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Novel TPR-containing subunit of TOM complex functions as cytosolic receptor for *Entamoeba* mitosomal transport

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Under anaerobic environments, the mitochondria have undergone remarkable reduction and transformation into highly reduced structures, referred as *m*itochondrion-*r*elated *o*rganelles (MROs), which include mitosomes and hydrogenosomes. In agreement with the concept of reductive evolution, mitosomes of *Entamoeba histolytica* lack most of the components of the TOM (*t*ranslocase of the *o*uter *m*itochondrial membrane) complex, which is required for the targeting and membrane translocation of preproteins into the canonical aerobic mitochondria. Here we showed, in *E. histolytica* mitosomes, the presence of a 600-kDa TOM complex composed of Tom40, a conserved pore-forming subunit, and Tom60, a novel lineage-specific receptor protein. Tom60, containing multiple tetratricopeptide repeats, is localized to the mitosomal outer membrane and the cytosol, and serves as a receptor of both mitosomal matrix and membrane preproteins. Our data indicate that *Entamoeba* has invented a novel lineage-specific shuttle receptor of the TOM complex as a consequence of adaptation to an anaerobic environment.

Mitochondria are highly divergent structures in eukaryotes, and often reveal degenerate morphology, function, and components in eukaryotes that have been adapted in anoxic or hypoxic environments. Such degenerated mitochondria with reduced or no organellar genome are called mitochondrion-related organelles (MROs), which include mitosomes and hydrogenosomes. While the minimal common function of MROs is still in debate[1–6], protein import of nuclear-encoded proteins into MROs is indispensable for the organisms that possess MROs. All organisms possessing MRO that have been investigated so far, indeed retain at least a gene encoding the core translocation channel Tom40 of the TOM (*T*ranslocase of the *O*uter membrane of *M*itochondria)[7–11]. However, in agreement with the concept of reductive evolution, other components of the canonical aerobic mitochondria such as subunits of TOM, SAM (*S*orting and *A*ssembly *M*achinery), TIM (*T*ranslocase of the *I*nner membrane of *M*itochondria), and small TIM complexes[8,12,13] are often missing in MROs. These data imply two possible scenarios of evolution of mitochondrial protein import: the majority of the import machinery of MROs has been secondarily lost[7,8], or the transport machinery or subunits were replaced with unique and possibly lineage-specific components[14].

The TOM complex is involved in the initial process of the import of nuclear-encoded mitochondrial preproteins into the mitochondria. Remarkable variation exists in the architecture of TOM complexes among eukaryotic lineages. In yeast and mammals, the translocation channel (Tom40), membrane-anchored receptors for the recognition of a targeting signal in preproteins (Tom22, Tom20, and Tom70), and accessory subunits (Tom5, Tom6, and Tom7) consist the TOM complex[15,16]. In plants, an 8-kDa truncated form of Tom22 serves as translocase[17] and chloroplast import receptor Toc64 homolog functions as a TOM component[18], while in trypanosomes, Tom40 appears to be replaced by Omp85 of archaic origin[19]. Tom20, a presequence binding receptor appeared to have independently evolved from two distinct ancestral genes in the animal and plant lineages[20]. Therefore, the investigation of the TOM complex may shed light on the evolution of the protein import machinery of endosymbiont-derived organelles.

Entamoeba histolytica is an anaerobic unicellular parasite, and causes hemorrhagic dysentery and extra intestinal abscesses that are responsible for an estimated 100,000 deaths in endemic areas annually[21]. This parasite possesses mitosomes, and is a good representative of mitochondrial diversification. *Entamoeba* MRO contains the sulfate activation pathway, which has been so far identified only in this organism[9]. Moreover, it lacks a

genome[22], has no membrane potential[22,23], and is devoid of an import system using the canonical transit peptide[9]. Furthermore, *E. histolytica* has none of the homologous subunits of the TOM complex except Tom40[9,13]. This fact, more specifically the lack of Tom20 and Tom70 receptors, suggests that import of mitosomal proteins does not depend on receptor recognition in *Entamoeba*, or that *Entamoeba* possesses an unprecedented receptor subunit undetectable by currently available *in silico* analysis. Here we show that the TOM complex in the *E. histolytica* mitosomes contains a lineage specific subunit, designated Tom60, which is associated with Tom40. Repression of Tom40 or Tom60 by gene silencing shows defects in protein import to mitosomes, and consequently retardation of proliferation. Tom60 is distributed to both the periphery of the mitosomal outer membrane and the cytosol. Moreover, our data strongly suggest that Tom60 is capable to bind *in vitro* to both mitosomal matrix proteins and membrane proteins.

Results

Demonstration of Tom40 localization on *Entamoeba* mitosomes. As we aimed to characterize TOM complex from *Entamoeba,* we first established an *E. histolytica* cell line expressing hemagglutinin (HA)-tagged *E. histolytica* Tom40 (EhTom40) at the carboxyl terminus (Tom40-HA). To verify the expression and mitosomal localization of Tom40-HA, we fractionated lysates from Tom40-HA-expressing trophozoites by two rounds of Percoll gradient ultracentrifugation and analyzed the fractions by immunoblot with anti-HA antibody and anti-Cpn60 antiserum[9]. Cpn60 served as a canonical mitosomal marker. The banding pattern of Tom40-HA among fractions was similar to that of Cpn60 (Supplementary Fig. S1). Next, we carried out the immunofluorescence assay (IFA) using anti-HA antibody and anti-Cpn60 antiserum (Supplementary Fig. S2). Fluorescence signals of Tom40-HA were observed as dotted pattern and were merged with fluorescence signals of Cpn60, suggesting that EhTom40 is localized in mitosomes. Moreover, mitosomal localization of EhTom40 was also supported by immunoelectron microscopy (immuno-EM) (Supplementary Fig. S3) showing that Tom40-HA is concentrated on mitosomal outer membranes.

