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Endoplasmic reticulum-resident Rab8A GTPase is involved in phagocytosis in the protozoan parasite *Entamoeba histolytica*

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Summary

Phagocytosis is indispensable for the pathogenesis of the intestinal protozoan parasite *Entamoeba histolytica*. Here, we showed that in *E. histolytica* Rab8A, which is generally involved in trafficking from the trans-Golgi network to the plasma membrane in other organisms but was previously identified in phagosomes of the amoeba in the proteomic analysis, primarily resides in the endoplasmic reticulum (ER) and participates in phagocytosis. We 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. 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. Further, 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. To our knowledge, this is the first report that the ER-resident Rab GTPase is involved in phagocytosis through the regulation of trafficking of a surface receptor, supporting a premise of direct involvement of the ER in phagocytosis.

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Introduction

Phagocytosis is a ubiquitous mechanism that is evolutionarily conserved in a wide range of eukaryotes. It comprises several key steps: receptor-mediated recognition of the particle (prey), signal transduction, rearrangement of the cytoskeleton and remodelling of the membrane and contents (Vieira *et al.*, 2002; Stuart and Ezekowitz, 2005). The surface receptors for a variety of targets activate a number of signalling cascades leading to target-dependent responses (Kinchen and Ravichandran, 2008). The subsequent maturation of phagosomes requires interactions between the phagosomes and various intracellular compartments, such as endosomes, lysosomes, the endoplasmic reticulum (ER) and the Golgi apparatus (Rogers and Foster, 2007; Fairn and Grinstein, 2012; Gutierrez, 2013). The membrane fusion and recycling between phagosomes and other intracellular compartments are regulated by specific Rab GTPases localized to each organelle (Stenmark, 2009).

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis in humans. Phagocytosis plays an important role in its pathogenesis as it participates in the killing of and evasion from host immune cells (Ralston and Petri, 2011; Faust and Guillen, 2012). Two types of ingestion of cells and particles by *E. histolytica* are proposed. Phagocytosis is a process by which dead cells and large particles such as bacteria are internalized as a whole. Induction of apoptosis to host cells by a contact of *E. histolytica* has been reported previously (Huston *et al.*, 2003; Faust *et al.*, 2011). The Gal/GalNAc lectin and cysteine protease A5 are required for phagocytosis (Mann *et al.*, 1991). Trophozoites induce apoptosis to host cells by contact (Huston *et al.*, 2003; Faust *et al.*, 2011), recognize phosphatidylserine exposed on the apoptotic and necrotic host cells (Boettner *et al.*, 2005) and internalize them by phagocytosis. In contrast, trogocytosis, recently described by Ralston *et al.* (2014), is a process in which a cell nibbles pieces of a living host cell, eventually leading to death by the loss of membrane integrity and mitochondrial potential (Guillen, 2014; Ralston *et al.*, 2014; Ralston, 2015). This process requires Gal/GalNAc lectin, actin rearrangement and PI3K signalling of trophozoites by the loss of membrane integrity and

mitochondrial potential of host cells (Ralston *et al*., 2014; Ralston, 2015). Adhesion and subsequent phagocytosis (trogocytosis) may also require additional surface receptors such as transmembrane kinases (TMK) (Beck *et al*., 2005; Boettner *et al*., 2008; Buss *et al*., 2010; Shrimal *et al*., 2010); serine-rich, threonine-rich and isoleucine-rich protein (STIRP) (MacFarlane and Singh, 2007); lysine-rich and glutamic acid-rich protein (KERP1) (Santi-Rocca *et al*., 2008); and serine-rich *E. histolytica* protein (SREHP) (Stanley *et al*., 1990).

During and after the engulfment of the prey such as human and bacterial cells, vesicular traffic mediated by Rab GTPases plays a pivotal role in the degradation of the prey. It has been demonstrated that Rab GTPases are highly diversified in *E. histolytica*, and 102 Rab genes are currently annotated (Saito-Nakano *et al*., 2005; Nakada-Tsukui *et al*., 2010). Among them, EhRabB, EhRab5, EhRab7A and EhRab7B have been shown to be sequentially involved in phagosomal maturation (Saito-Nakano *et al*., 2004; Nakada-Tsukui *et al*., 2005; Saito-Nakano *et al*., 2007; Juarez-Hernandez *et al*., 2013). First, EhRabB is recruited, along with phosphatidylinositol-3-phosphate, to the phagocytic cup (Rodriguez *et al*., 2000; Nakada-Tsukui *et al*., 2009; Juarez-Hernandez *et al*., 2013). Simultaneously, a unique vacuole called prephagosomal vacuole (PPV), is formed sequentially by EhRab5 and EhRab7A. Finally, the EhRab7A-positive PPV and EhRab7B-positive lysosomes/late endosomes fuse with the phagosome to allow its full maturation (Saito-Nakano *et al*., 2004; 2007).

Besides the aforementioned EhRab proteins, localization and/or functions of only four amoebic Rab proteins have been shown. It has been shown that EhRab11A is involved in encystation (McGugan and Temesvari, 2003), whereas EhRab11B is involved in the transport and secretion of cysteine protease (Mitra *et al*., 2007). It was shown that EhRabA and EhRab8A are localized to the leading edge of the cell and the plasma membrane (PM) respectively (Juarez *et al*., 2001; Welter and Temesvari, 2004; Welter *et al*., 2005). However, functions of other Rab proteins, particularly those discovered in previous phagosome proteomic studies, have not been elucidated (Okada *et al*., 2005; 2006; Okada and Nozaki, 2006). Among the 14 identified phagosomal EhRab proteins, EhRab8A, a homologue of Rab8 that was also identified in phagosomes of human macrophages and *Dictyostelium* (Trost *et al*., 2009; Urwyler *et al*., 2009), is the subject of the present study.

Here, we report that EhRab8 is primarily localized to the ER in the steady state and is likely involved in the transport of surface receptors necessary for target recognition. To the best of our knowledge, this is the first study to show that the ER-resident Rab8 is involved in phagocytosis through the transport of PM proteins.

Results

Down-regulation of phagocytosis by repression of Rab8 expression by gene silencing

Our previous proteomic study of isolated phagosomes has revealed that 14 amoebic Rab proteins are localized to phagosomes (Okada *et al*., 2005; 2006). In order to screen Rab proteins that are involved in the engulfment and phagosome maturation *per se*, *E. histolytica* strains in which expression of each EhRab protein of the all phagosome-associated EhRabs except for EhRab7E, which is not expressed in G3 strain, along with two additional Rab7 isotypes, EhRab7G and 7H, was silenced by antisense small RNA-mediated transcriptional gene silencing were created (Bracha *et al*., 2006; Mi-ichi *et al*., 2011; Zhang *et al*., 2011). Among 15 EhRab proteins, the specific repression of EhRab7B (EHI_081330), EhRab7D (EHI_082070), EhRab7G (EHI_187090), EhRab7H (EHI_005900), EhRab8A (EHI_199820), EhRab11B (EHI_107250) and EhRab11C (EHI_161030) was achieved. However, gene silencing of EhRab1A (EHI_108610), EhRab5 (EHI_026420), EhRab7A (EHI_192810), EhRab7C (EHI_189990), EhRabC1 (EHI_153690), EhRabC2 (EHI_045550), EhRabC3 (EHI_143650) and EhRabX17 (EHI_042250) was not successful despite repeated trials, presumably because of their essentiality (data not shown). In this study, we focused on the analysis of EhRab8A. Analyses of other *EhRab* gene-silenced strains will be reported elsewhere.

