An Evolutionarily Conserved Phosphatidate Phosphatase Maintains Lipid Droplet Number and ER Morphology But Not Nuclear Morphology

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KEY WORDS
Phosphatidic Acid Hydrolase (PAH), lipin, Tetrahymena thermophila, Lipid droplet, Nuclear membrane expansion, Endoplasmic Reticulum

ABBREVIATIONS
PAP Phosphatidic Acid Phosphatase
PAH Phosphatidic Acid Hydrolase
NEM1 Nuclear Envelope Morphology protein 1
SPO7 Sporulation-specific protein 7
HAD Haloacid Dehalogenase
NUP Nuclear pore
INO1 Inositol-3-phosphate synthase
OPI3 Phosphatidyl-N-methylethanolamine N-methyltransferase
MTT Metallothionein
SD Synthetic Complete Dextrose media
YPD Yeast Extract Peptone Dextrose
TAP Tandem Affinity Purification
DAPI 4′, 6-Diamidino-2-Phenyindole, Dihydrochloride
DHCC 3,3′-dihexyloxacarbocyanine iodide
SUMMARY STATEMENT

*Tetrahymena* possesses two PAH homologs *TtPAH1* and *TtPAH2*. *TtPAH1* regulates lipid droplet biogenesis and ER morphology in *Tetrahymena* and functionally replaces yeast *PAH1*.

ABSTRACT

Phosphatidic acid phosphatases are involved in the biosynthesis of phospholipids and triacylglycerol, and also act as transcriptional regulators. Studies to ascertain its role in lipid metabolism and membrane biogenesis are restricted only to Opisthokonta and Archaeplastida. Herein, we report the role of phosphatidate phosphatase (*PAH*) in *Tetrahymena thermophila*, belonging to the Alveolata clade. We identified two *PAH* homologs in *Tetrahymena*, *TtPAH1*, and *TtPAH2*. Loss of function of *TtPAH1* results in reduced lipid droplet number and increase in ER content. It also shows more ER sheet structure as compared to wild type *Tetrahymena*. Surprisingly, we did not observe a visible defect in nuclear morphology of Δ*Ttpah1* mutant. *TtPAH1* rescued all the known defects in yeast *pah1Δ* strain and is conserved functionally between *Tetrahymena* and yeast. The homologous gene derived from *Trypanosoma* also rescued the defects of yeast *pah1Δ* strain. Our results indicate that *PAH*, previously known to be conserved between Opisthokonts, is also present in a set of distant lineages. Thus, a phosphatase cascade is evolutionarily conserved and is functionally interchangeable across eukaryotic lineages.
INTRODUCTION

Eukaryotic cell organelles are enclosed by a membrane composed of the lipid bilayer and proteins. Phospholipids constitute the major structural components of lipid bilayers and play a central role in membrane biogenesis, lipid metabolism, and signaling (Van Meer et al., 2008). The lipid composition of the membrane is critical for maintaining shape, size, and number of organelles, and is established through synthesis, transport, and modification of phospholipids (McMahon and Gallop, 2005). The regulation of lipid synthesis and storage is critical for maintaining lipid homeostasis since both excess and poor fat storage results in various lipid-associated disorders (Klingenspor et al., 1999; Reue et al., 2000; Péterfy et al., 2001). However, the molecular mechanisms that link lipid production to organelle morphology remain unclear.

Pah/lipin proteins are Mg\(^{2+}\)-dependent phosphatidic acid phosphatases (PAP; 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) (Han et al., 2006). Members of the Pah/lipin protein family perform dephosphorylation of phosphatidic acid (PA) to generate diacylglycerol (DAG), the penultimate step in glycerolipid synthesis (Lin and Carman, 1989). DAG can be converted back to PA by DAG kinase. Phosphatidic acid (PA) and diacylglycerol (DAG) are the central precursors which control the levels of phospholipids, govern membrane structure and lipid storage. In yeast, phospholipid biosynthesis occurs by two pathways; the cytidine diphosphate diacylglycerol (CDP-DAG) pathway (de novo) and Kennedy pathway (salvage) (Fig. 1A) (Carman and Zeimetz, 1996; Carman and Henry, 1999). DAG is converted to triacylglycerol (TAG) (Han et al., 2006), which forms the lipid droplet. The dual function of TAG, as a reservoir of cellular energy and precursor for membrane phospholipids, makes it a key player in lipid homeostasis. DAG derived from PA is used for the synthesis of membrane phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) via the Kennedy pathway (Carman and Kersting, 2004). Through the CDP-DAG pathway, phosphatidic acid serves as the precursor for the synthesis of phospholipids PE, PC, and phosphatidylserine (PS). Apart from the synthesis of lipids, PA and DAG act as lipid second messengers in signaling events that trigger membrane expansion, secretion, and endocytosis (Kearns et al., 1997; Nadra et al., 2008). In yeast, PA positively regulates phospholipid synthesis through sequestration of a transcription repressor Opi1, thereby activating transcription of genes encoding lipid biosynthetic enzymes (White et al., 1991; Loewen et al., 2004).
Lipins are relatively large proteins close to 100 kDa and primarily found in the cytosol. These proteins contain a carboxy-terminal region (C-LIP) with a haloacid dehalogenase-like (HAD) domain possessing the DXDXT/V catalytic motif and an amino-terminal domain (N-LIP) of unknown function (Santos-Rosa et al., 2005). Lipin 1 was initially identified through positional cloning as the mutated gene in the fatty liver dystrophy (fld) mouse, which is characterized by abnormal development of adipose tissue that results in lipodystrophy and insulin resistance (Reue et al., 2000; Péterfy et al., 2001). In Saccharomyces cerevisiae, a single lipin orthologue, PAH1, is present whereas mammals express three lipin paralogues: LIPIN1, LIPIN2, and LIPIN3 exhibiting distinct but overlapping expression pattern (Han et al., 2006; Donkor et al., 2007). The first lipin protein shown to function as a Mg\(^{2+}\)-dependent phosphatidic acid phosphatase enzyme was S. cerevisiae Pah1 (Han et al., 2006). Deletion of PAH1 in yeast causes aberrant expansion of nuclear/ER membrane, increased phospholipid synthesis, decreased TAG level and lipid droplet number, and slow growth (Siniossoglou et al., 1998; Adeyo et al., 2011). In C. elegans, downregulation of lipin affects dynamics of peripheral ER and nuclear envelope (Golden et al., 2009; Gorjánácz and Mattaj, 2009). Defects in mammalian lipins lead to various metabolic disorders including lipodystrophy and insulin resistance, rhabdomyolysis, peripheral neuropathy, and inflammation (Reue et al., 2000; Müller-felber et al., 2010).

Besides serving enzymatic functions, lipins also act as transcriptional regulators (Finck et al., 2006; Zhang and Reue, 2017). Mammalian lipins regulate gene expression by modulating the activity of key transcription factors such as peroxisome proliferator-activated receptor γ, (PPARγ), PPAR co-activator 1α (PGC-1α), and sterol regulatory element binding protein (Phan and Reue, 2004; Peterson et al., 2011; Kim et al., 2013). Yeast Pah1 translocates to the nucleus where it interacts with the promoter of phospholipid synthesis genes (Santos-Rosa et al., 2005).