Identification of 600-kDa *Entamoeba* TOM complex and a novel subunit Tom60. The TOM complex exists in yeast as a ~400-kDa complex, composed of Tom40, Tom22, Tom5, Tom6, and Tom7[24]. To see if *Entamoeba* mitosomes contain TOM complex, and if so, to isolate the whole complex and identify proteins interacting with EhTom40, we investigated an EhTom40-containing complex by blue native polyacrylamide gel electrophoresis (BN-PAGE) followed by immunoblot with anti-HA antibody. Immunoblot analysis of the 100,000 × *g* organelle-enriched fraction of Tom40-HA-expressing trophozoites with anti-HA antibody showed a 600-kDa band (Fig. 1a). To isolate and identify proteins that are associated with the 600-kDa band, the complex was immunoprecipitated with anti-HA antibody (Fig. 1b) and electrophoresed on SDS-PAGE under reducing conditions. A band of approximately 60-kDa in size was detected exclusively in samples co-immunoprecipitated with lysates from the Tom40-HA-expressing trophozoites (Fig. 1c). The band was subjected to liquid chromatography-tandem mass spectrometric analysis (LC-MS/MS), identified to be XP_657124 (Supplementary Fig. S4 and S5, and Supplementary Table S1A), and designated as *E. histolytica* Tom60 (EhTom60). EhTom60 was also detected by LC-MS/MS analysis of the 600-kDa complex (Supplementary Table S1B). The protein was previously identified in our mitosome proteome[9].

Lineage specific distribution of Tom60. Tom60 appears to be uniquely present in the genus. Tom60 orthologs were found in *E. dispar* and *E. invadens* (EDI_218540 and EIN_149090, respectively)

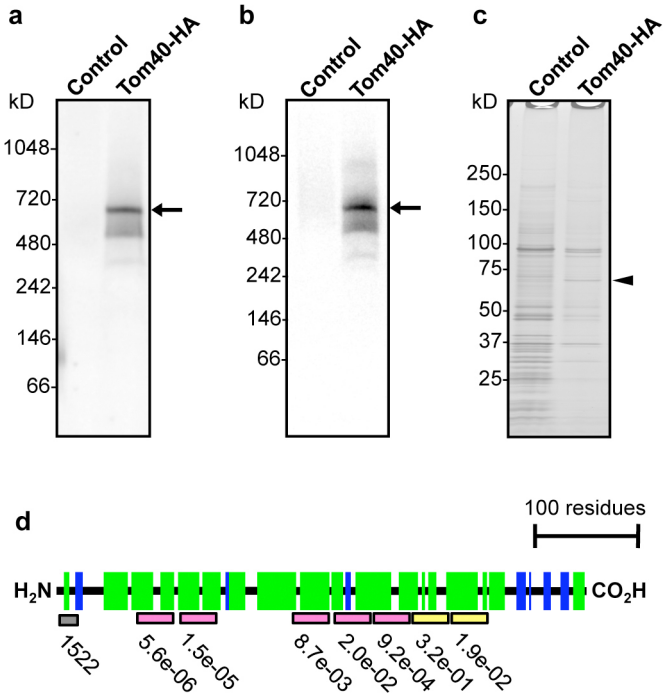


Figure 1 | Identification of the *Entamoeba* TOM complex and its novel subunit. (a), The TOM complex demonstrated by BN-PAGE and immunoblot analysis. (b), Immunoprecipitation of the TOM complex (arrows) from Tom40-HA transformant by BN-PAGE and immunoblot analysis with anti-HA antibody. (c), SDS-PAGE and silver stain of immunoprecipitated TOM complex from Tom40-HA. Arrowhead indicates Tom60. (d), Prediction of the secondary structure and the domain organization of EhTom60. Gray box indicates the hydrophobic cluster, while pink and yellow boxes depict putative tetratricopeptide repeats (TPRs) conserved among genus *Entamoeba* or those specific to *E. histolytica*, respectively. Probability and *E*-value are shown (Supplementary Fig. S4). Green and blue boxes indicate α-helices and β-strands, respectively, predicted by PSIPRED (Supplementary Fig. S6).

(Supplementary Fig. S4), whereas they were not identified in bacteria, archaea, and other eukaryotes. Among amoebozoan organisms, we confirmed by BLAST search (using the threshold of *E*-value < 0.1) that a Tom60 homolog is absent in the *Dictyostelium discoideum* (dictyBase: http://dictybase.org/) and *Acanthamoeba catellani* (https://www.hgsc.bcm.edu/content/acanthamoeba-castellani-neff) genomes, and the transcriptome of *Mastigamoeba balamuthi* (Spears, C. and Roger, A., personal communication).