The specific repression of *EhRab8A* expression was confirmed by real-time polymerase chain reaction (PCR) of the corresponding cDNA (Fig. 1A). The expression level of *EhRab8A* mRNA was decreased 35-fold ($P = 0.015$) in *EhRab8A* gene-silenced strain compared with that of the mock control. Moreover, expression of the paralogous Rab8 isotype, *EhRab8B* (EHI_127380), which has a 54% nucleotide identity to *EhRab8A*, was not affected by *EhRab8A* gene silencing ($P > 0.05$). Loss of EhRab8A protein was further confirmed by immunoblot analysis with a polyclonal antibody raised against recombinant EhRab8A protein (Fig. 1B). *EhRab8A* gene-silenced strain showed growth retardation compared with mock control (the doubling time: 34 ± 6.0 h for *EhRab8A* gene-silenced strain; 26 ± 2.4 h for mock control, $P < 0.05$) (Fig. 1C).

EhRab8A gene-silenced strain showed a remarkable defect in phagocytosis of various prey, that is, carboxylated latex beads, erythrocytes and *Escherichia coli* cells (Fig. 1D). Flow cytometric analysis revealed that *EhRab8A* gene-silenced trophozoites ingested carboxylated latex beads, erythrocytes and *E. coli* cells less efficiently by $46\% \pm 10\%$ (at 30 min, $P < 0.05$ compared with the mock control cells, $n = 3$), $26\% \pm 11\%$ (at 20 min, $P < 0.01$) and $83\% \pm 6\%$ (at 30 min, $P < 0.01$) respectively.

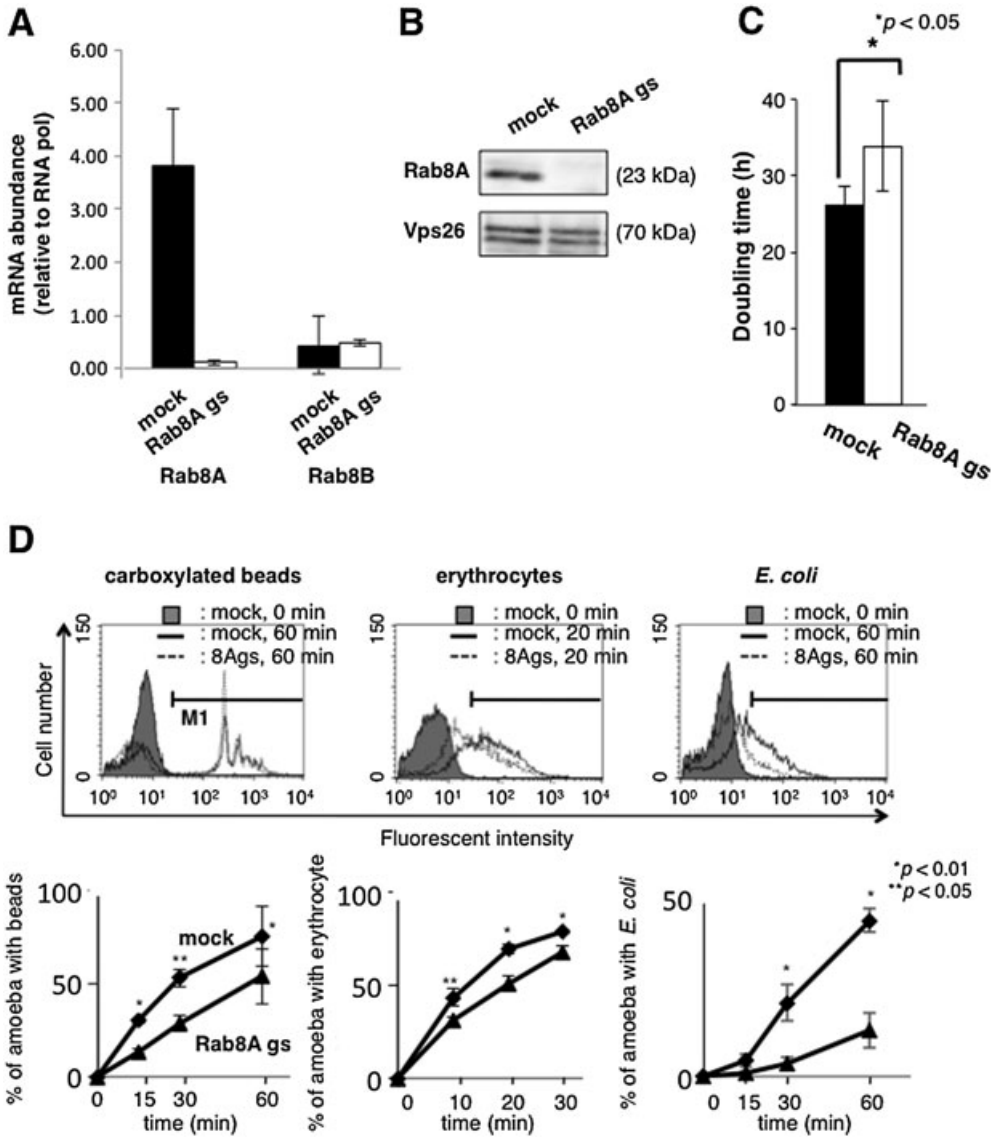

Fig. 1. The EhRab8A gene-silenced strain showed reduced phagocytic activity.
A. The specific down-regulation of expression of EhRab8A mRNA was confirmed by reverse-transcription PCR of the EhRab8A and EhRab8B genes in EhRab8A gene-silenced (Rab8A gs) and mock control cells. The amount of cDNA of each EhRab8 isotype was normalized relative to that of the gene encoding the 15 kDa subunit of RNA polymerase II.
B. Immunoblot analysis of the specific gene silencing of EhRab8A. The strains used were the same as those in Fig. 1A. Anti-EhRab8A and anti-Vps26 antibodies were used.
C. Doubling time of EhRab8A gs strain. The correlation coefficients were calculated using Student's *t*-test: $*P < 0.05$.
D. Phagocytosis assay of the EhRab8A gs strain. Upper panels: Representative histograms of the flow cytometry of the EhRab8A gs and control strains. The trophozoites of the EhRab8A gs (dotted lines) and mock control (black lines) strains were fed with fluorescent carboxylated latex beads (left), PKH26 red fluorescent-labelled erythrocytes (middle) and rhodamine-labelled *E. coli* (right) and subjected to flow cytometric analyses. Lower panels: Time-course of phagocytosis. The percentages of trophozoites that ingested fluorescent carboxylated latex beads (left), PKH26-labelled erythrocytes (middle) or rhodamine-labelled *E. coli* (right), which are labelled *M1* in the upper panel, are shown. The means and standard deviations of three independent experiments are shown. The correlation coefficients were calculated using Student's *t*-test: $*P < 0.01$, $**P < 0.05$.

Thus, EhRab8A is involved in phagocytosis of a broad range of targets.

