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Small phosphatidate phosphatase (*TtPAH2*) of *Tetrahymena* complements respiratory function and not membrane biogenesis function of yeast *PAH1*

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Phosphatidate phosphatases (PAH) play a central role in lipid metabolism and intracellular signaling. Herein, we report the presence of a low-molecular-weight *PAH* homolog in the single-celled ciliate *Tetrahymena thermophila*. *In vitro* phosphatase assay showed that TtPAH2 belongs to the magnesium-dependent phosphatidate phosphatase (PAP1) family. Loss of function of *TtPAH2* did not affect the growth of *Tetrahymena*. Unlike other known *PAH* homologs, *TtPAH2* did not regulate lipid droplet number and ER morphology. *TtPAH2* did not rescue growth and ER/nuclear membrane defects of the *pah1Δ* yeast cells, suggesting that the phosphatidate phosphatase activity of the protein is not sufficient to perform these cellular functions. Surprisingly, *TtPAH2* complemented the respiratory defect in the *pah1Δ* yeast cells indicating a specific role of *TtPAH2* in respiration. Overall, our results indicate that TtPAH2 possesses the minimal function of PAH protein family in respiration. We suggest that the amino acid sequences absent from TtPAH2 but present in all other known PAH homologs are critical for lipid homeostasis and membrane biogenesis.

Keywords. Lipid droplet; lipin; PAP; phosphatidate phosphatase; *Tetrahymena*

1. Introduction

Phosphatidate phosphatase (PAP) enzymes are involved in lipid synthesis, signaling, and also act as a key regulator of lipid metabolism and cell physiology. There are two distinct families of PAP enzymes, Mg^{2+}-dependent (PAP1) and Mg^{2+}-independent (PAP2), that serve different physiological functions in lipid metabolism (Sciorra and Morris 2002; Reue and Brindley 2008). PAP1 enzymes are required for lipid synthesis and play transcriptional regulatory roles, whereas PAP2 enzymes are associated with lipid signaling in yeast and mammals. Apart from the distinct cofactor requirement, they vary in their nature of membrane association. PAP1 enzymes are cytosolic or membrane associated proteins whereas PAP2 enzymes are integral membrane proteins.

PAP1 enzymes catalyse the penultimate step in triglyceride synthesis, the dephosphorylation of phosphatidic acid (PA), to generate diacylglycerol needed for the synthesis of TAG and phospholipids (PE and PC) (figure 1A) (Han *et al*. 2006). PAP1 enzymes in mammals are encoded by *LIPIN* genes whereas *PAH1* is the yeast homolog (Han *et al*. 2006). PAH proteins reside in the cytoplasm and translocate to the nuclear/ER membrane where it binds its substrate (PA) and catalyses the enzymatic reaction. Dephosphorylation of PAH induces membrane association and makes it active whereas phosphorylation inhibits membrane association and PAP activity. The amino-terminal (N-LIP) and carboxyl-terminal (C-LIP) regions of Lipin/PAH1 are highly conserved (Santos-Rosa *et al.* 2005). N-LIP domain of TtPAH2 contains a conserved glycine residue critical for lipin function; mutation of which causes lipodystrophy (fld) in the mouse. Haloacid dehalogenase (HAD)-like domain present in the C-LIP possesses the conserved DXDXT/V catalytic motif essential for PAP activity (Han *et al*. 2007). Apart from an enzymatic role, yeast PAH1 and mammalian lipin1 also act as transcriptional regulators (Santos-Rosa *et al*. 2005; Finck *et al.* 2006; Kim *et al.* 2013). Deletion of *PAH1* leads to

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Table 1. Primers used in this study.

Primer name	Sequence (5′ to 3′)
*TtPAH2*_del_5′UTR_F	GCGAGCTCCTAAATACTAAAATCAAATGCAATAGG
*TtPAH2*_del_5′UTR_R	GCGAATTCCGTTAATCATCTTTATCCTATAGTTTAG
*TtPAH2*_del_3′UTR_F	GCGAATTCAAGGAGCACTACTGAAAG
*TtPAH2*_del_3′UTR_F	GCCTCGAGATTTAATTAGAAAAAATAAAGAAATTAAC
*TtPAH2*_RT_F	CTGACGTTGATGGCACTGTT
*TtPAH2*_RT_R	GGAGATAGCAAGATAGGGCCA
Alpha tubulin_RT_F	CCTCCCCCTAAGTCTCAACC
Alpha tubulin_RT_R	CGAAGGCAGAGTTGGTGATT
*TtPAH2*_GFP_F	CACCATGCATCACCATCACCATCACATGATTAACGGAATTAAAAATC
*TtPAH2*_GFP_R	TCATTTTGGTAATTGCTTATT