Phosphorylation and dephosphorylation at multiple sites regulate the activity and subcellular localization of PAH proteins. In yeast, Cdc28 phosphorylation of Pah1 is critical for cell cycle progression while phosphorylation by Pho85 plays other roles; in mammals, mTOR kinases phosphorylate lipins (Laplante and Sabatini, 2009; Peterson et al., 2011; Choi et al., 2012). Dephosphorylation of Pah1 by a nuclear/ER membrane complex consisting of a catalytic phosphatase subunit Nem1, and its regulatory subunit, Spo7, activates its catalytic function and recruit it to the ER membrane where it acts on its substrate PA (Santos-Rosa et al., 2005; Karanasios et al., 2010).
Studies of phosphatidic acid phosphatase have focused on Opisthokonta (fungi, nematode, flies, and mammals) (Santos-Rosa et al., 2005; Han et al., 2006; Donkor et al., 2007; Golden et al., 2009; Gorjánácz and Mattaj, 2009; Ugrankar et al., 2011) and Plantae clades (Nakamura et al., 2009). In contrast, these enzymes and the regulatory cascades in which they participate are not reported in organisms including Amoebozoa, Alveolata, and Excavata. *Tetrahymena thermophila* belongs to the Alveolata, a major evolutionary branch of eukaryotic protists in which cells display functional complexity comparable to the cells of humans and other metazoans. In this study, we report the role of phosphatidic acid phosphatase (Pah) in regulating lipid homeostasis and membrane biogenesis in this ciliate. We also investigated the cellular functions of *PAH/LIPIN* homologs in Excavata to understand the evolutionary conservation of this cascade.

We found two homologs for *PAH* in the Tetrahymena Genome Database. The larger protein is TtPah1, and a smaller one is TtPah2. We investigated the role of *TtPAH1* in regulating lipid homeostasis, maintaining nuclear morphology and ER organization. We characterized the effects of loss of function of *TtPAH1* and also performed complementation studies in pah1Δ yeast strain. Deletion of *TtPAH1* in *Tetrahymena* led to a reduction in lipid droplet number, thus confirming its role in lipid homeostasis. However, unlike in yeast, *TtPAH1* was not required to maintain nuclear morphology. Overall, we provide evidence for the evolutionary conservation of this Mg$^{2+}$-dependent phosphatidic acid phosphatase in Alveolata and Excavata.

**RESULTS**

*Tetrahymena* harbors two *PAH* homologs

We identified two homologs of phosphatidic acid phosphatase (*PAH/LIPIN*) in Tetrahymena Genome Database and designated them as *TtPAH1* (TTHERM_00189270) and *TtPAH2* (TTHERM_00215970). TtPah1 contains 872 amino acids and is comparable to Pah1 proteins in other organisms whereas TtPah2 (335 amino acids) is smaller than other known lipins. Both TtPah1 and TtPah2 proteins possess two specific PAP domains, N-LIP and C-LIP, suggesting these are the Mg$^{2+}$-dependent phosphatidate phosphatases (Fig. 1B). All Mg$^{2+}$-dependent phosphatidic acid phosphatases contain an essential catalytic DXDXT/V motif in the HAD-like domain of the C-LIP region. This catalytic motif is present in the C-LIP domain of both TtPah1 (666 DIDGT 670) and TtPah2 (146 DVDGT 150) (Fig. 1B, C). While the amino acid sequence of TtPah1 has 31% identity with human lipin and 24% identity with
yeast Pah1, TtPah2 has 34% identity with human lipin and 22% with yeast Pah1. Similar to other phosphatidic acid phosphatases, the amino acids are more conserved in the N-LIP (50% and 49% identity for TtPah1, 35% and 30% identity for TtPah2 with yeast Pah1 and human lipin1 respectively) and C-LIP regions (49% identity for TtPah1 and 44% identity for TtPah2 with both yeast Pah1 and human lipin1). A conserved G residue in N-LIP is critical for PAH function since its mutation in mammalian lipin1 causes lipodystrophy. We have also identified the conserved G residue in N-LIP of both TtPah1 (G75) and TtPah2 (G79) (Fig. 1B, C).

**TtPAH1 localizes on ER and encodes functional phosphatidate phosphatase**

We focused our study on *TtPAH1*. To assess its localization, we overexpressed it bearing a GFP tag. Analysis of confocal images showed TtPah1-GFP was distributed throughout the cell (Fig. 2A). To evaluate if TtPah1 associates with ER membrane, *Tetrahymena* cells expressing TtPah1-GFP were labeled with ER-Tracker, and analyzed by confocal microscopy. The results revealed that TtPah1-GFP is localized to ER membrane in addition to the cytoplasm (Fig. 2B). To examine if *TtPAH1* encodes a functional phosphatidate phosphatase, we expressed a TAP-tagged fusion protein in *Tetrahymena*. We then purified the protein from lysates and measured phosphatidate phosphatase activity using a colorimetric assay. The purified protein migrated by SDS-PAGE at its expected size near 100 kDa but there were also more abundant smaller species, probably corresponding to proteolytic products (Fig. 2C). This purified protein dephosphorylated PA in a Mg\(^{2+}\)-dependent manner (Fig. 2D, E). Taken together these results confirm that TtPah1 is a functional PAH in *Tetrahymena*.

**TtPAH1 is dispensable for normal growth of Tetrahymena and loss of TtPAH1 does not affect expression of TtPAH2**

In many organisms such as *Saccharomyces cerevisiae*, *C. elegans*, and *Drosophila melanogaster*, PAH is required for normal growth (Santos-Rosa et al., 2005; Golden et al., 2009; Ugrankar et al., 2011). To assess whether *TtPAH1* is essential for normal growth of *Tetrahymena*, we generated the knockout strain by removing all 45 copies of *TtPAH1* from the macronucleus of wild-type *Tetrahymena* by homologous recombination. The knockout strains thus generated (Δ*Ttipah1*) were analyzed by semi-quantitative RT-PCR, which confirmed the absence of *TtPAH1* transcripts (Fig. 3A, B). The growth of Δ*Ttipah1* cells was not significantly different from that of wild-type cells (Fig. 3C). Moreover, there was no visible defect in the morphology of the knockout cells (data not shown). To rule out the possibility that the lack of growth defect in Δ*Ttipah1* is due to compensatory overexpression
of TtPAH2 in these cells, we compared the expression of TtPAH2 in ∆Tipah1 with wild-type cells. The expression of TtPAH2 was not enhanced in ∆Tipah1 cells (Fig. 3D, E). Taken together, these results suggest that TtPAH1 is dispensable for normal growth of Tetrahymena.

