A Putative NEM1 Homolog Regulates Lipid Homeostasis and Membrane Biogenesis via PAH1 in *Tetrahymena thermophila*

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Ms. **Sushmita Shukla** entitled "**A Putative NEM1 Homolog Regulates Lipid Homeostasis and Membrane Biogenesis via PAH1 in *Tetrahymena thermophila***" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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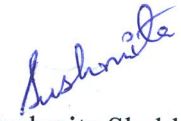

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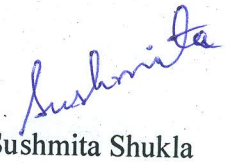
DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.



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List of Publications

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1. **Sushmita Shukla,** Anoop Narayana Pillai, and Abdur Rahaman. A Putative NEM1 Homolog Regulates Lipid Homeostasis via PAH1 in Tetrahymena thermophila. Journal of Biosciences. Journal of Biosciences Vol. 43, No. 4, September 2018, pp. 693-706.

2. Anoop Narayana Pillai*, **Sushmita Shukla***, Sudhanshu Gautam* and Abdur Rahaman. Small phosphatidate phosphatase (TtPAH2) of Tetrahymena complements respiratory function and not membrane biogenesis function of yeast PAH1. Journal of Biosciences Vol. 42, No. 4, December 2017, pp. 613–621 (* = Equal contribution).

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Sushmita

Sushmita Shukla

Dedicated to my parents

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SYNOPSIS OF Ph. D. THESIS

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SYNOPSIS

Neutral lipids are stored in cells in the form of specialized organelles called lipid droplets (LD), which are dynamic cellular organelles found ubiquitously in eukaryotic cells and in some prokaryotic cells (1)(2). Based on the requirement these stored lipids can either be used for the synthesis of membrane lipids or be hydrolyzed by lipases to yield free fatty acids, which may be utilized for energy production in a cell (3)(4). LDs are composed of a hydrophobic core which contains neutral lipids, mostly triacylglycerol (TAG) and a single phospholipid monolayer, which separates the aqueous phase and the hydrophobic core (2)(5)(6)(7)(8). Storage of lipid in the form of lipid droplet is critical since both excess and poor fat storage leads to various metabolic disorders (9)(10)(11)(12). Therefore, regulation of lipid droplet biogenesis is essential for maintenance of lipid homeostasis in a cell.

Phosphatidate (PA) acts as a precursor of phospholipids and triacylglycerols. Pah/Lipin plays a pivotal role in lipid metabolism by catalyzing the Mg^{2+} dependent dephosphorylation of phosphatidic acid to yield diacylglycerol, an elementary reaction of lipid biosynthesis (13)(14). DAG generated by the dephosphorylation of PA is acylated by diglyceride acyltransferases (DGATs) to produce TAG, and may also be used for the synthesis of membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (15). Phosphorylation and dephosphorylation of PAH enzymes are tightly regulated (16)(17). PAH1 lacks a transmembrane domain and is primarily located in the cytosol in its inactive form (18). A highly conserved membrane-associated protein phosphatase complex comprising Nem1 (catalytic subunit) and Spo7 (regulatory subunit), dephosphorylates and recruits Pah1 to the nuclear/ER membrane (18)(19). The dephosphorylation of Pah1 exclusively by Nem1-Spo7 acts as a rate-limiting step and is essential for its phosphatidate phosphatase function (18)(20). Thus, this cascade is essential in the regulation of lipid metabolism and membrane biogenesis.

Tetrahymena thermophila, unicellular ciliated protozoa, exhibits nuclear dimorphism, a transcriptionally inert diploid germline micronucleus (MIC) and a transcriptionally active polyploid somatic macronucleus (MAC) (21)(23)(24). MAC, the larger nucleus, consists of 45 copies of each gene (21)(24)(25). We have previously shown that *Tetrahymena* possesses two *PAH* homologs, *TtPAH1* and *TtPAH2*. *TtPAH1*, unlike *TtPAH2*, is required for lipid droplet biogenesis and maintaining tubular endoplasmic reticulum morphology (26)(27). NEM1 that regulates PAH has not been identified in *Tetrahymena thermophila*. The search in genome database of *Tetrahymena* (http://ciliate.org/index.php/home/welcome) exhibited the presence of various putative Nem1 homologs. We have studied four of these putative NEM1 homologs namely *NEM1A* (TTHERM_00262970), *NEM1B* (TTHERM_00473100), *NEM1C* (TTHERM_00685940), and *NEM1D* (TTHERM_00688650).

Based on the background information, the present study aims at elucidating the role of *Tetrahymena* NEM1 homologs in lipid homeostasis and membrane biogenesis of *Tetrahymena*. The objectives are -

1) Identification and elucidation of the role of *NEM1A, NEM1B, NEM1C* and *NEM1D* in lipid and membrane biogenesis.

2) Analysis of the functional conservation of *NEM1* in yeast and *Tetrahymena*.

3) Analyzing the evolutionary conservation between *Tetrahymena PAH2* and yeast *PAH1*.

The thesis consists of six chapters.

The first chapter includes the introduction and the literature survey related to the present work. Experimental methodologies are covered in the second chapter. The third chapter describes the generation of Nem1 knockout strains of *Tetrahymena* and elucidation of the role of *Tetrahymena NEM1* homologs in lipid and membrane biogenesis. The fourth chapter provides the analysis of functional conservation of *NEM1* in yeast and *Tetrahymena*. The fifth chapter covers analysis of the evolutionary conservation between *Tetrahymena PAH2* and yeast *PAH1*. Finally, summary and conclusion of the current study have been presented in the sixth chapter.

Chapter 1: Introduction and Literature Review

NEM1 and PAH1 of yeast are members of the haloacid dehalogenase (HAD) superfamily and harbors the conserved D*X*D*X*T catalytic motif important for its function (17)(20). SPO7 binds to the catalytic domain of NEM1 and is essential for the phosphatase activity of the complex (14)(27). Cells lacking either *nem1*Δ or *spo7*Δ subunit of the complex display phenotypes similar to those of *pah1*Δ cells including increased phospholipid synthesis, aberrant expansion of the nuclear/ER membrane, decreased lipid droplet number, and temperature sensitivity (14)(27). The *nem1*Δ *spo7*Δ, double mutants also show phenotypes similar to *pah1*Δ or *nem1*Δ or *spo7*Δ, suggesting that these three proteins work in unison (14)(27). Overexpression

of the NEM1-SPO7 phosphatase complex results in a lethal phenotype only in the presence of its substrate PAH1, indicating that an excess level of PAP activity is deleterious to cell growth (14). CTDNEP1 (C-terminal domain nuclear envelope phosphatase1, formerly known as Dullard) and NEP1-R1 (nuclear envelope phosphatase 1-regulatory subunit 1, initially termed as TMEM188) are the mammalian orthologs of NEM1 and SPO7 respectively (29)(30). Similar to yeast, CTDNEP1 also requires an endogenous binding partner, NEP1-R1, for its stabilization and dephosphorylation of LIPIN (19)(30). The human CTDNEP1-NEP1R1 complex can functionally replace yeast *NEM1-SPO7* in *nem1*Δ*spo7*Δ yeast double mutant with respect to nuclear membrane expansion and formation of lipid droplet, suggesting that NEM1-SPO7 phosphatase is evolutionarily conserved between yeast and mammals (20). Earlier studies have shown that PAH1 homolog of *Tetrahymena* (TtPAH1) also functionally replaces yeast PAH1 and is evolutionarily conserved across the eukaryotic lineages (25).

Chapter 2: Experimental Procedures

This chapter covers the methodologies used to accomplish the objectives of the present study. It includes the detailed description of various reagents and chemicals used for this study. This chapter also covers the culture techniques including the maintenance of *Tetrahymena* culture, yeast culture, and bacterial culture conditions. Various molecular biology techniques such as polymerase chain reaction (PCR), cloning, site-directed mutagenesis used in this study and confocal microscopy are also described in this chapter. Methodology for the generation of *Tetrahymena* knock-out mutants and other experimental details have also been described here.

Chapter 3: Role of *Tetrahymena NEM1* homologs in lipid homeostasis and membrane biogenesis

To evaluate the role of *NEM1* homologs, their subcellular localizations were investigated. For this purpose, strains were generated to overexpress individual GFP-tagged proteins from the cadmium-inducible metallothionein (*MTT1)* promoter. *NEM1B* exhibited evenly dispersed

distribution in the cytoplasm in addition to the punctate pattern, while *NEM1D* showed only the punctate distribution in the cytoplasm. The revelation of different subcellular localization indicates different functions of NEM1B and NEM1D. Co-localization of NEM1D with endoplasmic reticulum was performed by staining the NEM1D-GFP-expressing cells with ER-tracker Red. The result shows that unlike NEM1 of yeast, NEM1D of *Tetrahymena* does not co-localize with ER. To investigate the role of putative *NEM1* homologs in *Tetrahymena*, the generation of knockout strains of each of four *NEM1* homologs was attempted. Gene disruption was performed by replacing the endogenous open reading frame with the neo3 cassette (encoding paromomycin resistance) in the macronucleus through homologous recombination. All the 45 macronuclear copies of *NEM1B*, *NEM1C*, and *NEM1D* were replaced by the drug resistance cassette and confirmed by reverse transcription-PCR (RT-PCR), indicating that these genes are not essential for *Tetrahymena* growth. In contrast, multiple attempts to generate *NEM1A* knockout strains were not successful, suggesting that *NEM1A* is essential for *Tetrahymena* growth. The growth curves of the *Δnem1B*, *Δnem1C*, and *Δnem1D* mutant strains were similar to that of the wild-type cells, suggesting that these homologs are dispensable for the normal growth of *Tetrahymena*. Moreover, the lack of growth defect in one knock out strain is not due to compensatory overexpression of the other homologs as demonstrated by semi-quantitative reverse transcription-PCR (RT-PCR).

NEM1 proteins regulate the phosphatase activity of PAH1, which in turn regulates the lipid droplet biogenesis (17)(20). Of the *Tetrahymena* homologs, TtNem1C is most similar in size to yeast Nem1 and therefore the putative bona fide ortholog. To evaluate the role of TtNem1C in lipid droplet biogenesis, the lipid droplets of both wild-type and ΔTtnem1C *Tetrahymena* cells were stained with a neutral lipid dye Oil Red O. Nem1C in *Tetrahymena*, though of similar size to yeast Nem1, is not functionally equivalent and does not regulate lipid droplet number. To further investigate the role of other NEM1 homologs in lipid droplet biogenesis,

we checked the effect of loss of *NEM1B* and *NEM1D* on lipid droplet number. Lipid droplets in wild-type and knockout *Tetrahymena* strains (both Δ*nem1B* and Δ*nem1D*) were stained using a neutral lipid dye Oil Red O. Lipid droplet number was not significantly affected in Δ*nem1D* as compared to wild-type cells. However, loss of *NEM1B* in *Tetrahymena* showed significant reduction in lipid droplet number as compared to wild-type cells. The number of lipid droplet per cell was approximately 300 in case of wild-type and Δ*nem1D* cells whereas lipid droplet count was reduced to 200 per cell in case of Δ*nem1B* cells, suggesting that *NEM1B* homolog of *Tetrahymena* plays a role in lipid droplet biogenesis. In *S. cerevisiae*, Nem1 functions via Pah1 and overexpression of *PAH1* rescues defects due to loss of *NEM1* function. In *Tetrahymena,* we previously showed that loss of *TtPAH1* causes a severe reduction in lipid droplet number and overexpression of *TtPAH1* leads to increased lipid droplet number (26). To assess if Nem1B regulates lipid droplet biogenesis via Pah1 in *Tetrahymena*, we overexpressed *TtPAH1,* in Δ*Ttnem1B* cells, evaluated the lipid droplet numbers in these cells and compared with Δ*Ttnem1B* cells. The overexpression of *TtPAH1* in Δ*Ttnem1B* cells was confirmed by RT-PCR analysis. Analysis of confocal images after Oil Red O staining showed that the mean lipid droplet number was 257 per cell in Δ*Ttnem1B* cells overexpressing *TtPAH1* (n=65), 202 per cell in Δ*Ttnem1B* cells (n=54), and 304 per cell in the wild-type cells (n=50). Therefore, there was ~50% rescue of the defect in lipid droplet number in Δ*Ttnem1B* by *TtPAH1* overexpression. The rescue may be due to the limiting kinase activity needed to phosphorylate the large pool of overexpressed Pah1. These results indicate that TtPah1 together with TtNem1B forms a cascade for regulation of lipid homeostasis and membrane biogenesis in *Tetrahymena.*

To see if the *NEM1* homologs of *Tetrahymena* are essential for maintaining the normal nuclear envelope (NE) morphology, like yeast, we stained the isolated nucleus of Δ*nem1B,* Δ*nem1C,* Δ*nem1D* cells, and wild-type cells with a lipophilic dye (DHCC). The result

demonstrated that the loss of *NEM1B* or *NEM1C* or *NEM1D* does not lead to any visible defect in nuclear morphology suggesting that unlike yeast, *NEM1* is not essential for maintaining normal nuclear morphology in *Tetrahymena*.

To assess whether *NEM1* homologs have a direct role in ER morphology, *Δnem1B, Δnem1C, Δnem1D,* and the wild-type cells were stained with ER-Tracker Green. The results showed that there was no significant difference in ER morphology and total ER content between *Δnem1B, Δnem1C, Δnem1D,* and the wild-type cells. These results suggest that *NEM1* homologs do not regulate endoplasmic reticulum morphology in *Tetrahymena*.

Chapter 4: Functional conservation of *NEM1* between *Tetrahymena* and yeast

Yeast mutants lacking *NEM1* or *SPO7* exhibit slow growth and aberrant nuclear membrane morphology. In the mutants, the nucleus is elongated rather than spherical as in wild-type, and also has projections (17). Yeast Nem1 can be functionally replaced with its mammalian ortholog, CTDNEP1. Similarly, we previously found that *Tetrahymena TtPAH1* functionally complements yeast *PAH1* (26). To investigate if *TtNEM1B* complements the deletion of yeast *NEM1*, we overexpressed *TtNEM1B* in the *nem1*Δ yeast cells and evaluated its ability to rescue the slow growth and aberrant nuclear phenotypes. A nucleoplasmic marker PUS-GFP was expressed to evaluate nuclear morphology in yeast. Surprisingly, *TtNEM1B* failed to rescue either the slow growth or aberrant nuclear morphology of *S. cerevisiae nem1*Δ.

To examine if other *NEM1* homologs of *Tetrahymena* could replace yeast *NEM1*, we overexpressed *TtNEM1A* and *TtNEM1D* in *nem1*Δ yeast cells and monitored the nuclear morphology and growth phenotype. Neither *TtNEM1A* nor *TtNEM1D* rescued the defects. Interestingly, we found that the growth of *nem1Δ* yeast cells overexpressing *TtNEM1A* is slower than that of *nem1Δ* yeast cells. How *TtNEM1A* affects the growth of *nem1*Δ yeast cells is not known. It is possible that TtNem1A has an inhibitory effect on some essential process of yeast.

The *NEM1* homologs of *Tetrahymena* did not functionally replace *NEM1* in yeast, which might be due to the fact that the regulatory subunit *SPO7* of yeast *NEM1* does not recognize *Tetrahymena NEM1B*. Interestingly, we did not find the *SPO7* homolog in Tetrahymena Genome Database, which suggests that regulatory subunit of Nem1B has diverged significantly from Spo7.

Chapter 5: Evolutionary conservation of *TtPAH2*

The ciliates diverged quite early during the eukaryotic evolution (30). They belong to the kingdom chromalveolata (31). Thus, *Tetrahymena thermophila* is distantly related to higher eukaryotes from an evolutionary viewpoint. We have previously demonstrated that TtPAH1 is evolutionarily conserved ranging from *Tetrahymena* to higher eukaryotes (25). We complemented *pah1*Δ yeast cells with *TtPAH2* to check if *TtPAH2* rescues mutant phenotypes. We confirmed the expression of TtPAH2 in *pah1*Δ yeast cells transformed with TtPAH2 by RT-PCR analysis. Although TtPAH2 was expressed, it was unable to rescue the abnormal nuclear morphology as visualized by expression of PUS-GFP. It also failed to rescue the growth defect of *pah1*Δ yeast cells under normal growth conditions. The *pah1*Δ yeast cells are also known to exhibit respiratory deficiency phenotype (i.e., growth defect) on non-fermentable carbon sources. To evaluate the role of TtPAH2 in respiratory function, we complemented *pah1*Δ yeast cells with TtPAH2 and grew them on plates containing glycerol as the non-fermentable carbon source. The *pah1*Δ yeast cells expressing TtPAH2 grew faster than the *pah1*Δ cells in glycerol, suggesting that TtPAH2 rescues respiratory deficiency of *pah1*Δ yeast cells (26). These results suggest that TtPAH2 possesses the minimal function in respiration suggesting it to be one of the ancient proteins in this family.

Chapter 6: Summary and Conclusion

Tetrahymena possesses multiple *NEM1* homologs and we have used four *NEM1* homologs namely *NEM1A, NEM1B, NEM1C,* and *NEM1D* in this study. *NEM1B, NEM1C,* and *NEM1D*

are dispensable whereas *NEM1A* is essential for the optimal growth of *Tetrahymena*. The lack of growth defect in Δ*nem1B/C/D* is not due to compensatory overexpression of the other *NEM1* homologs. *TtNEM1B* is functionally related to *NEM1* of other organisms and is required for the lipid droplet biogenesis. Overexpression of *TtPAH1* partially restores lipid droplet biogenesis in the *Δnem1B* mutant. *NEM1* homologs of *Tetrahymena* cannot functionally replace yeast *NEM1*. Our results indicate that a conserved cascade comprising Nem1 and Pah1 functions in *Tetrahymena* to regulate lipid homeostasis and membrane biogenesis. We also show that *TtPAH2* rescues the respiratory deficiency of Δ*pah1* yeast cells suggesting a specialized role.

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2.	Dr. Abdur Rahaman	Guide/ Convener		29.6.18
3.	NIL	Co-guide (if any)		
4.	Dr. Praful Singru	Member		29.6.18
5.	Dr. Ramanujam Srinivasan	Member		29.6.18
6.	Dr. Narottam Acharya	Member		2.7.18

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ABBREVIATIONS

Δ	Gene deletion
µL	Microlitre
ml	Millilitre
Ade	Adenine
Amp	Ampicillin
ATP	Adenosine Triphosphate
bp	Base pair
BLAST	Basic Local Alignment Search Tool
C-LIP	Carboxy-terminal Lipin Domain
C-terminal	Carboxyl terminal
CDK	Cyclin dependent kinase
CDP-DAG	Cytidine diphosphate diacylglycerol
CDS	Phosphatidate cytidylyltransferase
CL	Cardiolipin
CTDNEP1	C-terminal domain nuclear envelope phosphatase 1
DAG	Diacylglycerol
DAPI	4', 6-Diamidino-2-Phenylindole, Dihydrochloride
DEPC	Diethylpyrocarbonate
DGAT	Diglyceride Acyltransferase
DGK	Diacylglycerol kinase
DHCC	3,3'-dihexyloxacarbocyanine iodide
DIC	Direct interference contrast
DMC	Dryl's Medium concentrate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide phosphates
DPP	Diacylglycerol pyrophosphate phosphatase
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
EST	Expressed sequence tags

Fld	Fatty liver dystrophy
EtBr	Ethidium Bromide
g	Gravity
GFP	Green fluorescent protein
HAD	Haloacid Dehalogenase
HAT	Histone acetyltransferase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
His	Histidine
hr	Hour
INO1	Inositol-3-phosphate synthase
Kan	Kanamycin
kb	Kilo base
kDa	Kilo Dalton
KO	Knockout
LB	Luria Bertani
LD	Lipid droplet
Leu	Leucine
LPP	Lipid phosphate phosphatase
MAC	Macronucleus
Met	Methionine
MIC	Micronucleus
Min	Minutes
mM	Millimolar
mm	Millimeter
MTT	Metallothionein
N-LIP	Amino-terminal Lipin Domain
N-terminal	Amino terminal
NE	Nuclear envelope
NEBD	Nuclear envelope breakdown
NEM1	Nuclear Envelope Morphology protein 1
NEO3	Neomycin
NEP1-R1	Nuclear envelope phosphatase 1-regulatory subunit 1
NLS	Nuclear localization signal

NPC	Nuclear pore complex
ns	Non-significant
NUP	Nuclear pore
OD	Optical density
OPI3	Phosphatidyl-N-methylethanolamine N-methyltransferase
PA	Phosphatidic acid
PAH	Phosphatidic Acid Hydrolase
PAP	Phosphatidic Acid Phosphatase
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PDH	Pyruvate dehydrogenase
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
PGC1α	Peroxisome proliferator activated receptor γ coactivator-1α
PI	Phosphotidylinositol
PMS	Paromomycin sulfate
PP2C	Protein phosphatase 2C
PPARα	Peroxisome proliferator activated receptor α
PS	Phosphatidylserine
PSA	Penicillin-Streptomycin-Amphotericin B Solution
RCF	Relative centrifugal force
rDNA	Ribosomal DNA
RPM	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Synthetic Complete Dextrose media
SPO7	Sporulation-specific protein 7
SPP	Sequestrin Proteose Peptone
StE	Steryl Esters
TAE	Tris-acetate buffer
TAG	Triacylglycerol

TE	Tris-EDTA
TGD	Tetrahymena Genome Database
Tris	Tris Hydroxymethylaminoethane
Trp	Tryptophan
Ura	Uracil
UTR	Un-translated region
v/v	Volume/volume
WT	Wild-type
YPD	Yeast Extract Peptone Dextrose
µg	Microgram

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Membrane biogenesis and lipid homeostasis

Membranes form the main structural component for the complex architecture of biological systems (32, 33). Membranes are composed of proteins and lipids and form a highly dynamic and complex interactive system (34). Membrane biogenesis involves the synthesis of molecular components of cell membrane and their assembly. It is a critical phenomenon in which the cells engage throughout their existence. It helps in dividing the cell into structural and functional compartments. Cell growth and division depend stringently on the production of sufficient membrane to equip the daughter cells. Lipids form the fundamental component of cellular membranes. Other than acting as a barrier, lipids are vital for carrying out other cellular functions like cell division, reproduction, and intracellular membrane trafficking by helping the membrane in budding, tubulation, fission, and fusion (32). Lipids are used for energy storage in the form of lipid droplets, principally as triacylglycerol esters and steryl esters. Lipids function as first and second messengers in various signal transduction pathways. They also help in the aggregation of proteins to the membrane. Maintaining the proper lipid composition of cellular membranes is critical for numerous biological processes (32). The matrix of the membrane is formed of polar lipids, consisting of a hydrophobic and a hydrophilic portion. The distribution of lipids across the cellular compartments is tightly regulated. The lipid composition of various organelles may vary dramatically. Membranes of the organelle are important to perform crucial cell functions like lipid production, protein synthesis, and cellular respiration. Membrane undergoes remarkable and dynamic changes in shape and size during the cell cycle that requires coordinated biosynthesis and remodeling of phospholipids (35). Maintenance of proper organelle morphology is highly significant for normal cell physiology.

1.1.1 Nuclear architecture

Nucleus undergoes significant changes during cell cycle progression and remodeling of the nuclear envelope is a highly dynamic and tightly regulated process (36). Nuclear morphology responds to various physiological conditions and may vary between different cell types. A characteristic feature of eukaryotic cells is the compartmentalization of chromatin inside the nucleus. The cytoplasm and nucleoplasm are separated by the nuclear envelope (NE). Nuclear envelope consists of a double lipid bilayer, the outer and inner nuclear membrane (37). The outer nuclear membrane is extended with the endoplasmic reticulum (ER) and plays a role in lipid biosynthesis and protein secretion while the inner nuclear membrane faces the nucleoplasm and serves as an anchoring site for chromatin at the nuclear periphery. In metazoans, the inner nuclear membrane is lined with the nuclear lamina (38). Nuclear pore complexes (NPCs) connect the inner and outer membranes and mediate the nucleocytoplasmic transport (39). Nuclear assembly involves coordinated biosynthesis, targeting and interaction of nuclear membrane with nuclear pores, inner nuclear membrane proteins and chromatin (40). Nuclear envelope undergoes dynamic structural changes that are critical for the proper nuclear division in all eukaryotes. Mammalian cells undergo an "open" mitosis, where the nuclear envelope breaks down at the onset of mitosis and reformed at the conclusion of mitosis (41). Conversely, yeast undergoes closed mitosis, where the NE remains intact during mitosis and the chromosomes are separated within a single intact nucleus that partitions between the mother and daughter cell (42). Yeast exhibits expansion of the nuclear envelope at the onset of mitosis since the surface area of the mother nucleus undergoing closed mitosis must increase to allow elongation of the intra-nuclear mitotic spindle (41). Although the method of nuclear division varies in yeast and animal cells, both exhibit

nuclear expansion during the cell cycle. Maintenance of proper nuclear structure is important for cell physiology, since changes in nuclear shape caused by mutations in the nuclear envelope proteins leads to several dominant heritable diseases (43–45). These diseases, referred to as laminopathies or envelopathies lead to dramatic alterations of the nuclear morphology. Previous studies in yeast and mammals have shown that phospholipid metabolism is linked to nuclear physiology since major phospholipid metabolic enzymes are localized in the nucleus and regulate the gene expression, nuclear organization, and nucleo-cytoplasmic transport (46–48). However, the molecular mechanism underlying the regulation of nuclear expansion is poorly understood.

Lipid homeostasis is maintained within a cell through a set of different proteins involved in the synthesis, transport, and degradation of lipids as well as the regulatory mechanisms that link lipid metabolism with the other cellular processes (49). Lipid homeostasis and membrane biogenesis are coupled together (50). The balance between lipid biosynthesis and its storage is crucial for maintaining metabolic homeostasis and normal cell physiology since both excess and poor fat storage leads to various disorders such as obesity, lipodystrophy, cardiovascular disease, diabetes, insulin resistance, hypertension, and cancer (12, 51–53). Elucidating the mechanism of this regulation is crucial for a better understanding of the disorders affecting lipid homeostasis and metabolism.

1.1.2 ER: the main site of lipid synthesis

Endoplasmic reticulum (ER) is the largest membrane-bound organelle in a eukaryotic cell and the main lipid biosynthetic organelle. ER plays a critical role in many cellular processes, such as lipid synthesis, protein synthesis, protein modification etc. (54).