reduced lipid droplet number, aberrant nuclear expansion, over developed ER membranes, and slow growth phenotype in yeast (Santos-Rosa *et al*. 2005; Adeyo *et al*. 2011). In metazoans, lipin regulates nuclear envelope breakdown, ER homeostasis, organelle morphology, phospholipid levels and lipid storage (Gorjánácz and Mattaj 2009; Golden *et al.* 2009; Ugrankar *et al*. 2011; He *et al.* 2017). Several human metabolic diseases are associated with mutations in genes encoding lipin protein (Müller-felber *et al*. 2010). Thus, PAH proteins play a crucial role in lipid storage, membrane biogenesis, and organelle morphology and are evolutionarily conserved.

Tetrahymena is a single-celled ciliate with two distinct nuclei, a transcriptionally active somatic macronucleus, and a transcriptionally silent germline micronucleus. It shows membrane complexity comparable to higher organisms (Rahaman *et al.* 2008). We identified two homologs of phosphatidic acid phosphohydrolase gene (*TtPAH1* and *TtPAH2*) in the genome database of *Tetrahymena thermophila*. The findings from our study show that *TtPAH1* encodes a protein of ~ 96kDa, similar in size to PAH proteins from other organisms. However, *TtPAH2* encodes a low molecular weight protein (37 kDa) and is the smallest PAH protein belonging to the PAP1 class. We have focussed our study on the small *PAH* homolog, *TtPAH2*, to understand the minimal function that a PAH protein can execute in this organism. Our results indicate a specific role of PAH in respiration. However, unlike other known PAH proteins, it does not regulate lipid droplet biogenesis or ER/nuclear morphology.

2. Materials and methods

2.1 *Strains and culture conditions*

Wild-type *Tetrahymena* strains B2086 and CU428 were obtained from Tetrahymena Stock Center at Cornell University (Ithaca, NY, USA). *Tetrahymena* cells were grown in SPP medium (2% proteose peptone, 0.2% glucose, 0.1% yeast extract and 0.003% Fe-EDTA), at 30°C with constant shaking (90 rpm). 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_2PO4, 0.1 mM Na_2HPO4, 0.65 mM $CaCl_2$, and 0.1 mM $MgCl_2$) for 16–24 h at 30°C.

2.2 *Gene synthesis*

The coding region of *TtPAH2* was commercially synthesized (Biotech Desk) after codon optimization and obtained in the pUC57 vector. This was used for expression in bacteria and complementation experiments in yeast.

2.3 *Cloning and expression of TtPAH2*

The genomic sequence of the *TtPAH2* gene (gene ID: TTHERM_00215970) was obtained from the *Tetrahymena* Genome Database (TGD). *TtPAH2* was amplified from genomic DNA using specific primers listed in table 1. The amplified product was cloned into pENTR/D-TOPO vector (Invitrogen), sequenced and subsequently introduced into the pIGF vector (*Tetrahymena* specific rDNA-based vector). *TtPAH2*-GFP was transformed into wild-type *Tetrahymena* cells by electroporation. Transformants were selected with 100 μg/mL paromomycin sulfate and induced with 1 μg/mL cadmium chloride.

2.4 *Generation of ΔTtpah2 Tetrahymena strain*

Macronuclear gene knockout was performed as previously described (Gaertig *et al.* 1994). To construct the disruption cassette of the Tt*PAH2* gene, 5′UTR of the Tt*PAH2* gene, the neomycin-resistance gene (*NEO3*) and 3′UTR of the Tt*PAH2* gene were sequentially introduced into the pCRII vector (Invitrogen). This disruption cassette was released by digestion with SacI, and XhoI restriction enzymes and the

digested product was ethanol precipitated before transformation. The transformation was performed by biolistic bombardment using PDS-1000/He particle delivery system (Bio-Rad) as previously described (Gaertig *et al.* 1994).

2.5 *Confirmation of knockout strain by RT-PCR analysis*

Total RNA was isolated from Δ*Ttpah2* 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 of cDNA for 25–40 cycles using *TtPAH2* specific primers (*TtPAH2*_RT) and alpha-tubulin primers (Alpha-tubulin_RT) as mentioned in table 1. As a control, parallel reactions were performed without adding reverse transcriptase.