In silico analyses indicate that *Entamoeba* Tom60 contains putative tetratricopeptide repeats (TPRs)[25] and an amino-terminal hydrophobic cluster (Fig. 1d and Supplementary Fig. S4). TPRs are implicated in protein-protein interactions, and are also present in Tom20 and Tom70, which are membrane-spanning receptors for mitochondrial import[26], suggesting that *Entamoeba* Tom60 may be a receptor for mitosomal import. However, in contrast to the above-mentioned TPR-containing mitochondrial receptors, which consist of only α-helices, *Entamoeba* Tom60 appears to contain β-strands, based on the secondary structure prediction by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Fig. 1d and Supplementary Fig. S6). The predicted structural differences argue against the premise that *Entamoeba* Tom60 has a common evolutionary origin with Tom20 and Tom70.

Furthermore, phylogenetic analyses of TPR elements from 23 yeast proteins, plant Toc64, human Tom34 (Supplementary Table S2), 36 *D. discoideum* proteins (Supplementary Table S3), and 28 TPR-containing proteins from *Entamoeba* (Supplementary

Table S4), showed that TPRs of *Entamoeba* Tom60s have no significant affinity with TPRs from other proteins. Therefore, we conclude that *Entamoeba* Tom60 is a genus-specific protein.

Localization and membrane topology of Tom60. We confirmed by IFA the mitosomal localization of Tom60 in an *E. histolytica* cell line expressing Tom40-Myc and Tom60-HA. EhTom40, EhTom60, and APS kinase[9] (APSK; XP_656278) were colocalized and concentrated in the mitosomes (Fig. 2a). However, faint cytosolic signals were also detected for EhTom60 (data not shown). Next, to verify localization, cellular fractionation of lysates was performed, followed by immunoblot analysis. EhTom60 was detected in both the 100,000 × *g* organelle fraction and the soluble supernatant fraction, suggesting that EhTom60 is present in both mitosomes and the cytosol. We next investigated the topology of EhTom60, EhTom40, and other mitosomal proteins by examining their sensitivity to proteinase K treatment followed by immunoblot analysis (Fig. 2b). Proteinase K sensitivity increased in the order of APSK-HA, AAC-HA (inner membrane protein[23,27])/Tom40-HA, and Tom60-HA (Fig. 2b). Furthermore, sodium carbonate treatment, which liberates soluble and peripheral membrane proteins from organelles[28], decreased the amount of organelle-associated Tom60-HA and increased that of soluble Tom60-HA, while Tom40-HA and CPBF1-HA (single-membrane spanning protein)[29] remained in the pellet fraction after the treatment (Fig. 2b, left and Fig. 2c). These data demonstrate that EhTom60 is a cytosolic protein which can associate with EhTom40 on the surface of the mitosomal outer membrane.

Phenotypes of *Tom40*- and *Tom60*-gene silencing. The importance of mitosomal matrix proteins, i.e., ATP sulfurylase (AS; XP_653570), APSK, inorganic pyrophosphatase (IPP; XP_649445), Cpn60, and AAC for *E. histolytica* proliferation was previously verified by gene silencing[27]. To demonstrate the biological importance of the mitosomal import machinery per se, we established *E. histolytica* strains in which *EhTom40* and *EhTom60* genes were silenced. Gene silencing was verified by quantitative real-time PCR (Fig. 3a). Repression of *EhTom40* and *EhTom60* genes caused a decrease in the transport of mitosomal matrix proteins, Cpn60,