Most of the lipid synthesis enzymes are transmembrane proteins and located in the ER membranes. The lipid metabolism is coordinated with ER growth (55, 56). The ER comprises of a complex network of tubules connected to the sheet-like cisternae that are continuous with the outer nuclear membrane, or 'nuclear ER' and extends throughout the cytoplasm to form the 'peripheral ER' (Figure 1.1) (57). ER is classified into two types, smooth ER (SER) and rough ER (RER). The RER has a sheet-like structure and is characterized by the presence of ribosomes associated with the synthesis of secretory and membrane proteins (Figure 1.1). Contrarily, the SER has more tubular morphology and is devoid of ribosomes (57, 58). The morphology of ER is critical for various intracellular events (54). The ER is a highly dynamic organelle that undergoes continuous rearrangements including tubule retraction, tubule branching, branchpoint sliding, tubule–tubule membrane fusion, ring closure and partitioning of ER during cell division (59, 60). The ER interacts with many organelles such as mitochondria, Golgi, endosomes, lysosomes, peroxisomes, and the plasma membrane and permits the transfer of lipids and intracellular signals. ER produces the structural phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol), cholesterol and significant levels of triacylglycerol and cholesteryl esters (61). Cholesterol is mainly synthesized in the ER and is transported to the other organelles (62). Hence, the ER harbours very low concentrations of sterols and complex sphingolipids. The lipids synthesized in ER are distributed to other organelles via secretory pathway and/or ER contact sites (63). TAG buds from the ER membrane and subsequently gets transferred to lipid droplet organelles (63, 64). ER is also involved in the transport of newly synthesized lipids and proteins (65). The ER also consists of lipids such as DAG, CDP-DAG, PA, lysophospholipids (32). Most of the cellular lipids are synthesized by enzymes

localized in the ER (66). The ER-localized lipid enzyme activities play a central role in maintaining lipid and membrane homeostasis across the cell. ER controls the production of different lipid classes and has an effect on cellular lipid biomass. Thus, ER is the key regulator of lipid levels across the cell (67, 68). To modify the activity of ER-localized enzymes various regulatory mechanisms have emerged. Some regulatory mechanisms alter the gene transcription, while others modify the levels of lipid by post-translational modification of the enzymes. These regulatory mechanisms help in maintaining cellular lipid homeostasis and crucial for modifying lipid synthesis in response to physiological stimuli (48, 69).

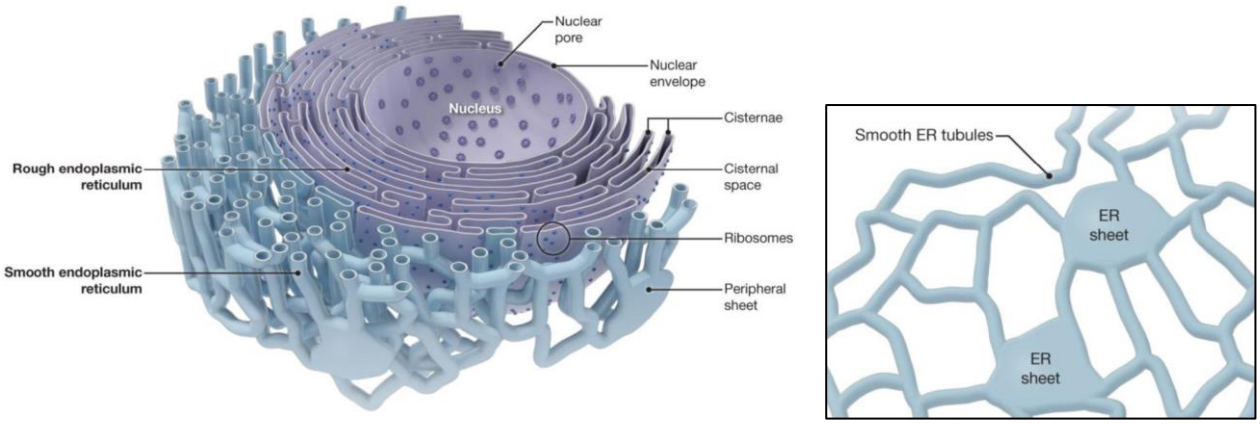

Figure 1.1: Schematic showing the different structural features of the ER network in animal cells
(Taken from Goyal et al, Untangling the web: Mechanisms underlying ER network formation Biochim Biophys Acta. 2013 Nov; 1833(11): 2492–2498)

In yeast, the peripheral ER forms a continuous tubular network underlying the plasma membrane (70, 71). The ER membrane is the main site of lipid biosynthesis and consists largely of phosphatidylinositol and phosphatidylcholine in yeast (72). In yeast, membrane biogenesis at the ER is regulated mainly by the intracellular concentration of the essential phospholipid precursors inositol and choline (73). During low inositol levels, a transcription factor complex composed of the Ino2p and

Ino4p proteins activates the expression of various genes encoding phospholipid, fatty acid, and sterol biosynthetic enzymes. Contrarily, high levels of inositol induce nuclear translocation of an ER-localized transcription factor, Opi1, to repress transcription of genes synthesizing phospholipid (74).

1.1.3 Lipid droplet

Lipid droplets (LDs) are unique intracellular organelles and composed of a hydrophobic core comprising triacylglycerol (TAG) and cholesterol ester (CE) (Figure 1.2)(2, 5, 7). LDs are surrounded by a phospholipid-cholesterol monolayer and associated proteins that prevent the exposure of the nonpolar contents to the aqueous cytoplasm (5, 75, 76). The phospholipids in the monolayer include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, lysoPC and lysoPE (5, 75, 77). Free cholesterol may also be present within the monolayer (78). LDs are thought to form *de novo*, however, after *de novo* formation LDs are not static with respect to size, localization or protein composition (79–82). Lipid droplets are associated with the ER and this interaction facilitates the transfer of newly synthesized neutral lipids into the lipid droplets (83). LDs play a central role in cellular lipid homeostasis (83, 84). The storage of free fatty acids in the form of TAG protects against fatty acid-induced lipotoxicity, and storage of cholesterol in the form of cholesterol esters prevents ER stress and eventual cell death (69, 85). LDs are present in most organisms, from bacteria and yeast to plants and mammals (86). The conversion and storage of free fatty acids in the form of lipid droplets are conserved from prokaryotes to eukaryotes (87, 88). LDs have a diverse morphological appearance, ranging in size from diameter 1 to 5 μm in non-adipocytes, but can be greater than 100 μm in white adipocytes (86, 89, 90). LDs have been observed to

move rapidly within cells (91, 92). LDs are usually dispersed throughout the cytoplasm (76, 93). However, under certain conditions, they can aggregate and form densely packed clusters, composed of many individual LDs (76, 94, 95). LDs are often found in close proximity to mitochondria, ER, and peroxisomes (96). LDs are more lipid-rich than the membrane-bound organelles and are composed of neutral lipids instead of polar lipids (phospholipids). Earlier, lipid droplets were considered to be fat storage sites in specialized cell types like adipocytes (97). Recent research reveals that LDs are highly dynamic and mobile structures with multiple and diverse functions (98, 99). Other than acting as energy reservoir, lipid droplets also play a role in signaling events, the regulation of cholesterol homeostasis, biosynthesis of membrane lipids, steroid hormones and eicosanoids, and acts as a transient storage compartment of proteins (100–105). The energy stored in the form of lipid droplet is mobilized during fasting by hydrolyzing the triacylglycerol and releasing the fatty acids (106–108). The overall mechanism of biogenesis and breakdown of LDs is not well understood. In humans, storage of triacylglycerol in large amounts, in the adipose tissue, resulting in obesity is often accompanied by the development of various metabolic diseases such as insulin resistance, type 2 diabetes, and atherosclerosis (12, 52, 53, 109). Human pathogens such as the hepatitis C virus (HCV) or *Chlamydia trachomatis* can hijack the lipid droplets as sites for assembly or replication (110, 111).

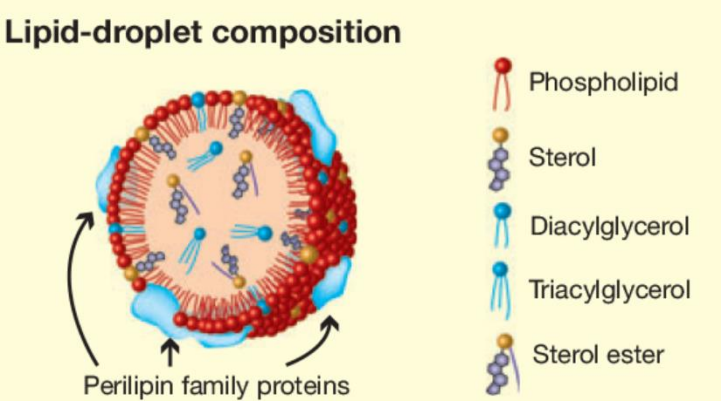

Figure 1.2: General lipid droplet structure and composition
(Taken from Guo et al, Lipid droplets at a glance, J Cell Sci. 2009 Mar 15; 122(6): 749–752.)

1.2 Phospholipids

Phospholipids are the major structural components of cell membranes, lipoproteins, and surfactants (32, 112). They are essential for vital cellular processes and function as dynamic building blocks for the nuclear membrane biogenesis. They also play important roles as molecular chaperones, reservoirs of second messengers and regulators of key physiological events such as cell migration endocytosis, exocytosis, and cytokinesis (113–116). In addition, they provide precursors for the synthesis of macromolecules and assist in the modification of membrane association (114).

The structure of the phospholipid molecule generally is composed of two hydrophobic fatty acids linked via ester bonds to a glycerol backbone containing a hydrophilic polar head-group. Phospholipids are categorized based on the nature of their head-group and the length and saturation of their fatty acyl chains. They can spontaneously form lipid bilayers in aqueous environments because of their amphiphilic characteristic. These lipid bilayers form the major constituent of cell membranes (117–119). The structural features of the constituent membrane phospholipids can affect the curvature, viscosity, and electrostatic charge of the lipid bilayer (120, 121).

Phospholipid membranes form the boundaries and barriers and separate organelles from the cytosol, and cells from their surrounding environment (122). Phospholipids confer plasticity to the membranes and help in maintaining the chemical and electrical gradients across the membranes (113). The amounts of the phospholipids vary with growth conditions (e.g., nutrient availability, carbon source, temperature, and growth phase) and with genetic variations. The relative distribution of specific phospholipid class varies among organelles (66). A balance in phospholipid synthesis, remodeling, transport, and degradation is required to maintain this distribution (123). The endoplasmic reticulum is the main site for the production of the structural phospholipids and sterols, which are later distributed for the biogenesis of the other membrane-bound organelles (66, 124–127).

1.3 Phospholipid biosynthesis

The regulation of phospholipid synthesis is well studied in budding yeast, *Saccharomyces cerevisiae* (128). The pathways for the synthesis of membrane phospholipids are generally common in yeast and higher eukaryotes. Phospholipid structural genes encode enzymes responsible for phospholipid synthesis and play a crucial role in the regulation of the cellular processes. Nearly all the structural and regulatory genes involved in *de novo* phospholipid synthesis are identified and characterized in yeast, leading to a better understanding of the regulation of phospholipid synthesis. Phospholipid synthesis in yeast is a complex process and is regulated by lipids, water-soluble phospholipid precursors, and products, by phosphorylation, the synthesis and regulation of other lipid classes like fatty acids, triacylglycerol, sterols, and sphingolipids (128, 129). The major phospholipids present in the cellular membranes of yeast include phosphatidylcholine (PC),

phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). Phosphatidylglycerol (PG) and cardiolipin (CL) are also major phospholipids found in the mitochondrial membranes (130). The minor phospholipids consist of intermediates such as phosphatidate (PA), CDP-diacylglycerol (CDP-DAG), lysophospholipids, and diacylglycerol pyrophosphate etc. (131).

Phosphatidate forms the branch point in the lipid biosynthesis and serves as a precursor of diacylglycerol (DAG) and phospholipids. The resulting DAG is either converted to TAG through the action of diacylglycerol acyltransferase (DGAT) enzymes encoded by DGA1 and LRO1 or used in phospholipid synthesis (13, 14, 128). The requirements of storage or membrane lipids vary during the proliferative phase or the quiescent phase of a cell. It is crucial to balance the metabolic flux either into the phospholipids (proliferative state) or into TAG (nutrient deprived state) (4, 132). The DAG generated can be converted back to PA by the DGK1-encoded DAG kinase (Figure 1.3) (133, 134). DAG can also be acylated to form TAG by the ARE1- and ARE2-encoded acyltransferase enzymes, which are primarily responsible for the synthesis of ergosterol esters (4, 13, 128, 135–137). The key metabolic enzymes involved in TAG synthesis are conserved between yeast and mammals. In yeast, two pathways for phospholipid biosynthesis are present, the cytidine diphosphate diacylglycerol (CDP-DAG) pathway (*de novo*) and the Kennedy pathway (salvage) (Figure 1.3) (130, 138). In both yeast and mammals, the glycerol backbone of the central phospholipid precursor, phosphatidic acid (PA), partitions the two major branches of the phospholipid biosynthetic pathway. In one branch, PA is dephosphorylated to generate diacylglycerol (DAG) that is used for the synthesis of the major phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC), through the CDP-ethanolamine and the CDP-choline arms of the Kennedy

pathway, respectively (Figure 1.3) (128, 130, 137). In the other branch, PA is converted to liponucleotide intermediate CDP-DAG that gives rise to phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin. The CDS1-encoded CDP-DAG synthase catalyzes the formation of CDP-DAG from PA. Phosphatidylserine (PS), PE and PC are also synthesized from PA via CDP-DAG in yeast cells. The ethanolamine or choline required for the Kennedy pathway is provided either by growth medium supplementation or from the phospholipase D-mediated turnover of the PE or PC synthesized by CDP-DAG. The CDP-DAG pathway defective mutants can synthesize PC by the Kennedy pathway. The Kennedy pathway mutants defective in both the CDP-ethanolamine and CDP-choline branches can synthesize PC only by the CDP-DAG pathway and do not exhibit any auxotrophic requirements unlike those defective in the CDP-DAG pathway. In metazoans, the pathway converting CDP-DAG to PC/PE is absent, therefore, they depend on the Kennedy pathway for phospholipid biosynthesis. In yeast, phospholipid metabolism is coordinately regulated based on the availability of the phospholipid precursor molecules, choline and inositol, in the growth medium (74, 139–142). Addition of phospholipid precursor molecules to the actively growing yeast cultures leads to change in the expression of genes and in the phospholipid synthesis (74, 139, 143, 144).

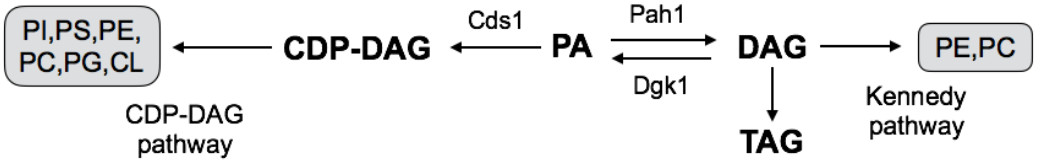

Figure 1.3: Pathways for the lipid biosynthesis
Two alternate pathways, the CDP-DAG pathway and the Kennedy pathway (salvage pathway) synthesize PE and PC in yeast. In metazoans, the CDP-DAG pathway does not exist. PA acts as the precursor for the synthesis of PE and PC through the CDP-DAG pathway. In the presence of choline and ethanolamine, these phospholipids are synthesized through the Kennedy pathway. PI, Phosphatidylinositol; PS, Phosphatidylserine; PE, Phosphatidylethanolamine; PC, Phosphatidylcholine; PG, Phosphatidylglycerol; CL, Cardiolipin; CDP-DAG, cytidine diphosphate diacylglycerol; cds1, Phosphatidate cytidylyltransferase 1; pah1, Phosphatidate phosphatase; dgk1 Diacylglycerol kinase 1; DAG, Diacylglycerol; TAG, Triacylglycerol.

PAH1-encoded PA phosphatase among the various enzymes (e.g., lysoPA acyltransferase, CDP-DAG synthase, PA phosphatase, DAG kinase, phospholipase D) is the key regulator of PA content that regulates the metabolism of PA (Figure 1.4) (128, 145). Cells lacking the PAH1-encoded PAP activity exhibit the derepression of phospholipid synthesis genes and elevated PA content (Figure 1.4). The elevated PA content triggers the expansion of the nuclear/ER membrane and also stimulates the PS synthase activity, which favors the synthesis of PC via the CDP-DAG pathway. The DGK1-encoded DAG kinase counteracts the role of PAH1-encoded PA phosphatase. The overexpression of DGK1-encoded DAG kinase causes an increase in PA content, the derepression of phospholipid synthesis genes, and the aberrant expansion of nuclear/ER membrane. CDP-DAG regulates the PA metabolic enzymes, PAH1-encoded PA phosphatase and DGK1-encoded DAG kinase. CDP-DAG stimulates the PA phosphatase activity but inhibits the DAG kinase activity. The increase in DAG levels and CDP-DAG-mediated regulation of PA phosphatase activities would lead to

the generation of TAG or phospholipids via Kennedy pathway (128, 133, 134, 145–147).

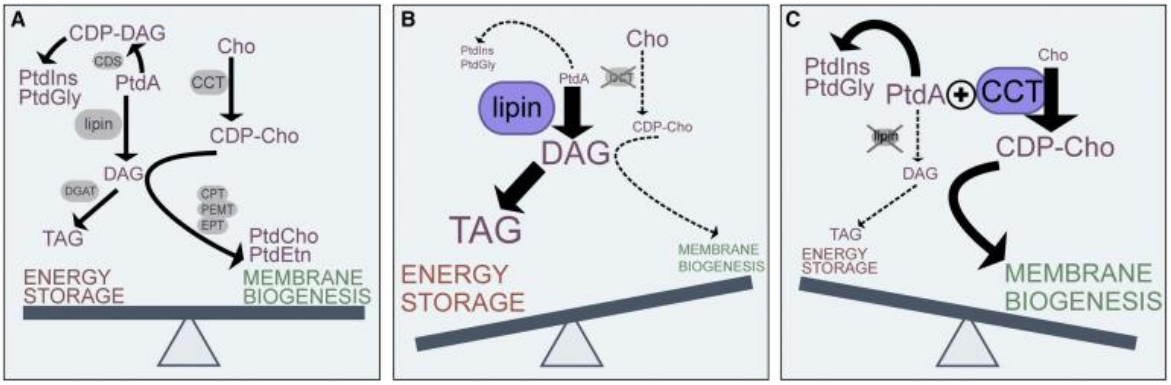

Figure 1.4: Lipid Metabolism Pathways

(A) Schematic diagram of cellular lipid metabolism. Key enzymes are denoted in gray circles and metabolites are denoted in purple colour. (B and C) Lipin generates DAG from PtdA (phosphatidic acid). Lipin is rate limiting for TAG production (Han et al., 2006; Ugrankar et al., 2011) and negatively regulates PtdA-derived lipids (PtdIns and PtdGly) (Bahmanyar et al., 2014). CCT is rate limiting for bulk membrane lipid synthesis (PtdCho production) (Cornell and Ridgway, 2015; Hermansson et al., 2011). PtdA stimulates CCT, denoted by + symbol, and through this lipin negatively regulates the level of membrane lipids (Craddock et al., 2015). CCT, CTP phosphocholine cytidylyltransferase; CDP, cytidine diphosphate; Cho, choline; CDS, PtdA cytidylyltransferase; CPT, CDP choline:diacylglycerol choline-phosphotransferase; DAG, diacylglycerol; DGAT, diglyceride acyltransferase; EPT, CDP-ethanolamine:DAG-ethanolamine-phosphotransferase; PEMT, phosphatidylethanolamine N-methyltransferase; PtdA, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGly, phosphatidyglycerol; PtdIns, phosphatidylinositol; TAG, triacylglycerol. (Taken from Grillet et al, Torsins Are Essential Regulators of Cellular Lipid Metabolism developmental cell, Volume 38, Issue 3, p235–247, 8 August 2016).

1.4 Phosphatidate phosphatase (PAP)

Phosphatidate phosphatases (PAP, 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) are the evolutionarily conserved enzymes that acts as key regulators of lipid metabolism and membrane biogenesis (13, 148). PAP enzymes catalyze the penultimate step in triglyceride synthesis, dephosphorylation of phosphatidic acid (PA) to diacylglycerol (DAG) (149). PAP enzymes have dual roles in lipid biogenesis and

the generation or degradation of lipid-signaling molecules (145, 150). They are classified as PAP1 (Mg^{2+}-dependent) and PAP2 (Mg^{2+}-independent) enzymes (151–154). These enzymes are differentiated based on their cofactor requirement for catalytic activity, substrate specificities, cellular locations, and physiological roles. PAP1 enzymes are associated with the de novo synthesis of phospholipids and TAG whereas PAP2 enzymes play a role in lipid signaling (13, 153, 155, 156). The PAP2 enzyme activity is encoded by the *DPP1* and *LPP1* gene (157, 158). Dpp1p and Lpp1p are relatively small integral membrane proteins localized to the vacuole and Golgi compartments, respectively and comprise of a three-domain conserved phosphatase motif composed of the consensus sequences KX6RP, PSGH, and SRX5HX3D (Figure 1.5) (150, 159–162). PAP2 enzymes have broad substrate specificity since, in addition to phosphatidic acid (PA), they also utilize other lipid phosphate molecules including DAG pyrophosphate, lysoPA, sphingoid base phosphates, and isoprenoid phosphates as substrates (157, 163–166) Dpp1p and Lpp1p do not require divalent cations, Mg^{2+}, for their activity (157, 158). The *DPP1* and *LPP1* genes are not essential for cell growth (158). Unlike PAP2 enzymes, PAP1 require Mg^{2+} ions for catalytic function and have a specific substrate phosphatidate (PA) (13). In *Saccharomyces cerevisiae,* phosphatidic acid phosphohydrolase (*PAH1*) gene encodes the PAP1 enzyme whereas *LIPIN* encodes PAP1 in mammals (13, 145). The members of PAP1 family possess a conserved DXDX(T/V) motif (Figure 1.5). PAP1 catalyzes the Mg^{2+}-dependent dephosphorylation of phosphatidate (PA) to form diacylglycerol (DAG), which can be acylated to generate triacylglycerol (TAG), the major form of energy and fat stored in lipid droplets (13, 149). Alternatively, DAG also serves as the immediate precursor of membrane phospholipids like phosphatidylcholine and phosphatidylethanolamine (167). The PAP1 enzyme is found

in the cytosolic and membrane fractions of the cell, and its association with the membrane is peripheral (13). PA is the precursor of membrane phospholipids and triacylglycerol and also functions as a lipid signaling molecule. PA is also involved in transcription, membrane proliferation, activation of cell growth, secretion, and vesicular trafficking (168–171). PAP1 activity regulates the PA flux towards TAG or phospholipid synthesis (172, 173). Phosphatidate phosphatase activity of PAH/lipin plays a crucial role in organelle biogenesis through the effects of PA and DAG as metabolic intermediates, signaling molecules and regulators of membrane structure (150, 174, 175). Studies in various model organisms have reported the important role of PAP1 in regulating the lipid metabolism, ER membrane organization, and nuclear morphology (176).

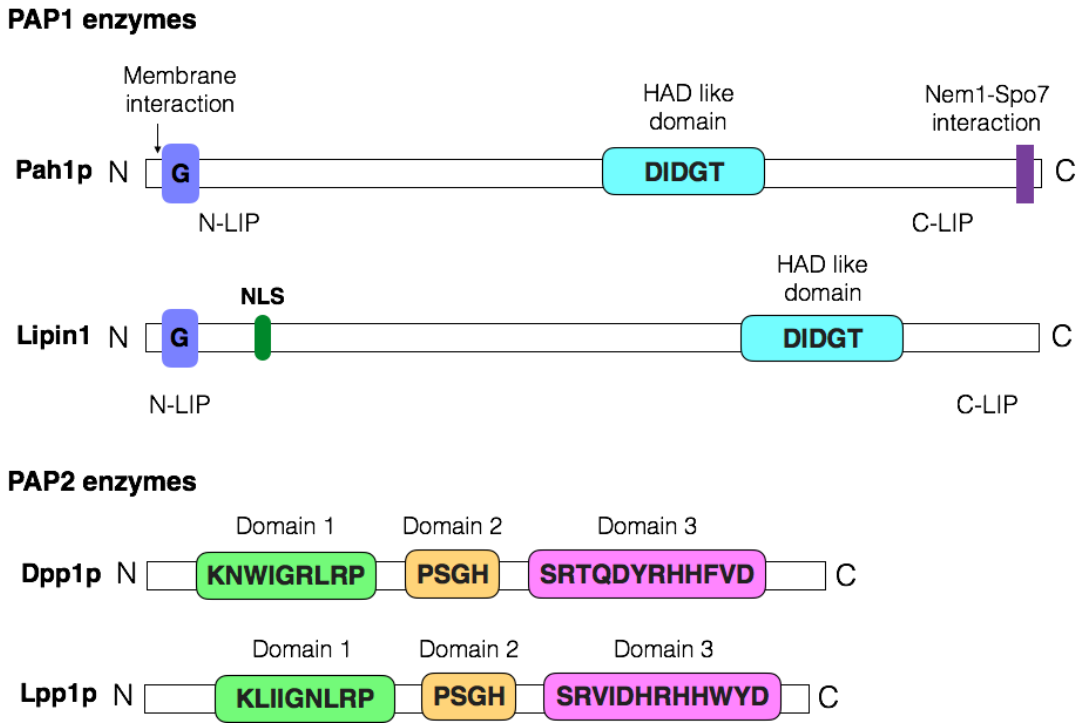

Figure 1.5: The domain organization of PAP1 and PAP2 enzymes
PAP1 enzymes consist of conserved amino-terminal domain (N-LIP) and a HAD like domain, harboring the conserved DIDGT catalytic motif, in the C-terminal region. A conserved glycine residue is present within the N-LIP domain. The yeast PAP1 enzyme contains an amino-terminal amphipathic helix mediating its membrane recruitment. The NLS is a lysine and arginine-rich nuclear localization signal present in the amino terminal region of mammals. Acidic tail at the C-terminus of PAP1 enzymes is essential for the binding of human lipin to Nem1-Spo7 protein phosphatase complex. PAP2 enzymes consist of three conserved catalytic domains. The conserved amino acid residues in the catalytic motifs are indicated by the single letter code.

Lipins/PAHs are relatively large proteins and consist of a conserved amino-terminal domain (N-LIP) and carboxy-terminal catalytic domain (C-LIP). C-LIP domain harbours a conserved DXDXT catalytic motif found in the members of Mg2+-dependent phosphatase superfamily (Figure 1.5) (17). This catalytic motif is essential for the Pah1p activity and mutation of the Aspartate residues in the catalytic motif results in loss of phosphatase function. The non-conserved C-terminal region of yeast Pah1 contains the sequence WRDPLVDID, which is essential for the in vivo function

of the enzyme (177). The tryptophan residue of the WRDPLVDID domain is conserved in Pah1/lipin proteins of yeast, mice, and humans and essential for its function in TAG synthesis. However, the mutation of the tryptophan residue did not abolish the catalytic function of Pah1, suggesting that the PAP activity is essential, but not sufficient for the physiological function of Pah1 (177). In yeast, other conserved residues (e.g. Gly-80, Asp-398, and Asp-400) of Pah1 are essential for the PAP activity and the *in vivo* function of Pah1. On the contrary, the Trp-637 residue is not required for the PAP activity but essential for the *in vivo* function suggesting that Trp-637 is a regulatory residue and not a catalytic residue (177).