Reduced phagocytosis in EhRab8A *gene-silenced strain because of decreased adherence*

Using a rosette formation assay described in the Experimental Procedures section, we evaluated whether the decreased phagocytosis in the *EhRab8A* gene-silenced cells was because of reduced adherence to the target (Fig. 2A). We quantified the number of trophozoites adherent to five or more erythrocytes after a 30 min coincubation on ice. Under that condition, where the amoebae did not ingest erythrocytes (Huston *et al.*, 2003), 30% ± 8% of the *EhRab8A* gene-silenced cells adhered to ≥5 erythrocytes,

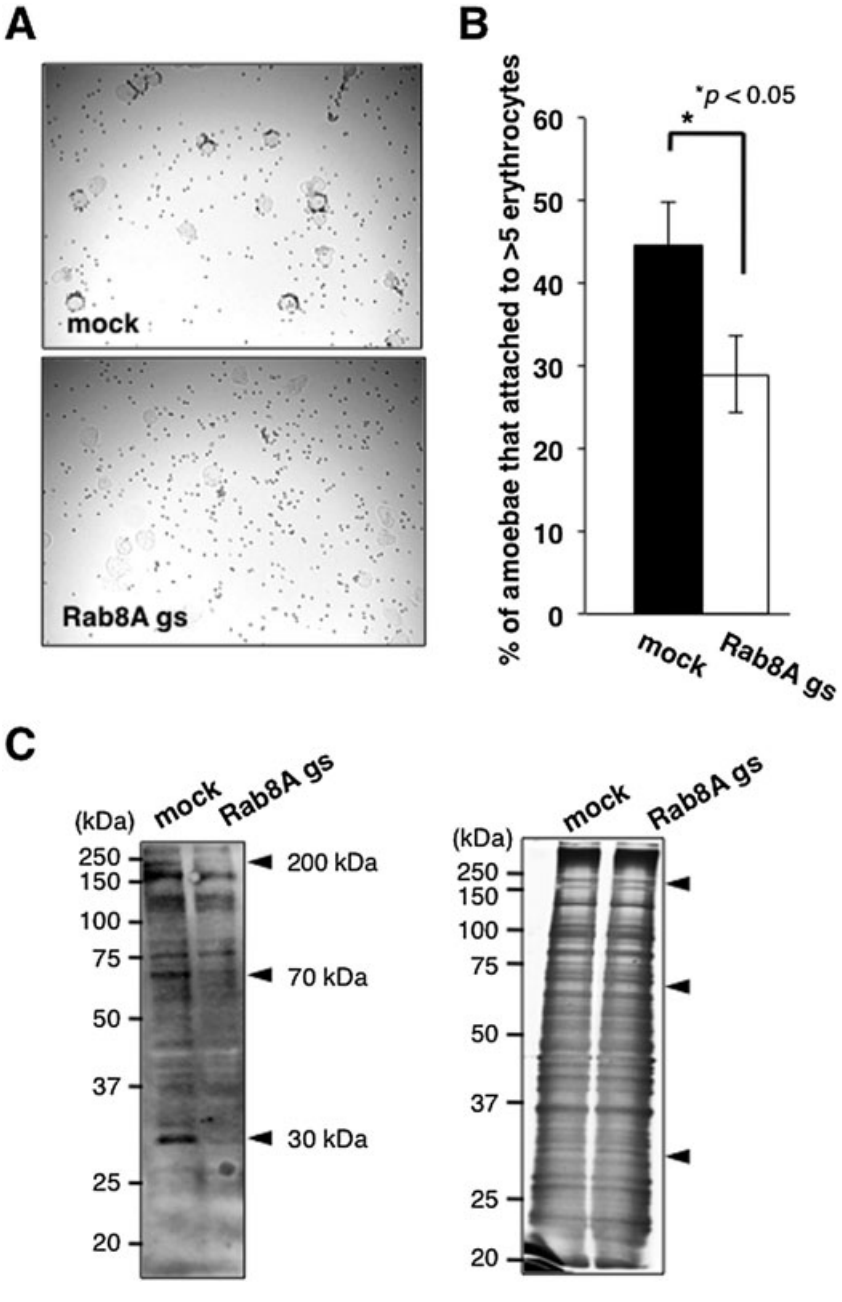

Fig. 2. Reduced adhesion to erythrocytes and surface protein expression by the EhRab8A gene-silenced strain.
A. Microscopic images of adhesion of the EhRab8A gs and mock control strains to erythrocytes. Only representative micrographs are shown.
B. The percentages of the amoebae that adhered to five or more erythrocytes are shown. The correlation coefficients were calculated using Student's *t*-test: $^{*}P < 0.05$.
C. Surface protein profile of the trophozoites of the EhRab8A gs and mock control strains. Live amoebae were labelled with biotin, solubilized and subjected to immunoblot analyses with streptavidin–HRP (left panel) and staining with SYPRO Ruby (right panel). Note that the intensities of the three bands corresponding to 200, 70 and 30 kDa (arrowheads) were reduced in the EhRab8 gs strain.

whereas 47% ± 3% of the mock control cells attached to ≥ 5 erythrocytes ($n = 100$ for each, $P = 0.026$) (Fig. 2B). These data suggested that the *EhRab8A* gene-silenced transformant was less capable of adherence to erythrocytes.

It has been shown that amoebic host recognition and attachment are mediated by a range of PM-associated proteins, including Gal/GalNAc lectin (Petri *et al*., 1989; Cheng *et al*., 1998), TMK96 (Boettner *et al*., 2008), STIRP (MacFarlane *et al*., 2007), KERP1 (Santi-Rocca *et al*., 2008) and SREHP (Stanley *et al*., 1990). To examine the surface protein profiles of *EhRab8A* gene-silenced and mock control cells, the PMs of live trophozoites were labelled with biotin and visualized on immunoblots using streptavidin–horseradish peroxidase (HRP) after SDS-PAGE (Zhao *et al*., 2004) (Fig. 2C). The intensities of at least three major bands, corresponding to 200, 70 and 30 kDa, decreased in *EhRab8A* gene-silenced cells compared with those in the mock control, indicating that these surface proteins are possibly responsible for the reduction in amoebic adherence to the ligands. To examine if these bands corresponded to the aforementioned proteins, an indirect immunofluorescence assay

was conducted. However, no difference was observed in the staining of the PM of the heavy (170 kDa) and intermediate (150 kDa) subunits of Gal/GalNAc lectin by the corresponding specific antibodies (Petri *et al*., 1990; Cheng *et al*., 2001) (Fig. S1A and B). Surface staining with anti-KERP1 (21 kDa) antibody also showed no difference between mock control and *EhRab8A* gene-silenced cells (Seigneur *et al*., 2005) (Fig. S1C). Furthermore, the apparent molecular mass of the missing bands in the *EhRab8A* gene-silenced strain did not match the sizes of the aforementioned proteins (TMK96, 146 kDa; STIRP, 280 kDa; SREHP, 50 kDa). No difference was observed in the profiles of the whole cell lysate stained with SYPRO Ruby after SDS-PAGE between the *EhRab8A* gene-silenced strain and mock control cells (Fig. 2C).