2.6 *Cloning, Expression, and Purification of recombinant TtPAH2 in E. coli*

TtPAH2 gene in pUC57 was sub-cloned into the pET33b vector using BamHI and NdeI restriction sites. The *TtPAH2*-pET33b construct was transformed into BL-21(DE3) strains of *E. coli* to express it as the N-terminal His_6 fusion protein. The transformants were grown in LB broth supplemented with 50 μg/mL kanamycin at 37°C and induced with 1 mM IPTG for 5 h. The cells were harvested by centrifugation at 5000 rpm at 4°C. The cell pellet was resuspended in ice-cold buffer containing 25 mM HEPES (pH 7.5), 300 mM NaCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol and 10% glycerol supplemented with EDTA-free protease inhibitor cocktail (Roche). The cells were lysed by sonication, and the clarified lysate was purified using Ni-NTA affinity column as described earlier (Rahaman *et al.* 2003). The purified protein was checked by the Coomassie-stained SDS-PAGE gel.

2.7 *Preparation of cell extracts and Western blotting*

Cell lysis was performed in ice-cold buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 10% glycerol, 0.02% NP-40 and 10 mM β-mercaptoethanol supplemented with protease inhibitors (1 μg/mL leupeptin, 2 μg/mL pepstatin, 1 μg/mL E-64, 1 μg/mL aprotinin and protease inhibitor cocktail). The cells were lysed by cell homogenizer (Isobiotec), and cell lysate was clarified by centrifugation at 13,000 rpm for 1 h. Equivalent volumes of supernatant and pellet fractions along with clarified total cell lysate were resolved by 10% SDS-PAGE. Protein was detected by Western blot analysis using anti-GFP polyclonal antibody (from Stefan Kircher, University of Freiburg).

2.8 *Growth assay*

Mid-log-phase cultures of wild-type and *ΔTtpah2 Tetrahymena* cells were used to inoculate 50 mL of fresh SPP medium at an initial cell density of 0.4×10^5. Cell numbers were determined every 2 h using haemocytometer. The averaged cell density was plotted against time.

2.9 *Staining and microscopy*

Tetrahymena cells were pelleted down by centrifugation (1100*g* for 2 min) at room temperature, washed with DMC and fixed with 4% paraformaldehyde. Fixed cells were washed with 10 mM HEPES and resuspended in the freshly prepared oil red O solution for staining the lipid droplets. Stained cells were washed thrice with 10 mM HEPES before imaging. For endoplasmic reticulum staining, *Tetrahymena* cells were grown to a density of 3×10^5 per mL, and ER-Tracker™ Green dye (1 μM final concentration) was added to the culture and incubated for 60 min before fixing with 4% paraformaldehyde. Imaging was carried out in a Zeiss LSM780 confocal microscope. Confocal images were analysed by LSM image analyser (Zeiss), and ER quantification was done by Image J (NIH).

2.10 *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 $MgCl_2$, 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 triplicates at 30°C for 20 min unless mentioned otherwise. The reaction was terminated by addition of 500 μL of 0.1 M HCl in methanol and 1 mL chloroform. To this 1 mL of water was added for phase separation, 1 volume of upper phase was mixed with two volumes of Biomol Green, and the color was allowed to develop. The absorbance was measured at 620 nm, and the amount of phosphate produced was quantified using a standard curve.

2.11 *Yeast culture conditions*

Yeast cells were grown in synthetic medium (SD) containing 2% glucose with appropriate amino acids. For growth analysis, yeast cells were grown in the SD medium to early logarithmic phase, serially diluted (10-fold) and 5 μL of each dilution was spotted onto the appropriate solid media in the plates and incubated at 30°C for 2–4 days. For checking the respiratory deficiency, glycerol (2%) was used as the carbon source in place of glucose.

2.12 *Complementation experiments in yeast*

The full-length coding sequence of *TtPAH2* was 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 YCplac111-*TtPAH2* or empty vector along with *PUS-GFP*. Transformants were screened on solid SD medium lacking uracil and leucine. The transformants were grown in SD media at 30°C to early log phase and analysed by confocal microscopy. The results from three independent experiments were used for analysis of nuclear morphology.