1.4.1 PAP in the phylogeny

The PAP activity was first studied in animal tissues by Smith et al. in 1957 (178). PAP enzymatic activity was identified in the yeast *S. cerevisiae* by Hosaka and Yamashita in 1984 (179). The pathways of lipid synthesis are common between yeast and higher eukaryotic cells and the function of PAP enzymes in yeast parallel those in mammals (130, 138). Studies in *Saccharomyces cerevisiae* led to the identification of the phosphatidate phosphohydrolase (PAH1) gene encoding PAP enzyme activity and Pah1p was purified and identified by George Carman in yeast in 2006 (13). Lipin proteins encode the phosphatidate phosphatase enzymes in mammals. The identification of the yeast PAH1 gene led to the discovery of mammalian lipins and subsequently to homologous genes from *C. elegans*, *Drosophila*, *Arabidopsis*, and *Tetrahymena*. Fungi (*S. cerevisiae*), nematodes (*C. elegans*) and insects (*D. melanogaster*) encode a single lipin ortholog, whereas mammals express three homologs and plants (*Arabidopsis thaliana*), and ciliate (*Tetrahymena thermophila)* express two homologs (180–184). The presence of lipin protein family in the

organisms from a wide evolutionary spectrum suggests that it plays a fundamental role. The sequence analysis of lipin homologs from organisms belonging to different clades suggests that it is conserved across evolution. PAH proteins play a role in lipid droplet biogenesis in all the organisms studied (181, 182, 185). PAH also regulates ER morphology in yeast, *C.elegans*, mammals, *Tetrahymena*, and *Arabidopsis* (25, 148, 181, 186). PAP is shown to regulate the level of phospholipid and neutral lipid, membrane proliferation, and transcriptional de-repression of phospholipid biosynthetic enzymes. PAH proteins from mammals, *Arabidopsis*, *Tetrahymena* functionally replace yeast PAH1 (25, 184, 187, 188). The function of phosphatidate phosphatase enzymes is evolutionarily conserved across the eukaryotic lineages.

1.4.2 Phosphatidic acid phosphohydrolase (PAH1) in yeast

In *Saccharomyces cerevisiae*, a single lipin orthologue, *PAH1*, encodes PAP1 protein (13). Pah1p consists of 862 amino acids. The yeast *PAH1* gene (previously known as *SMP2*) was identified by the reverse genetic method from the amino acid sequence information acquired from a purified fraction of the enzyme (13). Pah1 in yeast plays a major role in the TAG synthesis and regulates the *de novo* synthesis of membrane phospholipids derived from its substrate PA. Pah1p in yeast is required for lipid droplet biogenesis, nuclear/ER membrane biogenesis, and vacuole function (17, 185, 189). Pah1p play an important role in the nuclear organization by regulating the lipid biosynthesis (Figure 1.6) (13, 17, 173). It also acts as a transcriptional repressor of membrane phospholipid biosynthesis. Loss of Pah1 function leads to transcriptional up-regulation of phospholipid biosynthesis genes, *INO1* (encoding inositol-3-phosphate synthase) and *OPI3* (encoding phospholipid methyl transferase), resulting in aberrant expansion of the nuclear membrane (131, 190, 191). On contrary,

constitutive dephosphorylation of Pah1 represses *de novo* phospholipid synthesis resulting in inhibition of cell division. Mutation of *PAH1* in yeast results in deleterious phenotypes like aberrant nuclear expansion, reduction in lipid droplet formation, abnormality in the morphology of the ER, slow growth, respiratory deficiency, defect in vacuole homeostasis and temperature sensitivity (13, 173). Loss of PAH1 in yeast cells exhibits a two-fold reduction in the levels of ATP (192).

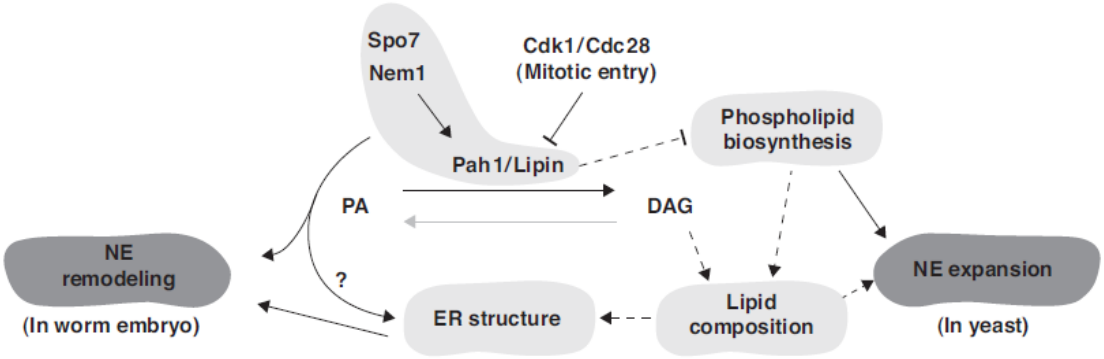

Figure 1.6: Schematic describing the link between lipin functions and nuclear envelope dynamics in yeast and C.elegans
Solid lines illustrate direct functions while dotted lines suggest indirect contributions. Taken from Zhang *et al* 2013.

1.4.3 Lipin in C.elegans

Caenorhabditis elegans has one putative lipin homolog, LPIN-1 (H37A05.1), essential for ER organization and NEBD (Nuclear Envelope Break Down) (193). Down-regulation of LPIN-1 homolog in *C. elegans* results in disruption of the ER morphology and the nuclear structure. As metazoans undergo open mitosis, the nuclear envelope is disassembled during the division. Loss of lipin leads to a defect in the ability of the nuclear envelope to break down, a decrease in lipid droplet formation, and disorganization of the ER. Lpin-1 is required for proper nuclear envelope disassembly but is dispensable for nuclear envelope assembly (Figure 1.6) (194).

1.4.4 Lipin in Drosophila

In *Drosophila melanogaster,* a single lipin orthologue, *dLipin*, plays an important role in normal adipose tissue development and energy metabolism. Loss of *lipin* in *Drosophila* results in severe reduction of larval fat body mass, whole-animal TAG content, and size of the lipid droplet (182). Though the fat body mass is reduced, the size of individual fat body cells and nuclei are increased. The cells also exhibit changes in organelle structure that affect mitochondria, autophagosomes, and nucleus. These defects are associated with lethality, reduced fertility, impaired eclosion and starvation resistance (182, 195, 196).

1.4.5 Lipin in Arabidopsis

Arabidopsis thaliana consists of two PAH homologs, *AtPAH1* (At3g09560) and *AtPAH2* (At5g42870). Both AtPAH1 and AtPAH2 have the conserved amino-terminal lipin (NLIP) and carboxy-terminal lipin (CLIP) domains. The catalytic motif or HAD-like domain is also present in both the isoforms. AtPAH1 and AtPAH2 encode Mg^{+2} -dependent PAPs with enzymatic features similar to those of yeast Pah1p (184). The loss of both the lipin homologs increases the level of phosphatidic acid and other phospholipids in *Arabidopsis* and causes expansion of the ER membrane and increased expression of phospholipid biosynthesis enzymes. However, no alterations in TAG content in seeds is observed. Phosphate is an essential nutrient for plant viability and disruption of AtPAH1 and AtPAH2 shows severe growth defect under Pi starvation (184). AtPAH1 and AtPAH2 play a key role in membrane lipid remodeling during Pi starvation, and this remodeling is an essential adaptation mechanism for plants to prevent phosphate starvation (184). AtPAH1 and AtPAH2 rescue different phenotypes exhibited by the yeast *pah1*Δ mutant strain, such as

temperature sensitivity and TAG synthesis (197). The conserved aspartate residues in the HAD-like motif of the plant enzymes are critical for the PAP activity. Mutations within the carboxy-terminal domain of PAH homologs of plants displayed a significant decrease in PAP activity, thus, their limited ability to complement the phenotypes of pah1 deficient yeast cells (197).

1.4.6 Lipin in Tetrahymena

Tetrahymena thermophila expresses two lipin homologs named as *TtPAH1* (TTHERM_00189270) and *TtPAH2* (TTHERM_00215970). The larger protein is TtPah1 containing 872 amino acids and the smaller one is TtPah2 containing 335 amino acids (25). Both TtPah1 and TtPah2 proteins belong to the Mg^{2+}-dependent phosphatidate phosphatase (PAP1) family and possess the characteristic N-LIP and C-LIP domains (25, 26). A conserved catalytic DXDXT/V motif is present in the HAD-like domain of the C-LIP region of TtPah1 (666 DIDGT 670) and TtPah2 (146 DVDGT 150). Both TtPAH1 and TtPAH2 are localized in both cytoplasm and membranes and are dispensable for the normal growth and survival of *Tetrahymena* (25, 26). TtPAH1 plays a role in regulating lipid homeostasis since deletion of TtPAH1 exhibits reduction in lipid droplet number. TtPAH1 is also needed for maintaining tubular ER in *Tetrahymena*. Loss of function of TtPAH1 results in an increase in the endoplasmic reticulum (ER) content and more ER sheet structure (25). However, TtPAH2 is not required to maintain lipid droplet number or ER morphology. TtPAH2 is the only PAH protein which does not regulate lipid homeostasis and membrane biogenesis (26). Contrary to yeast, PAH homologs of *Tetrahymena*, TtPAH1 and TtPAH2, are not required for regulating nuclear expansion and nuclear morphology (25, 26). Yeast *pah1Δ* cells exhibit aberrant nuclear expansion, slow

growth at 30°C, temperature-sensitive growth at 37°C and respiratory deficiency (i.e. growth defect) on non-fermentable carbon sources (13, 173). TtPAH1 restores different phenotypes of *pah1Δ* yeast cells. TtPAH1 also replaces yeast PAH1 in regulating the expression of phospholipid biosynthesis genes. Thus, TtPAH1 of *Tetrahymena* can functionally replace yeast PAH1 (25). Interestingly, TtPAH2 rescues only the respiratory deficiency phenotype of *pah1Δ* yeast cells in a non-fermentable substrate. Thus, TtPAH2 possesses the minimal function of respiration and the mere presence of catalytic activity is not sufficient for its function in regulating lipid homeostasis and membrane biogenesis (26).

1.4.7 Lipin in Mammals

The mammalian and mice lipin protein family comprises of three members, lipin-1, lipin-2, and lipin-3 (183). Lpin1 was identified in mice as the gene whose mutation at the glycine 80 residue results in transient fatty liver dystrophy (*fld)* phenotype of mice. A loss of lipin-1 results in lipodystrophy and insulin resistance, while an excess of lipin-1 promotes obesity and insulin sensitivity in mice (52, 53). In humans, the lipin-1 gene undergoes alternative mRNA splicing to generate two distinct isoforms, lipin-1α (891 amino acids) and lipin-1β (924 amino acids) (198). The three human lipin proteins have a non-redundant function with each protein showing a distinct but overlapping tissue expression pattern (183). Lipin contains two evolutionarily conserved regions, N-terminal (N-LIP) and C-terminal (C-LIP) domains. NLS, a lysine and arginine-rich nuclear localization signal, is present in the N-LIP region. The C-LIP domain of lipin 1α contains two functional motifs, the conserved DXDXT catalytic motif is essential for PAP enzyme activity, whereas the LXXIL motif is the transcriptional co-activator motif and required for its interaction with the nuclear

receptors and function as a transcriptional regulator. All three lipin isoforms contain the LXXIL motif (176, 199, 200). Lipin1 transcriptional coactivator activity was first identified in peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) knockout mice (201). The expression of lipin1 is induced by PGC-1α, and lipin1 forms a complex with PGC-1α and PPARα to control the expression of genes involved in fatty acid oxidation (Figure 1.7). The depletion of nuclear lipin1 by ethanol leads to impairment of the PPARα/PGC-1α axis, thereby contributing to the decrease of fatty acid oxidation and the development of alcoholic liver steatosis (202–204). Lipin1 also functions as a transcriptional repressor in some cases (28, 205). Thus, lipin1 serves as a molecular scaffold to activate or repress the expression of a gene depending on the cellular context.

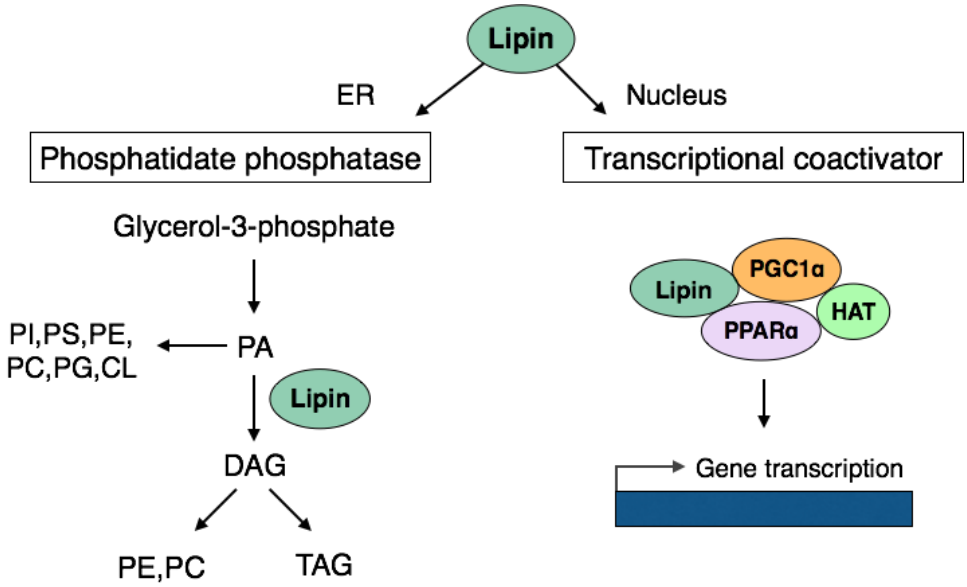

Figure 1.7: Schematic describing the dual molecular role of lipin proteins
Lipin proteins play dual molecular function in glycerolipid biosynthesis and transcriptional coactivation. Left: Lipin proteins act as PAP1 enzymes and catalyze the conversion of phosphatidate (PA) to diacylglycerol (DAG). DAG is a direct precursor of triacylglycerol (TAG) and of the phospholipids, phosphatidylethanolamine (PE), and phosphatidylcholine (PC). Lipin proteins reside in the cytosol and associate transiently with the ER membrane to perform the PAP1 function. Right: Lipin-1 also functions as a transcriptional coactivator and amplifies the effect of known coactivators on transcription of fatty acid oxidation genes. Lipin-1 interacts with PGC1α and the nuclear receptor PPARα in a co-activation complex that also includes other factors like histone acetyltransferase (HAT). HAT, histone acetyltransferase; PGC1α, peroxisome proliferator activated receptor γ coactivator-1α; PPARα, peroxisome proliferator activated receptor α.

Human lipin-1 and lipin-2 complement phenotypes exhibited by yeast *pah1Δ* mutant cells indicating that the phosphatidate phosphatase function of lipin/pah1 is highly conserved from human to yeast (187). The function of lipin-1 and lipin-2 proteins have been identified, however, the role of lipin-3 remains unclear (206–208). Mammalian lipin proteins display some differences from lipin protein of yeast (209). Various key phosphorylation sites that regulate the function of lipin in yeast are not found in the mammalian lipins (18, 210).

The target of rapamycin complex 1 (TORC1) controls various growth-related

processes such as protein, and lipid metabolism. Its deregulation is associated with various diseases like cancer, obesity, type 2 diabetes. Mammalian TORC1 directly phosphorylates and inhibits the phosphatidate phosphatase lipin-1. In addition, TORC1 inhibits the function of Pah1, hence, preventing the accumulation of triacylglycerol in yeast (211).

1.4.8 Lipin gene associated mutations and disease

Mutation in mouse *Lpin1* prevents normal adipose tissue development and leads to lipodystrophy and insulin resistance whereas overexpression of lipin-1 in adipose tissue or skeletal muscle causes obesity and insulin sensitivity (52, 53, 212–214). Loss of PAP activity leads to peripheral neuropathy in mice (215, 216). In humans, Lipin-1 plays a crucial role in balancing the lipid metabolism and is associated with various metabolic syndromes. Bi-allelic pathogenic mutations in human LPIN1 cause severe, potentially life-threatening rhabdomyolysis in early childhood because of the accumulation of PA in the muscles (217). Individuals having bi-allelic pathogenic LPIN2 mutation, S734L, develop an auto inflammatory bone disease known as Majeed syndrome and is associated with osteomyelitis and anemia. Majeed syndrome is characterized by recurrent fever, inflammatory skin lesions and bone lesions (218, 219). The diseases associated with lipin-1 or lipin-2 deficiency indicates that these proteins have unique function *in vivo*. The regulation of membrane lipid composition governed by PA phosphatase is crucial for maintenance of metabolic homeostasis and normal cell physiology as indicated by diseases caused by loss of PAP activity. Lipin-1 plays a crucial role in balancing the lipid metabolism and is associated with metabolic syndrome, obesity, type 2 diabetes, lipodystrophy, and childhood myopathy (52, 217, 220, 221).

1.5 Nem1-Spo7 protein phosphatase complex

Nuclear envelope morphology protein 1 (Nem1) and sporulation-specific protein 7 (Spo7) forms a transmembrane phosphatase complex and dephosphorylates the nuclear membrane-associated phosphatidic acid phosphatase, Pah1 in yeast (Figure 1.8) (20, 190, 222). Nem1 is the catalytic subunit whereas Spo7 is the regulatory subunit. Nem1 is a member of the haloacid dehalogenase superfamily and comprises the conserved catalytic motif DXDX(T/V) (20). The two membrane proteins were identified in yeast through their genetic interaction with the nucleoporins of the Nup84p complex and were shown to participate in nuclear membrane biogenesis (222). *NEM1* along with the phosphatidate phosphatase (*PAH1*) regulates lipid homeostasis and membrane biogenesis in yeast and mammals (20).

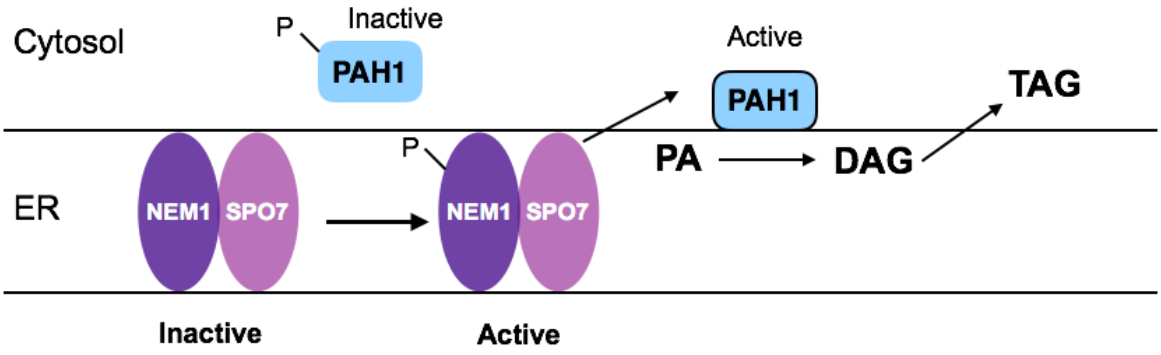

Figure 1.8: Schematic describing the role of NEM1 in lipid biosynthesis
Nem1-Spo7 protein phosphatase complex dephosphorylates PAH1 in yeast. The dephosphorylated PAH1 becomes active and membrane bound and subsequently catalyzes the dephosphorylation of PA to generate DAG. DAG is converted to TAG, the major constituent of lipid droplet. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PAH1*, active dephosphorylated PAH1; LD, lipid droplet; DAG, diacylglycerol; TAG, triacylglycerol.

1.5.1 NEM1: a regulator of PAH1 function

Pah1p is found mostly in the cytosol, whereas its substrate PA resides in the nuclear/ER membrane (190). All Lipins/PAHs lack transmembrane domains and must be translocated onto membranes in order to dephosphorylate PA. The translocation of the PAH1 onto the membrane is vital for its *in vivo* function (18, 190). The membrane association of PAH1 and its catalytic activity is regulated by phosphorylation-dephosphorylation (Figure 1.9). Phosphorylation-dephosphorylation also governs the sub-cellular localization of PAH1 (190). Phosphorylated Pah1p resides in the cytosol, whereas PAH1 in its dephosphorylated form is associated with the membrane (223, 224). Multiple kinases such as protein kinase A, Pho85p–Pho80p, and CDC28 (CDK1: cyclin-dependent kinase) function in a negative regulatory manner to phosphorylate Pah1 at multiple serine and threonine residues in a cell cycle-dependent fashion (Figure 1.9) (225–228). Pah1p is phosphorylated on seven multiple Serine/Threonine sites *in vivo* (225, 226). Positive activation occurs via a highly conserved membrane-associated protein phosphatase complex comprising Nem1 (catalytic subunit) and Spo7 (regulatory subunit), which dephosphorylates and recruits Pah1 onto the nuclear/ER membrane (20, 186, 190). Nem1-Spo7 protein complex plays a role in nuclear envelope morphogenesis by regulating Pah1 function. The dephosphorylation of Pah1 exclusively by Nem1-Spo7 acts as a rate-limiting step and is essential for its phosphatidate phosphatase function. Thus, this cascade is essential in the regulation of lipid metabolism and membrane biogenesis (18, 229). In yeast, Pah1p activity is regulated through membrane association with an amphipathic alpha-helix located in the amino-terminus (18). Dephosphorylation of Pah1 by the Nem1/ Spo7 phosphatase complex promotes membrane binding only in the presence of amino-terminal amphipathic helix. Conversely, phosphorylation of Pah1p prevents the amphipathic

helix from binding to membranes (18). Pah1p in yeast consists of an acidic stretch at their C-terminal ends (amino acid residues 837 to 862) which is crucial for its binding to the Nem1p-Spo7p phosphatase complex (19). The acidic tail is essential and sufficient for Nem1p-Spo7p–dependent recruitment of Pah1p close to lipid droplets and is important for droplet biogenesis (19). Phosphorylation-deficient Pah1p mutant leads to increase in PAP1 activity, suggesting that phosphorylation inhibits the PAP function (190).

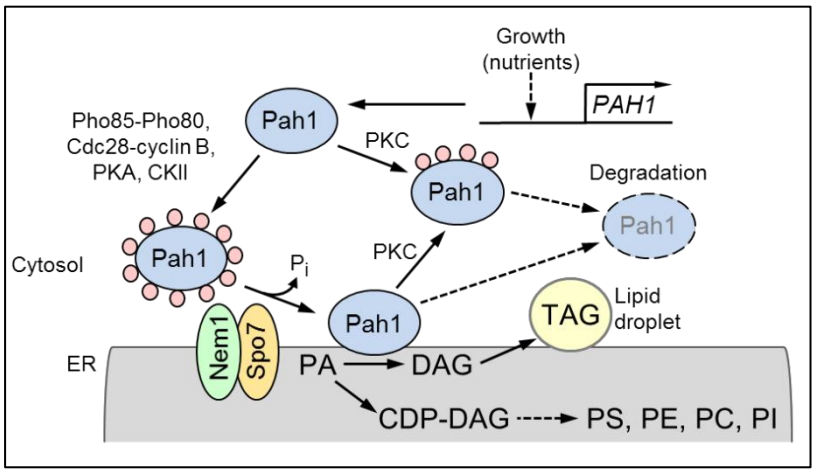

Figure 1.9: Model for the regulation of Pah1 by phosphorylation, dephosphorylation, and proteasomal degradation

PAH1 expression is regulated during growth by nutrients. Pah1 present in the cytosol is phosphorylated by multiple protein kinases. Phosphorylated Pah1 (indicated by *pink circles*) translocates to the ER membrane through its dephosphorylation by the Nem1-Spo7 protein phosphatase complex. Membrane associated dephosphorylated Pah1 catalyzes the conversion of PA to DAG, which is then acylated to form TAG. Dephosphorylated Pah1 or PKC-phosphorylated Pah1 that is not phosphorylated at the target sites for Pho85-Pho80/Cdc28-cyclin B is degraded by the proteasome (indicated by the *dashed line arrows* and *ellipse*). (Taken from Park *et al,* A conserved tryptophan within the WRDPLVDID domain of yeast Pah1 phosphatidate phosphatase is required for its in vivo function in lipid metabolism, The Journal of Biological Chemistry - *292, 19580-19589,* December, 2017).

1.5.2 NEM1 in yeast

In yeast, NEM1 (YHR004C) consists of 446 amino acids and SPO7 (YAL009W) encodes a protein of 259 amino acids. SPO7 was earlier identified as a gene required

for sporulation in yeast (230). Nem1p of yeast is a member of the haloacid dehalogenase (HAD) superfamily and harbors the conserved DXDXT catalytic motif (20). The amino terminus of Nem1p contains a transmembrane helix motif of 40 residues with two potential membrane-spanning regions and directs it to the nuclear envelope and endoplasmic reticulum (20). The conserved C-terminal domain of Nem1p is sufficient and necessary for its interaction with Spo7p (186). *NEM1* or *SPO7* is not essential for the cell viability, however, mutants show a slightly reduced growth rate at higher temperatures (222). Nem1-Spo7 complex dephosphorylates Pah1 and is essential for the formation of a spherical nucleus thus, regulates the nuclear membrane biogenesis (17, 19, 190, 223, 225, 226). Loss of Nem1p or Spo7p exhibits an aberrant nuclear morphology with long nuclear membrane extensions (222). Nem1p-Spo7p phosphatase complex interacts with the C-terminal acidic tail of Pah1p (229). Nem1p localizes close to the lipid droplets (185). The acidic stretch of Pah1 is necessary and sufficient for Nem1p-Spo7p dependent recruitment of Pah1p to the lipid droplets and is essential for the droplet biogenesis (229). Cells lacking either the Nem1 or Spo7 subunits of the complex mirror the phenotypes of yeast *pah1*Δ cells including increased phospholipid synthesis, aberrant expansion of the nuclear/ER membrane, decreased lipid droplet number, and growth inhibition (Figure 1.10) (28, 145, 175). The *nem1*Δ *spo7*Δ, double mutants in yeast also show phenotypes similar to *pah1*Δ or *nem1*Δ or *spo7*Δ, suggesting that these three proteins work in unison (14, 17, 186). Overexpression of the Nem1-Spo7 phosphatase complex in yeast results in a lethal phenotype only in the presence of its substrate Pah1, indicating that an excess level of PAP activity is deleterious to cell growth (17).