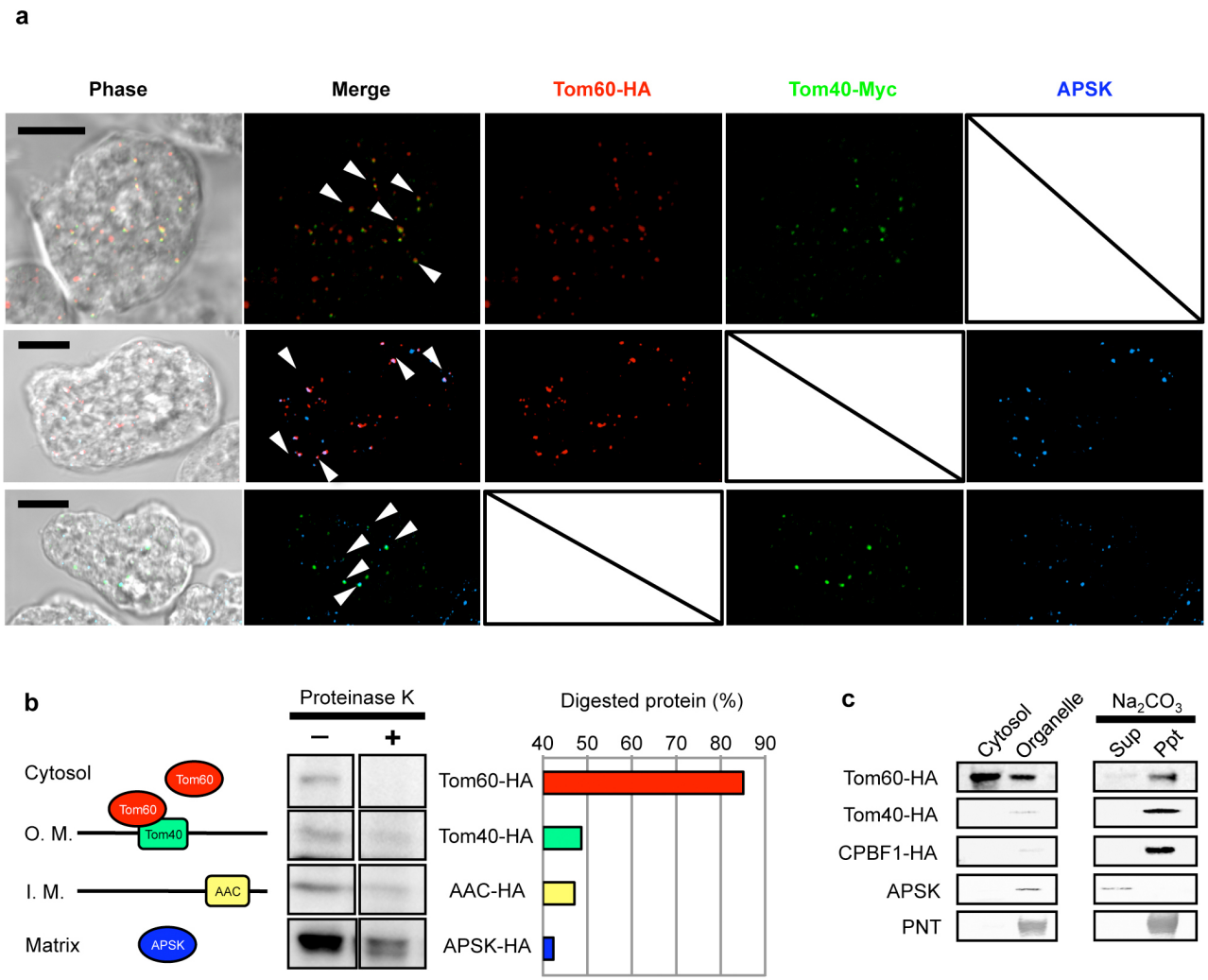


Figure 2 | Localization and topology of Tom40 and Tom60. (a), Indirect fluorescence analyses of Tom40-HA and Tom60-Myc. Anti-APSK antiserum was used as mitosomal marker. Scale bar = 10 μm. (b), Differential sensitivity of several mitosomal proteins to proteinase K treatment. Left panel shows expected topologies of Tom60, Tom40, AAC, and APSK. "O. M." and "I. M." indicate outer and inner membranes, respectively. Middle panel shows immunoblots of each organelle fraction treated (+) or untreated (−) with proteinase K. Right panel shows the ratio of digested protein to that of total undigested protein. (c), Fractionation of mitosomal components. Lysates from amoebae expressing Tom60-HA, Tom40-HA, and CPBF1 (cysteine protease binding family protein 1; XP_655218[29])-HA were fractionated. The three upper and two lower blots were reacted with anti-HA, anti-APSK, or anti-pyridine nucleotide transhydrogenase (PNT, XP_001914099[55]) antibody. CPBF1 and PNT serve as a control for single- and multi-membrane spanning proteins, respectively.

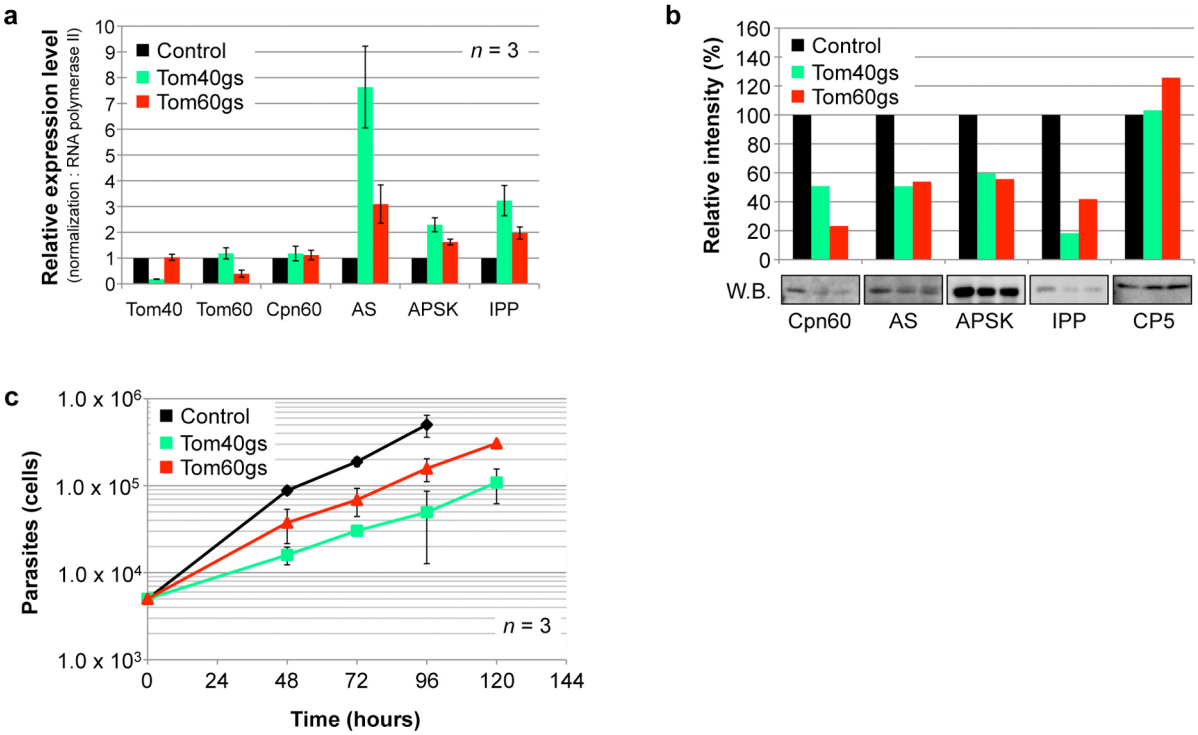


Figure 3 | Phenotypes of *Tom40*- and *Tom60*-gene silenced strain. (a), Effects on the relative mRNA expression levels of mitosomal proteins of *Tom40*- and *Tom60*-gene silencing. Error bars indicate standard deviations. (b), Effects on the amount of mitosomal proteins in organelle fractions from *Tom40*-gene silenced (gs) and Tom60gs strains. Cysteine protease 5 (CP5) was used as loading control. Relative levels of each transcript and protein are shown after normalization against control. (c), Growth kinetics of Tom40gs, Tom60gs, and control strains.