◀ **Figure 1.** TtPAH2 is a Magnesium-dependent phosphatidate phosphatase. (**A**) A schematic describing the role of PAH1 in lipid biosynthesis. PAH1 is dephosphorylated by the Nem1-Spo7 phosphatase complex in yeast. The dephosphorylated PAH1 catalyses the dephosphorylation of PA to generate DAG. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PAH1*, active dephosphorylated PAH1; LD, lipid droplet; DAG, diacylglycerol; TAG, triacylglycerol. (**B**) The domain organization of PAP1 and PAP2 enzymes with the essential catalytic motifs indicated. The conserved amino acid residues in the catalytic motifs are indicated by the single letter code. HAD denotes Haloacid dehalogenase domain and Dpp1 is Diacylglycerol pyrophosphate phosphatase, a member of PAP2 family. (**C**) Alignments of amino-acid sequences of a part of HAD-like domain of *Tetrahymena PAH2* (*TtPAH2*), yeast PAH1 and human LIPIN1. Sequence alignment was performed by ClustalW. The identical amino acid residues are indicated by asterisks. Box shows the conserved amino residues in HAD-like domain. (**D**) SDS-PAGE gel showing purified TtPAH2-His6 after staining with Coomassie blue. Left lane is molecular weight markers, and right lane is purified TtPAH2. Positions of the molecular weight markers (kDa) are indicated on the left. (**E**) The phosphatidic acid phosphatase activity of recombinant TtPAH2-His6 was measured with increasing protein concentrations for 20 min. (**F**) The phosphatidic acid phosphatase activity using 1 μM recombinant TtPAH2. (**G**) The phosphatidic acid phosphatase activity of 1 μM recombinant TtPAH2-His6 measured for 20 min either in the presence (Mg^{2+}) or in the absence (No Mg^{2+}) of magnesium.

3. Results

3.1 *TtPAH2 belongs to the PAP1 family*

Unlike other PAH proteins, small phosphatidate phosphatase of *Tetrahymena* (TtPAH2) is a low molecular-weight protein consisting of 336 amino acid residues. The predicted sequence of TtPAH2 comprises two conserved regions, N-LIP and C-LIP domains present in all PAP1 proteins. TtPAH2 also consists of a conserved catalytic motif (DXDXT) found in the members of the Mg^{2+}-dependent phosphatase superfamily (figure 1B and 1C). Moreover, TtPAH2 lacks three signature motifs of PAP2 proteins (figure 1B). This suggests that TtPAH2 is a potential Mg^{2+}-dependent PAP1 enzyme. To determine whether TtPAH2 is a Mg^{2+}-dependent (PAP1) or Mg^{2+}-independent (PAP2) phosphatidic acid phosphatase, we examined the enzymatic activity of recombinant TtPAH2. For this purpose, we expressed *TtPAH2* in *E. coli* and purified TtPAH2 as N-terminal His6 fusion protein. The purity of protein, migrating at ~37 kDa, was confirmed by SDS-PAGE analysis (figure 1D). Phosphatase activity was measured following the release of inorganic phosphate (Pi) from PA. The recombinant TtPAH2 dephosphorylated PA in a concentration and time-dependent manner (figure 1E and F). We did not observe any detectable phosphatase activity of TtPAH2 in the absence of magnesium ions (figure 1G). Our results showed that TtPAH2 is a Mg^{2+}-dependent phosphatidic acid phosphatase belonging to the PAP1 family.

3.2 *TtPAH2 is localized in both cytoplasm and membranes and is dispensable for growth of Tetrahymena*

We generated a *TtPAH2* knockout strain of *Tetrahymena* by disrupting all the macronuclear copies of *TtPAH2* by homologous recombination (figure 2A). Expression analysis by reverse transcription (RT)-PCR confirmed the complete disruption of *TtPAH2* in the knockout strain (figure 2B). To determine if *TtPAH2* is essential for growth in *Tetrahymena*, we assessed the growth rates of the *ΔTtpah2* cells and the wild-type cells cultured in parallel under optimal growth conditions. *ΔTtpah2* cells displayed no visible growth alteration as compared to wild-type cells (figure 2C). Also, we did not observe any apparent defect in cell morphology in the *ΔTtpah2* cells. These findings suggest that the *TtPAH2* gene is not essential for the normal growth and survival of *Tetrahymena.*

To examine the sub-cellular localization of TtPAH2, we expressed *TtPAH2-GFP* in the wild-type *Tetrahymena*. Confocal image analysis showed the distribution of TtPAH2-GFP throughout the cell (figure 2D). We fractionated the total lysate of cells expressing TtPAH2-GFP into the membrane and cytosolic fractions. TtPAH2 was present in both the soluble fraction and the membrane fraction (figure 2E).