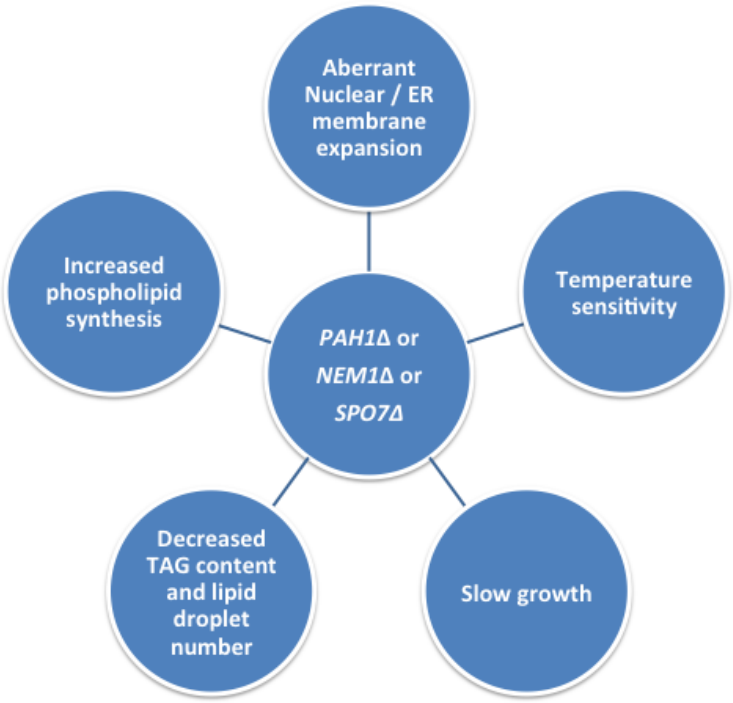

Figure 1.10: Phenotypes shown by yeast pah1Δ or nem1Δ or spo7Δ mutant yeast strains

In yeast, *pah1*Δ or *nem1*Δ or *spo7*Δ mutant strains show elevated levels of PA, phospholipids, fatty acids, and sterol esters and decreased TAG content. These mutants display phenotypes such as slow growth, aberrant nuclear expansion, respiratory deficiency, and decrease in lipid droplet number, fatty acid induced lipotoxicity, and temperature sensitivity.

1.5.3 NEM1 in mammals

CTDNEP1 (C-terminal domain nuclear envelope phosphatase1, formerly known as Dullard) and *NEP1-R1* (nuclear envelope phosphatase 1-regulatory subunit 1, initially known as *TMEM188*) are the mammalian orthologs of *NEM1* and *SPO7* respectively (29). *CTDNEP1* is the member of the DXDX(T/V) phosphatase family and localizes to the nuclear envelope. It is a protein Ser/Thr phosphatase and dephosphorylates the mammalian phosphatidic acid phosphatase, lipin (20). Thus, CTDNEP1 plays a crucial role in the regulation of nuclear membrane biogenesis (29). Human CTDNEP1p and yeast Nem1p share 50% amino acid sequence similarity. The amino terminus of CTDNEP1 possesses two membrane-spanning regions and C-terminus

consists of DLDET phosphatase domain. Mutation of the catalytic aspartate residue abolishes the phosphatase activity. The human *CTDNEP1-NEP1R1* complex can functionally replace yeast *NEM1-SPO7* suggesting that Nem1-Spo7 phosphatase is evolutionarily conserved between yeast and mammals. However, the catalytically inactive *CTDNEP1* does not rescue the morphological phenotype of mutant yeast Nem1 (20, 29).

1.6 Regulation of lipin mediated nuclear remodeling in yeast

The exact method by which nuclear architecture is maintained in yeast is not understood. However, several factors that influence the nuclear morphology in mutant form have been identified. Most of these mutants are loss-of-function alleles of proteins associated with the nuclear pore complex suggesting that the NPC might be involved in the maintenance of nuclear shape (222, 231, 232). Nuclear envelope morphology is associated with the nuclear pore dynamics. Two additional factors that influence the nuclear morphology are Nem1 and Spo7 integral membrane proteins and were identified through genetic interaction with nup84p (186). The absence of Nem1 or Spo7 leads to the arrangement of the nucleus into two large lobes connected by a narrow bridge, and long nuclear envelope extensions that extrude into the cytoplasm (222). Interestingly, the altered nuclear structure is devoid of the genetic material. Overexpression of certain membrane proteins can also result in alterations in the nuclear shape. A balance is required between membrane proliferation factors and mechanisms to prevent aberrant membrane formation. Lipins play an important role in the nuclear organization in yeasts, where inactivation of the Nem1p–Spo7p phosphatase complex or Pah1p leads to nuclear membrane expansion (175, 190). Inactivation of only Pah1p among the other enzymes associated with phospholipid

biosynthesis results in aberrant nuclear expansion (13, 173). Nuclear expansion caused by a catalytically inactive Pah1p can be phenocopied by the overexpression of a catalytically competent Dgk1p, a nuclear/ER transmembrane DAG kinase that generates PA (13, 134, 233, 234). However, the role of PA in maintaining nuclear shape is unknown. The inactivation of Pah1p, resulting in the PA-dependent de-repression of phospholipid biosynthesis via the CDP-DAG pathway, is necessary but not sufficient for nuclear expansion in *pah1Δ, nem1Δ* or *spo7Δ* yeast cells (190). In addition to increased phospholipid synthesis, other factors are also involved in nuclear membrane expansion.

Inactivation of Pah1p function results in a significant alteration of phospholipid composition of membranes, such as an increase in PA, suggesting that it is possible that changes in the composition of nuclear/ER membrane are also involved in promoting the nuclear expansion (174, 190). Since yeast undergoes closed mitosis, the nuclear envelope undergoes dramatic structural changes during the cell cycle progression (41, 235, 236). Regulation of the phospholipid composition of the nuclear/ER membrane by Pah1p may play a key role nuclear remodeling (48, 237). Constitutive dephosphorylation of Pah1p in yeast represses the *de novo* phospholipid biosynthesis and inhibits nuclear envelope expansion during mitosis (17, 190). The underlying mechanism by which Pah1p activity regulates nuclear remodeling is not known.

Also, in *Drosophila* and *Caenorhabditis elegans*, lipin is critical for maintenance of proper nuclear morphology (182, 194). Downregulation of lipin 1 in *C. elegans* leads to defects in lamina depolymerization, NEBD and chromosome segregation in addition to altered ER membrane organization with the appearance of the more sheet-like structure. Since ER morphology can affect the nuclear envelope dynamics, the

role of lipin in NEBD may be an indirect consequence of its function on ER membrane organization. The defects in NEBD mediated by lipin1 are significantly rescued by co-depletion of lamins without affecting the ER defects, suggesting that the role of lipin 1 in NEBD may be distinct from those in peripheral ER (194, 238, 239).

1.7 *Tetrahymena thermophila* as a model organism

Tetrahymena thermophila is a single-celled eukaryote that belongs to the phylum Ciliophora and live in freshwater systems (229, 230). Its dimension is about 50 μm in length and 20 μm in width. As apparent from the name, it has four (tetra) membrane-like (hymen) oral structures and can survive at high temperatures (231). The preferred temperature for optimal growth is 30℃. Ciliates diverged quite early during the eukaryotic evolution and belong to the kingdom chromalveolate (229). Although *T. thermophila* is distantly related to humans; it has around 2,280 human orthologs making it a suitable model organism to study the evolution of human gene function (232). It serves as an excellent model organism to conduct molecular genetic and cell biology studies and can be grown easily in lab-scale. They are apathogenic, fast to grow with a short replication time of 2 to 3 hours and can be easily grown in relatively inexpensive media. For long-term storage, a method for freezing *Tetrahymena* cells has been developed (239). It can be manipulated genetically using biolistic bombardment or electroporation to introduce gene targeting constructs (242, 243). The annotation of the fully sequenced Macronuclear genome is available at Tetrahymena genome database (TGD: http://ciliate.org/index.php/home/welcome). The expression profiles for all the predicted protein-coding genes are also available during growth, starvation, and conjugation in the Tetrahymena genome database.

Vectors are available for gene targeting via homologous recombination that allows the creation of gene knock out strains (22). RNAi technology can also be used to knock down the genes of interest (244). There is significant difference concerning the codon bias between humans and ciliates. *Tetrahymena* uses an alternative genetic code in which canonical stop codons UAA and UAG encode glutamine whereas UGA is the only stop codon (245).

Nuclear dimorphism

The distinct characteristic of ciliates is the presence of nuclear dimorphism which makes it a unique model system to study membrane biogenic pathways. *Tetrahymena* possesses a diploid germline micronucleus (MIC) and a polyploid somatic macronucleus (MAC) (Figure 1.11) (22). MIC is mostly inert transcriptionally, whereas MAC is transcriptionally active (246). MIC consists of two complete haploid genome sets Coordination of Lipid Metabolism in Membrane Biogenesis. capable of both mitosis and meiosis whereas MAC possesses multiple copies of a rearranged subset of the complete MIC genome (22, 246). Micronucleus encompasses ~120 Mb sequence and contains 5 pairs of metacentric chromosomes that are held in a heterochromatic state and is transcriptionally silent during asexual reproduction. During vegetative cell division (asexual reproduction), the cell undergoes fission and the micronucleus divides mitotically but during sexual reproduction, it divides meiotically. Macronucleus contains ~104 Mb of DNA with roughly 27,000 putative protein-coding genes and the average chromosome copy number is 45 (247, 248). The predicted protein-coding genes in *Tetrahymena* are over four times more than that of *Saccharomyces cerevisiae* and even more than many metazoans. During asexual reproduction, the macronucleus divides through an amitotic process and the

chromosomes are randomly segregated into daughter cells. This random chromosomal segregation is of significant consideration during genetic transformation and selection of transgenes. Although MAC genome is derived from the MIC, the two genomes are quite distinct (22). MAC genome is around 10-20% smaller than that of the MIC. During MAC differentiation, various DNA rearrangements occur. Internal eliminated sequences (IESs) are the segments of the MIC genome that are excised in a site-specific manner and eliminated during MAC differentiation (249). Approximately 6,000 IESs are removed during MAC development.

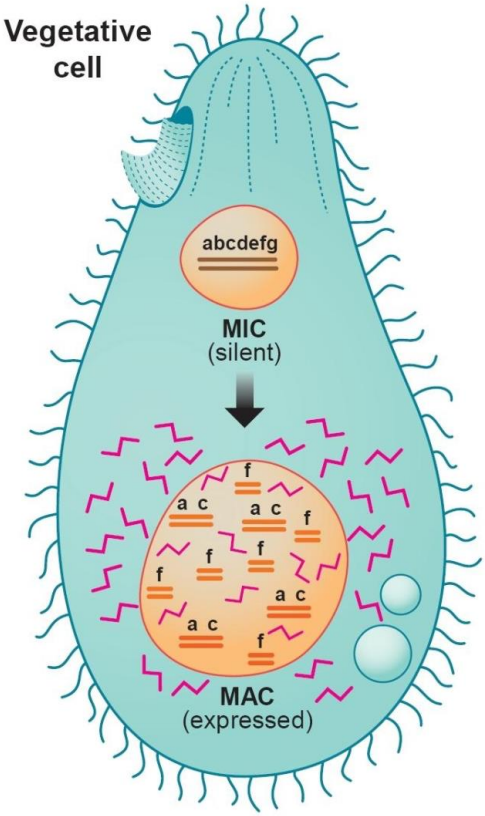

Figure 1.11: The vegetative state of *Tetrahymena*
Tetrahymena possess two copies of their genome in functionally distinct nuclei. The somatic polyploid macronucleus, and a silent germline diploid micronucleus (Taken from O'Connor, C., Telomeres of Human Chromosomes, Nature Education 1(1):166, 2008).

1.7.1 Tetrahymena as a heterologous expression system

Tetrahymena being a complex eukaryote has several advantages over bacteria as a molecular and cellular biological system in terms of protein folding, processing, and targeting. It is an excellent expression host and several foreign proteins have been expressed (250). The nuclear dimorphism exhibited by ciliates provides different possibilities of altering the organism phenotype. Manipulating the phenotype requires genetic engineering of the vegetative MAC (246). It is a suitable model organism to study membrane biogenic pathways since it exhibits nuclear dimorphism. For the heterologous protein expression, a basic requirement is to transform the expression host. Various DNA transformation techniques have been developed for *Tetrahymena* such as electroporation, microinjection, and biolistic bombardment (251). Electroporation of vegetatively growing cells results in a lower level of macronuclear transformation whereas efficient high-frequency macronuclear transformation is obtained when transformation vectors are introduced directly into the mating cells. Conjugant electrotransformation (CET) yields high-frequency macronuclear transformation using high copy number vectors containing the selectable markers. The most efficient method for the mass transformation of the MAC and the MIC is biolistic bombardment (251, 252). The conjugating cells of *Tetrahymena* are bombarded and used to generate macronuclear transformants using rDNA-based vectors. The plasmids used take advantage of the amplification of the rDNA (Ribosomal DNA) gene during the MAC development. Episomal presence of the plasmid depends on the presence of antibiotics in the growth medium and the plasmids often recombinate homologously and non-directionally into the endogenous rDNA. To manipulate the genome, the stable integration of knock out or expression cassettes into the diploid MIC is used, since after conjugation of two different mating

cell types the old MACs are disintegrated and new ones are formed that carry the new information derived from the recombinant MIC. Episomal high-copy vectors including a rDNA origin or integrative vectors available. The expression vectors depend on large double rDNA origin stretches to ensure a stable propagation in the cells or on the large flanking integration sites of non-coding regions that are essential for efficient homologous recombination into the gene loci of the MAC or MIC (248). For the selection of transformants, the vector backbone codes for the paromomycin resistance. A neomycin resistance cassette optimized for the codon usage of *T. thermophila* has been developed. A blasticidin resistance gene is used for the selection of transformants. A beta-tubulin marker is also used that uses the resistance to the mitotic toxin taxol. The regulation of expression of a recombinant protein can be regulated using constitutive as well as inducible promoters. A region of the cadmium inducible MTT1 promoter is capable of driving high-level gene expression in *T. thermophila*. Metallothioneins (*MTTs*) are the ubiquitous proteins with the ability to efficiently bind heavy metal ions, mainly cadmium, zinc or copper (253, 254).

1.7.2 Lifecycle

Similar to other ciliates, the life cycle of *T. thermophila* comprises of two major phases, the vegetative growth (asexual phase) and the conjugation (sexual phase).

1.7.2.1 Vegetative growth

Tetrahymena reproduces asexually through binary fission during the vegetative growth. The MIC divides mitotically to produce two daughter cells having the identical genetic content. After the conjugation, the cells are immature and lack the ability to conjugate. After ~100 vegetative divisions, the cells become mature and

capable of mating and conjugating. During vegetative growth, cells reach a point of infertility and are no longer capable of mating. MIC is not under any selective pressures against the loss of chromosomes since no gene expression takes place from it. However, MAC divides by amitosis and does not have equal segregation of alleles (255). Hence, the genetic material is randomly distributed to the daughter nuclei, which provides the foundation for phenotypic assortment. The phenomenon of phenotypic assortment generally takes approximately 62 vegetative generations and can be increased through selection (256).

1.7.2.2 Conjugation (Sexual Reproduction)

In *T. thermophila* the sexual reproduction is called conjugation and does not involve cell reproduction and is stimulated when cells experience nutrient deprivation or other environmental stress. Seven mating types have been identified in *Tetrahymena*, and when cells are subjected to prolonged starvation, each mating type is capable of mating with the cells of other mating type but not with the same mating type (257, 258). Each of the seven types can be generated from the mating of any two different parental mating types. Mating is inducible in the laboratory when the cells of opposite mating types are starved for ~18 hours and subsequently mixed in equal numbers (259). The cells of two opposite mating types come close together and fuse their oral apparatuses during conjugation and produce a genetically unique diploid nucleus. In each cell, the micronucleus undergoes several rounds of meiosis and mitosis to generate four haploid micronuclei (gamete micronuclei). At the beginning of meiosis, the MIC elongates and adopts a crescent shape. The cells then complete meiosis I, and meiosis II reductional divisions resulting in the production of four haploid nuclei. Out of four, three nuclei are degraded and one remains in the anterior of the cell that later undergoes mitosis resulting in two identical nuclei called pro-nuclei. One of the nuclei

is present in the anterior and is called the migratory nucleus whereas the one in the posterior of the cell is called stationary nucleus. The migratory pro-nuclei of the fused cells are exchanged and subsequently exchanged migratory nuclei are fused with the stationary nuclei within each mating partner. The resulting diploid nucleus in each mating partner is called as zygote nucleus (Figure 1.12). The zygotic nucleus undergoes two mitotic divisions to generate four genetically identical diploid nuclei. The two anterior nuclei differentiate into macronuclei (also termed Anlagen nuclei), while the posterior nuclei remain as diploid micronuclei (258). During this stage, site-specific DNA rearrangements and mating type determination occur in the MAC. As the daughter MACs start developing (anlagen 1 stage), the parental MAC moves toward the posterior end of the cell and begins to degrade via a process analogous to lysosomal autophagy (260). Hence, the gene expression switches from the old, parental macronucleus to the developing macronucleus also called the zygotic MAC. The cells then enter the anlagen II stage during which the developing MACs align in the center with a perpendicular orientation with two MICs on each side. The mating cells separate during the late stages of macronuclear development. The parental MAC is apoptotically destroyed until after the cells have separated (called as ex-conjugants). The old MAC and one of the two new MICs are degraded in the ex-conjugants. Later, ex-conjugants undergo the first postzygotic cell division to form 4 karyonide cells (258). Resulting cells have single macronuclei and micronuclei and are capable of resuming normal, asexual reproduction by binary fission (Figure 1.12).

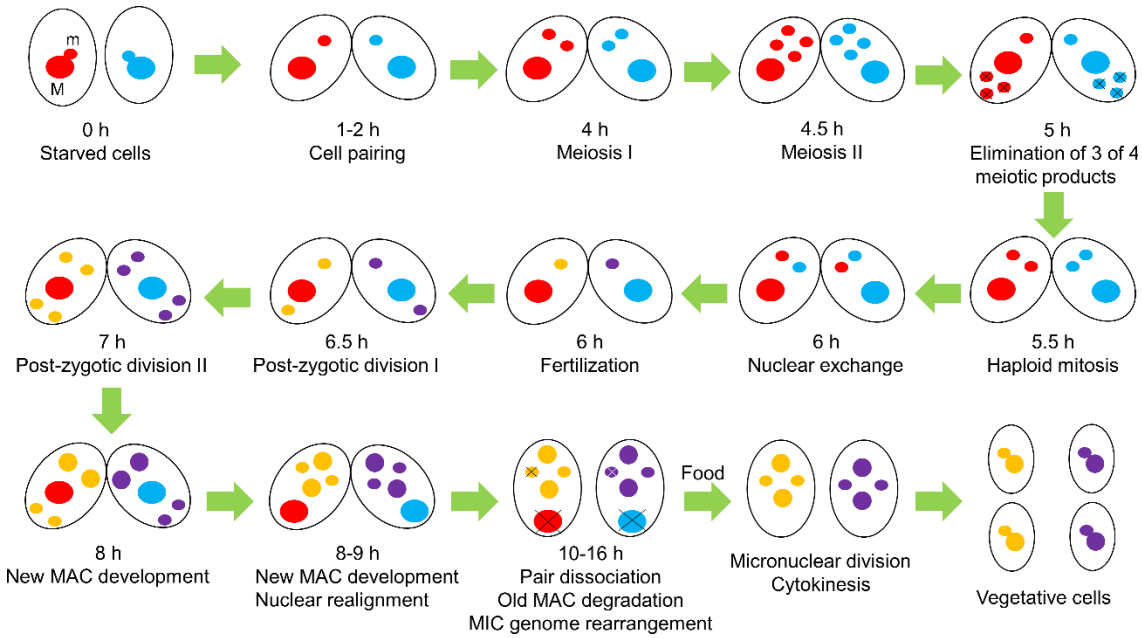

Figure 1.12: Conjugation stages of *Tetrahymena*.
The small circle represents micronucleus (m) and macronucleus (M) is denoted by large circle. After prolonged starvation, two cells of complementary mating types pair with each other and begin conjugation (the sexual reproduction). Their micronuclei (m) undergo meiosis and generates four haploid products, out of which three are resorbed by the programmed nuclear death. The functional meiotic product divides mitotically generating two gametic nuclei, one haploid migratory (anterior) and other stationary (posterior) gamete pronuclei. The migratory gametic nuclei are reciprocally exchanged. The incoming migratory and the resident stationary pronuclei fuse to produce a diploid zygotic nucleus. The fertilization nucleus undergoes two mitotic divisions producing four diploid anlagen, two of the products differentiate as new polyploid MACs (anterior two) whereas the other two form the new diploid MICs (posterior two). Newly generated MACs and MICs take up the central position and the parental macronucleus migrates posteriorly and is degraded. Subsequently, the exconjugant cells separate and one of the two micronuclei is resorbed. When the nutrients are available again, the progeny restores the vegetative growth by cytokinesis with micronuclear (without macronuclear) division. (Taken from Aslan et al, A comparative in-silico analysis of autophagy proteins in ciliates, PeerJ 5:e2878, January 2017)

1.8 Objectives of the present study

The present study aims at elucidating the role of *NEM1* homologs in lipid homeostasis and membrane biogenesis of *Tetrahymena*. The major objectives are -

1) Generation of individual knockout strains of *NEM1* homologs of *Tetrahymena*, analyzing their growth effect and elucidating their role in lipid and membrane biogenesis.
2) Analysis of the functional conservation of *NEM1* in yeast and *Tetrahymena*.
3) Analyzing the functional conservation of *Tetrahymena PAH2*.

Chapter 2

MATERIALS AND METHODS

This chapter describes the materials and experimental procedures that were used in the following chapters.

2.1 MATERIALS

2.1.1 Plasmids

Tetrahymena specific expression vector pIGF was a gift from Doug Chalker, Washington University. *Tetrahymena* expression vector pVGF was procured from Meng-Chao Yao, University of Washington. pCRII vector was obtained from Invitrogen. pRS316-URA3-PUS1-GFP plasmid was provided by Symeon Siniossoglou (University of Cambridge). Yeast centromeric plasmid YCplac111 with a LEU2 marker was obtained from Dr. Pankaj V. Alone (NISER, Bhubaneswar).

2.1.2 Gene Synthesis

Because of the unusual genetic code of *Tetrahymena* (245), the coding region of *TtNEM1A, TtNEM1B,* and *TtNEM1D* were commercially synthesized by Invitrogen with codon optimization for yeast expression and required restriction sites were added at both the ends. The TAA and TAG codons in the coding region of the *Tetrahymena NEM1* homologs were replaced with CAA and CAG respectively since TAA and TAG which codes for glutamate in *Tetrahymena* but are stop codons in yeast. The codon optimized *TtNEM1C* gene was commercially synthesized and obtained in the pUC57 vector from Eurofins. The commercially synthesized genes were used for complementation studies in yeast.

2.1.3 Bacterial strain and culture media

Escherichia coli DH5α *(fhuA2 Δ (argF-lacZ) U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi- 1hsdR17)* was used for plasmid amplification and for

all the recombinant DNA cloning procedures. Luria bertani broth and agar for bacterial culture were obtained from HiMedia. Antibiotics used were- Ampicillin sodium salt and obtained from MP Biomedicals, USA.

2.1.4 *Tetrahymena thermophila* strains and culture media

Wild-type *Tetrahymena thermophila* strains CU428.1 and B2086 were procured from *Tetrahymena* Stock Centre, Cornell University, USA. Proteose peptone and yeast extract were purchased from BD Biosciences, USA. Dextrose and PSA (Penicillin-Streptomycin-Amphotericin B Solution) were obtained from MP Biomedicals.

2.1.5 Yeast strains and culture media

BY4741 (genotype MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0), *nem1*Δ and *spo7*Δ yeast strains were kindly provided by Ramon Serrano (Polytechnic University of Valencia, Spain). Δ*pah1* yeast strain (RS453 smp2Δ: ade2 his3 leu2 trp1 ura3 smp2::TRP1) was obtained from Symeon Siniossoglou (University of Cambridge). Dextrose, yeast nitrogenous base, yeast extract, peptone and agar were obtained from MP Biomedicals.

2.1.6 Other reagents

RNA Isolation kit was purchased from Promega. Superscript II reverse transcriptase for cDNA synthesis was from Invitrogen. Antibiotics were from MP Biomedicals. ER-Tracker Green, ER Tracker Red, pENTR/D-TOPO kit, DHCC and DAPI were from Invitrogen. Oil Red O dye was from Hi-Media. All the restriction enzymes and DNA modifying enzymes for cloning were purchased from New England Biolabs, UK unless specified otherwise. Other chemicals and media components were from Sigma-Aldrich unless mentioned otherwise.

2.2 METHODS

2.2.1 Sequence Analysis

Amino acid sequences of Nem1 homologs were obtained from TGD database (http://ciliate.org/index.php/home/welcome). *Tetrahymena* Genome Database annotations for *NEM1* sequences are as follows: TTHERM_00262970 (*NEM1A*), TTHERM_00473100 (*NEM1B*), TTHERM_00685940 (*NEM1C*), and TTHERM_00688650 (*NEM1D*). Protein sequence analysis and identification of phosphatase domain (characteristic of Nem1) were performed using Interpro program (https://www.ebi.ac.uk/interpro/).

2.2.2 Primer designing

The primers were designed by using Integrated DNA Technology (IDT) software. The list of primers used in the study is given in Table 2.1.