**TtPAH1 is required to maintain lipid droplet number in Tetrahymena**

Lipid droplets are ubiquitous eukaryotic organelles mainly used for storing lipids (Murphy, 2001). They consist of a hydrophobic core of neutral lipids such as triacylglycerol, sterols and sterol esters surrounded by a phospholipid monolayer originating from the ER (Tauchi-Sato et al., 2002; Farese and Walther, 2009; Radulovic et al., 2013). Lipid droplets growth occurs either by localized synthesis of lipids or by fusion with other lipid droplets (Thiele and Spandl, 2008). Since Pah proteins are required for the synthesis of triacylglycerol, we compared lipid droplet numbers between ∆Tipah1 and wild-type cells. Lipid droplets were visualized by staining with Oil Red O, and the number of lipid droplet was counted after analyzing confocal images by LSM Image analyzer. The number of lipid droplets decreased significantly in ∆Tipah1 (Fig. 4A, B). Although there was no visible difference in the size of lipid droplets, quantitative analysis showed ~60% reduction in lipid droplet numbers compared to wild-type (Fig. 4B). To provide further evidence that TtPAH1 is involved in lipid droplet biogenesis, we overexpressed TtPAH1-GFP in wild-type Tetrahymena cells. Overexpression of TtPAH1 resulted in ~ 20% increase in lipid droplet number compared to wild-type (Fig. 4C, D). To demonstrate the specificity of this effect, we similarly overexpressed DRP6-GFP (a dynamin-related protein in Tetrahymena) and observed that it did not affect the lipid droplet number (Fig. 4D). Hence, we conclude that TtPAH1 is required to maintain normal lipid droplet number in Tetrahymena. Decreased lipid droplet accumulation in ∆Tipah1 was not due to decreased nutrient uptake since we saw a similar reduction when the comparison between ∆Tipah1 and wild-type was done under starvation conditions (Fig. 4E, F). Under starvation conditions, we observed ~ 60% reduction in lipid droplet number in ∆Tipah1 cells. Moreover, the size of lipid droplet in ∆Tipah1 was smaller than wild-type cells (Fig. 4E). Taken together these results suggest that TtPAH1 influences the number and size of the lipid droplets in Tetrahymena.

**TtPAH1 is needed for maintaining tubular ER in Tetrahymena**

The ER is a complex network consisting of flat sheets and highly curved tubules, and their abundance varies with cell cycle stages. The ER serves as the primary site for *de novo* lipid biosynthesis. We hypothesized that PAH regulates ER morphology since phosphatidic acid, a major component of ER is converted to DAG by PAH. To determine whether TtPAH1 is important in maintaining ER morphology, we stained both ∆Tipah1 and wild-type cells with
ER-tracker dye (Invitrogen) and analyzed morphology by confocal microscopy (Fig. 5A, B and S1). The ER content increased significantly in cells lacking \textit{TtPAH1}, as measured by the mean density of ER-tracker red staining (Fig. 5C). Moreover, in wild-type cells, the ER appeared mainly as a network of fine tubules with occasional small patches, likely to represent ER sheets (Fig. 5A, B and S1). These patches seemed larger and more abundant in the absence of functional \textit{TtPAH1}. This result suggests that \textit{TtPAH1} is required for creating and/or maintaining the ER structure.

\textbf{Loss of \textit{TtPAH1} does not manifest visible nuclear envelope defect in \textit{Tetrahymena}}

\textit{Tetrahymena} harbors one polyploid, phenotypically active macronucleus (MAC) and a diploid transcriptionally silent germline micronucleus (MIC). To see if the \textit{TtPAH1} function is necessary to maintain normal NE morphology, we analyzed the NE by expressing and visualizing \textit{NUP3-GFP} (a nuclear pore component marker specifically localizing to macronucleus) in \textit{ΔTtpah1} cells and wild-type cells. This comparison did not reveal any visible defect in size or shape of the NE in \textit{ΔTtpah1} cells (Fig. 6A). Like in wild-type, the DAPI-stained DNA appeared round, compact, and non-fragmented (Fig. 6A). Consistent with this, isolated DAPI-stained nuclei from wild-type and mutant cells expressing \textit{NUP3-GFP} seemed identical (Fig. 6B). To further confirm that deletion of \textit{TtPAH1} did not affect nuclear morphology, we stained isolated nuclei (both MAC and MIC) with a lipophilic dye (DHCC) to visualize nuclear membrane. As with Nup3-GFP, we did not observe any visible defect in nuclear membranes of MAC (Fig 6C). We did not observe any detectable change in MIC structure as well (Fig 6C). These results suggest that \textit{TtPAH1} is not essential for maintaining normal nuclear morphology in \textit{Tetrahymena}. Our results are in contrast to findings in \textit{S. cerevisiae}, where cells lacking \textit{PAH1} showed abnormal expansion of nuclear envelope that appeared as a nuclear membrane projections lacking DNA. Our results, taken together with our analysis of the ER, suggest that defects in ER morphology in \textit{Tetrahymena} do not necessarily affect nuclear morphology, unlike the coupling in other organisms.

\textit{TtPAH1} restores different phenotypes of \textit{pah1Δ} yeast cells

Though \textit{TtPAH1} is not required for regulating nuclear expansion and nuclear shape in \textit{Tetrahymena}, we asked whether the ciliate protein could rescue the nuclear defects in \textit{S. cerevisiae pah1Δ}, which might be expected if the homologous proteins retain the same enzymatic activity. To assess nuclear morphology in budding yeast, we expressed nucleoplasmic protein \textit{PUS} as a GFP-fusion and visualized \textit{pah1Δ} cells expressing \textit{TtPAH1}. In \textit{pah1Δ}, the nuclei in non-dividing cells often appeared as two lobes interconnected by a long nuclear membrane extension (Fig. 7A) (Santos-Rosa et al., 2005). In contrast, \textit{pah1Δ}
expressing *TtPAH1* showed nearly normal nuclear morphology (Fig. 7A). This result suggests that *TtPAH1* can substitute for one or more functions of the yeast homolog.

*pah1Δ* also exhibits slow growth at 30°C, temperature sensitive growth at 37°C (Han et al., 2006) and respiratory deficiency (i.e., *growth* defect) on non-fermentable carbon sources (Han et al., 2007). Along with rescue of the nuclear morphology defect, expression of *TtPAH1* restored growth both at 30°C and 37°C (Fig. 7B). To evaluate the role of *TtPAH1* in rescuing respiratory deficiency, we grew cells on plates containing glycerol as non-fermentable carbon source. The *pah1Δ* expressing *TtPAH1* grew faster than control *pah1Δ* cells (Fig. 7B).

Nuclear expansion in yeast is linked to the induction of phospholipid biosynthetic genes (Santos-Rosa et al., 2005). Deletion of *PAH1* induces the expression of *INO1, INO2, and OPI3* involved in the induction of phospholipid biosynthetic genes leading to overly developed ER and aberrant expansion of nuclear membrane (Santos-Rosa et al., 2005). To test if *TtPAH1* inhibits abnormal nuclear expansion in *pah1Δ* yeast by inhibiting the phospholipid biosynthesis genes, we have analyzed the mRNA levels of *INO1, OPI3,* and *INO2* by quantitative real-time PCR using Sec 63 (a resident ER membrane protein unaffected by *PAH1* deletion) as a control (Santos-Rosa et al., 2005). *TtPAH1* repressed expression of all the three genes tested suggesting that *TtPAH1* could replace yeast *PAH1* in regulating expression of phospholipid biosynthesis genes (Fig. 7C). Taken together these results suggest that *TtPAH1* retains all the known functions of yeast *PAH1*, and hence is functionally conserved between yeast and *Tetrahymena*.