3.3 *TtPAH2 does not regulate lipid droplet biogenesis and ER morphology in Tetrahymena and complements respiratory defect in pah1Δ yeast cells*

PAH proteins control lipid droplet number in all the organisms studied (Golden *et al.* 2009; Adeyo *et al.* 2011; Ugrankar *et al.* 2011). To evaluate whether *TtPAH2* has a direct role in lipid droplet biogenesis, we stained lipid droplets of *ΔTtpah2* and wild-type *Tetrahymena* cells with Oil Red O. Confocal image analysis showed no visible difference in lipid droplet number between wild-type and *ΔTtpah2* cells (figure 3A, left panel). The mean value for the number of lipid droplets in wild-type cells (mean = 291) was comparable to that of *ΔTtpah2* cells (mean = 281) (figure 3A, right panel). Thus, deletion of *TtPAH2*, unlike other lipins/PAHs previously characterized, did not affect lipid droplet number in *Tetrahymena*. *PAH* also regulates ER morphology in organisms like yeast, *C.elegans*, mammals, and *Arabidopsis* (Siniossoglou *et al.* 1998; Campbell *et al.* 2006; Golden *et al.* 2009). To find out if *TtPAH2* regulates ER morphology, we stained both *ΔTtpah2* and wild-type cells with ER-Tracker dye and evaluated the ER structure and