Table 2.1: The list of primers used in the study

S.No.	Oligo name	Sequence
1	S105_Nem1B_W	CACCATGCATCACCATCACCATCACATGAAAAAA AGCAAGATACAG
2	S106_Nem1B_C	ATTTTATCCTTAAATGTTCTTTG
3	S107_Nem1C_W	CACCATGCATCACCATCACCATCAC ATGGATAAG TTATTTCACTT
4	S108_Nem1C_C	TCATATTCCTAACTCTTCT
5	S109_Nem1A_W	CACCATGCATCACCATCACCATCACATGAAGAAT AATCAGAATAATTC
6	S110_Nem1A_C	TCATATTCCTAACTCTTCT
7	5'UTR.Nem1A.F	GCGAGCTCGATTTATATTTTCAAAGATACAAAAG TG
8	5'UTR.Nem1A.R	GCGAATTCAAAACTTATTGATTGATTGTATTATCT AT
9	3'UTR Nem1A.F	GCGAATTCCAAATACTAAAACTAAAAAATTAAGA TTATG
10	3'UTR Nem1A.R	GCCTCGAGCAAAATAATAAAATTAGTAAAACAA GAGG
11	5' UTRNem1D.F	GCGAGCTCGATGATACTGATGATGAAGTAATGA
12	5'UTRNem1D.R	GCGAATTCTAATAATCTTTTTGACAAACTTGATTT AC

13	3’UTR Nem1D.F	GCGAATTCGTATAGATAGATAGAAATATAAATAGATC
14	3’UTR Nem1D.R	GCCTCGAGTAATAGGATATTTATGTAAAGATTTTG
15	S121_5’Nem1B_F	GCGAGCTCGACAATGATTTTGAAGTAATATATGTAAATC
16	S122_5’Nem1B_R	GCGAATTCGTAAAAATAATAATCTGCCTTGATGAG
18	S123_3’Nem1B_F	GCGAATTCCTCTAATAGCTCGACAAATCAATA
19	S124_3’Nem1B_R	GCCTCGAGGATAAAGTCACTAAATTAAGAATTTTCA
20	S125_5’Nem1A_F	GCGAGCTCACATTAAATCATCAATTCAAAAACATAT
21	S126_5’Nem1A_R	GCGAATTCTAATGCTACTATTATTGCTGTTGAA
22	S127_3’Nem1A_R	GCCTCGAGCAAGAGGATAAGTGGAGAAATTA
23	S132_Nem1A_F	AGTTAACAAAGATGACTCCAATGT
24	S133_Nem1A_R	CCGGTTTCTGCTTTGGTGTT
25	S134_Nem1B_F	AGACTTACAAAGAGACTTCTCGA
26	S135_Nem1B_R	GAGAAGCCATAGAAGGAGGCT
27	S140_Nem1A genesyn_F.P.	GCCATATGGAATTCCTCGAGGTCGACATGAAGAATAATCAGAATAATTCAGG
28	S141_Nem1A genesyn_R.P.	GCGCGGCCGCAAGCTTGGGCCCGGATCCTCATAAGCTCTTTTTATAGAGATTTTC
29	S142_Nem1B genesyn_F.P.	GCCATATGGAATTCCTCGAGGTCGACATGAGTTCTTTCAAGAGTTCTTCT
30	S143_Nem1B genesyn_R.P.	GCGCGGCCGCAAGCTTGGGCCCGGATCCTCAATATTGATTTGTCGAGCTATTAG
31	S144_Nem1D genesyn_F.P.	GCCATATGGAATTCCTCGAGGTCGACATGAAAATGAACAAAGAATTTGGAG
32	S145_Nem1D genesyn_R.P.	GCGCGGCCGCAAGCTTGGGCCCGGATCCTCAATTGTTGCTATCTTTGATAAGTT
33	S146_smp2.l_F	GCGTTTAAACCTCGAGATGAGTGTTTTAAAAAAACTACAG
34	S147_smp2.l_R	GCGGGCCCTCATTCGCTTAATAGCTGGTTAAT
35	S148_smp2_F	CAGCAGGTAGCTCAGC
36	S149_smp2_R	AAATCTTGACTGGGTCCTG
37	S172_5’Nem1C_F	GCGAGCTCTTCTAAAGAATTAAATGATACAACCATT
38	S173_5’Nem1C_R	GCGAATTCCTGCTTAAAAGTGAAATAACTTATCC
39	S174_3’Nem1C_F	GCGAATTCAGAAGAGTTAGGAATATGATTAGT
40	S175_3’Nem1C_R	GCCTCGAGAATTGTAGTTTACTTGCTAAAGTT

41	S176_Nem1C.F	AGGCCATAATACTCAAAAGGAA
42	S177_Nem1C.R	GTGGAACTGCTTGCTGTGAA
43	S178_ScNem1.F	CAGGGTCACTTGGTGGAAGT
44	S179_ScNem1.R	AACTGGATGGGAAGGAGCTT
45	Alpha tubulin_F	CCTCCCCCTAAGTCTCAACC
46	Alpha tubulin_R	CGAAGGCAGAGTTGGTGATT

2.2.3 Bacterial Strains and Culture Conditions

Escherichia coli DH5α cells were grown in LB medium.

Media preparation was done by following method- 25 g of LB powder (Himedia) was dissolved in 1 L of Milli Q water and autoclaved. To prepare LB agar plates, 25 g of LB agar powder (Himedia) was dissolved in 1 L of Milli Q water and autoclaved. It was cooled to about 55℃ and 1 ml of Ampicillin antibiotic was added to it and the media was poured into sterile petri plates (~25 ml/100 mm plate). The stock concentration of Ampicillin was 100 mg/ml (Filter sterilized using 0.22 μm membrane), dissolved in water and stored at -20 °C. The working concentration of Ampicillin was 100 μg/ml.

2.2.4 Preparation of ultra-competent *E. coli*

Higher competency is crucial to ensure the high transformation efficiency for cloning. DH5α was made ultra-competent for the transformation of recombinant plasmids. For the transformation of plasmid, *Escherichia coli* DH5α was used and the competent cells of these strains were prepared by the following procedure. DH5α from freezer stock was streaked on to LB-agar plate and incubated overnight at 37°C. A single isolated colony was inoculated into 25 ml of LB media in a 250 ml flask and grown at 37°C for 6-8 hours with constant shaking at 220 rpm. The grown culture was sub-cultured in 100 ml of LB media in a 500 ml flask to get an OD_{600} of 0.025. The flask was incubated at 18°C and OD of the culture was constantly monitored after 24 hours

so as to collect cells at OD_{600} of 0.55. The culture flask was transferred onto the ice for 10 minutes with occasional mixing. The cells were harvested by centrifuging at 3500 rpm for 10 minutes at 4°C. The cell pellet was re-suspended in 16 ml ice-cold transformation buffer containing 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, 10 mM PIPES (pH 6.7). Cells were pelleted down at 3500 rpm for 10 minutes and the residual buffer was removed. Cells were slowly re-suspended in 4 ml of ice-cold transformation buffer and 300µl of DMSO. The bacterial suspension was mixed by swirling and incubated on ice for 15 minutes. Aliquots were dispensed into pre-chilled centrifuge tubes and snap frozen in liquid nitrogen to store at -80°C.

2.2.5 Bacterial Transformation

A 50µL aliquot of ultra-competent cells was taken from -80°C and thawed on ice. Approximately 50-100 ng of DNA was added to 50µl thawed competent cells (the tube was tapped gently and avoided disturbing the cells by pipetting), incubated on ice for 30 minutes, subjected to heat shock in water-bath at 42°C for 40 seconds and immediately placed on ice for 2 minutes. One ml of LB media was aseptically added to the vial and incubated at 37°C with shaking at 220 RPM for 60 minutes. The cells were plated on LB agar plate containing appropriate antibiotic and incubated at 37°C for 12-16 hours for the colonies to appear.

2.2.6 Plasmid DNA isolation

For plasmid purification, E. coli DH5α cultures harboring the plasmids of interest were grown overnight in 4 ml LB media supplemented with ampicillin. Plasmid DNA was purified according to the protocol supplied by the manufacturer and stored at -20^0C for subsequent use. Plasmids used in this study are given in Table 2.2.

Table 2.2: Plasmids used in the study

S. No.	Plasmid name	Description	Comments
1	SS6	Nem1D KO plasmid	This study
2	SS11	Nem1A KO plasmid	This study
3	SS12	Nem1B KO plasmid	This study
4	SS17	pVGF-GFP + Nem1A	This study
5	SS18	pVGF-GFP + Nem1B	This study
6	SS19	pIGF -GFP + Nem1D	This study
7	SS25	YCplac111 + Nem1A	This study
8	SS26	YCplac111 + Nem1B	This study
9	SS27	YCplac111 + Nem1D	This study
10	SS28	NCVB + TtPAH1	This study
11	SS31	YCplac111 + TtPAH2 + TtPAH1 mid	This study
12	SS32	NCVB + Yeast Nem1	This study
13	SS33	NCVB + Tt Nem1B	This study
14	SS39	Nem1C KO plasmid	This study
15	SS41	YCplac111+TtNem1B+yeast Nem1 CTD fragment	This study
16	SS42	YCplac111 + Nem1C	This study
18	PUS-GFP	PUS-GFP expression vector	Ref
19	YCplac111	YCplac111 empty vector	Ref

2.2.7 *Tetrahymena* Strains and Culture Conditions

Wild-type *Tetrahymena* strains, B2086, and CU428.1 were grown at 30℃ with constant shaking at 90 rpm rotation speed in SPP medium that consists of 2% proteose peptone, 0.2% glucose, 0.1% yeast extract and 0.003% ferric EDTA sodium salt (261). Penicillin G & Streptomycin sulfate + Amphotericin (250 µg/ml pencillin and streptomycin and 1.25 µg/mL Fungizone amphotericin B) is also added. For passaging, the cells were transferred to fresh medium at maximum of 1:100 dilution. The stocks were maintained at room temperature in test tubes in low aeration in 2% SPP for short-term storage 3 to 4 months. For long-term storage, the cells were starved and frozen in liquid nitrogen in 4% DMSO.

To induce conjugation, cells of different mating types (B2086 and CU428.1) growing in mid-log phase were washed and starved in DMC, (0.17 mM sodium citrate, 0.1 mM NaH_2PO4, 0.1 mM Na_2HPO4, 0.65 mM $CaCl_2$, 0.1 mM $MgCl_2$) for 16-24 h at 30°C. An equal number of cells of the complementary mating types were mixed and incubated at 30°C without shaking to initiate conjugation. The conjugation efficiency was checked at the fourth hour post mixing by fixing the cells with formalin and counting the number of paired *Tetrahymena* cells versus number of unpaired cells. The conjugation cultures having more than 80% of conjugation efficiency were used for transformation.

2.2.8 *Tetrahymena* Genomic DNA Extraction

For *Tetrahymena* genomic DNA isolation, 25 ml of stationary culture ($1X10^6$ cells/ml) was harvested. The supernatant was quickly poured off to avoid the cells to swim out of their pellet. Cells were re-suspended in 3.5 ml Urea buffer (42% urea, 0.35 M NaCl, 0.01M Tris pH 7.4, 0.01M EDTA, 1% SDS) by pipetting up and down several times to mix and completely re-suspend the cells, then gently inverted the tube for 5 minutes until the suspension clears. The cell lysate was extracted with equal volume of phenol: chloroform: isoamylalcohol (25:24:1) by inverting the tube for several seconds (no vortexing) to mix phenol throughout the entire sample. The mixture was centrifuged for 5 minutes at maximum speed in a microcentrifuge and the top (aqueous) layer was transferred to a fresh microcentrifuge tube. The phenol extraction was repeated one more time and the cell lysate was further extracted with chloroform: isoamyl alcohol (1:1) one time. 1ml of 5M NaCl was added to approximately 3ml of the extracted cell lysate since the addition of salt helps to reduce the carbohydrate content of the final precipitate. The DNA was precipitated using an equal volume of

isopropyl alcohol to the lysate and inverting the lysate-alcohol several times, the mix was allowed to stand at room temperature for 10 minutes. The precipitate was collected by centrifuging the mixture at maximum speed for 10 minutes. The precipitated DNA was spooled onto a glass capillary tube, washed with 70% ethanol to solubilize salts and remove them from the DNA pellet and dried. The DNA was re-suspended in 600µl TE containing 6µl RNase A (10mg/ml) and kept in a heating block at 55^0C for overnight and further used for setting up the PCR reactions.

2.2.9 Disruption of Tt*NEM1* Homologs

To generate the knockout constructs, the genomic regions flanking (5'UTR and 3'UTR) of *TtNEM1* homologs were PCR amplified from the wild-type *Tetrahymena* cells 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 2.1). For amplification of 3'UTR, EcoRI and XhoI restriction sites were included in the forward and reverse primer respectively (Table 2.1). Finally, the *NEO3* cassette, which confers paromomycin resistance to *Tetrahymena* cells, was introduced between 5'UTR and 3'UTR of corresponding homologs using EcoRI restriction site. The resulting knockout construct was linearized by digesting with SacI and XhoI restriction enzymes and introduced biolistically into vegetative *Tetrahymena* by particle bombardment(262). The complete replacement of endogenous *TtNEM1* homologs 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. The knockout strains were confirmed by reverse transcription PCR after passaging (8 passages) them in the absence of drug.

2.2.10 Growth Rate

The wild-type, *ΔTtnem1B, ΔTtnem1C,* and*ΔTtnem1D* cells were incubated at 30℃ with shaking at 90 rpm. When the cell number reached 1×10^5/ml, cells were counted using a hemocytometer at 2 h intervals after fixation with formalin. The averaged cell density, using biological triplicates for each cell type, was plotted against time.

2.2.11 *Tetrahymena* Transformation by electroporation method

TtNEM1A-GFP, TtNEM1B-GFP, and *TtNEM1D-GFP* constructs were transformed in wild-type *Tetrahymena* cells through electroporation (263).

Conjugated *Tetrahymena* cells (3 X 10^5 cells/ml) were pelleted at 1100 RCF for 1 minute and supernatant was discarded. The cells were washed with 10 mM HEPES pH 7.5 and resuspended in approximately 500 µl of 10 mM HEPES pH 7.5. 125 µl of the conjugated *Tetrahymena* cells are mixed with 125 µl of plasmid DNA having concentration of 20-25 µg in an electroporation cuvette with 0.2-mm gap (Thermo scientific). Electro-pulsing was done exactly at the tenth hour of conjugation (post-mixing of the two complementary wild-type strains). Electro-pulsing was done using Gene Pulser (Biorad) electroporation unit at 250 V for 5 milli seconds under time constant protocol. After one minute of pulsing, the elecro-pulsed cells were diluted in SPP medium and cells were aliquoted in 96 well plates and incubated at 30^0C. After 16 hours, paromomycin sulphate was added to a concentration of 100 µg/ml. The positive transformants, which grow efficiently in the presence of paramomycin sulfate, were selected after 3 to 5 days post drugging. Contrarily, the drug sensitive cells divide slowly, show less movement and are swollen.

2.2.12 Reverse transcription PCR (RT-PCR) Analysis

Total RNA was isolated from 3×10^5 vegetative wild-type as well as Δ*Ttnem1B*, Δ*Ttnem1C*, and Δ*Ttnem1D* strains using the SV Total RNA isolation kit (Promega) following the manufacturer's guidelines. For standard reverse transcriptase-polymerase chain reaction (RT-PCR), cDNA synthesis was carried out from 2 μg of total RNA with SuperScriptII reverse transcriptase and random hexamer primers (Invitrogen). PCR reactions were performed with 100 ng cDNA using *TtNEM1* specific primers and alpha-tubulin (*ATU1*) primers (Table 2.1) in the same reaction for 28 or 35 cycles using Taq polymerase.

2.2.13 Isolation of nucleus

The wild type, Δ*Ttnem1B,* Δ*Ttnem1C,* and Δ*Ttnem1D* cells were used for nuclear isolation. 50 ml of *Tetrahymena* cells (5 x 10^5 cells/ml) were centrifuged at 1100 rcf for 5 min at 4°C. The cell pellet were washed with pre-chilled Solution A (sucrose 0.1M, gum arabic 4% v/v, MgCl2 0.0015M, Spermidine Hydrochloride 0.01% v/v) and resuspended in pre-chilled Solution B (sucrose 0.1M, gum arabic 4% v/v, MgCl2 0.0015M, Spermidine Hydrochloride 0.01% v/v, octanol 24mM). The cell suspension was shaken vigorously for 5 min followed by centrifugation at 1100 rcf for 5 min at 4°C. The nuclear pellet were resuspended in Buffer A and cells were imaged after staining with DAPI using a fluorescence microscope.

2.2.14 Staining and Microscopy

Fixation the cells with Paraformaldehyde

Tetrahymena cells were fixed in 4% paraformaldehyde (PFA) suspended in 50mM HEPES pH 7.5. The cells were centrifuged at 3000 rpm and the supernatant was

discarded. The cell pellet was re-suspended in the remaining fluid by flicking the tube and PFA was added to the cells by dispensing swiftly and incubated at room temperature for 30 min. Cells were centrifuged and washed with 10mM HEPES pH 7.5. The fixed cells were resuspended 10 mM HEPES pH 7.5.

Lipid droplet staining

For staining lipid droplets, *Tetrahymena* cells were pelleted by centrifugation (1100g for 2 min) at room temperature, washed with DMC and fixed with 4% paraformaldehyde for 30 minutes. After 30 min the 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 rotating 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.

Endoplasmic Reticulum staining

For Endoplasmic Reticulum staining, *Tetrahymena* cells were grown to a density of 3-4 $x10^5$, 0.5 µM ER-Tracker Green dye (Invitrogen) was added to the culture and incubated for 60 minutes before fixing with 4% paraformaldehyde (50 mM HEPES pH 7.5).

To quantitate ER content, the stacked images of ER-tracker Green stained cells were analyzed by image after sum intensity projection. The mean intensity values were plotted for both wild-type (n= 70) and Δ*Ttnem1C* (n= 60) cells using box plot.

2.2.15 DHCC Staining

For staining the isolated *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 (sucrose 0.1M, gum arabic 4% v/v, MgCl2 0.0015M, Spermidine Hydrochloride 0.01% v/v) and was re-suspended in the same solution before imaging. The slides were viewed under a under a 60X oil immersion lens in a Nikon Eclipse Ti fluorescence microscope.

2.2.16 Imaging

The imaging was done using a Zeiss LSM780 confocal microscope. For Oil Red O staining, the images were taken at 543 nm excitation / 619 nm emission. For ER-Tracker Green staining, the images were taken at excitation /emission – 504/511 nm. 3-5µl of cells were mounted on glass slide, covered with cover glass, and sealed with nail polish and imaged with a Zeiss LSM780 confocal microscope. To visualize the GFP tagged proteins, the images were taken at excitation/ emission at 488 /500 nm and for visualizing DAPI, the images were taken at excitation /emission- 405/ 460 nm.

2.2.17 Yeast Strains and culture Conditions

Yeast cells were grown either in minimal (**S**ynthetic **D**efined) media consisting of 2% glucose, 0.17% yeast nitrogen base, 2.5% ammonium sulphate, along with the appropriate amino acid and nucleoside base or in rich media (YPD) consisting of 1% **Y**east Extract, 2% **P**eptone and 2% **D**extrose at 30℃ (264). 2% agar was also added to SD or YPD media to make solid media plates.

For growing Δ*nem1* and Δ*spo7* cells leucine, uracil, methionine, and histidine were added and for Δ*pah1* cells adenine, leucine, uracil, and histidine were added as supplements to complete the synthetic defined media. To select the transformants, cells were grown in synthetic defined media containing adenine/methionine and histidine but lacking leucine and uracil. To check respiratory deficiency, dextrose was replaced by 2% glycerol as the carbon source.

Table 2.3: The stock and working concentration of amino acid for growing yeast cells in SD media

S. No.	Amino acid	Stock concentration/100 ml of MQ Water	Amount needed for 250 ml media
1	Adenine	0.135 gm	3.75 ml
2	Histidine	2.09 gm	0.75 ml
3	Leucine	1.31 gm	5 ml
4	Methionine	0.746 gm	5 ml
5	Uracil	0.224 gm	2.5 ml
6	Tryptophan	0.8 gm	2.5 ml

2.2.18 Generation of Yeast Expression Constructs

Because of the unusual genetic code of *Tetrahymena* (245), the coding region of *TtNEM1A*, *TtNEM1B*, and *TtNEM1D* were commercially synthesized by Invitrogen with codon optimization for yeast expression and required restriction sites were added at both the ends. TtNem1 genes were cloned into yeast expression vector YCplac111 using SalI and BamHI restriction sites.

2.2.19 Yeast transformation

50ml *nem1*Δ cells were inoculated in SD media containing histidine, methionine, leucine and uracil with an overnight grown pre-culture to an OD_{600} of 0.05 and grown at 30^0C at 220 rpm until an OD_{600} of 0.6. Cells were harvested at the mid-log phase (OD_{600} around 0.5-0.7), in a sterile 50 ml centrifuge tube at 2000 g for 5 min at RT. The supernatant was poured off and the cells were washed once with 25 ml sterile TE (10 mM Tris-HCl) buffer. The cells were centrifuged at 2000 g for 5 min at RT. Supernatant was removed and cell pellet was re-suspended in 25 ml of sterile 100mM lithium acetate. Cells were again pelleted down and the final pellet was further re-suspended in 500µl of 100mM lithium acetate and was incubated at 30^0C for 30 minutes in a water bath. 3µg of each plasmid were mixed with 10 µl of calf thymus

DNA in a sterile 1.5 ml centrifuge tube followed by addition of 50µl of yeast competent cells and mixed by tapping. 300µl of 40% polyethelene glycol (PEG) was added to the transformation mix and vortexed for 5 seconds followed by horizontal incubation at 220 rpm, 30^0C for 30 minutes. The samples were given heat shock at for 20 minutes at 42^0C in a water bath. The cells were pelleted down for at 2000 g for 4 min, dissolved in 150µl of water and plated on selective SD plates (containing histidine and methionine but lacking leucine and uracil) with 2% glucose and incubated at 30°C for three to four days. The growth of transformants colonies was observed after 48 hours of plating. The single isolated colonies were grown on SD media with the corresponding amino acids.

2.2.20 Yeast Complementation Assay

The functional conservation of Nem1p from *Tetrahymena* to yeast was assessed using plasmid complementation study by testing the ability to rescue the aberrant nuclear phenotype of the yeast *nem1*Δ cells. All the yeast strains used in this study are derivatives of BY4741 (genotype MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0) and were kindly provided by Ramon Serrano (Polytechnic University of Valencia, Spain). To examine the nuclear envelope morphology, *nem1*Δ cells of yeast expressing *pUS-GFP-URA* were transformed with single copy LEU2 plasmids harboring *TtNEM1A* or *TtNEM1B* or *TtNEM1C* or *TtNEM1D* gene of *Tetrahymena* or empty vector YCplac111. Transformations in yeast were performed by the standard lithium acetate protocol (265). The transformants acquiring both the constructs were grown in the synthetic media containing histidine, methionine, and 2% glucose at 30°C for 3 days. Images of approximately 100 cells from three independent experiments were acquired

by confocal microscope (Zeiss, LSM-780) for analyzing the nuclear membrane morphology.

2.2.21 Spotting assay

For spotting assays, Δ*nem1* and Δ*spo7* yeast transformants were grown in SD media containing histidine and methionine but lacking leucine and uracil for plasmid selection. The exponential phase cells were obtained from overnight culture and sub-cultured to early logarithmic phase (OD_{600} = 1.0). The O.D. of each culture was tested and normalized to 1.0 to start with an equal number of cells and was further serially diluted to 4 tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) in sterile water. Drop of 5µl of each dilution were spotted onto the solid SD media containing histidine and methionine and incubated at either 30°C or 37°C for 3 days. The plates were then examined and photographed.

2.2.22 RNA isolation from yeast cells

All the glasswares used were washed with diethylpyrocarbonate (DEPC) treated water and nitrile gloves were used to prevent RNase contamination. For RNA isolation, 4 ml liquid yeast culture was grown till OD 2.0. The cells were pelleted down at 3000g for 4 min at room temperature. Supernatant was removed and pellet was re-suspended in 375 µl of Trizol and glass beads were added to $1/10^{th}$ volume of cell suspension. The cells were lysed using a fast prep bead beater with alternate 30 sec lysis and 1 minute incubation at ice and repeated five times. Again, 375 µl of Trizol was added and mixed thoroughly and incubated at room temperature for 5 minutes. 150µl of chloroform: isoamyl alcohol (1:1) was added and mixed by vigorous shaking for 15 seconds and incubated for 2 minutes at room temperature after which cells were spun down at 12,000 g for 5 minutes at 4^0C. The upper aqueous phase containing the RNA

was transferred to another 1.5 ml microcentrifuge tube to which 375µl of isopropanol was added, mixed by flicking, and incubated for 10 min. The pellet obtained after centrifugation at 12,000 g for 5 minutes at 4^0C was washed with 1 ml of 75% ethanol. The sample was centrifuged at 7,500g for 5 min at 4^0C and pellet was collected. The pellet was dried thoroughly by removing the residual ethanol and re-suspended in 27µl of nuclease free water. The RNA was further treated with DNAaseI (Table 2.4).

Table 2.4: DNaseI treatment of RNA isolated from yeast

Components	Volume
RNA	25µl
10x DNAaseI Buffer	3µl
DNAaseI	1 µl

The reaction mixture was incubated at 37^0C for 2 hours then 1 µl of 0.5 M EDTA was added and heated at 75^0C for 10 minutes in water bath to stop the reaction.

Then, 70 µl of nuclease free water, 250 µl of RLT Buffer (RNA Isolation Kit Qiagen) and 350 µl of 70% ethanol were added. The mixture was loaded on to a column (RNA Isolation Kit Qiagen) and eluted as per manufacturer's (RNA Isolation Kit Qiagen) instruction. RNA was eluted in 30µl nuclease free water and quantitated by reading at A260. The purity of RNA was confirmed by ratio of A260/A280 (1.9-2.0) and A260/A230 (2.0-2.2). The RNA was stored at -80°C till further use.

Chapter 3

ROLE OF *TETRAHYMENA NEM1* HOMOLOGS IN LIPID HOMEOSTASIS AND MEMBRANE BIOGENESIS

3.1 INTRODUCTION

Biological membranes serve as selective barriers, compartmentalize biochemical processes and protect the cellular environment. Membrane biogenesis requires the coordinated synthesis of lipids and membrane proteins (266). Membranes undergo remodeling to facilitate critical cellular functions such as organelle biogenesis, and cell division (267, 268). Cellular homeostasis depends on the stringent and coordinated use of lipids as energy reservoir or building blocks for membrane biogenesis (50). Cell expansion and cell division depend on membrane biogenesis and are the two important mechanisms underlying cell growth (267). Cell expansion requires the production of plasma membrane and division of the nucleus also needs the production of nuclear-endoplasmic reticulum (ER) membrane. The outer membrane of the nucleus is in continuation with the endoplasmic reticulum and any defect in the ER morphology is generally reflected in the nuclear membrane (269). ER plays a key role in membrane biogenesis since it is a major site for glycerophospholipid biosynthesis and membrane protein folding (270, 271). ER regulates lipid metabolism by establishing contact sites with the plasma membrane, the Golgi, lipid droplets, mitochondria and peroxisomes (269, 272). ER regulates the relative abundance of lipids and proteins in the cellular membranes (64).

Lipid droplets (LDs) are dynamic organelles that store lipid building blocks and play important role in the regulation of lipid metabolism (2, 7, 273, 274). LDs are involved in various aspects of cellular physiology and control the metabolic flux and availability of fatty acids and sterols (2, 46, 59, 63, 265, 266). A defect in LD biogenesis causes deregulation of lipid fluxes and lipid homeostasis. LD biogenesis is driven by the availability of its precursors, TAG and SE, and cells are devoid of LDs in the absence of these lipids (8, 15, 88, 185). Formation of LDs is critical for cell

survival in the presence of excess fatty acids (5, 7, 8, 273). Lipid droplet biogenesis and membrane biogenesis are tightly coupled. Regulation of lipid homeostasis and membrane biogenesis is crucial for normal cell physiology (2, 5, 7, 8, 273, 274).