EhRab8A localized to the ER, but not to the trans-Golgi network nor to lysosomes

EhRab8A is homologous to yeast Sec4p and human Rab8, with 49–53% amino acid identity (Juarez *et al*., 2001; Saito-Nakano *et al*., 2005) (Fig. S2). Yeast Sec4p and human Rab8 are known to be involved in trafficking from the trans-Golgi network (TGN) to the PM (Salminen and Novick, 1987; Huber *et al*., 1993). Further, colocalization of Rab8 with Rab11, which is the upstream regulator of Rab8, has also been reported (Feng *et al*., 2012; Wang *et al*., 2012). Thus, we examined whether EhRab8A was colocalized with EhRab11B, which was previously demonstrated to be on the TGN (Mitra *et al*., 2007). The transformant cells expressing EhRab8A with the amino-terminal 3myc-tag were established and subjected to immunoblots using anti-EhRab8A and anti-myc antibodies (Fig. 3A). 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; 26.4 kDa, 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. We 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 (Nakada-Tsukui *et al*., 2012), 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 (Sato *et al*., 2006) (Fig. 3B). The amount of exogenously expressed myc-EhRab8A protein was 2.1 ± 0.64-fold higher than that of the endogenous protein (Fig. 3A). An

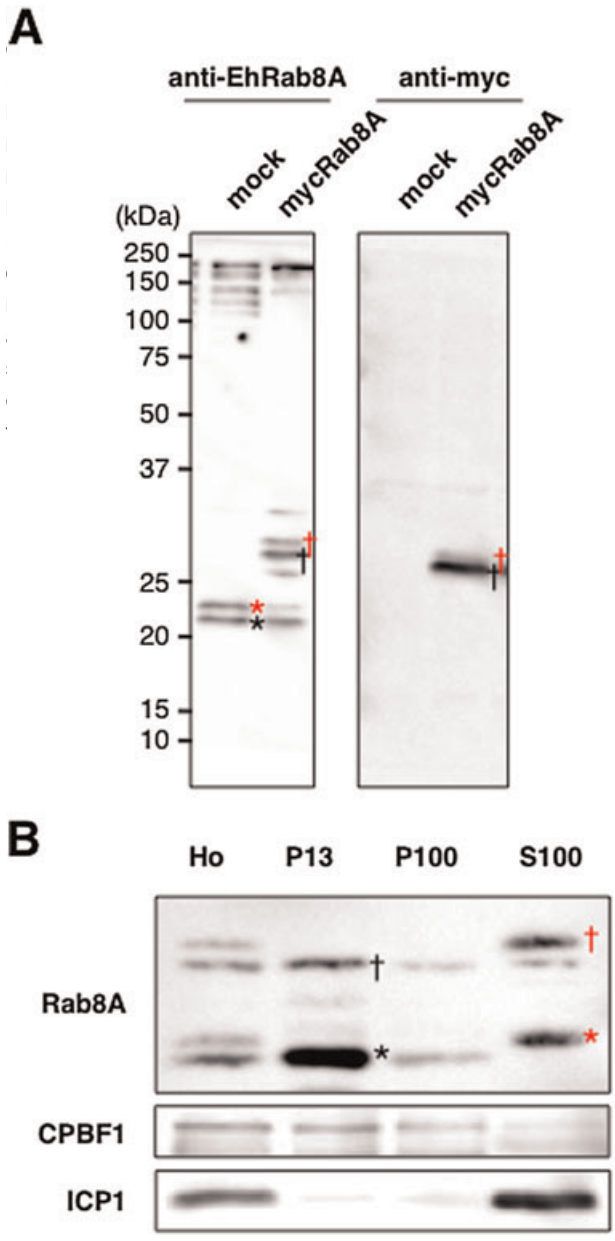

Fig. 3. Subcellular fractionation of EhRab8A.
A. Immunoblot analysis of endogenous (wild-type) and myc-EhRab8A detected by anti-EhRab8A (left) and anti-myc (right) antibodies. In mock cells, two bands corresponding to endogenous unmodified (a black asterisk) and modified EhRab8A (a red asterisk) were 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 × g centrifugation), the high-speed pellet (p100, the pellet fraction of 100 000 × g centrifugation) and the supernatant fractions (s100, the supernatant fraction of 100 000 × 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).

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 (Mitra *et al*., 2007) ($R = 0.24$, Fig. 4B). Furthermore, EhRab8A did not colocalize with Vps26, 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 (Nakada-Tsukui *et al*., 2005; Rojas *et al*., 2008; Cullen and Korswagen, 2012) ($R = 0.05$, Fig. 4C). Local-