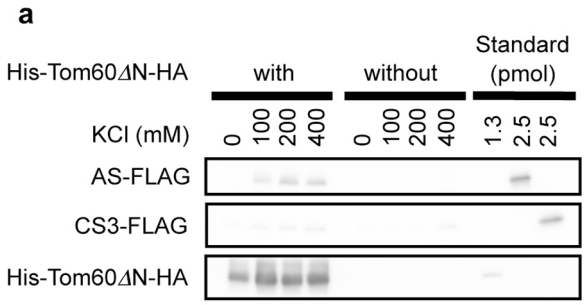
AS, APSK, and IPP (Fig. 3b). On the contrary, we observed a remarkable accumulation of AS, APSK, and IPP transcripts in *EhTom40*- and *EhTom60*-gene silenced strains (Fig. 3a). Finally, repression of *EhTom40* and *EhTom60* genes caused growth retardation when compared to control (Fig. 3c), suggesting that EhTom40 and EhTom60 are important for proliferation. These data also suggest that gene transcription of matrix proteins was upregulated by compensatory mechanisms, but was not sufficient to overcome undesirable effects caused by the repression of proteins involved in the mitosome import. Taken together, we conclude that EhTom40 and EhTom60 play essential roles in the import of matrix proteins to mitosomes.

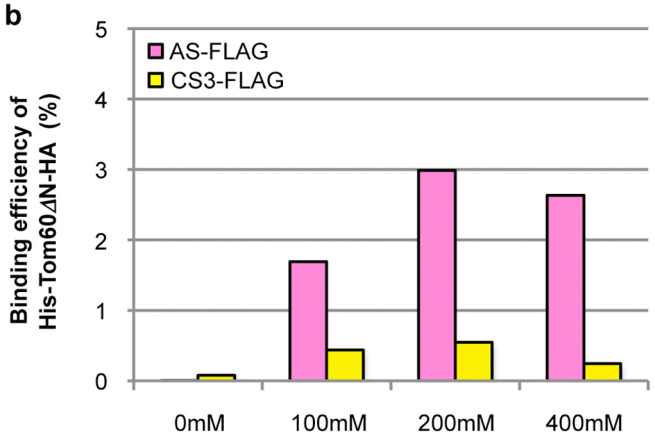
Tom60 serves as a cytosolic receptor of mitosomal proteins. To verify whether EhTom60 functions as a receptor subunit of the TOM complex, we performed an *in vitro* binding assay, using recombinant AS and cysteine synthase isotype 3 (CS3, XP_653246; control for an irrelevant cytosolic protein) that have the FLAG-tag at the carboxyl terminus, and recombinant His-Tom60ΔN-HA, which lacks the amino-terminal hydrophobic region (a.a. 1–30) of EhTom60, and contained the His-tag at the amino terminus. We removed the amino-terminal region of EhTom60 because it negatively affected solubility of the recombinant protein. His-Tom60ΔN-HA showed a higher binding affinity towards AS-FLAG than CS3-FLAG (Fig. 4a and b; Supplementary Fig. S7). Moreover, the binding efficiency of His-Tom60ΔN-HA to AS-FLAG, but not CS3-FLAG, increased at higher KCl concentrations, which agreed well with the salt dependence of the binding between mitochondrial preproteins and the yeast Tom20[30]. These results strongly suggest that EhTom60 functions as a receptor for soluble proteins imported into the mitosomal matrix.

It has been demonstrated that in fungi, metazoa, and plants, mitochondrial transport of matrix and membrane proteins is mediated by different receptors, Tom20 and Tom70, respectively. Tom20 directly recognizes the amino-terminal presequence of soluble matrix proteins. However, Tom70 interacts with membrane preproteins directly, or indirectly via cytosolic heat shock protein 70 (Hsp70) and Hsp90 chaperones. In the latter case, Hsp70 and Hsp90 that are bound to mitochondrial membrane preproteins[31] further bind to the TPR domains of Tom70 via their conserved tetrapeptide "EEVD" motif at the carboxyl terminus[32]. We thus tested if *Entamoeba* TPR-containing Tom60 can also recognize the tetrapeptide motif present in *E. histoltyica* Hsp70 and Hsp90. His-Tom60ΔN-HA was mixed with recombinant CS3-FLAG or its engineered form (CS3-FLAG-EEVD), which has the tetrapeptide motif at the carboxyl terminus. CS3-FLAG-EEVD, but not CS-FLAG, efficiently bound to His-Tom60ΔN-HA (Fig. 4c). These results indicate that the EhTom60 is involved in the mitosomal transport of membrane proteins via cytosolic Hsp70 and Hsp90.