Lipid homeostasis and membrane biogenesis are regulated by a cascade comprising Pah1 and Nem1/Spo7 complex in yeast and a similar cascade is also present in other organisms including plants, mammals, worms, and flies (175, 181, 184, 275). Phosphatidate phosphatase (PAH1 in yeast and lipin in mammals) is a key regulator of lipid homeostasis and membrane biogenesis. Pah1p is mostly cytosolic but becomes membrane-localized upon dephosphorylation by the membrane associated protein phosphatase complex, Nem1p-Spo7p (17, 18, 223, 229). The dephosphorylation of Pah1 exclusively by Nem1-Spo7 complex acts as a rate-limiting step and is essential for its phosphatidate phosphatase function. The dephosphorylation of Pah1p allows it to interact with the ER membrane, where it converts PA into DAG (18, 229). DAG serves as a substrate for both phospholipid and triacylglycerol (TAG) biosynthesis. The loss of *PAH1* or *NEM1* results in a decrease in DAG and a concomitant increase in PA (13). Deletion of *PAH1* results in growth defect, reduced lipid droplet number, hyperproliferation of the nuclear membrane, and dramatic abnormalities in the ER structure due to the elevated levels of phospholipids (27, 175, 185, 239). Cells lacking either the Nem1 or Spo7 subunits of the phosphatase complex mirror the phenotypes of *pah1*Δ cells including increased phospholipid synthesis, aberrant expansion of the nuclear/ER membrane, decreased lipid droplet number, and growth inhibition (28, 145, 175). Thus, this cascade is essential for the regulation of lipid metabolism and membrane biogenesis (18, 229). Yeast and mammals contain a single Nem1 homolog, which is reviewed in detail in chapter1. *Tetrahymena thermophila* is a unicellular protozoan ciliate belonging to

Alveolata, and therefore much more distantly related from mammals or yeast than these are to one another (30, 31). *Tetrahymena* serves as a great model organism to study the membrane biogenesis in lower eukaryotes since it has membrane complexities comparable to those of higher eukaryotes. *Tetrahymena* possesses two *PAH* homologs *TtPAH1* and *TtPAH2*. Similar to yeast PAH1, TtPAH1 and TtPAH2 contain DXDX(T/V) catalytic motif within the haloacid dehalogenase-like domain present in the C-LIP region (25). Loss of TtPAH1 in *Tetrahymena* causes a severe reduction in lipid droplet count and affects the endoplasmic reticulum morphology but does not manifest visible defect in nuclear morphology (25). In contrast, *TtPAH2* does not regulate lipid droplet biogenesis or ER/nuclear morphology but has a specific role in respiration (26). The change in the structural organization of the ER does not alter the nuclear envelope morphology in *Tetrahymena*. *TtPAH1* plays a role in regulating lipid homeostasis and maintaining ER/nuclear morphology and is evolutionarily conserved across eukaryotic lineages (25). *Tetrahymena* possesses four putative *NEM1* homologs *NEM1A*, *NEM1B*, *NEM1C*, and *NEM1D*. In this chapter, the role of the putative *Tetrahymena NEM1* homologs in lipid homeostasis and membrane biogenesis, and subcellular localization of these homologs are discussed. NEM1 in other organisms is known to play a role in the regulation of lipid homeostasis and membrane biogenesis. We hypothesized that the *Tetrahymena NEM1* homologs might be playing a role in lipid droplet biogenesis and membrane biogenesis. For this purpose, we generated individual knockout strains of *TtNEM1* homologs by deleting all the macronuclear copies of the respective gene. The role of putative *TtNEM1* homologs in regulating lipid droplet biogenesis, ER structure, and nuclear morphology are discussed in this chapter.

3.2 RESULTS

3.2.1 Sequence analysis of TtNem1 homologs

Three genes, namely, TTHERM_00262970 (*NEM1A*), TTHERM_00685940 (*NEM1C*), and TTHERM_00688650 (*NEM1D*) are designated as the putative *NEM1* homologs in the genome database of *Tetrahymena thermophila*. Another gene, TTHERM_00473100 (*NEM1B*), was also included in our study since it was designated as putative *NEM1* homolog in the same database previously. We analysed the sequence of TtNem1 homologs using Interpro: protein sequence analysis & classification (https://www.ebi.ac.uk/interpro/) to predict the presence of domains and conserved residues. The amino acid sequence analysis of *Tetrahymena* Nem1 homologs showed that all the four homologs harbor a conserved DXDX(T/V) catalytic phosphatase motif in the HAD-like domain of the the C-terminal domain (Figure 3.1). However, the four homologs differ significantly in their overall sizes. Nem1A encodes a protein of 937 amino acids; Nem1B encodes a protein of 926 amino acids; Nem1C codes for a protein of 426 amino acids; and Nem1D consists of 1121 amino acids (Figure 3.1). In comparison, the Nem1p of yeast consists of 446 amino acids while human Nem1p consists of 244 amino acids. TtNem1C protein is a small in size comparable to the Nem1 proteins of yeast and mammals. The *Tetrahymena NEM1* homologs except TtNem1C are larger in size compared to the yeast and mammalian proteins. The amino acid sequences of the NEM1 homologs are mostly conserved in the C-LIP region (Figure 3.2).

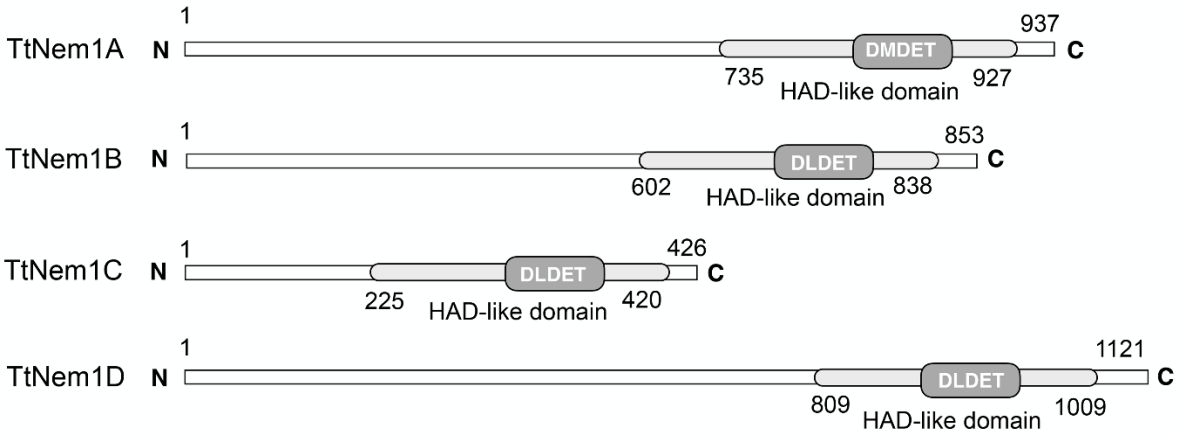

Figure 3.1: Schematic of the primary structure of *Tetrahymena* NEM1 homologs with the position of key motifs/domains shown.
Positions of the haloacid dehalogenase (HAD)-like domain harboring the conserved DXDXT motifs in the C-LIP region of TtNem1A, TtNem1B, TtNem1C and TtNem1D are also shown.