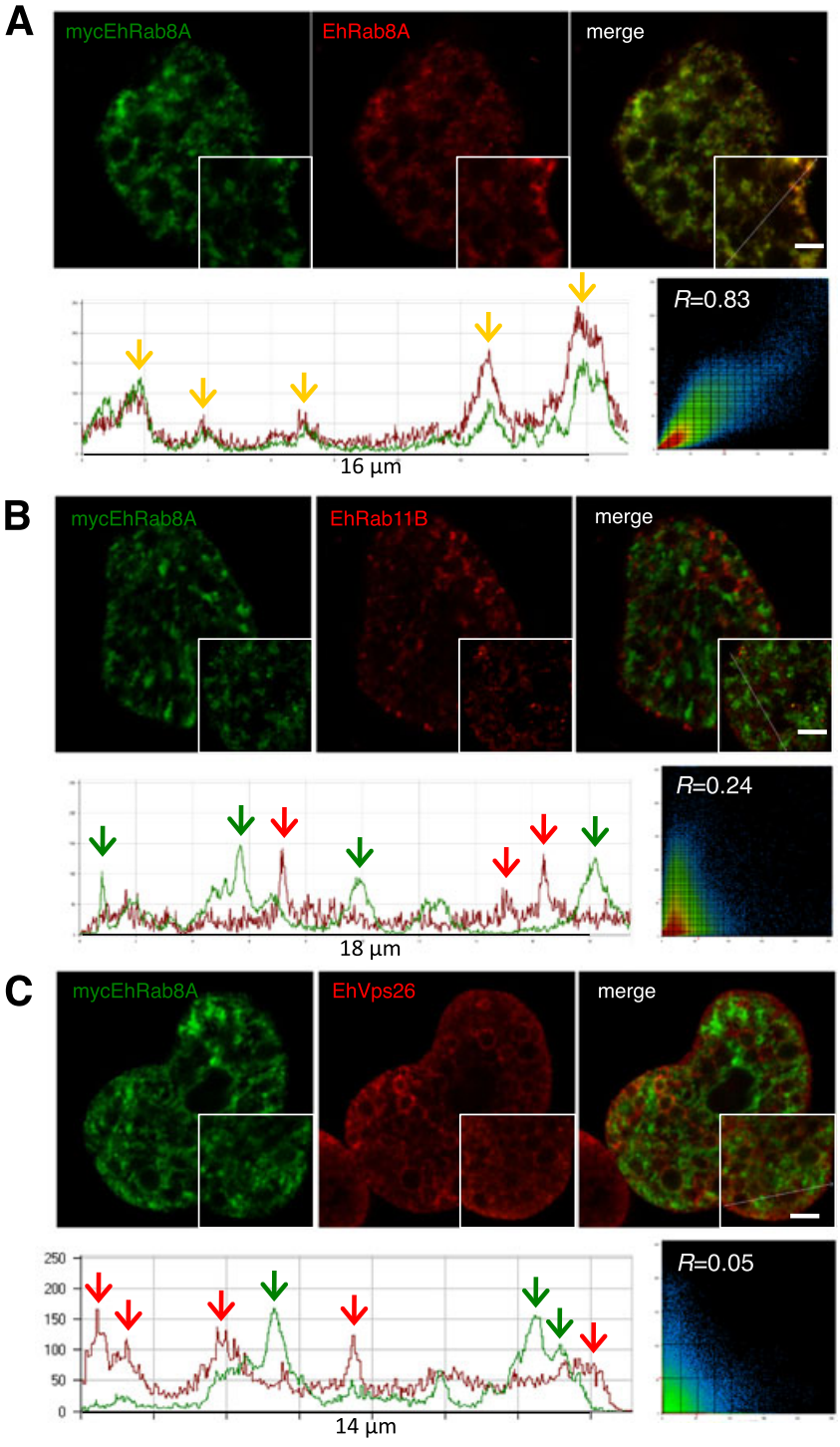

Fig. 4. Lack of colocalization of exogenously expressed myc-EhRab8A and the trans-Golgi network markers.
A–C. Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and endogenous EhRab8A, stained with anti-EhRab8A (A, red), anti-EhRab11B (B, red) or anti-EhVps26 (C, red) antibodies. Histograms of the green and red intensities along the lines indicated in the islets on the bottom right of each image are shown at the bottom left of panels A–C. 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, scatter plots showing the results of colocalization analyses are shown. R = Pearson's correlation coefficient. Thick bars, 2 µm.

ization of EhRab8A also clearly differed from that of EhRab7B and EhRabB, which is localized to lysosomes or the phagocytic mouth respectively (Rodriguez *et al*., 2000; Saito-Nakano *et al*., 2007) (Fig. S4). This observation was in contrast to other organisms (Knodler *et al*., 2010; Wang *et al*., 2012). In mammals, Rab10, which shares 70% amino acid identity with Rab8 (Chen *et al*., 1993; Pereira-Leal and Seabra, 2001) (Fig. S2), is known to localize to endosomes (e.g. in Madin–Darby canine kidney epithelial cells) (Babbey *et al*., 2006; Cardoso *et al*., 2010). In addition, Rab10 is localized to the ER in *Xenopus laevis* egg, COS-7 and HeLa cells (English and Voeltz, 2013); we next examined if EhRab8A localizes to the ER using antibodies raised against bacterial recombinant proteins of the ER luminal chaperone BiP (EHI_199890, 64% amino acid identity to human BiP, E-value = 0) (Munro and Pelham, 1986) and a component of the COPII vesicle, Sec13 (EHI_001050, 38% amino acid identity to human Sec13, E-value = 6e−46) (Barlowe *et al*., 1994). Monospecificities of the anti-BiP and anti-Sec13 antibodies were verified by immunoblot analyses of amoebic lysates (Fig. S3). An indirect immunofluorescence assay performed using the anti-BiP antibody revealed partial colocalization of myc-EhRab8A and BiP, suggesting that EhRab8A was localized to the ER or a compartment adjacent to the ER ($R = 0.7$, Fig. 5A). A subdomain of the ER, where COPII vesicle budding occurs, was named the ER exit site (ERES) (Jensen and Schekman, 2011), and it was visualized with the anti-Sec13 antibody. Sec13 did not colocalize with EhRab8A ($R = 0.15$, Fig. 5B). These indirect immunofluorescence assay data were also supported by the data of physical separation by two rounds of discontinuous Percoll gradient ultracentrifugation (Mi-ichi *et al*., 2009), followed by immunoblot assay, in which the distribution pattern of EhRab8A was similar to that of Sec13 (Fig. S5).

Overexpression of wild-type Rab8A enhanced phagocytosis, whereas a dominant-negative Rab8 mutant inhibited phagocytosis

Nucleotide-fixed state mutations can be engineered to alter the activity of Rab proteins (Stenmark *et al*., 1994).

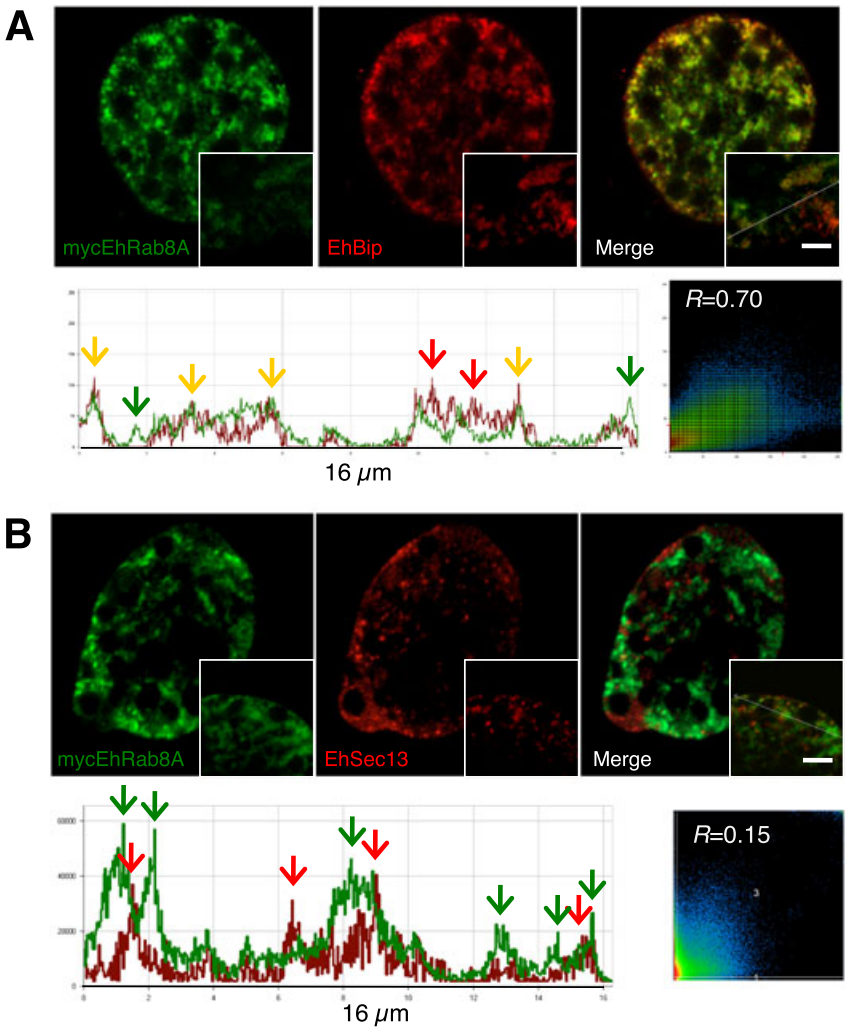

Fig. 5. Colocalization of exogenously expressed myc-EhRab8A and endoplasmic reticulum markers.
A and B. Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and the anti-EhBiP (A, red) or anti-EhSec13 (B, red) antibodies. Histograms of the green and red intensities along the lines indicated in the islets on the bottom right of each image are shown at the bottom left of panels A and 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 and B, scatter plots showing the results of the colocalization analyses are shown.
R = Pearson's correlation coefficient. Thick bars, 2 μm.

Two mutants of myc-EhRab8A, a GTP-fixed Q66L mutant and a GDP-fixed S21N mutant, were generated and introduced into the amoeba 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) (Der *et al*., 1986; Stenmark *et al*., 1994). 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 functions as a constitutively negative (GDP-fixed) form (S21N) (Feig and Cooper, 1988; Stenmark *et al*., 1994).