Discussion

We have demonstrated that *Entamoeba* possesses Tom60, a novel genus-specific peripheral membrane component of the TOM complex, that functions as a receptor/carrier to transport mitosomal proteins from the cytoplasm to mitosomes. One of the striking features of Tom60 is its bipartite localization, which allows Tom60 to function as a carrier of *de novo* synthesized mitosomal preproteins in the cytoplasm and a structural component of the TOM complex on the mitosomal membrane. In this respect, *Entamoeba* Tom60 resembles a mammalian peripheral membrane protein, Tom34, which serves as a co-chaperone of Hsp70 and Hsp90 in a Tom70-dependent transport[33]. However, there is a clear difference between *Entamoeba* Tom60 and mammalian Tom34. Tom60 has direct physical interaction with TOM complex, whereas Tom34 is indirectly associated with Tom40 via Tom22 and Tom70[33,34]. Moreover, *Entamoeba* Tom60 appears to play an indispensable role, judged from the severe growth defect caused by gene silencing (knock down), similar to yeast Tom20 and Tom70[35], whereas Tom34-deficient mice were





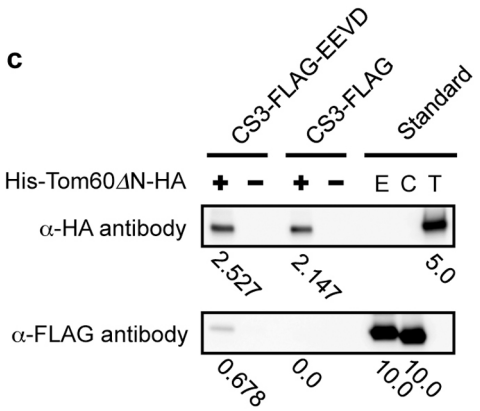


Figure 4 | *In vitro* binding assay of Tom60 and a mitosomal protein. (a), Immunoblotting of proteins bound to His-Tom60ΔN-HA. AS-FLAG is a mitosomal matrix protein, while CS3-FLAG is a cytosolic protein as a control. Approximately 40% of the whole eluates and 1.3 or 2.5% of standards used for the binding assay were subjected to SDS-PAGE and immunoblot analyses with anti-HA and anti-FLAG mouse monoclonal antibody (clone M2, Sigma-Aldrich Japan). (b), Relative binding efficiency of His-Tom60ΔN-HA towards a mitosomal matrix protein. The data were quantitated based on the result shown in Fig. 4a. Vertical and horizontal axes indicate the binding efficiency of His-Tom60ΔN-HA towards substrates and the KCl concentrations, respectively. Low bars in the graph are described numerically while measurements and calculations are described in Supplementary Fig. S7 and Supplementary Methods. (c), The verification of interaction between His-Tom60ΔN-HA and the "EEVD" motif. CS3-FLAG-EEVD is an engineered cytosolic protein in which the "EEVD" motif was added to the carboxyl terminus, like in cytosolic Hsp70 and Hsp90, while CS3-FLAG is a negative control. Approximately 50% of the whole eluates and 5.0 or 10.0 pmol of standards used for the binding assay were subjected to SDS-PAGE and immunoblot analysis, as described above. "E", "C", and "T" stand for CS3-FLAG-EEVD, CS3-FLAG, and His-Tom60ΔN-HA, respectively. Values below panels indicate protein amounts (pmol) estimated by densitometric scanning of the blots.

viable, grew normally, and had a normal Mendelian inheritance pattern[36]. Thus, *Entamoeba* Tom60 represents an unprecedented essential bipartite-localized receptor/carrier for the protein import to MROs. It was presumed that Tom20 and Tom70 are loosely associated with other components of TOM complex, mobilized on the entire mitochondrial surface, and capable of interacting with preproteins[37]. Similarly, we assume that cytosolic localization of the *Entamoeba* Tom60 also maximizes the chance of its interaction with mitosomal preproteins. The mechanisms of the recruitment of Tom60 to the mitosomal outer membrane remain unsolved. One possibility is that Tom60 loaded with a precursor protein docks to the TOM complex, whereas free unloaded Tom60 remains dissociated from the TOM complex in the cytosol. Another possibility is the post-translational modifications. It has been recently reported that the binding of mammalian Tom20 and Tom70 toward preproteins is regulated by phosphorylation[38].

Tom60 is a robust receptor for the mitosomal transport. Tom60 seems to transport both soluble and membrane mitosomal proteins. It has been shown in Opisthokonta that mitochondrial soluble matrix and membrane preproteins are transported via binding with distinct TPR-containing mitochondrial receptors, namely Tom20 and Tom70, which recognizes the amino-terminal transit peptide or the internal (cryptic) targeting signals, respectively[31]. Subsequently, these preproteins are passed from Tom20 and Tom70 to Tom22 and inserted into the Tom40 channel[31]. Therefore, Tom22 plays a role as a receptor for both mitochondrial soluble matrix and membrane preproteins. Similarly, *Entamoeba* Tom60 binds to a soluble mitosomal matrix protein, AS, as well as the "EEVD" motif, which is conserved in cytosolic Hsp70 and Hsp90 from three *Entamoeba* species. It was demonstrated that in mammals, cytosolic Hsp70 and Hsp90 are involved in the Tom70-dependent transport of mitochondrial membrane preproteins to TOM complex[39]. Among MRO-containing eukaryotes, no organism that possesses both Tom70 and Tom20 has been discovered. *Encephalitozoon*[1] and *Blastocystis*[40,41] encodes only a Tom70 homolog, suggesting that the Tom70 homolog may play a bifunctional role similar to *Entamoeba* Tom60. In contrast, in *Giardia*[42], *Trichomonas*[43], and *Cryptosporidium*[44], no potential receptor component of TOM complex has been identified. These organisms most likely possess a lineage-specific receptor like *Entamoeba* Tom60. Further investigation is needed to clarify if such lineage-specific functional Tom60 homologs also contain the TPR domains for the cargo interaction.