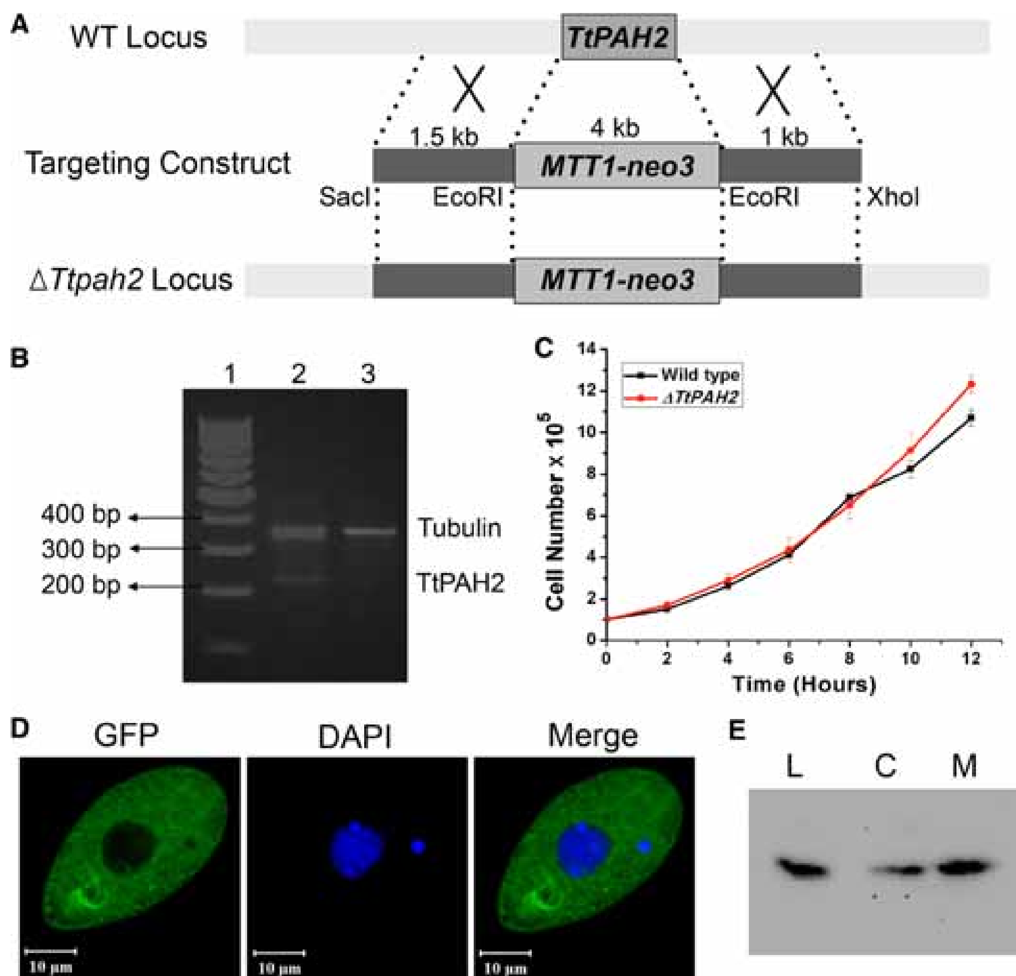

Figure 2. TtPAH2 localizes to both cytosol and membranes and is not required for normal growth of *Tetrahymena*. (**A**) Schematic showing the knockout construct used to disrupt *TtPAH2* in *Tetrahymena*. The *neo3* cassette (*MTT1-neo3)* includes a gene encoding paromomycin resistance under the control of the cadmium-inducible *MTT1* promoter. (**B**) RT-PCR analysis of wild-type and *ΔTtpah2* cells. Standard molecular weight marker (lane1); amplified products of cDNA from wild-type cells (lane2) and *ΔTtpah2* cells (lane3). The top band in lane 2 is alpha tubulin (387bp), and the lower band represents TtPAH2 (238bp). The absence of 238 bp band in *ΔTtpah2* confirms that knockout is complete. (**C**) Growth analysis of *Tetrahymena* wild-type and *ΔTtpah2* cells. (**D**) Sub-cellular localization of TtPAH2 in *Tetrahymena* cells. Fixed *TtPAH2-GFP* expressing *Tetrahymena* cells were imaged after DAPI staining. TtPAH2-GFP (left panel), DAPI stained nuclei (middle panel) and merged image (right panel). (**E**) Western blot analysis of the total lysate (**L**), its cytosolic fraction (**C**) and its membrane fraction (**M**) isolated from *Tetrahymena* cells expressing *TtPAH2-GFP* using anti-GFP antibody.

content by analysing confocal images. The ER morphology of *ΔTtpah2* cells was similar to that of the wild-type cells (figure 3B and S1). Moreover, total ER content of *ΔTtpah2* cells was comparable to that of wild-type cells (figure 3B). These results lead us to conclude that TtPAH2 is the first functional phosphatidate phosphatase that does not regulate lipid droplet biogenesis or ER morphology.

Yeast *PAH1* is functionally replaceable with *PAHs* from mammals and *Arabidopsis* (Grimsey *et al.* 2008; Nakamura *et al*. 2009; Craddocka *et al*. 2015). We complemented *pah1Δ* yeast cells with *TtPAH2* to check whether *TtPAH2* rescues mutant phenotypes. We confirmed the expression of *TtPAH2* in *pah1Δ* yeast cells transformed with *TtPAH2* by RT-PCR analysis (figure 3C). Although *TtPAH2* was expressed, it failed to rescue the abnormal nuclear morphology as visualized by expression of PUS-GFP (figure 3C). It was also unable to rescue the growth defect of *pah1Δ* yeast cells under normal growth condition (figure 3D). The *pah1Δ* yeast cells are also known to exhibit respiratory deficiency phenotype (i.e., *growth* defect) on non-fermentable carbon sources (Han *et al.* 2007). To evaluate the role of *TtPAH2* in rescuing respiratory