Confocal fluorescence micrographs revealed that 40–60% of the trophozoites were stained with the anti-myc antibody (Fig. 6A). Localization of myc-EhRab8A Q66L mutant was similar to that of wild-type Rab8A and also well colocalized to BiP, whereas S21N mutant revealed dispersed cytosolic staining like the Rab8 SN mutant in other organisms (Walworth *et al*., 1989; Peranen *et al*., 1996) (Fig. S6). The localization of Q66L and S21N mutants is consistent with presumed characteristics of GTP-fixed and GDP-fixed mutants. We quantified the amount of fluorescein-labelled erythrocytes ingested by the trophozoites expressing either wild-type myc-

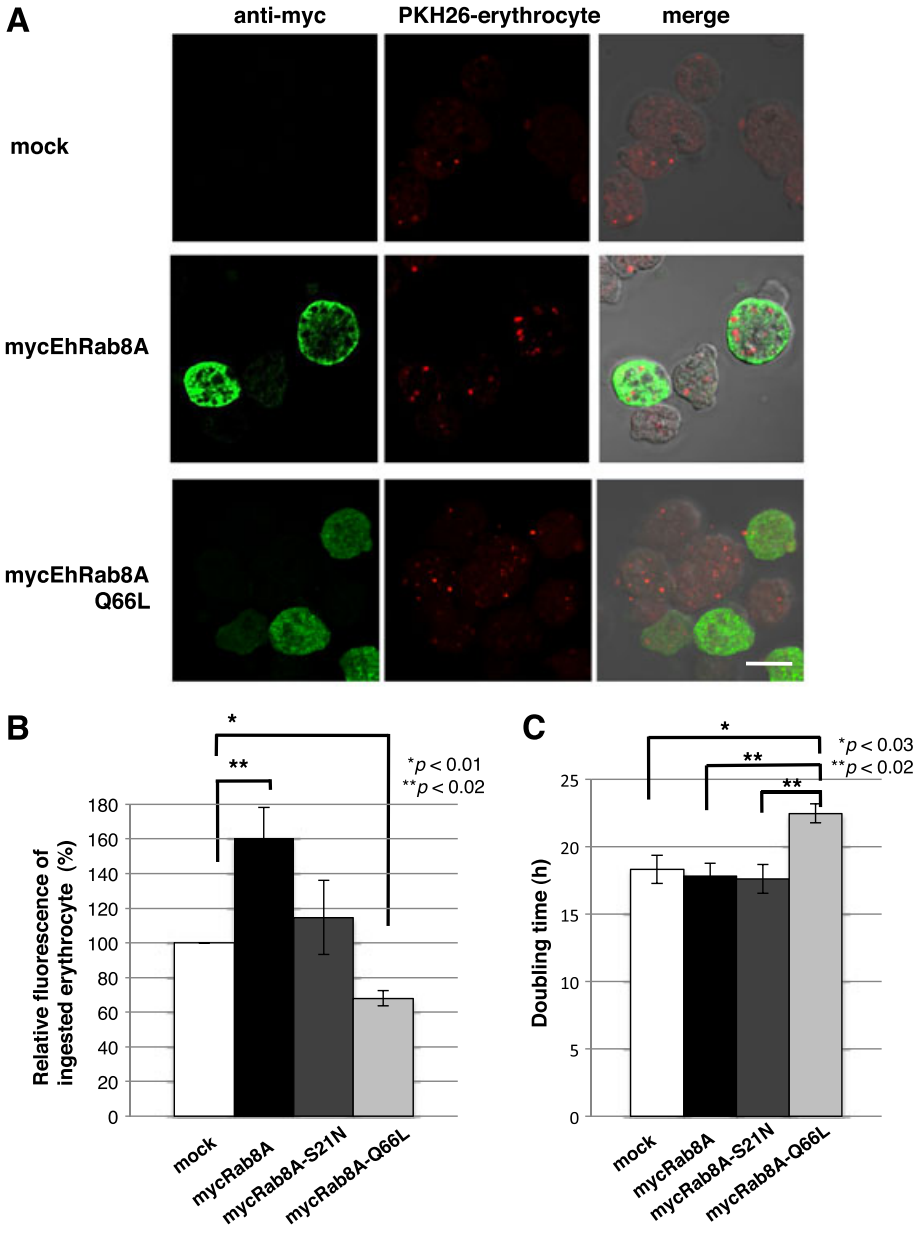

Fig. 6. Expression of EhRab8A GTP-bound mutant caused a defect in phagocytosis.
A. Immunofluorescence imaging of EhRab8A and erythrophagocytosis. Transformant trophozoites expressing myc-EhRab8A wild-type or myc-EhRab8A-Q66L mutant and mock transformant cells were mixed with PKH-labelled erythrocytes 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-labelled erythrocytes ingested by a single trophozoite were measured, and the average of the total fluorescence intensities per trophozoite for each strain is shown. Note that the 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 were calculated using Student's *t*-test.

EhRab8A or two mutant forms of myc-EhRab8A (Fig. 6A and B). The intensity of fluorescence reflecting the amount of ingested erythrocytes significantly increased in wild-type myc-EhRab8A-expressing cells compared with the mock control (160% ± 17% compared with the mock control; $P < 0.02$, Fig. 6B). Expression of the GDP-fixed mutant showed a slight increase in erythrophagocytosis (114% ± 21% compared with the control; $P > 0.5$). In contrast, expression of the GTP-fixed mutant had a negative effect on erythrophagocytosis (68% ± 4.3% compared with 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. 6C) (mock, 18.3 ± 1.03 h; wild-type EhRab8A, 17.8 ± 0.95 h; GDP-fixed mutant, 17.6 ± 1.06 h; GTP-fixed mutant, 22.4 ± 0.71 h; $P < 0.03$ between GTP-fixed mutant and each of mock, GDP-fixed mutant transformant or wild type). Expression of GTP-fixed or GDP-fixed mutants did not affect the ER morphology, which is visualized with anti-BiP antibody (Fig. S6).