It was hypothesized that the TOM complex in early eukaryotes is composed of Tom40, Tom22, and Tom7[17]. It was also shown that the TOM complex of the aerobic free-living social amoebozoan *D. discoideum* consists of Tom40, Tom22, Tom7, and Tom6, and lacks Tom20 and Tom70[7,13]. These data indicate that a common ancestor of amoebozoan species also contained Tom40, Tom22, and Tom7 in its TOM complex. This presumption is also supported by the existence of Tom40 homologs in the genome of other amoebozoan species including *Polysphondylium*[45], and *Acanthamoeba*[45], and the transcriptome of *Mastigamoeba* (Stairs, C. and Roger, A., personal communication), and Tom7 homologs in *Polysphondylium* (EFA78398) and *Acanthamoeba* (Contig6955 in the *Acanthamoeba* genome database). However, we did not detect Tom22 homologs in these amoebozoa. These data are consistent with the premise that *Entamoeba* probably secondarily has lost Tom22 during separation within Amoebozoa. A key question regarding a lineage-specific presence of Tom60 in *Entamoeba* is why and how the loss of the

canonical subunit Tom22 and gain of Tom60 occurred. We presume that it is related to the lack of mitosomal targeting sequences in *Entamoeba*[9]. In the general model of mitochondrial matrix protein import, Tom22 interacts with the positive-charged surface in the amphiphilic α-helix of presequences[30,46]. In contrast, such ionic interaction does not appear to mediate the binding between *Entamoeba* Tom60 and mitosomal proteins. An alternative explanation of the loss of Tom22 is that in the *Entamoeba* ancestor the mitosomal proteins that were acquired by lateral gene transfer, such as sulfate activation enzymes, were poorly imported into mitosomes by Tom22.

Our current hypothesis as to how a novel TOM complex evolved in *Entamoeba* mitosomes is as follows: The *Entamoeba* ancestor was exposed to anaerobic environments, under which oxygen-dependent energy generation became unusable. Under these conditions, the mitochondrion lost its electron transport chain, membrane potential, and other aerobic mitochondrion-related functions. The loss of membrane potential across the inner membrane promoted an elimination of the canonical membrane potential-dependent TIM23 and TIM22 complexes[31]. In agreement with this hypothesis, membrane potential-dependent AAC, that is present in the aerobic mitochondria, became non-reliant on the membrane potential in *E. histolytica*[23]. Moreover, as described above, *Entamoeba* mitosomal proteins lack a canonical positively-charged transit peptide[9], which is utilized for the electrophoretic import via the membrane potential[31]. Alterations of the TIM complex led to the rearrangement of the TOM complex, more specifically loss of Tom22, which is associated with the TIM23 complex in a typical aerobic mitochondrion. Finally, loss of Tom22 must have been compensated with the invention of a new targeting mechanism dependent on Tom60. It was also suggested that subunit replacement might have occurred in the TIM complex of *Trichomonas vaginalis*[43] and *Giardia intestinalis*[42]. It is worth further investigating how commonly replacement of subunits occurred in anaerobic MRO-possessing eukaryotes.

Methods

Organisms. Trophozoites of *Entamoeba histolytica* HM-1:IMSS cl6[47] and G3[48] strains were cultivated axenically in Diamond BI-S-33 medium[49].

RNA and cDNA preparation. Total RNA was isolated from various strains by TRIZOL® reagent (Invitrogen, Carlsbad, San Diego, CA). mRNA was purified using GenElute™ mRNA Miniprep Kits (Sigma-Aldrich Japan). cDNA was synthesized from mRNA using SuperScript™ III RNase H⁻ reverse transcriptase (Invitrogen), oligo(dT)$_{20}$ primer, and primer 1 (Supplementary Table S5).

Plasmid construction. *E. histolytica Tom40* and *Tom60* genes were PCR-amplified from cDNA using Phusion DNA polymerase (New England Biolabs, Beverly, MA) and corresponding primer sets (Supplementary Table S5). After restriction digestion, amplified fragments were ligated into pEhEx/HA[50] and pEhEx/Myc[29] using Ligation-Convenience Kit (Nippongene, Tokyo, Japan). To generate the plasmid for Tom40-Myc/Tom60-HA double-expression, a fragment containing the Tom40-Myc protein coding region flanked by the upstream and downstream regions of the *CS1* gene was PCR-amplified from pEhEx/Tom40-Myc by primers 6/7 (Supplementary Table S5), and inserted into the *Spe* I-digested pEhEx/Tom60-HA using In-Fusion® system (TaKaRa, Shiga, Japan). For gene silencing, a 400-bp fragment corresponding to the amino terminus of Tom40 and Tom60 was PCR-amplified with appropriate primers (Supplementary Table S5). Restriction-digested fragments were ligated into *Stu* I/*Sac* I double-digested psAP-2-Gunma plasmid[27].