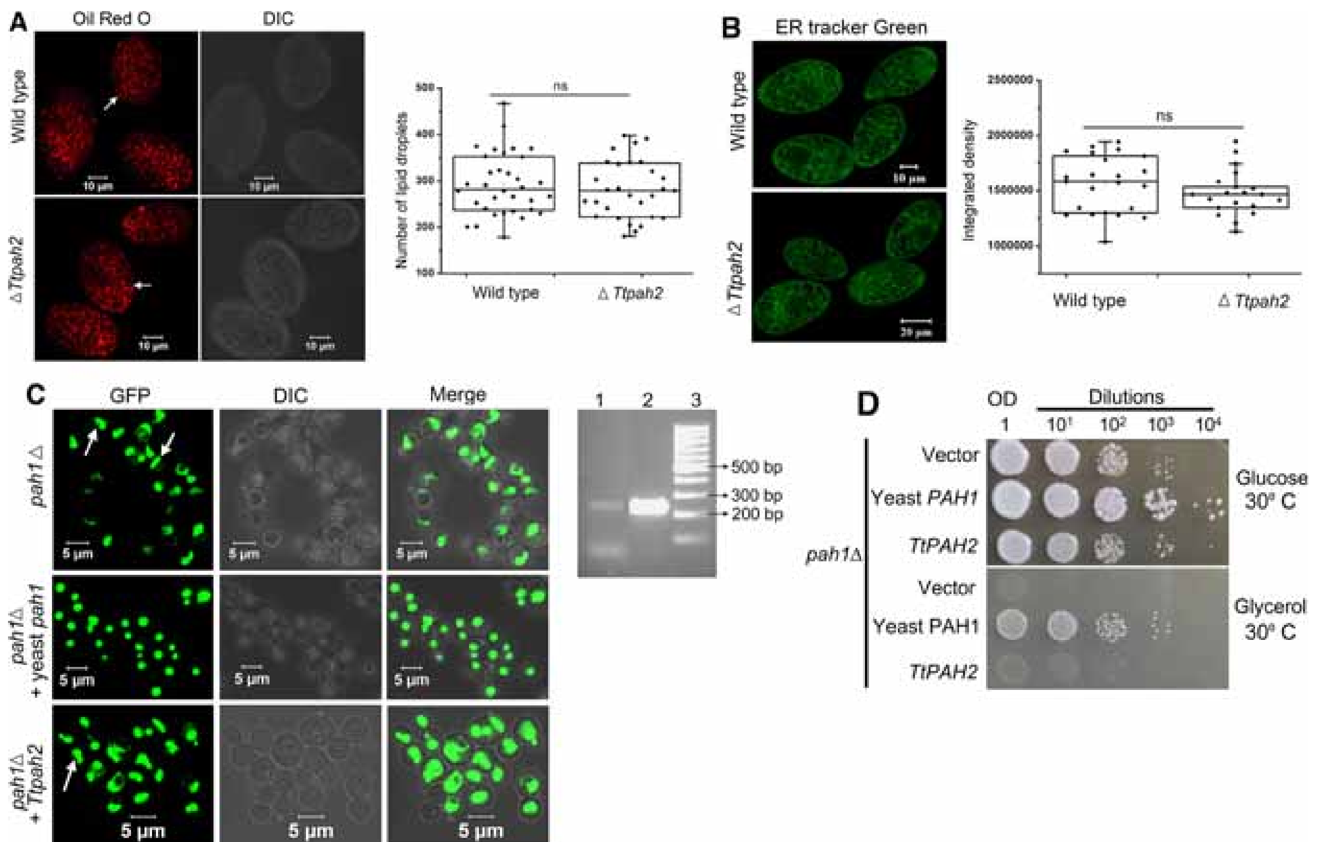

Figure 3. *TtPAH2* does not regulate lipid droplet biogenesis or ER morphology and does not functionally replace yeast *PAH1* in fermentable substrate. (**A**) Oil Red O-stained confocal microscopic pictures and DIC images of wild-type and *ΔTtpah2 Tetrahymena* cells to show the lipid droplets (white arrows). Total numbers of lipid droplets per wild type (n = 34) and *ΔTtpah2* (n = 29) cells are shown in the graph on the right. There was no significant difference (indicated as 'ns') in the number of lipid droplets as analysed by Kruskal-Wallis test ($p < 0.01$). (**B**) Confocal images of wild-type and *ΔTtpah2* cells stained with ER-Tracker Green showing ER morphology (left). Box plot showing the mean density of ER in wild-type and *ΔTtpah2* cells as measured by sum intensity projection of the stacked images (right). The mean intensity of *ΔTtpah2* (n=20) is similar to that of wild-type (n = 23) as analysed by Kruskal-Wallis test ($p < 0.01$). ns is not significant. (**C**) Confocal and DIC microscopic images of PUS1-GFP-expressing yeast *pah1Δ* cells transformed with empty vector (top), yeast *PAH1* (middle) or *TtPAH2* (bottom). Note that the aberrant structure (arrows) of the *pah1Δ* nuclei was rescued by yeast *PAH1* but not by *TtPAH2*. RT-PCR analysis of the total RNA isolated from the yeast *pah1Δ* cells transformed with *TtPAH2* (lane 1) and from the wild-type *Tetrahymena* cells (lane 2). Lane 3, molecular weight markers. The positions of the molecular weight markers are indicated on the right. (**D**) Cultures of *pah1Δ* cells transformed with empty vector (indicated as vector), yeast *PAH1* or *TtPAH2* were adjusted to $OD_{600} = 1$ followed by 10-fold serial dilutions. Five microliters of each spotted on SD plates containing either glucose (top panel) or glycerol (bottom panel) but lacking leucine and uracil were incubated for 3 days at 30°C. The respective dilutions are indicated on the top.