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ScNEM1   --SSNTVFGTKRMGRFLFP----KKLIPKSVLNTQKKKKLVIDLDETLIHSASRSTTHSN 274
TtNem1A LQTCQGVLYS-NLARPVNPKDLVAKRVQLGPRNPKYKKTLIFDMDETLIHCNESAS---- 754
TtNem1B ---FQKMYYNLQVNPKLENKFHQISPVFL-PPLTSNKKTIFFDLDETLIHCIDIFTDPQQ 637
TtNem1C TLVYRGLVYSTRCLKGPSEKYIESKKVNMKRPQYSKGKTLLLDLDETLIHTCSLKE---- 259
TtNem1D VQSFNALNFC-RSLEPIDPLVLESKLIEL-PLNPNKKKTIIFDLDETLIHCNEDPN---- 829
HsNem1   --QIRTVIQYQTVRYDILP----LSPVSRNRLAQVKRKILVLDLDETLIHSHHDGVLRPT 84
            . :                 . :          * :.:*:******
```

Figure 3.2: Multiple sequence alignment showing partial amino-acid sequences of Nem1 proteins.
Sequences of NEM1 protein from *S. cerevisiae* (ScNEM1), *T. thermophila* (TtNEM1A, TtNEM1B, TtNEM1C, TtNEM1D), and *H. sapiens* (HsNEM1) were analyzed. Conserved catalytic motif (DXDXT) in the C-LIP region is indicated in the box.

The amino acid sequence identity of full length sequence and sequence of HAD-like domain of TtNem1A, TtNem1B, TtNem1C, and TtNem1D with *S. cerevisiae* Nem1p and mammalian Nem1 is shown in Table 3.1 and Table 3.2.

Table 3.1: The percent identity of full length sequence between the putative *Tetrahymena* Nem1 homologs and orthologs in yeast and mammals

Sequence	% identity with yeast Nem1	% identity with mammalian Nem1
TtNem1A	24.16 %	31.62 %
TtNem1B	22.75 %	29.63 %
TtNem1C	24.22 %	28.63 %
TtNem1D	23.88 %	26.69 %

Table 3.2: The percent identity of sequence of HAD-like domain between the putative *Tetrahymena* Nem1 homologs and orthologs in yeast and mammals

Sequence	% identity with yeast Nem1	% identity with mammalian Nem1
TtNem1A	32.98%	35.52%
TtNem1B	32.49%	30.10%
TtNem1C	30.26%	32.63%
TtNem1D	32.28%	30.32%

3.2.2 Subcellular localization of TtNem1A, TtNem1B, and TtNem1D

To evaluate the subcellular localizations of *NEM1* homologs, strains were generated to overexpress individual GFP-tagged proteins from the cadmium-inducible metallothionein (*MTT1)* promoter. Analysis of confocal images of *Tetrahymena* cells expressing either *NEM1B* or *NEM1D* showed that both are localized mostly as cortical puncta (Figure 3.3). However, *NEM1B* also was present in the cytoplasm (Figure 3.3). The revelation of similar yet different subcellular localization pattern indicates that NEM1B and NEM1D perform different cellular functions. Like *NEM1B*, *NEM1A* also localizes in the cytosol in addition to the punctate pattern (Figure 3.3).

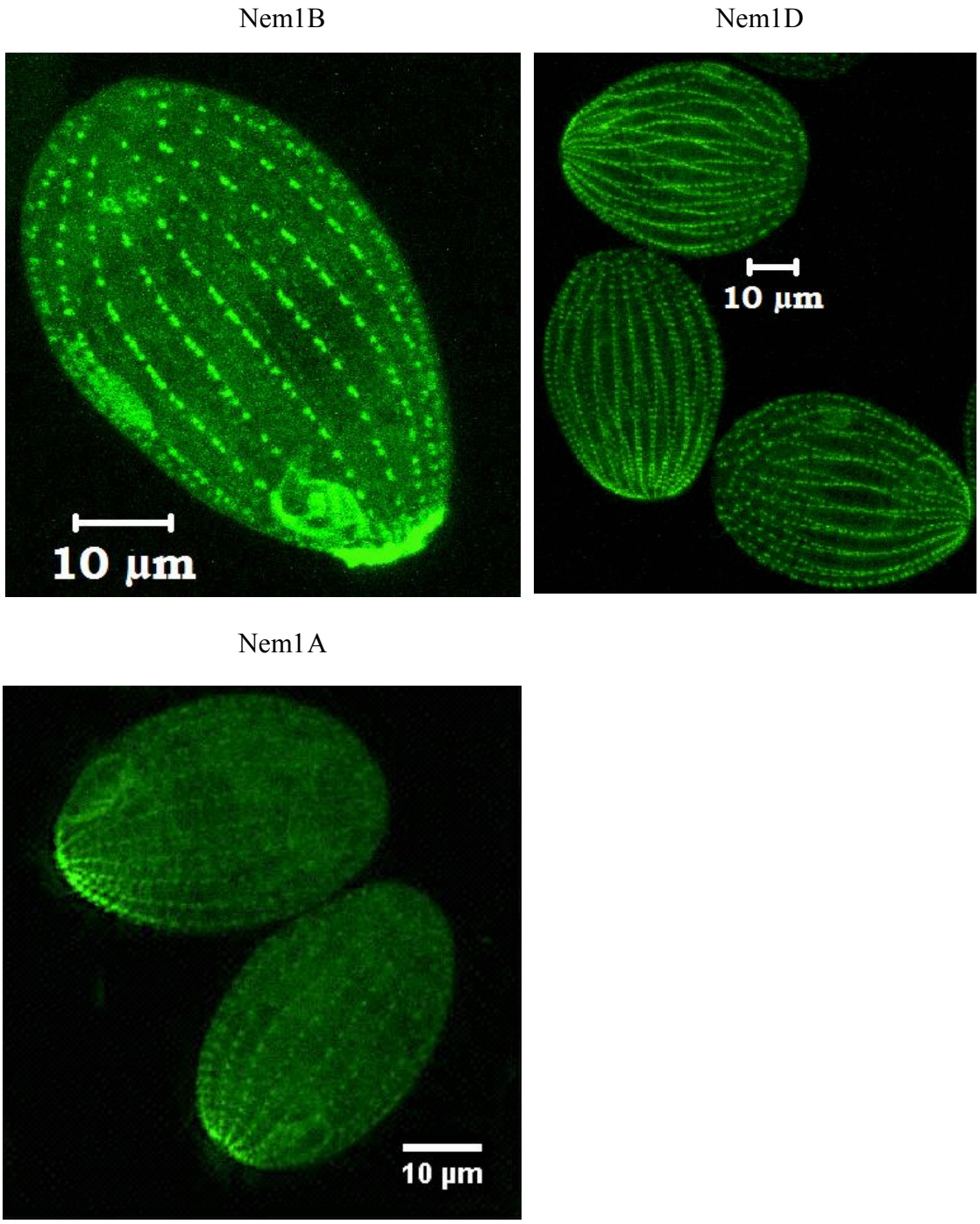


Figure 3.3: Localization of *Tetrahymena* NEM1 homologs
Fixed *Tetrahymena* cells expressing TtNEM1A-GFP, TtNEM1B-GFP and TtNEM1D-GFP were imaged using a confocal microscope.

3.2.3 Generation of Δ*TtNEM1B*, Δ*TtNEM1C*, and Δ*TtNEM1D* strains

To check which of the four *NEM1* genes of *Tetrahymena* might play a role in regulation of lipid homeostasis and membrane biogenesis, we targeted the disruption

in the somatic macronucleus of each of these genes. The disruption of gene was performed by replacing the endogenous macronuclear open reading frame with the *NEO3* cassette via homologous recombination (Figure 3.4).

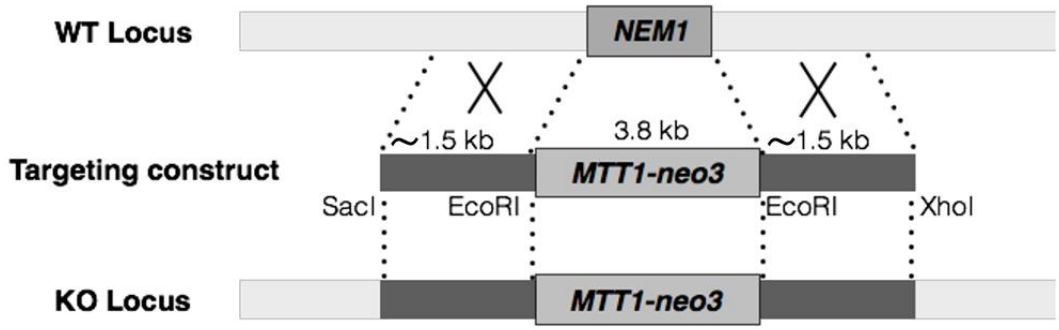


Figure 3.4: Diagram of the knockout construct and wild-type locus.
Gene disruption was done by replacing the entire coding region of TtNEM1 homologs with a drug resistance marker (neo3). The *neo3* cassette encodes a gene conferring paromomycin resistance under the control of the cadmium-inducible *MTT1* promoter. The resulting construct was linearized by digesting with SacI and XhoI restriction enzymes and introduced biolistically into vegetative *Tetrahymena* by particle bombardment to replace the corresponding endogenous gene by homologous recombination. To generate knockout cells by complete gene replacement, the initial transformants were grown in the presence of increasing concentrations of paromomycin.

The initial transformants harbor the drug resistant *NEO3* cassette in substitution of only a few of the 45 copies of each wild-type MAC chromosome. Since the macronucleus divides by amitosis, cells can receive additional copies of the transformed locus, by chance, during the subsequent cell divisions. Increasing the selective pressure by increasing the drug concentration results in the selection of cells with increasing number of copies of the exogenous gene construct resulting in complete replacement of the endogenous locus of the non-essential genes (Figure 3.5) (276). Strains targeted to disrupt a non-essential gene possess only the recombinant chromosomes, whereas strains targeted to disrupt an essential gene would retain some of the wild-type chromosome copies that can lead to back-assortment and increase in

the copy number after release from selection pressure.

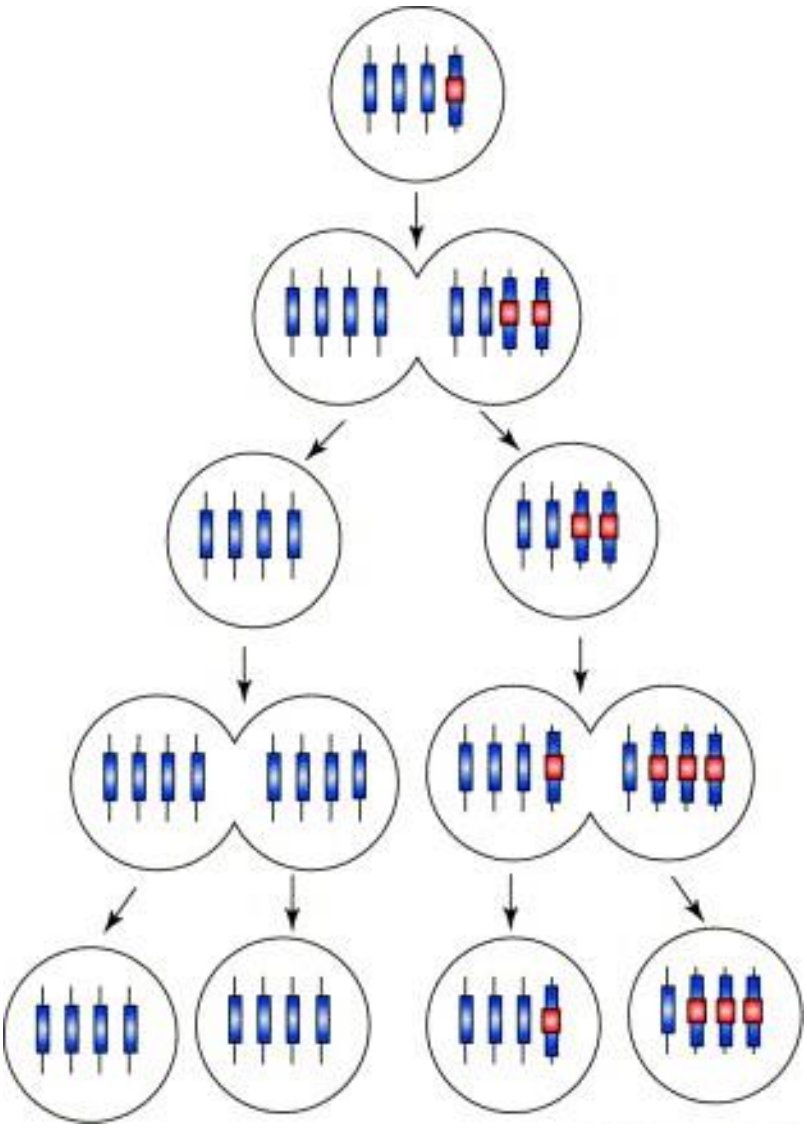


Figure 3.5: Schematic illustration of phenotypic assortment.
The phenotypic assortment is illustrated during two cell cycles. The top circle represents a heterozygous G1 macronucleus (Mac) generated after DNA-mediated transformation followed by integrative recombination. For clarity, the Mac shows only four allele copies (instead of 45). Three alleles are wild-type and the fourth is mutant, a knockout allele where the gene is disrupted by insertion of a neomycin-resistance cassette (red). The fused ovals represent Macs undergoing division after DNA replication. The allele copies are partitioned randomly at each Mac division. If neither allele has selective advantage, the assortants pure for either allele are ultimately generated. In the presence of drug, the vegetative descendants that acquire (by chance only) a higher fraction of the Mac copies with the disrupted allele will be selected. If the disrupted gene is essential, then both the alleles will be maintained by balanced selection. (Taken from Turkewitz et al, Functional genomics: the coming of age for Tetrahymena thermophila, Trends in Genetics, Volume 18, Issue 1, Pages 35-40, January 2002).

The replacement of all the 45 Macronuclear copies of *NEM1B*, *NEM1C*, and *NEM1D* genes by the drug resistance neo cassette was confirmed by the reverse transcription-PCR (RT-PCR), indicating that these genes are not essential for the normal growth of *Tetrahymena*. The generated knockout lines are indicated as Δ*Ttnem1B*, Δ*Ttnem1C*, and Δ*Ttnem1D*. Reverse-transcription PCR analysis of wild-type and Δ*Ttnem1B*, Δ*Ttnem1C*, and Δ*Ttnem1D* cells was carried out using cDNA from wild-type cells, Δ*Ttnem1B*, Δ*Ttnem1C*, and Δ*Ttnem1D* cells. The band corresponding to alpha tubulin (387 bp) is present in wild-type cells as well as Δ*Ttnem1B*, Δ*Ttnem1C*, and Δ*Ttnem1D* cells (Figure 3.6). The presence of *TtNEM1C*, *TtNEM1B* or *TtNEM1D* band in wild-type cells and absence of the corresponding band in Δ*Ttnem1C*, Δ*Ttnem1B*, and Δ*Ttnem1D* cells respectively, even after 34 cycles of PCR, confirms that the individual knockouts are complete (Figure 3.6).

In contrast, in multiple independent selections, we were unable to obtain a knockout of *NEM1A*. The failure in generating the *NEM1A* knockout strain is not attributed to the lack of recombination in this locus since we were able to obtain the initial transformants, suggesting that one or few copies of *NEM1A* were replaced with the drug resistance cassette. This result indicates that *NEM1A* is essential for vegetative growth of *Tetrahymena*.

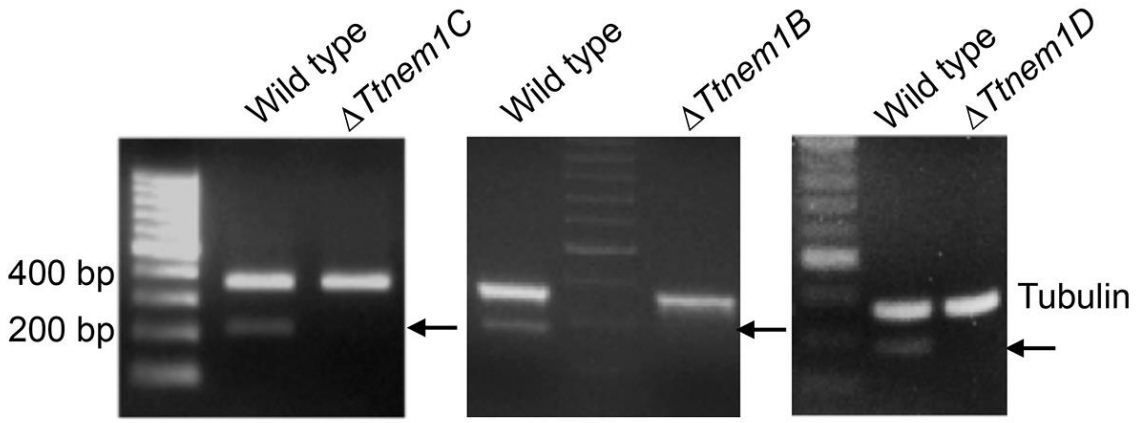


Figure 3.6: RT-PCR analysis for knockout confirmation.
Agarose gel image of RT-PCR products of Δ*Ttnem1C* cells (left), Δ*Ttnem1B* cells (middle) and Δ*Ttnem1D* cells (right) along with wild-type cells. The top band just below 400 bp marker corresponds to alpha-tubulin (387 bp). The absence of band of the respective gene (indicated by arrow) in Δ*Ttnem1C,* Δ*Ttnem1B,* and Δ*Ttnem1D* cells but their presence in the wild-type cells confirms that knockout is complete.

3.2.4 Growth analysis of *ΔTtnem1B, ΔTtnem1C, and ΔTtnem1D*

In *Saccharomyces cerevisiae, NEM1* is not essential for cell viability however null mutants exhibit a slightly reduced growth rate at higher temperatures (37°C) (186). To assess whether *TtNEM1* homologs are essential for normal growth of *Tetrahymena* we measured the growth rates of *ΔTtnem1B*, *ΔTtnem1C*, and *ΔTtnem1D* mutant cells and compared them to the wild-type cells. The growth of all the NEM1 mutant cells was similar to that of the wild-type cells (Figure 3.7), suggesting that these homologs are not essential for the normal growth of *Tetrahymena*. Moreover, Δ*Ttnem1B,* Δ*Ttnem1C,* or Δ*Ttnem1D* cells did not show any visible defect in their morphology.

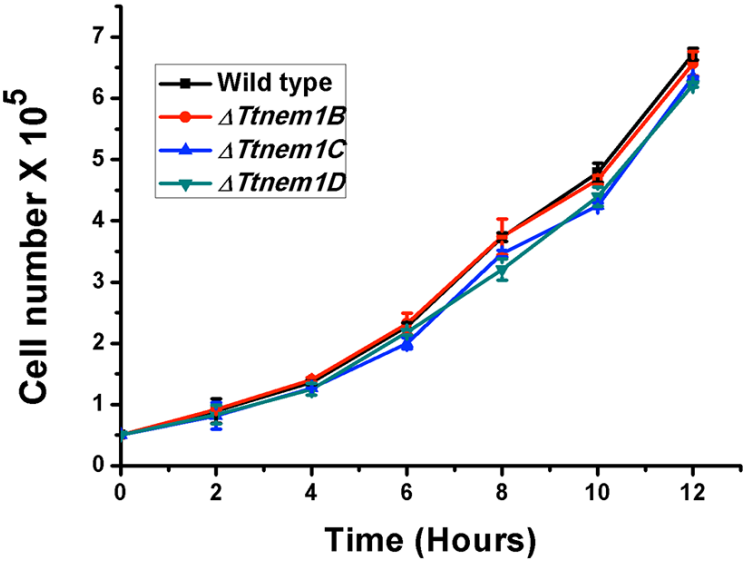


Figure 3.7: Growth curve of *Tetrahymena* NEM1 homologs
The cell numbers of wild-type, *ΔTtnem1B, ΔTtnem1C*, and *ΔTtnem1D* cells were counted every two hours, and the number of cells were plotted against time.

3.2.5 Lack of growth defect in *ΔTtnem1B* cells is not due to the overexpression of the other homologs

To evaluate if the lack of growth defect observed in NEM1 knockout cells is due to the compensatory overexpression of the other homologs, we carried out semi-quantitative reverse transcription-PCR (RT-PCR). To check the expression of *TtNEM1C* and *TtNEM1D* in Δ*Ttnem1B* and wild-type cells, *TtNEM1C* and *TtNEM1D* were amplified with the respective specific primers using cDNA from Δ*Ttnem1B* and wild-type cells as the template (Figure 3.8). Primers corresponding to alpha-tubulin were used as control. The band corresponding to *TtNEM1A*, *TtNEM1C* and *TtNEM1D* from Δ*Ttnem1B* and wild-type cells were quantitated and normalized with respect to the alpha-tubulin bands respectively. The expression of *TtNEM1A*, *TtNEM1C* or *TtNEM1D* was not enhanced in Δ*Ttnem1B* cells as compared to the wild-type cells (Figure 3.9). Similarly, the expression of *TtNEM1A*, *TtNEM1B* and *TtNEM1D* was checked in Δ*Ttnem1C* cells (Figure 3.10 and Figure 3.11) and the expression of

TtNEM1A, *TtNEM1B* and *TtNEM1C* was evaluated in Δ*Ttnem1D* (Figure 3.12 and Figure 3.13). These results suggest that the lack of growth defect in *ΔTtnem1B,* Δ*Ttnem1C* or Δ*Ttnem1D* mutant cells is not due to the compensatory overexpression of the other homologs.

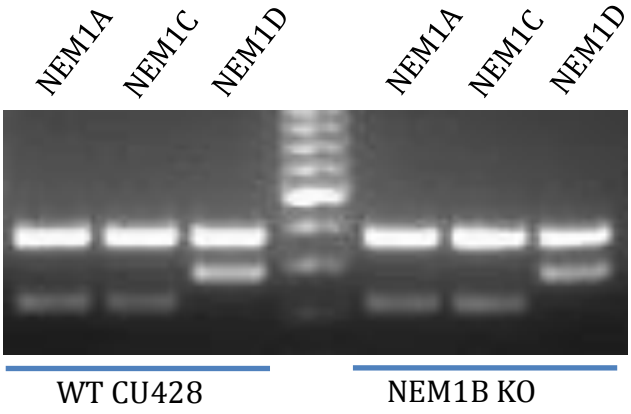


Figure 3.8: Semi-quantitative RT-PCR showing expression of NEM1 homologs in wild-type and Δ*Ttnem1B* cells.
Semi-quantitative RT-PCR showing expression of *TtNEM1A*, *TtNEM1C*, and *TtNEM1D* in wild-type (WT CU428) and Δ*Ttnem1B* cells (NEM1B KO). Lane 4, standard molecular weight marker; lane 1 to 3, amplified products of cDNA from wild-type cells; lane 5 to 7, amplified products of cDNA from Δ*Ttnem1B* cells. The top band in lanes 1 to 3 and 5 to 7 corresponds to alpha-tubulin (387 bp). The bottom band in lane 1 and 5 corresponds to TtNEM1A (221 bp), in lane 2 and 6 corresponds to TtNEM1C (220 bp), in lane 3 and 7 corresponds to TtNEM1D (282 bp).

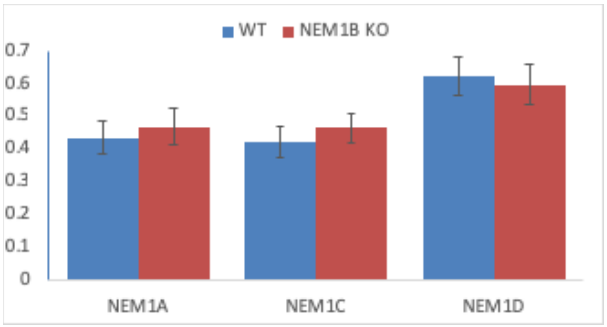


Figure 3.9: Quantitation of expression of NEM1 homologs in wild-type and Δ*Ttnem1B* cells.
The graph shows quantitation of TtNEM1A, TtNEM1C, and TtNEM1D after normalization with the alpha-tubulin. The expression of TtNEM1A, TtNEM1C, and TtNEM1D is not enhanced by the loss of TtNEM1B.

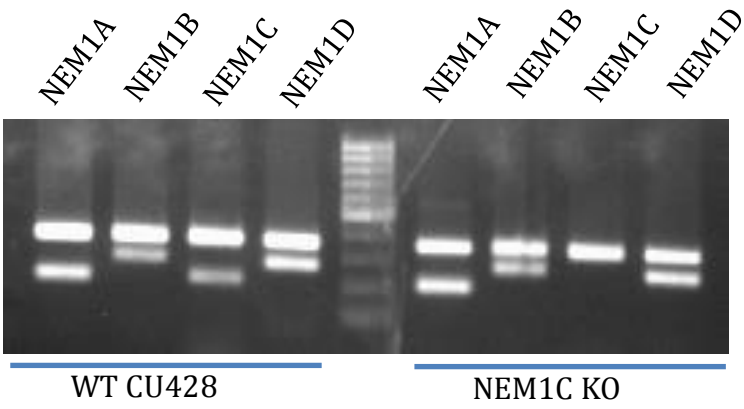


Figure 3.10: Semi-quantitative RT-PCR showing expression of NEM1 homologs in wild-type and Δ*Ttnem1C* cells.

Semi-quantitative RT-PCR showing expression of *TtNEM1A*, *TtNEM1C*, and *TtNEM1D* in wild-type (WT CU428) and Δ*Ttnem1C* cells (NEM1C KO). Lane 5, standard molecular weight marker; lane 1 to 4, amplified products of cDNA from wild-type cells; lane 6 to 9, amplified products of cDNA from Δ*Ttnem1C* cells. The top band in lanes 1 to 4 and 6 to 9 corresponds to alpha-tubulin (387 bp). The bottom band in lane 1 and 6 corresponds to TtNEM1A (221 bp), in lane 2 and 7 corresponds to TtNEM1B (295 bp), in lane 3 corresponds to TtNEM1C (220 bp) and in lane 4 and 9 corresponds to TtNEM1D (282 bp).

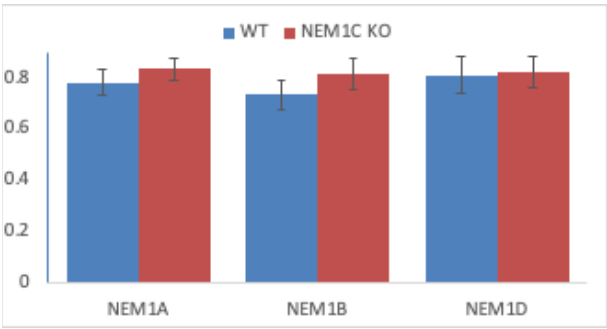


Figure 3.11: Quantitation of expression of NEM1 homologs in wild-type and Δ*Ttnem1C* cells.

The graph shows quantitation of TtNEM1A, TtNEM1B, and TtNEM1D after normalization with the alpha-tubulin. The expression of TtNEM1A, TtNEM1B, and TtNEM1D is not enhanced by the loss of TtNEM1C.

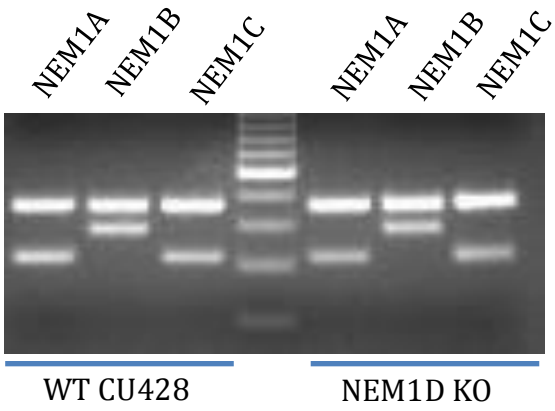


Figure 3.12: Semi-quantitative RT-PCR showing expression of NEM1 homologs in wild-type and Δ*Ttnem1D* cells.

Semi-quantitative RT-PCR showing expression of *TtNEM1A*, *TtNEM1B*, and *TtNEM1C* in wild-type (WT CU428) and Δ*Ttnem1D* cells (NEM1D KO). Lane 4, standard molecular weight marker; lane 1 to 3, amplified products of cDNA from wild-type cells; lane 5 to 7, amplified products of cDNA from Δ*Ttnem1D* cells. The top band in lanes 1 to 3 and 5 to 7 corresponds to alpha-tubulin (387 bp). The bottom band in lane 1 and 5 corresponds to TtNEM1A (221 bp), in lane 2 and 6 corresponds to TtNEM1B (295 bp), in lane 3 and 7 corresponds to TtNEM1C (220 bp).

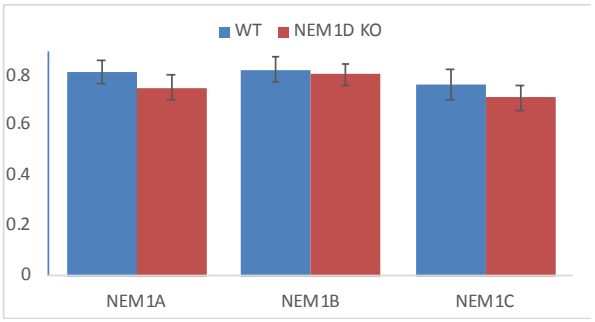


Figure 3.13: Quantitation of expression of NEM1 homologs in wild-type and Δ*Ttnem1D* cells.

The graph shows quantitation of TtNEM1A, TtNEM1B, and TtNEM1C after normalization with the alpha-tubulin. The expression of TtNEM1A, TtNEM1B, and TtNEM1C is not enhanced by the loss of TtNEM1D.

3.2.6 Nem1C in *Tetrahymena*, though of similar size to yeast Nem1, is not functionally equivalent.