Amoeba transformation. Lipofection of trophozoites, selection, and maintenance of transformants were performed as previously described[9].

Immunofluorescence assay. IFA[9,51] was performed as previously described.

Preparation of organelle fraction. Amoeba strains that expressed HA-tagged Tom60-HA, Tom40-HA, AAC-HA, APSK-HA, and CPBF1-HA[29] proteins, strains in which *Tom40* and *Tom60* genes were silenced, and mock transformants (pEhEx/HA and psAP2-Gunma) were washed three times with 2% glucose/PBS. After resuspension in lysis buffer (10 mM MOPS-KOH, pH7.2, 250 mM sucrose, protease inhibitors), cells were disrupted mechanically by a Dounce homogenizer. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min, and the supernatant centrifuged at 100,000 × *g* for 60 min to separate the organelle and cytosolic fractions. The 100,000 × *g* organelle fractions were resuspended with lysis buffer, and were recollected by the centrifugation at 100,000 × *g* for 60 min.

Immunoprecipitation of the TOM complex. Organelle fractions were solubilized with IP buffer (2% digitonin/50 mM BisTris-HCl, pH7.2/50 mM NaCl/10% [W/V] glycerol, protease inhibitors). The lysate was mixed with Protein G-Sepharose 4 Fast Flow (GE Healthcare), and Sepharose beads were removed by centrifugation. Precleared lysates were mixed with anti-HA mouse monoclonal antibody conjugated with agarose (Sigma-Aldrich Japan) at 4°C for 3 h. Agarose was washed three times with IP buffer containing 1% digitonin. Bound protein was eluted by IP buffer containing 1% digitonin and 600 μg/ml HA peptide (Sigma-Aldrich Japan).

Blue native polyacrylamide gel electrophoresis (BN-PAGE). Organelle fractions were solubilized by either 2% digitonin or n-dodecyl-β-D-maltoside (DDM) at 4°C for 30 min, and centrifuged at 20,000 × *g* for 30 min at 4°C. BN-PAGE was performed using NativePAGE™ Novex® Bis-Tris Gel System (Invitrogen) according to manufacturer's protocol. Immunoprecipitated samples were mixed with 0.25% Coomassie® G-250 (Invitrogen) before electrophoresis.

Liquid chromatography-tandem mass spectrometric analysis. In-gel trypsin digestion of protein bands of interest and LC-MS/MS were performed as previously described[52,53].

Proteinase K treatment. Organelle fractions (50 μg protein each) were treated with or without final 2.8 μg/ml proteinase K (Roche) at 4°C for 15 min, followed by SDS-PAGE and immunoblot analysis with anti-HA mouse monoclonal antibody. Band intensities were evaluated using the Analysis Toolbox in ImageQuant TL software (GE Healthcare).

Na_2CO_3 treatment. Organelle fractions (1 mg protein) in lysis buffer were diluted 20 times with ice-cold 100 mM Na_2CO_3, pH 11.5 and 150 mM NaCl, kept at 4°C for 30 min, and centrifuged at 100,000 × *g* for 60 min. The 100,000 × *g* supernatant was transferred to a fresh tube and the precipitate washed once with Na_2CO_3 solution. Immunoblot analysis was performed as described above. Anti-PNT (1 : 1,000) and anti-APSK (1 : 1,000) rabbit antisera were used as primary antibodies. Alkaline phosphatase-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody.

Quantitative real-time PCR. Quantitative real-time PCR analysis was performed as described[27] using primer sets (primers 12-25; Supplementary Table S5) for *Tom40*, *Tom60*, *Cpn60*, *AS*, *APSK*, *IPP*, and *Rnapol* (XM_643999) genes.

Recombinant proteins. To generate recombinant histidine tagged (His$_6$)-Tom60*Δ*N-HA, AS-FLAG, CS3-FLAG, and CS3-FLAG-EEVD proteins, we amplified *Tom60*, *AS*, and *CS3* genes using appropriate primers sets (Supplementary Table S5) and pEhEx/Tom60-HA, pEhEx/AS-HA[9], and pET15b/CS3[54] as templates. Fragments were digested by appropriate sets of restriction enzymes and ligated into pCold I (TaKaRa). These plasmids were transformed into BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* (Invitrogen) and expression of recombinant proteins was induced by 1 mM IPTG. After lysis of bacteria and purification by Ni-NTA system (QIAGEN GmbH, Hilden, Germany), the His$_6$-tag was removed from His$_6$-AS-FLAG, His$_6$-CS-FLAG and His$_6$-CS-FLAG-EEVD by AcTEV™ protease (Invitrogen).

***In vitro* binding assay of Tom60.** The binding efficiency of His$_6$-Tom60*Δ*N-HA was calculated and shown as the ratio of eluted AS-FLAG or CS3-FLAG to that of eluted His-Tom60*Δ*N-HA in the *in vitro* binding assay (Supplementary Methods). The hydrophobic nature of the amino terminus of Tom60 negatively affected solubility, thus it was removed prior to the binding assay. To verify the interaction between His$_6$-Tom60*Δ*N-HA and the "EEVD" motif, we carried out the assay with CS3-FLAG-EEVD or CS3-FLAG. Assay condition was identical to *in vitro* binding assay as described in Supplementary Methods except that the assay buffer contained 50 mM KCl.

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Author contributions

T.M. did the experiments. T.M. and T.N. wrote the manuscript. T.M., F.M., K.N.-T. and T.N. interpreted the data.

Additional information

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