Discussion

ER-localized EhRab8A *is required for phagocytosis*

In this study, we have shown using two genetic approaches that in *E. histolytica* unique ER-localized Rab8 is involved in phagocytosis. First, we have demonstrated that a specific repression of the *EhRab8A* gene expression by transcriptional gene silencing reduced amoebic adherence to and phagocytosis of prey and also surface presentation of a few PM proteins (Figs. 1 and 2). Further, we have also shown that overexpression of the wild type or GTP-fixed mutant of EhRab8A enhanced or reduced phagocytosis respectively (Fig. 6). Although statistically insignificant, expression of the GDP-fixed mutant caused a slight increase (~15%) on phagocytosis compared with the mock control. EhRab8A GDP-fixed mutant caused no defect in growth, unlike GTP-locked Q66L mutant (Fig. 6C). In general, overexpressed GTP-fixed mutant Rab, but not GDP-fixed mutant, is able to compete with the intrinsic wild-type Rab for downstream effectors and elicit phenotypes. Thus, we have no good explanation for the observed slight increase of phagocytosis caused by the expression of GDP-fixed mutant. This observation may indicate that the cycle of GTP hydrolysis and guanine nucleotide exchange is essential for the completion of EhRab8A function like in other organisms (Walworth *et al*., 1989; Peranen *et al*., 1996). Rab8 is a member of the highly conserved Rab subfamily in eukaryotes, including Metazoa (*Caenorhabditis elegans* and *Nematostella vectensis*), Choanozoa (*Monosiga brevicollis*), Fungi (*Saccharomyces cerevisiae*), Amoebozoa (*Dictyostelium discoideum*), Viridiplantae (*Arabidopsis thaliana*), Stramenopiles (*Phytophthora sojae*), Ciliophora (*Tetrahymena thermophila*) and Schizopyrenida (*Naegleria gruberi*), but missing in Diplomonadida (*Giardia lamblia*) and Trichomonadida (*Trichomonas vaginalis*) (Pereira-Leal and Seabra, 2001; Saito-Nakano *et al*., 2010; Diekmann *et al*., 2011). In mammals and yeasts, Rab8 is involved in trafficking from the TGN to the PM (Hutagalung and Novick, 2011). In free-living *D. discoideum*, Rab8 is localized to a contractile vacuole, which is a specialized osmoregulatory organelle involved in water expulsion (Essid *et al*., 2012). A line of evidence provided in this study suggests that EhRab8A plays a unique function in *Entamoeba*: EhRab8A is involved in the amoeba's adherence to prey by mediating the transport of candidate receptor(s) to the PM (Fig. 2). Until now, many ER proteins including Rab1 have been identified in proteome analyses of macrophage phagosomes (Rogers and Foster, 2007; Gutierrez, 2013); the results suggested that the ER may supply membranes to the PM to engulf large foreign particles and to recruit major histocompatibility complex class I molecules to phagosomes (Gagnon *et al*., 2002; Guermonprez *et al*., 2003). 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 (Boettner *et al*., 2008) and COPII components (Marion *et al*., 2005). 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 PM are dynamically interconnected in *E. histolytica* (Marquay Markiewicz *et al*., 2011; Vaithilingam *et al*., 2012) (also see below).

The transport of PM receptors is mediated by EhRab8A

Among seven Rab proteins that we successfully silenced by gene silencing, *EhRab8A* and *EhRab7B* gene-silencing strains showed a defect in phagocytosis. Interestingly, adhesion of *EhRab7B* gene-silencing strain to erythrocytes was comparable with that of mock transformant, indicating that EhRab8A and EhRab7B are involved in distinct stages of phagocytosis (our unpublished result). Surface biotinylation has shown that a few major surface proteins (200, 70 and 30 kDa) were apparently missing in the *EhRab8A* gene-silenced strain (Fig. 2C). Although their identity remains unknown, they are probably involved in the adherence to various extracellular prey and transported from the ER to the PM in an EhRab8A-dependent mechanism. The amoebic cell

surface proteome, comprising nearly 700 proteins (Biller *et al*., 2014), possibly contains these potential surface receptors. Our attempt to identify and characterize these potential surface receptors is underway. It is noteworthy that the transport of at least three proteins, the heavy and intermediate subunits of the Gal/GalNAc lectin and KERP1, was not mediated by EhRab8A as their surface expression remained unchanged by *EhRab8A* gene silencing (Fig. S1). The light subunit of Gal/GalNAc lectin (30–35 kDa) (Petri *et al*., 1989), which is coded by five independent genes (Bracha *et al*., 2007), is an alternative candidate for the 30 kDa surface protein transported by EhRab8A.

EhRab8A *may be involved in a non-conventional traffic pathway from the ER to the PM*

The absence of EhRab8A at the ERES (Fig. 4C) 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 (Demmel *et al*., 2011; Herzig *et al*., 2012). 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 (Venditti *et al*., 2014). Thus, our 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. S2) and forms a specialized domain linked to phospholipid synthesis involving phosphatidylinositol synthase (PIS) and ethanolamine phosphotransferase 1 (CEPT1) (English and Voeltz, 2013). 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 (English and Voeltz, 2013). The *E. histolytica* genome encodes both PIS and CEPT1 (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 PM proteins via the lipid biosynthesis. Our 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.

Lack of the Rab11–Rab8 cascade for transport from the TGN to the PM in E. histolytica

In the mammalian secretory pathway, Rab11 functions upstream of Rab8. The GTP-bound active form of Rab11 binds its effector molecule, Rabin8, which is a guanine nucleotide exchange factor (GEF) of Rab8 (Hattula *et al*., 2002). Consistently, partial colocalization of Rab8 and Rab11 in the TGN has been reported in mammalian cells (Hattula *et al*., 2006; Knodler *et al*., 2010). This Rab cascade, comprising Ypt32p and Sec2p, homologues of Rab11 and Rabin8, respectively, is also conserved in the budding yeast (Walch-Solimena *et al*., 1997). In contrast, in *E. histolytica*, EhRab8A and EhRab11B were not colocalized (Fig. 4B), indicating that EhRab8A and EhRab11B regulate non-overlapping steps of the same pathway or independent pathways. We have 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 PM in *E. histolytica* (Mitra *et al*., 2007). Lack of the Rab11–Rab8 cascade in *E. histolytica* is also consistent with the finding that Sec2, Rab8 GEF, is only conserved in Opisthokonts, with exceptions of Dipterans and Microsporidia, and absent in Amoebozoa (Elias, 2008). 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.

Experimental procedures

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 (Diamond *et al*., 1972; 1978).

Establishment of an EhRab8A *gene-silenced strain, a 3myc-tagged EhRab8-expressing strain and strains expressing dominant-active and dominant-negative EhRab8A mutants*

A plasmid for the gene silencing of EhRab8A was constructed as follows. A 480-bp-long 5′ end of the *EhRab8A* protein-coding region was amplified by PCR from cDNA using the following oligonucleotides: 5′-AAA AGG CCT TCG GAG AAG GAT TCA ACA ATT-3′ and 5′-GCC GAG CTC ACA GTC ATC AAC TTT TAA TGA-3′ (the StuI and SacI restriction sites are underlined). The PCR-amplified DNA fragment was digested with StuI and SacI and ligated into StuI and SacI double-digested pSAP2-Gunma (Mi-ichi *et al*., 2011) to produce pSAP2-EhRab8A. The EhRab8A gene-silenced strain was established by transfection of the G3 strain (Bracha *et al*., 2006) by liposome-mediated transfection, as previously described (Nozaki *et al*., 1999; Saito-Nakano *et al*., 2004); stable transformants were cultured in the medium containing 10 μg ml^{-1} Geneticin (Life Tech Oriental).

Moreover, we established a control mock transformant by transfection of the G3 strain with pSAP2-Gunma.

A plasmid was constructed as follows for the creation of a transformant wherein EhRab8A, with the 3myc-tag 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 (Saito-Nakano *et al*., 2004) 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 (Landt *et al*., 1990) using a PrimeSTAR Mutagenesis Basal Kit (Takara). These plasmids were introduced into trophozoites as described earlier.

Quantitative real-time PCR

mRNA expressions of *EhRab8A* and *EhRab8B* were analysed by quantitative real-time PCR analyses, essentially as previously described (Gilchrist *et al*., 2006; Saito-Nakano *et al*., 2007), with some modifications. RNA polymerase II served as an internal control (GenBank accession number XP_649091). The primers used were as follows: 5′-ATT CCA TTC TTA GAA ACA TCT GCC AAA AAT-3′ and 5′-GAA GAA GAA GGA AAT GTG ATT AAC AAC ATC-3′ (*EhRab8A*); 5′-GAG CTT GCT GAT AAA TTA GGT ATT CCT TTC-3′ and 5′-ACA ACA GCC ACT TTG ACC TTC TTG TGA AGA-3′ (*EhRab8B*); and 5′-GAT CCA ACA TAT CCT AAA ACA ACA-3′ and 5′-TCA ATT ATT TTC TGA CCC GTC TTC-3′ (RNA polymerase II). The following parameters were used: an initial step of denaturation at 95°C for 9 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 65°C for 1 min. A final step at 95°C for 9 s, 60°C for 9 s and 95°C for 9 s was used to remove the primer dimers.