A conserved DXDXT/V motif at C-LIP is essential for the catalytic activity of Pah1/lipin in yeast and mammals (Finck et al., 2006; Han et al., 2007). We identified a similar motif (666 DIDGT 670) in the predicted C-LIP of *TtPah1* and evaluated if the motif is important for the function of *TtPAH1* by mutating two aspartate residues (D666,668E) (*TtPAH1*mut). Since *TtPAH1* functionally replaces yeast *PAH1*, we attempted to complemented *pah1Δ* yeast cells with *TtPAH1mut* and evaluated nuclear morphology, growth in different temperatures and media. The mutant protein did not rescue aberrant nuclear morphology, slow growth at 30°C, and the respiratory defect to the wild type level (Fig. 7A, D). These results suggest that catalytic activity of *TtPah1* is important for its function.

**Phosphatidate phosphatase is conserved across eukaryotic lineages**

Prior studies on the role of PAH proteins in the regulation of lipid homeostasis and membrane biogenesis have focused mainly on the Opisthokont and Archaeplastid clades. The cellular function of *PAH* is not yet known in organisms belonging to clades distantly related
to Opisthokont, like the Excavata. Fig. 8A shows an evolutionary tree with representative organism for each clade. The sequence analysis of PAH homologs from organisms belonging to different clades suggest that it is conserved across eukaryotic lineages (Fig. 8B). In this study, we established the role of PAH1 in regulating lipid homeostasis and membrane biogenesis in Tetrahymena, an Alveolate. By complementation of pah1Δ yeast cells with Trypanosoma PAH1 (TbPAH1), we further show that conservation appears to extend to another group, the Excavates. TbPAH1 rescued growth, respiratory and nuclear defects of pah1Δ yeast cells (Fig. 8C, D).

The Arabidopsis PAH homolog AtPAH2 has previously been shown to possess some functions of S. cerevisiae PAH1, based on its ability to rescue the slow growth phenotype of pah1Δ yeast. However, it was not reported whether the plant homolog also rescues the nuclear envelope defect (Nakamura et al., 2009; Mietkiewska et al., 2011). We used AtPAH2 to complement the pah1Δ yeast strain. In addition to rescuing the growth phenotype, AtPAH2 mitigated the aberrant nuclear morphology of pah1Δ yeast cells, confirming conservation between Opisthokonta and Archaeplastida (Fig. 8C, D). Taken together, these results along with results from earlier reports suggest that the PAH phosphatase cascade is functionally conserved across eukaryotic lineages, indicating that it originated before the lineages diverged very early in eukaryotic evolution.

DISCUSSION

In this study, we have identified two homologs of LIPIN/PAH in Tetrahymena thermophila. We report that TtPAH1 is a phosphatidic acid phosphatase involved in the regulation of lipid droplet biogenesis and ER morphology in Tetrahymena. Regulation of lipid homeostasis and membrane biogenesis is fundamental to all eukaryotes, and the presence of a regulation cascade comprising Pah1 and its phosphatase complex Nem1-Spo7 has been shown in yeast (Siniossoglou et al., 1998; Péterfy et al., 2001; Han et al., 2006; Golden et al., 2009; Nakamura et al., 2009). Similar cascades are also reported in plants, (Nakamura et al., 2009; Mietkiewska et al., 2011) mammals, (Kim et al., 2007) worms, (Golden et al., 2009) and flies (Ugrankar et al., 2011). All the studies are restricted to Opisthokonta and Archaeplastida clades. The presence of such a cascade is not reported in the distantly related lower eukaryotic clades like Alveolata and Excavata. In the present study, we observed that PAH from the clades belonging to Excavata and Alveolata functionally replaces yeast PAH1. We, therefore, conclude that this phosphatidic acid phosphatase cascade regulating membrane biogenesis and lipid homeostasis is conserved across the eukaryotic evolutionary tree.
Fungi (Saccharomyces cerevisiae), nematodes (C.elegans), and insects (Drosophila melanogaster) express one PAH homolog, (Han et al., 2006; Golden et al., 2009; Ugrankar et al., 2011) whereas mammals express three and plants (Arabidopsis thaliana) express two homologs (Donkor et al., 2007; Nakamura et al., 2009). The presence of two PAH homologs in a lower eukaryote, like Tetrahymena, is unusual since multiple homologs are mainly found in higher organisms. Previous studies have shown that deletion of PAH leads to growth and development defects in yeast, (Han et al., 2006; Han et al., 2007; Adeyo et al., 2011) D.melanogaster, (Ugrankar et al., 2011) and C.elegans (Golden et al., 2009). In contrast, loss of PAH1 in Tetrahymena did not result in growth defect. The normal growth and development of ΔTtpah1 mutant cells may be attributed to the presence of another homolog TtPAH2.

TtPah1 displays cytoplasmic as well as membrane localization consistent with previously characterized mammalian lipin and yeast Pah1(Péterfy et al., 2001; Han et al., 2006). Dephosphorylation of Pah1 regulates its subcellular localization and promotes its translocation from the cytoplasm into ER where it converts PA to DAG (Karanasios et al., 2010). PA phosphatase regulates lipid droplet number by generating its precursor TAG from the substrate PA (Adeyo et al., 2011). The role of PAH/ LIPIN in lipid droplet biogenesis or lipid storage has been established in yeast (Adeyo et al., 2011), Drosophila, (Ugrankar et al., 2011) and C.elegans (Golden et al., 2009). By generating the deletion of TtPAH1 in Tetrahymena, we demonstrated its role in lipid droplet biogenesis. Overexpression of TtPAH1 in wild-type cells leads to an increase in lipid droplet number, further corroborating its role in lipid droplet biogenesis.

The role of PAH proteins in maintaining ER structure is well established in yeast and C.elegans (Siniossoglou et al., 1998; Campbell, 2006; Golden, Liu & Cohen-Fix, 2009). The loss of PAH produces a morphological change in many organelles in Drosophila, but perturbation of ER morphology was not reported (Ugrankar et al., 2011). Interestingly, deletion of macronuclear copies of TtPAH1 in Tetrahymena alters ER morphology resulting in an increased proportion of sheet to tubule structure. One possibility for the altered ER morphology could be the change in phospholipid flux arising from the loss of PAH1 leading to change in the phospholipid composition of ER. We observed increased intensity of ER-tracker dye in ΔTtpah1 cells, indicating higher levels of sulphonyl urea receptor (SUR) of ATP-sensitive K+ channel in these cells. The expansion of the ER by deletion of PAH1 is in general associated with increased expression of membrane proteins that include ER resident proteins in other organisms like S. pombe, C. elegans and Y. lipolytica (Tange, 2002; Golden
et al., 2009; Guerfal et al.; 2013). Therefore, we speculate that the increased ER membrane synthesis in ΔTipah1 cells concomitantly increases the production of ER associated proteins and might include ER resident proteins like SUR. However, this remains to be tested in *Tetrahymena*.