deficiency, we complemented *pah1Δ* yeast with *TtPAH2* and grew cells on plates containing glycerol as non-fermentable carbon source. The *pah1*Δ yeast cells expressing *TtPAH2* grew faster than control *pah1*Δ cells in glycerol (figure 3D), suggesting that *TtPAH2* rescues respiratory deficiency of *pah1*Δ cells. These results along with our analysis of catalytic activity of TtPAH2 suggest that mere presence of catalytic activity is not sufficient for cellular function of PAH proteins in lipid homeostasis and membrane biogenesis, and TtPAH2 possesses the minimal function in respiration suggesting it to be one of the ancient protein in this family.

4. Discussion

All Mg^{2+}-dependent phosphatidate phosphatases previously characterized are relatively large proteins, close to 100 kDa and low molecular weight proteins showing PA phosphatase activity are usually Mg^{2+}-independent. We report a low molecular weight (37 kDa) *PAH* homolog in *Tetrahymena* (TtPAH2) exhibiting Mg^{2+}-dependent phosphatidate phosphatase activity. TtPAH2, being the PAP1 enzyme, is anticipated to regulate lipid droplet biogenesis and ER/nuclear morphology. Studies in yeast, *C. elegans*, and *Drosophila* show that changes in PA homeostasis are responsible

for controlling the morphology of ER, nucleus, and other cell organelles (Carman and Han 2009; Gorjánácz and Mattaj 2009; Ugrankar *et al.* 2011; Bahmanyar *et al*. 2014). In contrast, deletion of *TtPAH2* does not affect lipid droplet number and ER morphology in *Tetrahymena.* This might be due to the presence of the other homolog, *TtPAH1*. However, *TtPAH1* and *TtPAH2* shows non-overlapping expression in different cell cycle stages (supplementary figure 2) suggesting that *TtPAH2* may have a specialized role in *Tetrahymena*. TtPAH2 is the first example of phosphatidate phosphatase which does not regulate lipid droplet or membrane biogenesis. Previous studies show that catalytic activity of PAH proteins is essential for its function in lipid homeostasis and membrane biogenesis (Han *et al.* 2007). But this study shows catalytic activity alone is not sufficient. These results suggest that the amino acid sequences absent in *TtPAH2* but present in other *PAH* homologs may be critical for performing these cellular functions.

It is interesting to note that the function of *PAH1* in yeast is more prominent during respiration since the growth of *pah1Δ* yeast cells is inhibited more severely in the non-fermentable substrate as compared to its growth in the fermentable substrate. The exact role of PAH1 in respiration remains ambiguous. However, a recent study in yeast showed a two-fold reduction in the levels of ATP in *pah1Δ* yeast cells compared to the wild-type (Park *et al.* 2015). Therefore, it is conceivable that PAH1 is required for ATP production via TCA cycle since in non-fermentable substrates the cell is highly dependent on TCA cycle for energy.

We discuss here a possible link between PAH1 and ATP production. The loss of *PAH1* results in accumulation of PA (Han *et al*. 2006). In *Arabidopsis* and mammals, it has been shown that PA accumulation leads to inhibition of PP2C family of protein phosphatases (Wang *et al.* 2006). Interestingly, a PP2C family member Ptc5/Ptc6 in yeast, activates pyruvate dehydrogenase by dephosphorylation (Gey *et al.* 2008). Therefore, we speculate that loss of *PAH1* which results in accumulation of PA could potentially inactivate Ptc5/Ptc6 leading to the inhibition of pyruvate dehydrogenase. Pyruvate dehydrogenase is the first enzyme linking the glycolysis and TCA cycle and is essential for ATP production via TCA cycle. This is in accordance with previous observations that loss of *PAH1* causes a severe growth defect in non-fermentable substrate where ATP production by TCA cycle is the primary source of energy.

In this study, we report the presence of a low molecular weight PAH protein in *Tetrahymena*, the first PAH protein shown not to regulate lipid homeostasis and membrane biogenesis. Our results show that TtPAH2 is a protein that possesses the minimal respiratory function of this family of proteins and that the extra amino acids present in other homologs are perhaps acquired to perform additional cellular functions like lipid homeostasis and membrane biogenesis. Further studies are required to understand the specific role of *TtPAH2* in *Tetrahymena*.

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