Production of recombinant EhRab8A, EhBiP, EhSec13 and EhRab11B

In order to generate a plasmid expressing the maltose-binding protein-fused EhRab8A-recombinant protein, the full-length EhRab8A open reading frame was inserted into the pMAL-c2X vector (New England Biolabs, Ipswich, MA, USA). Further, to generate a plasmid expressing glutathione-*S*-transferase-fused BiP or Sec13, the C-terminal domain of BiP (amino acids 435–661) or full-length Sec13 was inserted into the pGEX-6p1 vector (GE Healthcare). The full length of EhRab11B was inserted into the pColdI vector (Takara) for expressing the histidine-tagged recombinant EhRab11B. Recombinant proteins were expressed using the *E. coli* strain BL21 (DE3) and purified according to the manufacturer's instructions.

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 maltose-binding protein-conjugated-*EhRab8A* according to the manufacturer's instruction. The antiserum was purified using a HiTrap protein G kit (GE Healthcare).

Phagocytosis assay

Phagocytosis was assayed by either flow cytometry (FACSCalibur) or microscopy. The flow cytometry assay for amoebic ingestion was adapted from the method previously described by Huston *et al*. (2003), with some modifications. FluoSpheres (2 μm of carboxylated Nile Red-labelled beads, Invitrogen), PKH26 red fluorescent-labelled (Sigma) erythrocytes and fluorescein-conjugated *E. coli* K-12 strain BioParticles (Invitrogen) were incubated with 1×10^5 amoebic trophozoites in 200 μl of BIS medium in a well of a 12-well plate at 37°C for 10–60 min. Following the incubation, the amoebae and beads or cells were resuspended in 2% D-galactose in ice-cold phosphate-buffered saline (PBS) and analysed by flow cytometry. The amoebae were distinguished from beads, erythrocytes and *E. coli* cells on the basis of differences in the forward-scatter and side-scatter signal characteristics and background fluorescence. Efficiency of the amoebic ingestion of targets was determined by the percentages of amoebae with fluorescence levels above the background. For the microscopy assay to examine the phagocytosis efficiency, the myc-EhRab8A dominant-negative mutant-expressing cells were attached to the slide glass and engulfed with PKH26-labelled 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 analysed with ZEN software (Zeiss).

Adherence assay

Entamoeba histolytica adherence was assayed as previously described (Huston *et al*., 2003) with some modifications. Approximately 1×10^5 amoebae and 1×10^7 erythrocytes were resuspended in 1 ml Opti-MEM I medium (Life Technologies) supplemented with 5 mg ml^{-1} of cysteine and 1 mg ml^{-1} of ascorbic acid (pH 6.8), centrifuged (440 × g, 5 min, 4°C) and incubated for 30 min on ice. After the incubation, the supernatant was poured off, and the pellet was resuspended in 100 μl of transfection medium. The sample was then examined under a microscope.

Surface biotinylation

Approximately 10^7 live *E. histolytica* trophozoites were biotinylated in 500 μl of Opti-MEM, pH 8.0, for 15 min at 4°C with EZ-Link Sulfo-NHS-Biotin (Pierce), as previously described (Berro *et al*., 2007). After the suspension, the cells were centrifuged at 500 × g for 5 min in 0.5 ml of cold Dulbecco's PBS with 0.1% lysine that

was added to the cell pellet, and the cells were further washed two times with PBS. Next, the cells were lysed by 1× SDS sample buffer and subjected to immunoblot analysis with avidin–HRP and SYPRO Ruby (Life Technologies) staining.

Subcellular fractionation

The accessibility by proteinase K assay was performed as reported previously with some modifications (Saito-Nakano *et al*., 2007). Approximately 3×10^5 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^{-1} of 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 × g for 2 min, the supernatant was centrifuged at 13 000 × 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 × g at 4°C for 1 h to obtain soluble (s100) fractions. These fractions were subjected to immunoblot analyses with anti-EhRab8A, anti-CPBF1 (Nakada-Tsukui *et al*., 2012) or anti-ICP1 antibodies (Sato *et al*., 2006).

Confocal microscopy

An indirect immunofluorescence assay was conducted, essentially as previously described (Saito-Nakano *et al*., 2004). Trophozoites were transferred to 8 mm round wells 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-Vps26 (Nakada-Tsukui *et al*., 2005), anti-BiP (this study), anti-Sec13 (this study) polyclonal rabbit sera and the anti-heavy (clone 7F4) and intermediate (clone EH3015) subunits of Gal/GalNAc lectin, the last two of which were a gift from William A. Petri, Jr., and Hiroshi Tachibana respectively (Petri *et al*., 1990; Cheng *et al*., 2001). Anti-KERP1 rabbit serum is a gift from Nancy Guillén (Seigneur *et al*., 2005).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Staining of the surface Gal/GalNAc lectin subunit in EhRab8A gene-silenced (gs) cells. The EhRab8A-gs and mock cells were fixed and stained with the anti-lectin heavy (A) and intermediate (B) subunits with the 7F4 and EH3014 monoclonal antibody clones and anti-KERP1 antibody (C) respectively.

Fig. S2. Sequence alignment (A) and percentage identity (B) of EhRab8A, EhRab11B, *Homo sapiens* HsRab8A, HsRab10 and *Saccharomyces cerevisiae* Sec4p. Amino acid 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 (A). Amino acid percentage identity matrix was created by CLUSTALW (B).

Fig. S3. Immunoblot analysis using newly raised antibodies. The wild-type trophozoite was suspended with 1× SDS sample buffer supplemented with 0.5 mg ml^{-1} of E64, a cysteine protease inhibitor; the total cell lysate was then prepared by freeze–thawing. The lysate was applied to SDS-PAGE without boiling in order to avoid protein degradation, particularly for BiP. The following antibody dilutions were used: anti-Sec13 at 1:1000, anti-BiP at 1:1000 and anti-Rab11B at 1:1000.

Fig. S4. EhRab8A did not localize to the lysosome and phagocytic cup. A. Lysosomes were stained with Lyso Tracker Red (red), and myc-EhRab8A cells were subjected to immunofluorescence assay using anti-myc antibody (green). B. After 5 min of phagocytosis of erythrocytes, myc-EhRab8A cells were fixed and subjected to immunofluorescence assay using anti-myc antibody (red). Phagocytic cup was stained with phalloidin-Alexa Fluor 488 (green). Bars, 10 μm.

Fig. S5. 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 (Mi-ichi *et al*., 2009) and subjected to immunoblot using anti-Cpn60 (mitosome), anti-EhRab8A and anti-Sec13 antibodies respectively. The first Percoll fractions that are positive for Cpn60 were subjected to the second Percoll fractionation. Membrane-bound Sec13 was fractionated to J to L, and Cpn60 was detected from the L to M fractions. EhRab8A was co-fractionated with the Sec13-positive fraction (J and K).

Fig. S6. 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 pattern was similar to that of the wild-type myc-EhRab8A. GDP-bound myc-EhRab8A S21N showed cytosolic staining.