Loss of PAH in mammals and *C.elegans* results in a defect in nuclear envelope break down (NEBD) without any nuclear expansion (Golden et al., 2009; Gorjánácz and Mattaj, 2009). The regulation of nuclear expansion by PAH is restricted to yeast, which could be explained by the presence of CDP-DAG pathway in yeast and absence in mammals and *C.elegans* (Bahmanyar et al., 2014; Bahmanyar, 2015; ). The accumulation of PA due to loss of PAH1 leads to the excess synthesis of phospholipids PE and PC via the CDP-DAG pathway, resulting in a massive nuclear expansion in yeast (Santos-Rosa et al., 2005; Han et al., 2006; Bahmanyar et al., 2014). It is interesting to note that *Tetrahymena*, though possessing the CDP-DAG pathway for phospholipid synthesis, nuclear expansion was not visible in ΔTipah1. Although we have used only NUP-GFP as a marker to detect nuclear expansion, it might be useful to test with other nuclear markers as well. However, we believe that NUP3-GFP is also a reliable marker since nuclear membrane flares seen in yeast contain assembled nuclear pore structures (Siniossoglou et al., 1998). Further, by staining the nuclear membrane with a lipophilic dye that should stain any membranous structure, we failed to detect any visible flares in both micronucleus and macronucleus of ΔTipah1. These results suggest that unlike yeast, where the expansion of nuclear membrane is very prominent, there is no extensive expansion of the nuclear membrane in *Tetrahymena* upon deletion of *TtPAH1*. Nuclear volume in *Tetrahymena* is variable presumably due to differential ploidy level in the MAC (Raikov IB, 1976; Gorovsky MA, 1980; Bodenbender J, Prohaska A, Jauker F, Hipke H, 1992). Therefore, one could speculate a different mechanism that allows plasticity in nuclear expansion to accommodate different nuclear volumes.

The NE is connected with the ER, and changes in ER structure lead to defects in the NE. For example, while overexpression of reticulons and *DP1* inhibits nuclear envelope formation, and nuclear expansion, loss of their functions enhances nuclear envelope assembly (Anderson and Hetzer, 2008). In yeast, loss of PAHI leads to over developed ER membrane which in turn results in the nuclear expansion (Siniossoglou et al., 1998; Tange, 2002). As discussed above, loss of PAH1 in *Tetrahymena* demonstrates a change in ER content and structure. Although the loss of *TtPAH1* increases the ER sheet structure (Fig. 5A), it does not manifest visible defect in the nuclear envelope. It appears that in *Tetrahymena*, unlike other organisms, ER content and structure are functionally isolated from mechanisms underlying nuclear
expansion. However, further studies are required to clearly understand the regulation of nuclear expansion and its relation to ER in *Tetrahymena*.

Mutation of the catalytic motif in TtPah1 leads to loss of function suggesting that the catalytic activity is necessary for its function. The role of *PAH*, other than catalytic function is identified in other organisms. For example, *PAH* acts as transcriptional co-activator in mammals and as a transcription factor in yeast (Santos-Rosa et al., 2005; Finck et al., 2006; Kim et al., 2013). However, further studies are required to show if *Tetrahymena PAH1* has a direct role in transcription.

*PAH* homolog is functionally conserved (interchangeable) between Opisthokonta and Plantae (Nakamura et al., 2009; Mietkiewska et al., 2011). We have now extended functional conservation of *PAH* to lower eukaryotic lineages such as Alveolata and Excavata. Though there is no report of the presence of such a cascade in Amoebozoa, the *PAH* homolog is present in the genome sequence of *Dictyostelium* (an Amoebozoan). Therefore, it can be concluded that cascade comprising *PAH* for regulation of lipid homeostasis and membrane biogenesis was present in common ancestor before the divergence of lineages and this cascade remained functionally conserved without allowing change or modification in these functions since lipid homeostasis and membrane biogenesis regulation is important for the normal growth of all eukaryotes. *PAH*, in addition to lipid homeostasis and membrane biogenesis, has an additional role such as NEBD (in *C.elegans*) (Golden et al., 2009; Bahmanyar et al., 2014) and nuclear expansion (in yeast) (Santos-Rosa et al., 2005), suggesting that lineage-specific role of *PAH* is adopted after divergence from the common ancestor. However, *PAH* homologs from all the lineages discussed here rescue abnormal nuclear expansion. Therefore, it can be concluded that though all the known functions of *PAH* were present before lineage divergence, different lineages have adopted these functions to regulate various cellular processes.

Overall, our results along with results from previous studies as discussed above clearly demonstrate a common regulatory cascade across eukaryotic lineages and may have appeared before the divergence of lineages. Our results also show that unlike other known *PAH* homologs, *TtPAH1* does not regulate nuclear morphology.
MATERIALS and METHODS

Strains and culture conditions
Wild-type CU428.1 and B2086 strains of T. thermophila were grown at 30°C in SPP medium (2% proteose peptone, 0.2% dextrose, 0.1% yeast extract, 0.003% ferric EDTA). For conjugation, cells of different mating types were grown to log phase, washed and starved in DMC (0.17 mM sodium citrate, 0.1 mM NaH$_2$PO$_4$, 0.1 mM Na$_2$HPO$_4$, 0.65 mM CaCl$_2$, and 0.1 mM MgCl$_2$) for 16-24 hours at 30°C (Orias et al., 2000). For long term storage, wild-type or knockout cells were starved and frozen in liquid nitrogen in 4% DMSO (Bruns et al., 2000).

Construction and expression of TtPAH1-GFP, TtPAH1-TAP, and NUP3-GFP
To generate the TtPAH1-GFP construct, full-length TtPAH1 was amplified from genomic DNA using specific primers (Table S1). The amplified product was cloned into an entry vector using pENTR/D-TOPO kit (Invitrogen). This was further cloned into the destination vector pIGF (Tetrahymena specific rDNA-based vector, a gift from Doug Chalker, Washington University) using LR clonase. For expressing TtPAH1 as TAP-tagged protein, full-length TtPAH1 was PCR amplified using specific primers with XhoI restriction site in forward primer and ApaI restriction site in the reverse primer (Table S1) and the amplified product was cloned into Tetrahymena specific vector pVGF (from Meng-Chao Yao, University of Washington) using XhoI and ApaI restriction sites. TtPAH1-TAP and TtPAH1-GFP were transformed into wild-type Tetrahymena cells using 20 μg of the plasmid by electroporation (Gaertig et al., 1994). Transformants were selected with 100 μg/ml paromomycin sulfate and induced with 1μg/ml cadmium chloride for 4-5 hours to stimulate transcription of the transgene from the MTTI promoter. The NUP3-GFP in NCVB vector (from Aaron Turkewitz, University of Chicago) was linearized and introduced biolistically into vegetative Tetrahymena by particle bombardment, and the transformants were selected using 60μg/ml blasticidin in the presence of 1μg/ml cadmium chloride (Rahaman et al., 2008).

Disruption of TtPAH1
5’UTR and 3’UTR of TtPAH1 were PCR amplified and cloned into the pCRII vector (Invitrogen). To amplify 5’UTR, SacI and EcoRI restriction sites were incorporated in forward and reverse primers respectively (Table S1). For amplification of 3’UTR, EcoRI and XhoI restriction sites were included in the forward and reverse primer respectively (Table S1). Finally, the NEO3 cassette was introduced between 5’UTR and 3’UTR using EcoRI.
restriction sites. The resulting knockout construct was linearized by digesting with SacI and XhoI restriction enzymes and introduced biolistically into vegetative *Tetrahymena* by particle bombardment as previously described (J. Gaertig, L. Gu, B. Hai, 1994; Cassidy-Hanley, 2003). The complete replacement of endogenous *TtPAH1* was achieved by growing the transformants in the presence of increasing concentrations of paromomycin sulfate (up to 1.2 mg/ml) with 1 µg/ml cadmium chloride.

