport, is reported in other eukaryotes such as *H. sapience*, *S.*

cerevisiae, *and D. discoideum*. The cascade is that Rab11 effector, named Rabin8 in mammals, is Rab8 GEF, that is activated Rab11 subsequently activates Rab8 in the same pathway [74]. Interestingly, *Entamoeba* dose not possesses a homolog of Rabin8 in their genome, supporting that EhRab8A plays a different roll from other organisms. In human cases, Rabin8 activates several isotypes of Rab GTPases, HsRab8, HsRab8B, and HsRab10, and also regulates several pathways, such as primary ciliogenesis and cystogenesis in other organisms. Besides of the lack of Rabin8 homolog in *E. histolytica*, Rab10 is not conserved in *Entamoeba* and function of EhRab8B is not well elucidated. These observations indicated that the Rab GTPase function and transport pathways are diversified, and then the regulation system of Rab is also significantly altered in *E. histolytica.*

It is unresolved whether cargo proteins sorted by EhRab8A target to the Golgi and then modified with Golgi resident enzyme, but otherwise transport to the plasma membrane directly. It needs to identify the true effector or more cargo proteins of EhRab8A for the clarification of EhRab8A-dependent pathway. The EhRab8A binding protein, EhCdc50, reported in this study was modified with N-linked sugar, which is transferred to the EhCdc50 into the ER lumen. Currently, it is still unknown whether EhCdc50 is modified with Golgi resident glycosylation, that is only glycosyltransferase, galactosyl transferase in *E. histolytica*. My result also shows that EhRab8A regulates several surface proteins in addition to the EhCdc50, and those surface proteins might be involved in host cell attachment and phagocytosis. Thus, the identification of the additional cargo proteins will contribute to the understanding of amoebic virulence. In conclusion, the discovery of effector proteins will support the identification of the new transport pathway regulated by EhRab8A, and may contribute to the generation of new insights on eukaryotic membrane trafficking.

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