**Semi-quantitative RT-PCR**

Total RNA was isolated from Δ*Ttpah1* cells and wild-type cells using Rneasy Mini Kit (Qiagen). QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA. PCR reactions were performed with 100 ng cDNA using alpha-tubulin (*ATU1*) and *TtPAH1* specific primers (Table S1) in the same reaction for 25-40 cycles.

**Purification of TtPah1-TAP**

For purification of TtPah1-TAP, *Tetrahymena* cells harboring *TtPAH1*-pVGF were grown to a density of 3x10^5 cells per ml. The culture was induced with 1µg/ml cadmium chloride for 5 hours at 30°C, and cells from 300 ml cultures were collected by centrifugation. The cell pellet was resuspended in 10 ml lysis buffer (20 mM Tris-HCl (pH 8.00), 100 mM NaCl, 0.5% NP-40, 10% glycerol) supplemented with a mixture of protease inhibitor (pepstatin, E-64, aprotinin, and protease inhibitor cocktail). The lysate was clarified by ultracentrifugation (Beckman Coulter Optima L100K, 70Ti rotor) for 1 hour at 250,000 g. To minimize proteolysis, all subsequent steps were carried out at 4°C unless mentioned otherwise. Rabbit-IgG agarose slurry (Sigma) pre-equilibrated with wash buffer was added to the clarified lysate and was kept for binding for 2 hours. Resin was collected by centrifugation (1 min at 3,000 g) and washed with 50 bed volumes of wash buffer (20 mM Tris-HCL (pH 8.00), 2 mM MgCl₂, 0.2 mM EGTA, 0.1 % Tween 20, 10 % glycerol, 1 mM DTT, 0.1mM PMSF). Resin was incubated with 2µl of TeV protease in 200 µL cleavage buffer (10 mM Tris-HCl (pH 8.00), 0.1 M NaCl, 0.1% Tween 20, 0.5 mM EDTA, 1 mM DTT) for 1.5 hours at room temperature followed by further incubation at 4°C overnight. The eluate after proteolytic cleavage was adjusted to 3 mM CaCl₂ and mixed with 3 volumes of calmodulin binding buffer (10 mM Tris-HCl (pH 8.00), 100 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM CaCl₂, 0.1 % Tween 20, 10 mM βME). This was incubated with 100 µL of calmodulin resin (GE Biosciences) at 4°C for 1 hour. The resin was recovered by centrifugation and washed with calmodulin binding buffer. Protein was eluted with calmodulin elution buffer (10 mM Tris-HCl (pH 8.00), 100 mM NaCl, and 1 mM Mg acetate, 1 mM imidazole, 10 mM EGTA, 0.1 % Tween 20, 10 mM 2-mercaptoethanol)(Witkin and Collins, 2004). Eluted
fractions were loaded on 10 % SDS polyacrylamide gel, and the protein was detected by silver staining.

Growth analysis


tipa1

knockout cells and wild-type cells were grown in triplicate. When cell number reached 1 x 10^5/ml, cells were counted using a hemocytometer at 2 hours interval after fixation with formalin. The averaged cell density was plotted against time.

Isolation of nuclei

50 ml of Tetrahymena cells (5 x 10^5 cells/ml) were centrifuged (5 min at 1100g) at 4°C and cell pellets were washed with pre-chilled Solution A (sucrose 0.1M, gum arabic 4% v/v, MgCl_2 0.0015M, Spermidine Hydrochloride 0.01% v/v) and resuspended in pre-chilled Solution B (sucrose 0.1M, gum arabic 4% v/v, MgCl_2 0.0015M, Spermidine Hydrochloride 0.01% v/v, octanol 24mM). The suspension was shaken vigorously for 5 min followed by centrifugation(A llen, 2000). The nuclear pellet was resuspended in Buffer A and was imaged by fluorescence microscope after staining with DAPI.

Staining and microscopy

For staining lipid droplets, Tetrahymena cells were pelleted down by centrifugation (1100g for 2 min) at room temperature, washed with DMC and fixed with 4% paraformaldehyde. Fixed cells were washed with 10mM HEPES and resuspended in the freshly prepared oil red O solution. Cells were tapped briefly and incubated in the dark in a nutating mixer at room temperature for 10 min. Stained cells were washed thrice with 10mM HEPES and were resuspended in 10mM HEPES before imaging in a confocal microscope (Binns et al., 2006). For Endoplasmic Reticulum staining, Tetrahymena cells were grown to a density of 3-4 x10^5 cells/ml, 0.5 µM ER-Tracker™ Red dye (Invitrogen) was added to the culture and incubated for 60 minutes before fixing with 4% paraformaldehyde (50 mM HEPES pH 7.5). To rule out any effect of differential pressure (during placing coverslips) on ER morphology in different samples, we imaged both wild-type cells and knockout cells simultaneously. For Oil Red O staining images were taken at 543 nm excitation / 619 nm emissions and for ER-Tracker Red images were taken at 587 nm excitation /615 nm emissions. 3-5µl of cells were mounted on glass slides, covered with cover glasses, and sealed with nail polish and imaged with a Zeiss LSM780 confocal microscope.

Staining Tetrahymena nucleus, it was incubated with 5µg/ml of 3,3’- dihexyloxacarbocyanine iodide (DHCC) and 0.5 µg/ml of 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 10 minutes in dark, washed thrice with Solution A and was
resuspended in the same solution before imaging in a Nikon Eclipse Ti fluorescence microscope.

To quantitate ER content, the stacked images of ER tracker red stained cells were analyzed by image J after sum intensity projection. The mean intensity values were plotted for both wild-type (n= 34) and ΔTtpah1 (n=32) cells using box plot.

**Gene synthesis**

The coding region of *TtPAH1* was commercially synthesized (Eurofins) after codon optimization and obtained in the pUC57 vector. This commercially synthesized gene was used for expression in bacteria and complementation assays in yeast.

**Yeast culture conditions**

Yeast cells were grown either in YPD media or synthetic medium (SD) containing 2% glucose with appropriate amino acids (Sherman, 2002). For growth analysis, yeast cells were grown in the SD media lacking leucine and uracil to early logarithmic phase, serially diluted (10-fold) and 5µl of each dilution was spotted onto the solid SD media lacking leucine and uracil and incubated at either 30°C or 37°C for 2–4 days. To check respiratory deficiency, glycerol (2%) in place of dextrose was used as the carbon source.

**Site-directed mutagenesis**

Point mutations (D666,668E) at the corresponding sites of *TtPAH1* coding region in *YCplac111-PAH1* fusion construct were introduced using Quick Change Site-Directed Mutagenesis protocol (Stratagene), and the mutations were confirmed by DNA sequencing.

**Yeast complementation assay**

The full-length coding sequence of *TtPAH1* (*T. thermophila PAH1*), *AtPAH2* (*Arabidopsis thaliana PAH2*) and *TbPAH1* (*Trypanosoma brucei PAH1*) were amplified using specific primers and cloned into *YCplac111* (LEU) using SalI/ BamHI restriction sites. To assess nuclear membrane morphology and growth rescue, *pah1Δ* yeast cells (*RS453 smp2Δ: ade2his3leu2trp1ura3 smp2::TRP1*) were transformed with either *TtPAH1* or *TtPAH1*mut or *AtPAH2* or *TbPAH1* along with *PUS-GFP* by standard Lithium acetate protocol (Gietz and Woods, 2001). Transformants were screened on solid SD medium lacking uracil and leucine. The transformants were grown in the same media at 30°C to early log phase and analyzed by confocal microscopy. The results from three independent experiments were used for analysis of nuclear morphology.

**Analysis of gene expression**

Gene expression was analyzed by reverse transcription-PCR by isolating total RNA from cells grown in SD media containing adenine and histidine. The isolated RNA was used to
synthesize single-stranded cDNA using Superscript II reverse transcriptase (Invitrogen). For quantitative analysis, RT-PCR was performed using the SYBR Green qPCR (Roche) in 7500 Real-Time PCR System (Applied Biosystems) following manufacturer’s instructions. All the primer sequences used are compiled in Table S1. The relative expression level was calculated using the comparative Ct method after normalizing to SEC 63 as a control gene.

**Phosphatase Assay**

Phosphatidic acid phosphatase activity was measured by following the release of water-soluble Pi from chloroform-soluble PA. The standard reaction contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM MgCl₂, 10 mM Triton X 100, 10 mM 2-mercaptoethanol and 1 mM phosphatidic acid in a total volume of 100 μl. Reactions were initiated by the addition of recombinant proteins and carried out in triplicate at 30°C for 20 min. The reaction was terminated by adding 500μl of 0.1M HCl in methanol and 1ml of chloroform. To that mixture, 1 ml of water was added for phase separation, and 1 volume of upper phase was mixed with two volumes of Biomol Green to develop color. The absorbance was measured at 620 nm, and the amount of phosphate produced was quantified using a standard curve (Gil-Soo Han and George M. Carman, 2010).

**Sequence Analysis**

Sequences of *Tetrahymena PAH* homologs (TITHERM_00189270 and TITHERM_00215970) were retrieved from Tetrahymena Genome Database and domains were predicted with Interpro protein sequence analysis and classification tool (EMBL-EBI). Multiple sequence alignment was performed with PRALINE. Percent identity matrix was calculated using Clustal2.1. The sequences of PAH used in this study are; S000004775 for *Saccharomyces cerevisiae*, NM_001203528.1 for *Arabidopsis thaliana* (AtPAH2) and XM_841075 for *Trypanosoma brucei*, FBgn0263593 for *Drosophila melanogaster*, BC030537.1 for *Homo sapiens* and DDB_G0271730 for *Dictyostelium discoideum*.

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**Competing interests**

The authors declare no competing or financial interests.

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References


Farese, R. V. and Walther, T. C. (2009). Lipid Droplets Finally Get a Little R-E-S-P-E-C-


Kim, Y., Gentry, M. S., Harris, T. E., Wiley, S. E., Lawrence, J. C. and Dixon, J. E.


Phan, J. and Reue, K. (2004). Lipin Expression Preceding Peroxisome Proliferator-activated...


Fig. 1. Domain organisation, sequence analysis and function of PAH protein

(A) Schematic representation of the role of PAH in lipid biosynthesis. PA is a key precursor used for the synthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) through CDP-DAG pathway. In the presence of choline and ethanolamine, these phospholipids are synthesized through Kennedy pathway. In metazoans, the pathway that converts CDP-DAG (cytidine diphosphate diacylglycerol) to PC/PE (CDP-DAG pathway)
does not exist whereas both the pathways are present in yeast. G-3-P-Glycerol-3-phosphate, LPA-Lysophosphatidate, PI3P-Phosphatidylinositol-3-phosphate, PI4P- Phosphatidylinositol-4-phosphate, PI (3, 5) P2- Phosphatidylinositol-3, 5-biphosphate, PS- Phosphatidylserine.

(B) Domain Organization of TtPAH1 and TtPAH2. Predicted N-LIP and C-LIP domains are indicated in the boxes. Also shown are the positions of a conserved glycine residue in N-LIP and the haloacid dehalogenase (HAD) with its conserved DXDXT/V motif in C-LIP.

(C) Multiple sequence alignment showing partial sequences of N-LIP (Top) and C-LIP (Bottom) of PAH proteins from *Tetrahymena thermophila, Saccharomyces cerevisiae*, and *Homo sapiens*. Assigned colors of the particular residues are based on alignment consensus. Conserved Glycine residue in N-LIP and catalytic motif (DXDGT/V) in C-LIP are indicated inside the box.
Fig. 2. TtPAH1 localizes on ER and encodes functional phosphatidate phosphatase

(A) Localization of TtPAH1-GFP in Tetrahymena cells. Confocal image of fixed Tetrahymena cells expressing TtPAH1-GFP after DAPI staining. DAPI stained nuclei (left panel), TtPAH1-GFP (middle panel) and DIC image of the fixed growing cell (right panel).
(B) TtPAH1 associates with ER. Confocal images of fixed cells expressing \textit{TtPAH1-GFP} after staining with ER tracker Red; TtPAH1-GFP (upper left panel), ER tracker Red (upper middle panel) and merge (upper right panel). Confocal stack of a different \textit{Tetrahymena} cell expressing \textit{TtPAH1-GFP} and stained with ER tracker Red is shown in lower panel.

(C) TtPAH1 purified as TAP-tag fusion in \textit{Tetrahymena}. The silver stained gel of purified TtPAH1 along with standard molecular weight marker is shown. The molecular weights are indicated on the left. In addition to the expected band of \(~100\) kDa, many smaller species are likely to reflect partial proteolysis of the full-length protein.

(D) TtPAH1 displays phosphatidate phosphatase activity. TtPAH1 protein (1\(\mu\)M) purified from \textit{Tetrahymena} was used to measure phosphatidic acid phosphatase activity, using a colorimetric assay. The average phosphate released (nmol) (\(n=3\)) was plotted against time.

(E) The phosphatidate phosphatase assay performed either in the presence (+Mg\(^{2+}\)) or absence (-Mg\(^{2+}\)) of magnesium. The assay was carried out for 30 minutes before measuring the activity. TtPAH1 showed activity only in the presence of Mg\(^{2+}\), confirming it to be a PAP1 enzyme. The average phosphate released (nmol) (\(n=3\)) is shown.
Fig. 3. *TtPAH1* is dispensable for normal growth of vegetative *Tetrahymena* cells.

(A) Schematic showing organization of the knockout construct used to disrupt *TtPAH1* in the macronucleus. Gene disruption was done by replacing the *TtPAH1* ORF with *NEO3* gene cassette, by homologous recombination. The *NEO3* cassette confers resistance to paromomycin.

(B) Reverse transcription PCR analysis of wild-type and ∆*Ttpah1* cells. Lane1, standard molecular weight marker; lane2, amplified products of cDNA from wild-type cells; lane3, amplified products of cDNA from ∆*Tipah1* cells. The top band just below 400 bp marker
corresponds to alpha-tubulin (387 bp), and the band near 300 bp represents *TiPAH1*. The absence of 300 bp band corresponding to *TiPAH1* confirms that knockout is complete.

(C) Growth curve of *Tetrahymena* wild-type versus Δ*Ttpah1* cells. The cell numbers were counted every two hours, and the number of cells/ml was plotted against time. Loss of *TiPAH1* does not affect *Tetrahymena* growth significantly.

(D) Semi-quantitative RT-PCR showing expression of *TiPAH2* in wild-type and Δ*Ttpah1* cells. Lane1, standard molecular weight marker; lane2, amplified products of cDNA from wild-type cells; lane3, amplified products of cDNA from Δ*Ttpah1* cells. The top band in lane 2 and 3 corresponds to alpha-tubulin (387 bp), and the band near 238 bp represents *TiPAH2*.

(E) The graph shows quantitation of *TiPAH2* after normalization with alpha tubulin band. The expression of *TiPAH2* is not enhanced by the loss of TtPAH1.
Fig. 4. TtPAH1 maintains lipid droplet number in *Tetrahymena*

(A) Confocal images of *Tetrahymena* cells showing lipid droplets stained with Oil Red O dye. Wild-type cells and knockout cells were imaged together simultaneously. The wild-type cells were stained with DAPI to distinguish them from knockout cells.

(B) Box plot showing the distribution of lipid droplet numbers in wild-type (n=35) vs. ∆Ttpah1 (n=38) cells.
(C) Confocal images of wild-type and *TtPAH1-GFP* expressing cells showing lipid droplets after staining with Oil Red O dye.

(D) Box plot showing lipid droplet numbers in wild-type cells, cells overexpressing *TtPAH1-GFP* (n=20), and cells overexpressing *GFP-DRP6* (n=20). The increase in lipid droplet number is observed in cells expressing *TtPAH1-GFP*.

(E) Confocal images of *Tetrahymena* cells showing lipid droplets stained with Oil Red O dye. Wild-type and knockout cells after starvation were imaged together simultaneously. Knockout cells were stained with DAPI to distinguish them from wild-type cells. Both size and number of lipid droplet are reduced in ΔTtpah1 cells (KO). Lipid droplet size in wild-type cells appears to be larger than in the knockout cells as indicated by arrows.

(F) Box plot showing lipid droplet numbers in wild-type (n=22) and ΔTtpah1 (n=22) cells under starved condition.
Fig. 5. *TtPAH1* is needed for maintaining tubular ER

(A) Top panel shows wild-type (WT) and ∆*Ttpha1* (KO) cells imaged simultaneously in the same field after staining with ER tracker Red. While left panel represents confocal stack, right panel is a single mid plane confocal slice. Wild-type cells were stained with DAPI to distinguish them from ∆*Ttpha1* cells. The enlarged images of ∆*Ttpha1* (bottom left panel) and wild-type (bottom right panel) cells are shown indicating ER sheet (arrow) and ER tubule (arrowhead) structures. To rule out the effect of DAPI staining on ER morphology, we also stained ∆*Ttpha1* cells with DAPI and imaged simultaneously with wild type cells and found similar results.

(B) Box plot showing the mean density of ER tracker stain. The mean intensity of ∆*Ttpha1* (n=32) is significantly higher than that of wild-type (n=25).
Fig. 6. Loss of *TtPAH1* does not show visible nuclear envelope defect in *Tetrahymena*.

(A) Confocal images of wild-type and *ΔTtpah1* cells expressing *NUP3-GFP* after DAPI staining. In both wild-type and *ΔTtpah1*, upper panel is Z-stack and lower panel is a single slice. The enlarged nucleus from Z-stack is shown on the right side.
(B) Confocal images of DAPI stained nuclei isolated from wild-type (upper panel) and ∆Ttpah1 (lower panel) cells expressing NUP3-GFP.

(C) Fluorescence images of Tetrahymena nuclei of wild type (Top panel) and ∆Ttpah1 (Bottom panel) after staining with DHCC and DAPI. The images are deconvoluted using NIS Advanced Research software.
Fig. 7. \textit{TiPAH1} rescues the nuclear structure defect, slow growth phenotype and respiratory deficiency of \textit{pah1Δ} yeast strain and catalytic motif is essential for its function

(A) Confocal images of \textit{pah1Δ} yeast cells transformed with either empty vector (top panel) or \textit{TiPAH1} (middle panel) or \textit{TiPAHmut} (bottom panel) along with \textit{PUS1-GFP} (an intra-nuclear reporter). \textit{TiPAH1} but not \textit{TiPAHmut} restores aberrant nuclei of \textit{pah1Δ} yeast to wild-type spherical shape. Three different transformants per strain were analyzed and the number of cells counted for each transformant was 200-250 (n = 600 to 750). The percentage of cells containing round nucleus is indicated on the right.

(B) The growth of \textit{pah1Δ} yeast cells transformed with either \textit{TiPAH1} or empty vector grown on SD media containing either glucose or glycerol (lacking leucine and uracil) at either 30°C or 37°C as indicated. The experiment was repeated three times.

(C) Quantitative RT-PCR analysis of \textit{INO1}, \textit{INO2}, and \textit{OPI3} mRNAs in \textit{pah1Δ} yeast cells transformed with either empty vector or \textit{TiPAH1}. Amplification of each sample was
performed in triplicates and normalized to a control gene SEC63 in three independent experiments.

(D) The growth of *pah1Δ* yeast cells transformed with either with empty vector, *TtPAH1* or *TtPAHmut* grown on SD media lacking leucine and uracil and containing either glucose or glycerol as indicated.
Fig. 8. Phosphatidate phosphatase is conserved across eukaryotic lineages

(A) Eukaryotic evolutionary tree. Five clades with representative organisms from each clade are shown.

(B) Multiple sequence alignments showing parts of N-LIP (Top) and C-LIP (Bottom) of PAH proteins from various organisms. Assigned colors of the specific residues are based on alignment consensus. The boxes indicate conserved Glycine at the N-LIP and conserved catalytic motif (DXDXT) at the C-LIP.

(C) Confocal images of pah1Δ yeast cells complemented either with Trypanosoma PAH (TbPAH1) or with Arabidopsis PAH (AtPAH2). Both TbPAH1 and AtPAH2 rescued nuclear defect of pah1Δ yeast cells. The nucleus is visualized by expression of PUS-GFP. Two different transformants per strain were analyzed and the number of cells counted for each one was 200 (n=400). The percentage of cells containing round nucleus is indicated on the right.

(D) Rescue of growth defect of pah1Δ yeast cells by TbPAH1 and AtPAH2 on SD media containing glucose but lacking leucine and uracil at 30°C or 37°C as indicated. Two different transformants per strain were spotted and analyzed.
Figure S1: Confocal stack of ΔTpaH1 (KO) and wild-type (WT) cells stained with ER-tracker Red. Arrow indicates ER sheet and arrowhead indicates ER tubule structures.

Table S1: Oligonucleotides used in this study. (The underlined sequences represent sequences of restriction site added to the oligonucleotides.)

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<td>GCCTCGATGCTGCCATGACAGCTGGAGGAG</td>
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<tr>
<td>TbPAH1 FP</td>
<td>GCCTCGAGATGATATCTGTTTGCAGATTTTC</td>
</tr>
<tr>
<td>TbPAH1 RP</td>
<td>GCCTCGACACAGTGTCACCTTGGTG</td>
</tr>
</tbody>
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