Nem1p regulates lipid homeostasis and membrane biogenesis by dephosphorylating Pah1p (20, 190, 222). Of the *Tetrahymena* homologs, TtNem1C is most similar in

size to yeast Nem1 and therefore the putative *bona fide* ortholog. To evaluate the role of TtNem1C in lipid droplet biogenesis, the lipid droplets of both wild-type and Δ*Ttnem1C Tetrahymena* cells were stained with a neutral lipid dye Oil Red O. No significant reduction in the lipid droplet count was observed in Δ*Ttnem1C* as compared to the wild-type cells (Figure 3.14) as analyzed by the confocal images. The average lipid droplet count per cell was 304 in wild-type cells (n= 50) and 310 in Δ*Ttnem1C* (n= 50). The lipid droplet size of the wild-type and *ΔTtnem1C* cells was also quantitated using the Image J software. No significant difference was observed in the size of the lipid droplet in the wild-type and *ΔTtnem1C* cells. To find out if *TtNEM1C* regulates the ER morphology, we stained both Δ*Ttnem1C* and wild-type cells with ER-Tracker Green dye and evaluated the ER morphology by analyzing the confocal images. We did not observe any visible defect in the ER morphology and the ER content of Δ*Ttnem1C* cells as compared to the wild-type cells (Figure 3.15). These observations lead us to conclude that though TtNem1C is similar to yeast Nem1 in size, it is not functionally equivalent to yeast Nem1.

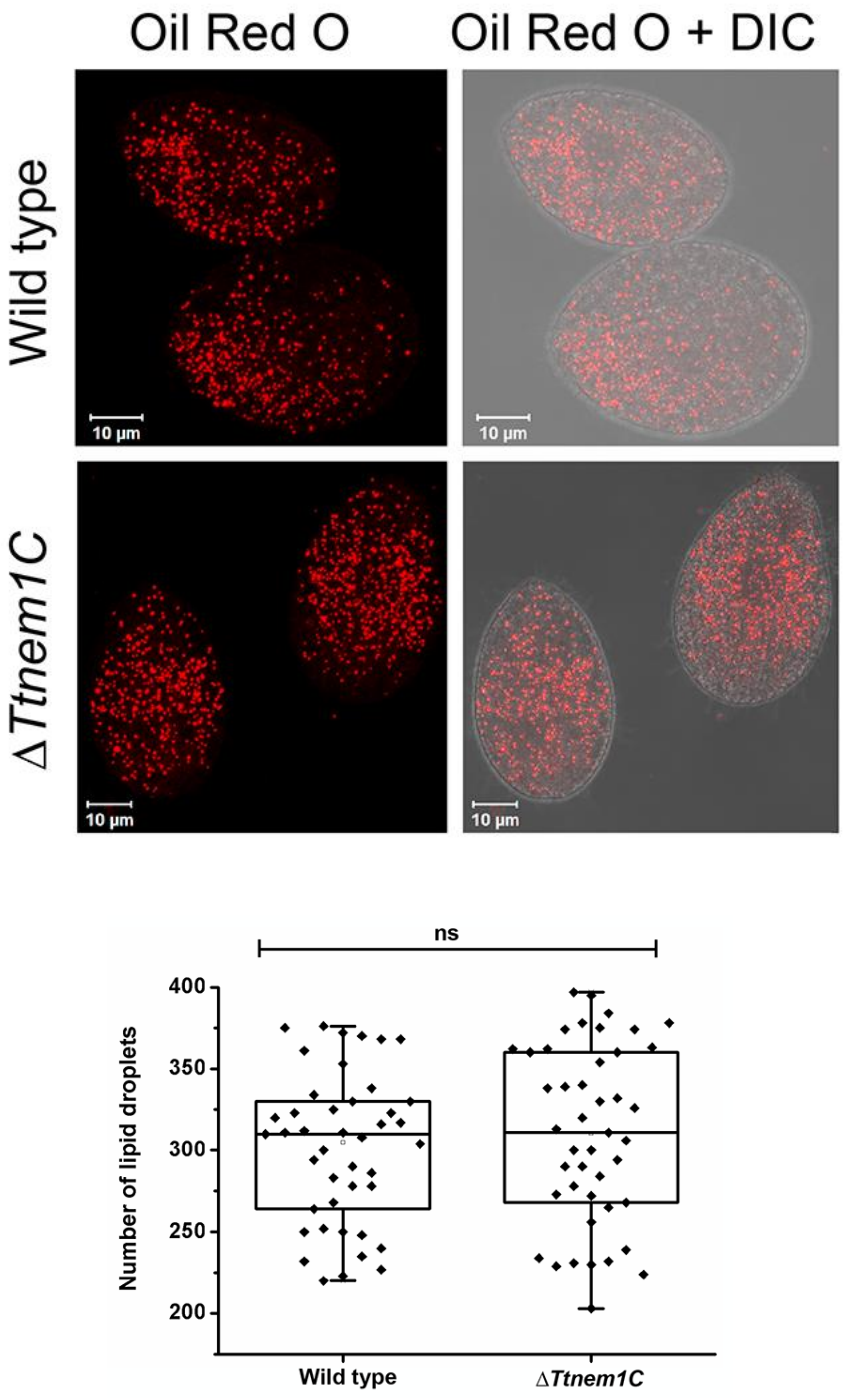


Figure 3.14: TtNem1C does not regulate the lipid droplet number in *Tetrahymena*.

Top panel- Confocal images of wild-type and, *ΔTtnem1C Tetrahymena* cells showing lipid droplets after staining with Oil Red O dye. Bottom panel- Box plot showing the distribution of lipid droplet numbers in wild-type and *ΔTtnem1C Tetrahymena* cells. No significant difference (indicated as 'ns') in the number of lipid droplets between *ΔTtnem1C* and wild-type cells was observed as analyzed by Kruskal-Wallis test ($p < 0.01$).

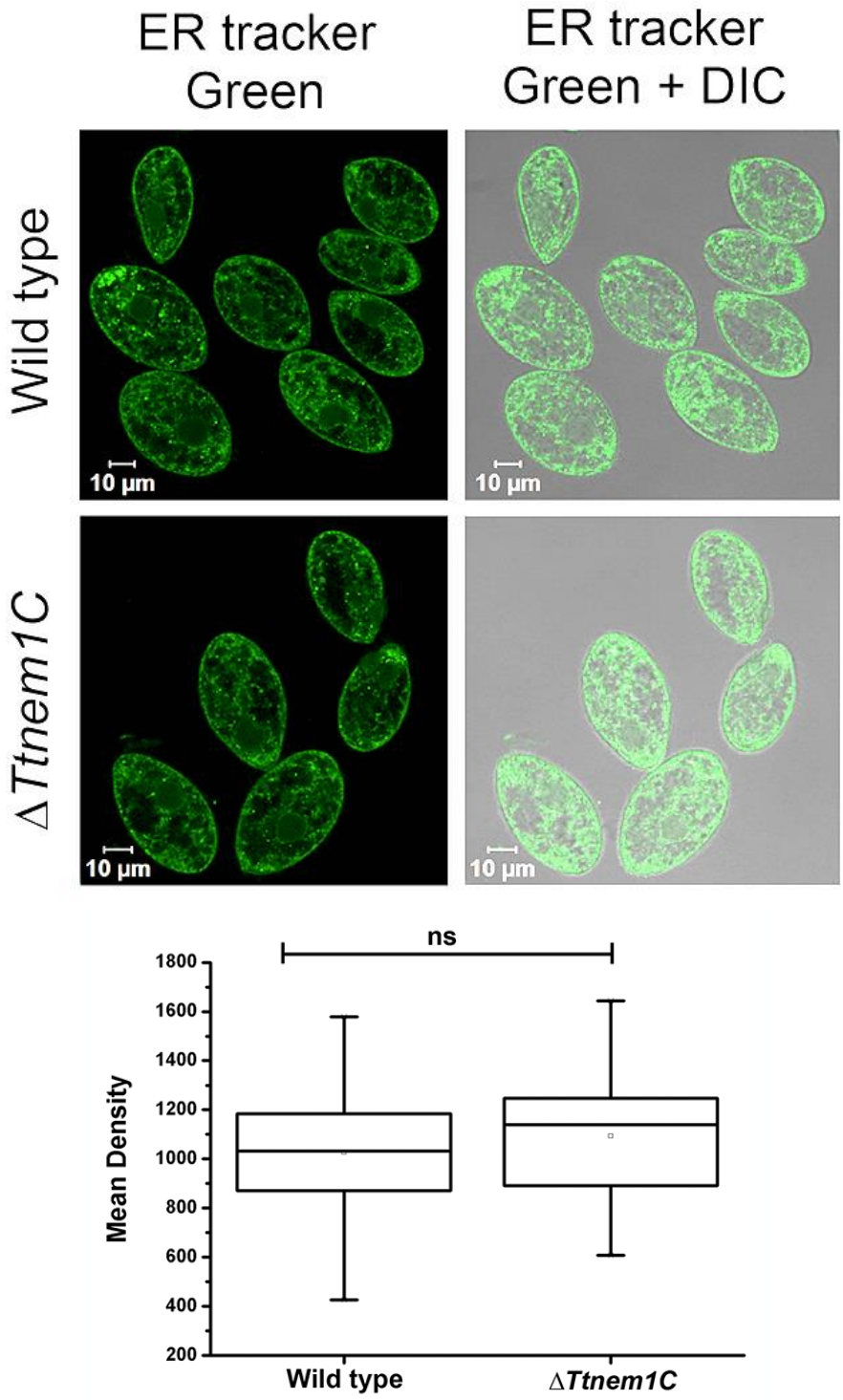
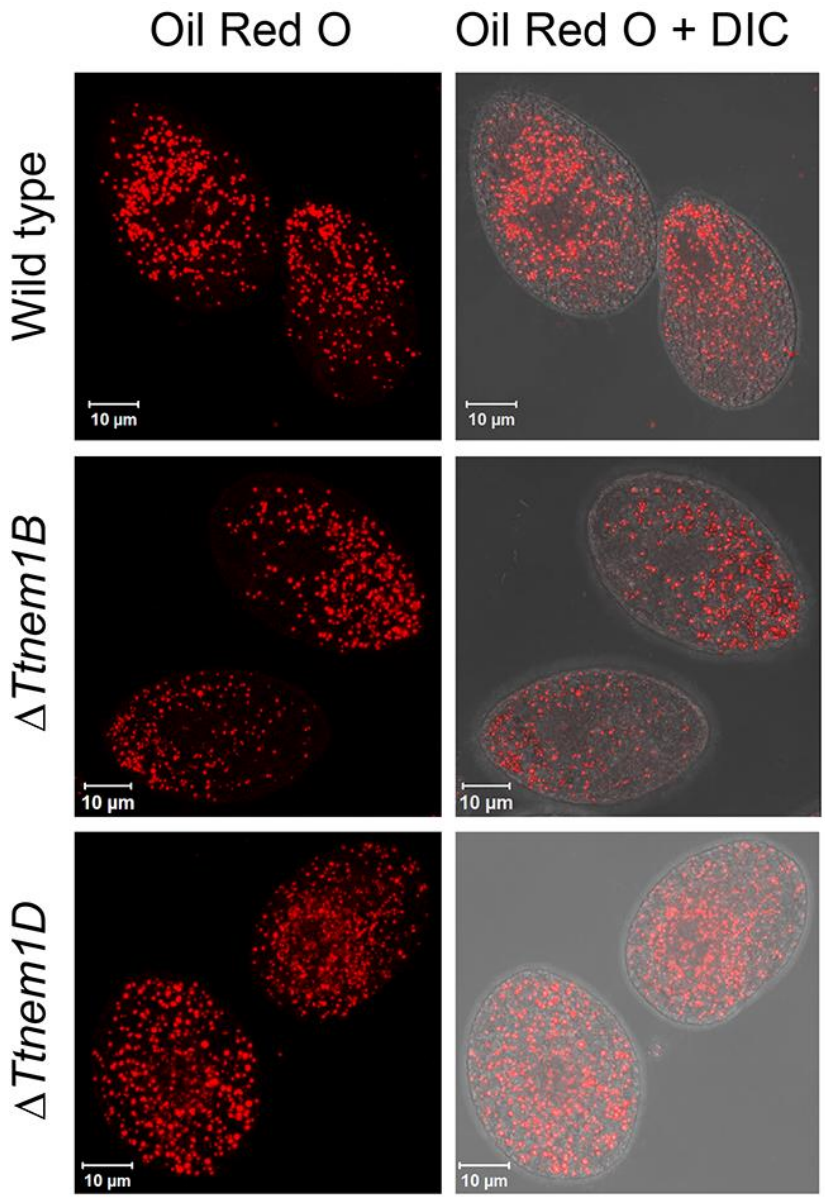
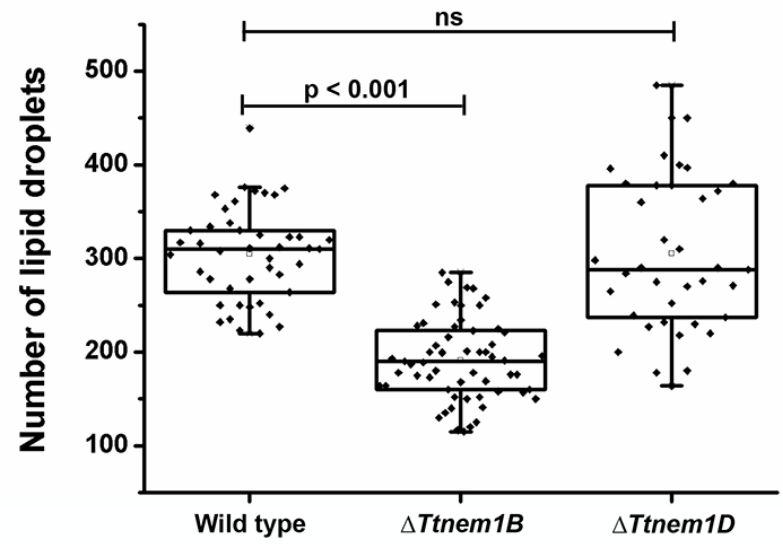


Figure 3.15: TtNem1C does not regulate ER morphology in *Tetrahymena*.
Top panel- Confocal images of wild-type and *ΔTtnem1C Tetrahymena* cells stained with ER-Tracker Green showing ER morphology. Bottom panel- Box plot showing the mean ER content in wild-type and, *ΔTtnem1C* cells as measured by sum intensity projection of the stacked images. There was no significant difference in the mean ER content between *ΔTtnem1C* and wild-type cells as analyzed by Kruskal-Wallis test ($p<0.01$). ns in the figure represents not significant.

3.2.7 *TtNEM1B* regulates lipid droplet biogenesis in *Tetrahymena*

Since, *TtNEM1C* did not regulate the lipid droplet biogenesis and ER morphology we investigated the potential role of other *Tetrahymena NEM1* homologs in this process. The lipid droplets of wild-type, Δ*Ttnem1B,* and Δ*Ttnem1D* cells, were stained with Oil Red O dye and visualized by confocal imaging (Figure 3.16). The average lipid droplet number per cell was 304 in wild-type (n= 44), 202 in Δ*Ttnem1B* (n= 59), and 305 in Δ*Ttnem1D* (n= 38) cells. The loss of TtNem1B led to a significant reduction in lipid droplet numbers (33%) whereas no reduction in lipid droplet number was observed in Δ*Ttnem1D* cells. Moreover, no visible difference was observed in the size of lipid droplets between Δ*Ttnem1B* and wild-type cells (Figure 3.16). These results suggest that Nem1B regulates lipid droplet biogenesis in *Tetrahymena*.


Oil Red O
Oil Red O + DIC
Wild type
ΔTtnem1B
ΔTtnem1D
10 µm


ns
p < 0.001
Number of lipid droplets
500
400
300
200
100
Wild type
ΔTtnem1B
ΔTtnem1D

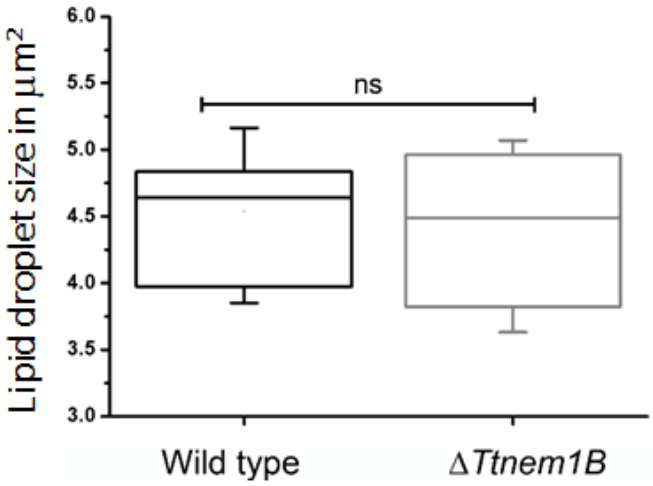


Figure 3.16: TtNem1B maintains lipid droplet number in *Tetrahymena*.
Top panel- Confocal images of wild-type, *ΔTtnem1B,* and *ΔTtnem1D Tetrahymena* cells showing lipid droplets after staining with Oil Red O dye. Middle panel- Box plot showing the distribution of lipid droplet numbers in wild-type, *ΔTtnem1B,* and *ΔTtnem1D Tetrahymena* cells. A significant difference was observed in the number of lipid droplets between *ΔTtnem1B* and wild-type cells ($p < 0.001$) whereas no significant difference was observed in the number of lipid droplets between *ΔTtnem1D* and wild-type cells as analyzed by Kruskal-Wallis test ($p < 0.01$) Bottom panel- Box plot showing the size distribution of lipid droplet in wild-type (n=20), and *ΔTtnem1B* (n=20) cells. No significant difference was observed in the size of lipid droplets as analyzed by Kruskal-Wallis test ($p < 0.01$).

3.2.8 Overexpression of *TtPAH1* partially restores the lipid droplet biogenesis in Δ*Ttnem1B* mutant *Tetrahymena* cells

In yeast, Nem1 functions via Pah1 and overexpression of *PAH1* rescues the defects observed due to loss of *NEM1* function (19, 277). We previously showed that loss of *TtPAH1* causes a severe reduction in lipid droplet number in *Tetrahymena* and overexpression of *TtPAH1* leads to increase in lipid droplet number (25). To assess if TtNem1B regulates lipid droplet biogenesis via Pah1 in *Tetrahymena*, we overexpressed *TtPAH1* in Δ*Ttnem1B* cells and evaluated the lipid droplet numbers in these cells and compared it with the Δ*Ttnem1B* cells. The expression of TtPAH1 was

confirmed by RT-PCR analysis (Figure 3.17). The analysis of Oil Red O stained confocal images showed that the mean lipid droplet number was 257 per cell in Δ*Ttnem1B* cells overexpressing *TtPAH1* (n=65), 202 per cell in Δ*Ttnem1B* cells (n=54), and 304 per cell in the wild-type cells (n=50) (Figure 3.18). We observed a ~50% rescue of the defect in lipid droplet number in Δ*Ttnem1B* by *TtPAH1* overexpression. These results suggest that TtNem1B regulates lipid droplet biogenesis via TtPah1.We further quantitated the levels of TAG in wild-type, Δ*Ttnem1B* and Δ*Ttnem1B* cells overexpressing *TtPAH1* by thin layer chromatography. We observed a reduction in TAG level in Δ*Ttnem1B* cells as compared to the wild-type cells that was increased upon the over-expression of *TtPAH1* (Figure 3.19). These observations suggest that *TtNEM1B* regulates lipid droplet biogenesis via *TtPAH1* by regulating the TAG levels.

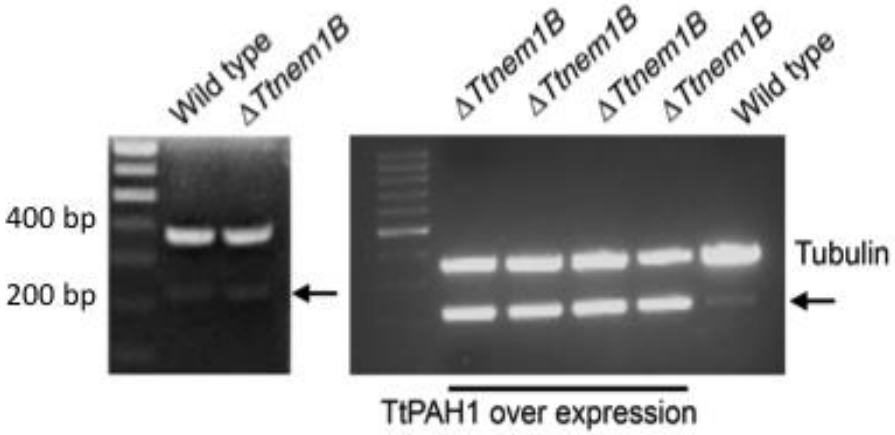


Figure 3.17: RT-PCR analysis of TtPAH1
Left panel shows amplified products of cDNA from wild-type cells (lane 2) and *ΔTtnem1B* cells (lane 3); lane1 is standard molecular weight marker. In lane 2 and 3 the top band corresponds to alpha-tubulin (387 bp) and the bottom band to *TtPAH1* (220 bp). Right panel shows amplified products of cDNA from *ΔTtnem1B* cells transformed with *TtPAH1* (lane 2 to 5*)*, from wild-type *Tetrahymena* cells (lane 6); lane 1 is standard molecular weight marker. In lane 2 to 6 the top band corresponds to alpha-tubulin (387 bp) and the bottom band to *TtPAH1* (220 bp). No visible difference in expression of *TtPAH1* was observed upon deletion of *TtNem1B* (left panel). As expected, significant overexpression of *TtPAH1* was observed in *ΔTtnem1B* cells transformed with *TtPAH1* (right panel).

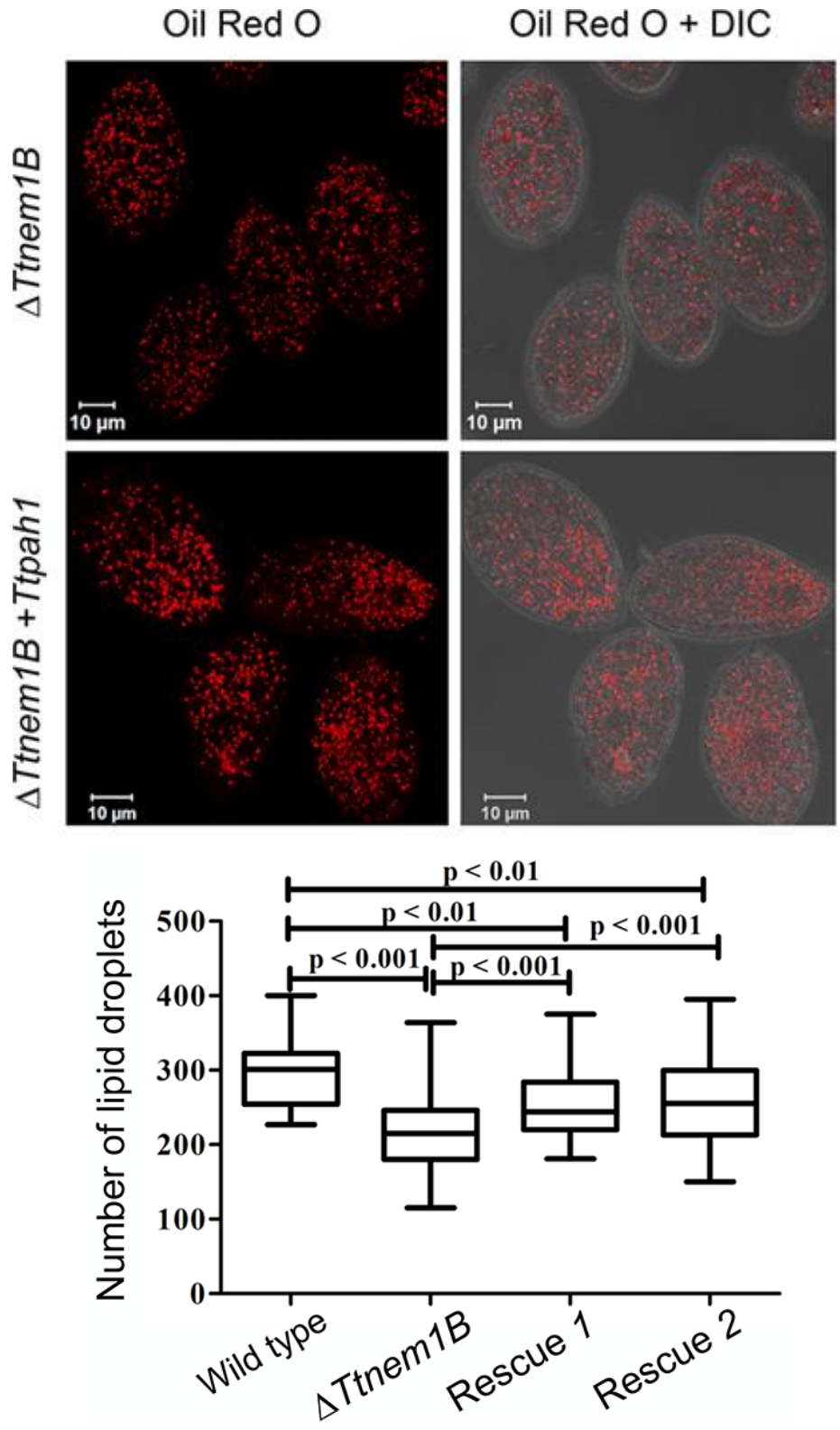


Figure 3.18: Overexpression of TtPAH1 partially restores lipid droplet number in Δ*Ttnem1B Tetrahymena* cells.

Top panel- Confocal images of *ΔTtnem1B* and *ΔTtnem1B* cells over-expressing *TtPAH1* showing lipid droplets after staining with Oil Red O dye. Bottom panel- Box plot showing lipid droplet numbers in wild-type cells, *ΔTtnem1B* cells and cells

overexpressing *TtPAH1* in *ΔTtnem1B* cells. Rescue 1 and rescue 2 are independent transformants overexpressing *TtPAH1* in *ΔTtnem1B* cells. An increase in lipid droplet number was observed upon overexpression of *TtPAH1* in *ΔTtnem1B* cells. P values are indicated in the figure. No significant difference was observed in the number of lipid droplets as analyzed by Kruskal-Wallis test ($p < 0.01$).

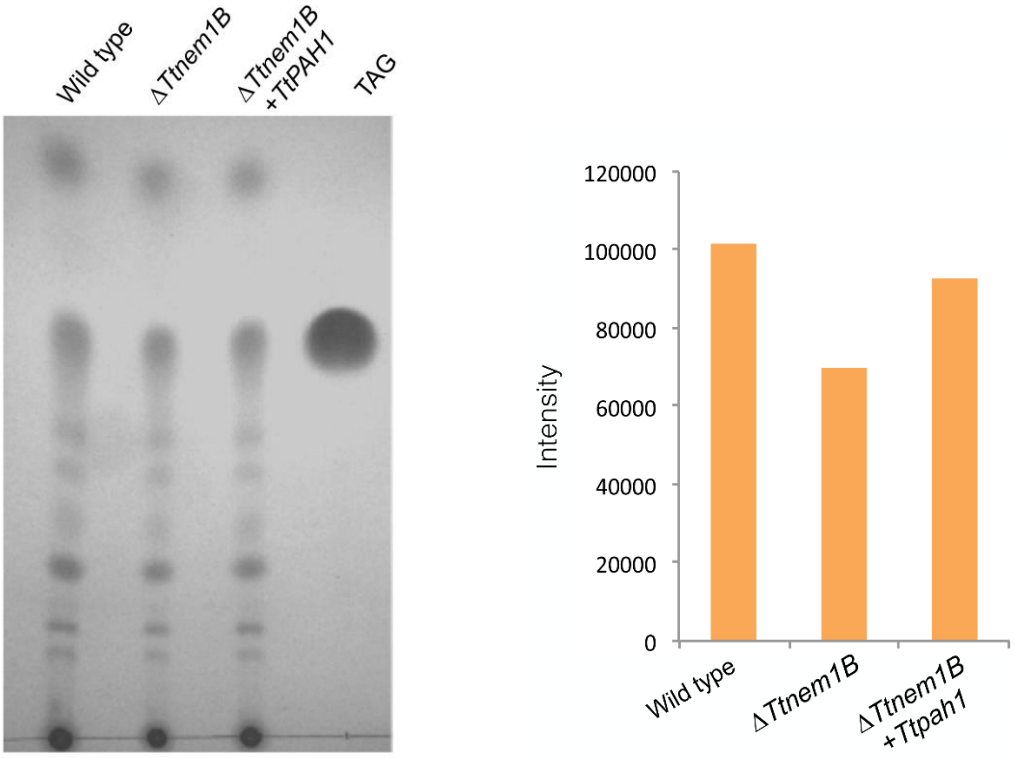
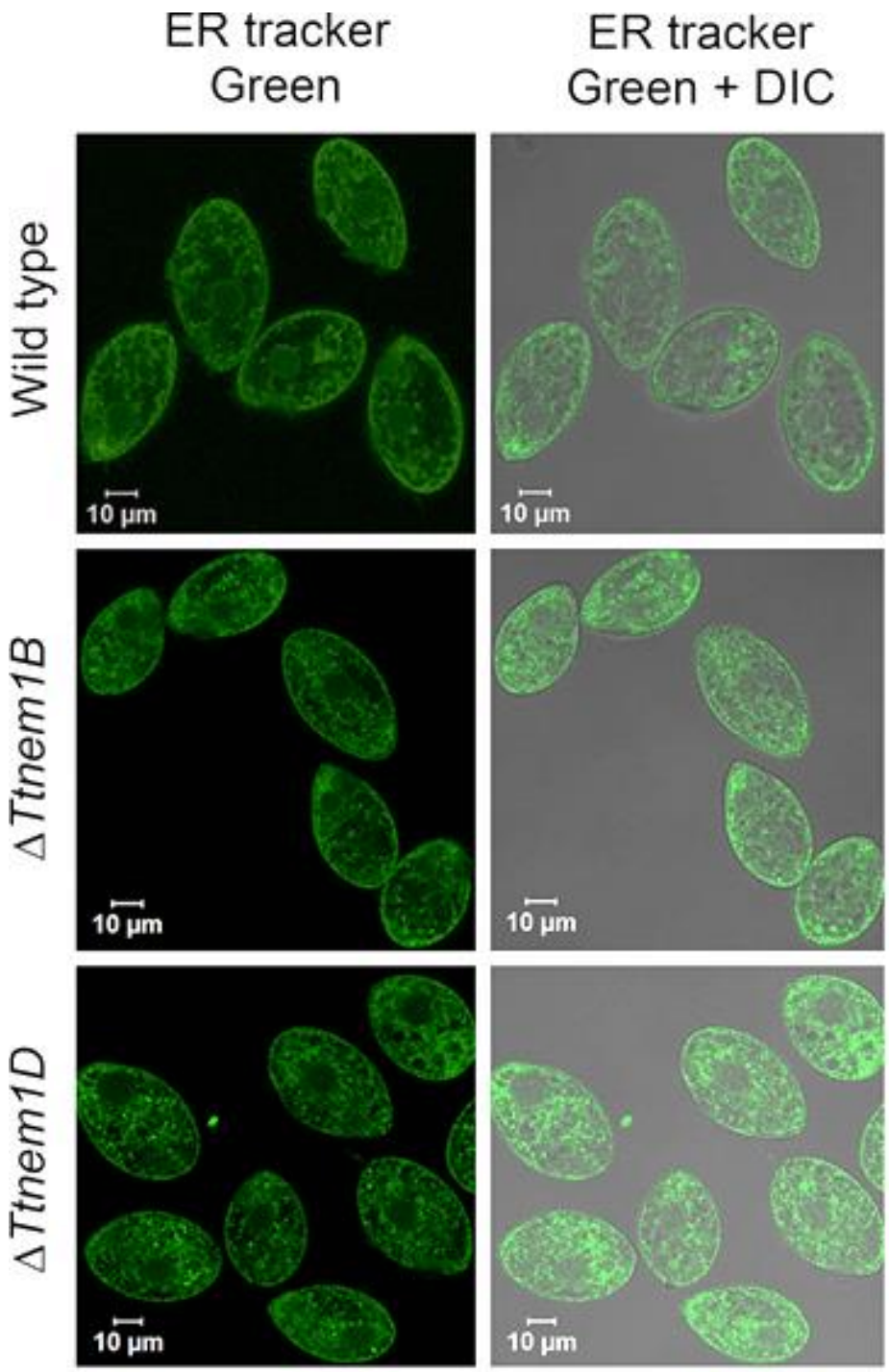


Figure 3.19: Overexpression of TtPAH1 partially restores the level of TAG in Δ*Ttnem1B Tetrahymena* cells.
Left panel- Total lipids were extracted from the wild-type, *ΔTtnem1B,* and *ΔTtnem1B* cells transformed with *TtPAH1* and subjected to thin layer chromatography to separate the neutral lipids. TAG standard was used to identify the appropriate position of TAG in the experimental samples. Right panel- The intensity of the bands corresponding to TAG of all three samples in the TLC plate was measured using the quantity-one software (Bio-Rad). Before plotting the graph, the intensity was normalized by dividing it with the respective total lipid.

3.2.9 *TtNEM1B* or *TtNEM1D* does not regulate ER structure

The ER is a complex network consisting of sheet and tubule like structures and serves as the primary site for *de novo* lipid biosynthesis (48, 54). In yeast and mammals, Nem1 regulates ER morphology (277). In *Tetrahymena TtPAH1*, but not *TtPAH2*,

regulates ER morphology (25, 26). To investigate if TtNem1B also regulates the ER morphology in *Tetrahymena*, we stained both the wild-type and Δ*Ttnem1B* cells with ER-Tracker Green dye and analyzed the ER morphology by confocal microscopy. The ER content was quantitated using ImageJ software (https://imagej.nih.gov/ij/). Surprisingly, both ER morphology and ER content of the Δ*Ttnem1B* cells was similar to that of the wild-type cells (Figure 3.20). Therefore, even though Nem1B in *Tetrahymena* regulates the lipid droplet biogenesis, it does not regulate the ER morphology. Since TtNem1B did not regulate the ER morphology, we, therefore, checked whether a different homolog, TtNem1D, might play that role by analyzing the Δ*Ttnem1D* cells stained with the ER tracker Green dye. No visible defect was observed in the ER morphology and ER content of the Δ*Ttnem1D* cells compared to the wild-type cells (Figure 3.20). Overall, these results suggest that neither Nem1B nor Nem1D is required to regulate the ER structure in *Tetrahymena*.


ER tracker Green
ER tracker Green + DIC
Wild type
ΔTtnem1B
ΔTtnem1D
10 µm
10 µm
10 µm
10 µm
10 µm
10 µm

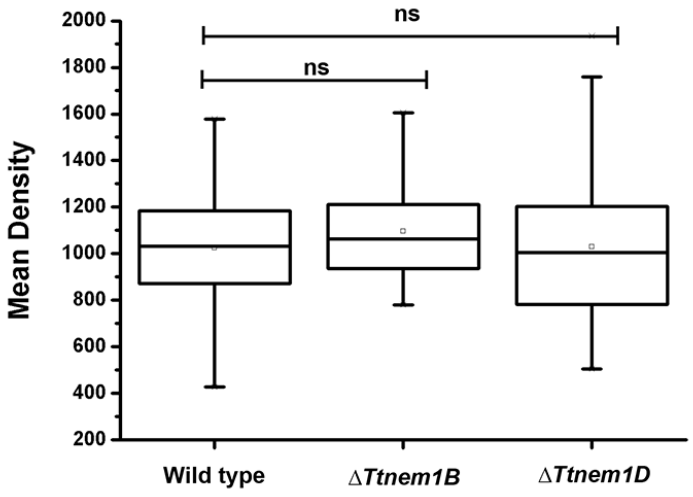


Figure 3.20: TtNEM1B and TtNEM1D do not regulate ER morphology.
Top panel shows confocal images of wild-type, *ΔTtnem1B,* and *ΔTtnem1D* cells stained with ER-Tracker Green showing ER morphology. Bottom panel- Box plot showing the mean density of ER-Tracker dye in wild-type, *ΔTtnem1B,* and *ΔTtnem1D* cells as measured by sum intensity projection of the stacked images. ns indicates non-significant.

3.2.10 Loss of *TtNEM1B, TtNEM1C* or *TtNEM1D* does not manifest visible nuclear morphology defect in *Tetrahymena*

In most eukaryotic cells, the nuclei are generally round or oval in shape. Nevertheless, aberrant nuclear morphology is observed in various physiological processes and disease pathologies (278–280). The exact means by which nuclear morphology is maintained in yeast is not well understood. Various factors associated with alteration in nuclear shape have been identified (45, 232). Nem1 and Spo7 integral membrane proteins influence the nuclear morphology in yeast (222). Loss of Nem1 or Spo7 causes the nucleus to divide into two large lobes connected by a narrow bridge, and long nuclear envelope extensions (222). Interestingly, the genetic material in these cells is excluded from the altered nuclear shape. *Tetrahymena thermophila* harbors one polyploid, somatic macronucleus (MAC) and a diploid transcriptionally silent germline micronucleus (MIC) (22, 281, 282).

In yeast, cells lacking *NEM1, SPO7* or *PAH1* cause abnormal expansion of nuclear envelope that appears as nuclear membrane projections (145, 283). Lipin inactivation in higher organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* also cause nuclear defects (194, 275).

To detect any defect in nuclear morphology upon deletion of *Tetrahymena NEM1* homologs, the isolated nuclei (both MAC and MIC) of Δ*Ttnem1B,* Δ*Ttnem1C,* Δ*Ttnem1D,* and the wild-type cells were stained with a lipophilic dye (DHCC), and DAPI and the nuclear membrane was visualized using a fluorescence microscope. The nuclear envelope of MAC or MIC did not show any visible defect in the structure (Figure 3.21) suggesting that unlike yeast, there is no nuclear membrane expansion observed in *Tetrahymena* upon deletion of *NEM1*.

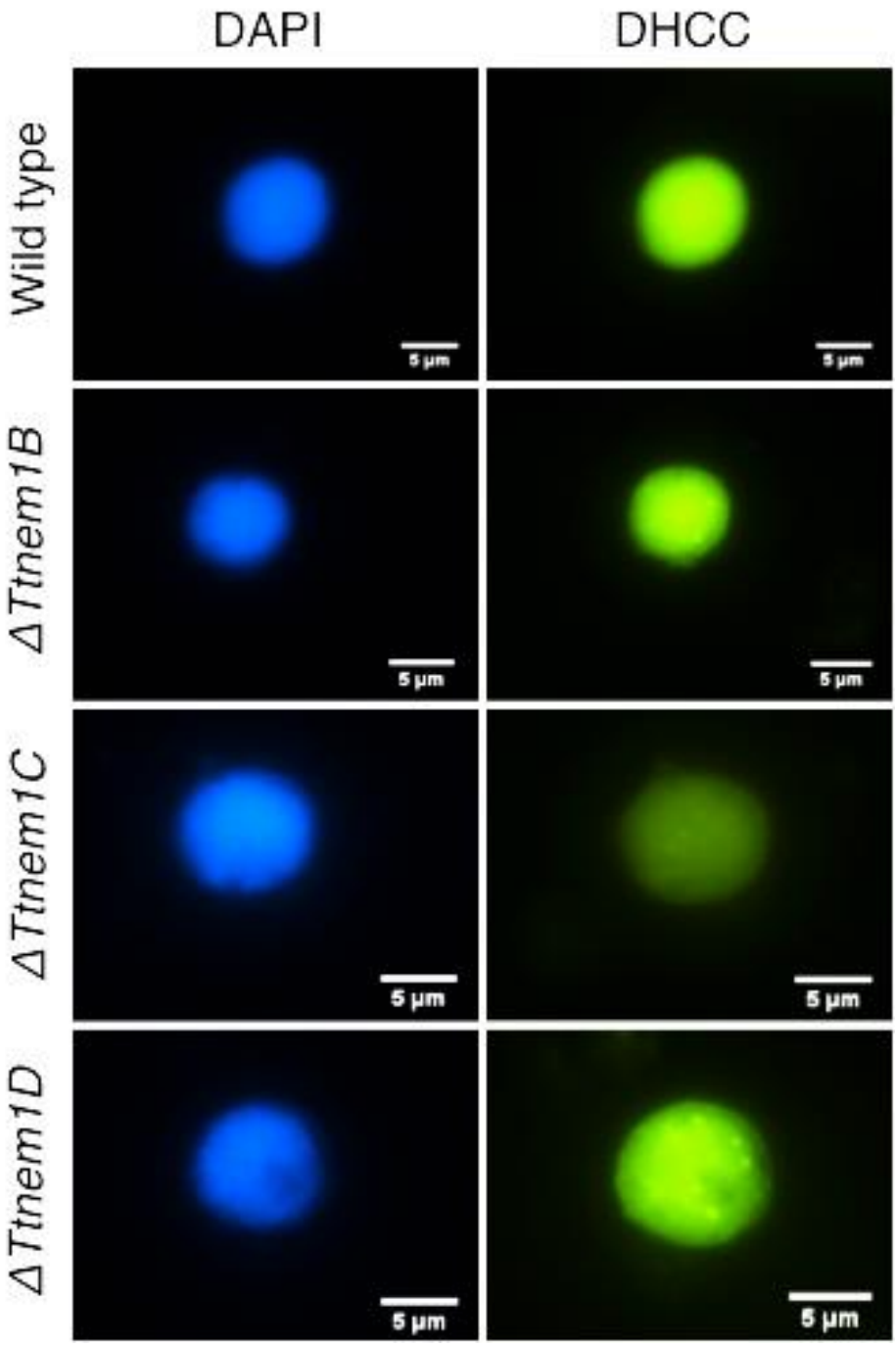


Figure 3.21: Loss of NEM1 homologs does not show nuclear defect in *Tetrahymena*

Fluorescence images of *Tetrahymena* nuclei of wild type, Δ*Ttnem1B,* Δ*Ttnem1C,* and Δ*Ttnem1D* cells after staining with DHCC and DAPI.

3.3 DISCUSSION

In yeast, lipid homeostasis and membrane biogenesis are regulated by a cascade comprising of Pah1 (a phosphatidate phosphatase) and Nem1-Spo7 (a cell cycle-regulated protein phosphatase) complex and a similar cascade is also reported in the other organisms like plants, mammals, worms, and flies (181, 184, 197, 275). The loss of NEM1 results in growth defect, reduced lipid droplet number and ER/nuclear membrane defect in yeast and metazoans (28, 175, 181, 185, 239). In this study, we investigated the role of four putative *Tetrahymena* NEM1 homologs and established that TtNem1B along with TtPah1 is involved in the regulation of lipid droplet biogenesis.

It has been shown previously that the loss of *NEM1* in yeast leads to a growth defect (14, 20). We found that TtNem1B, TtNem1C, and TtNem1D are not essential for normal growth of *Tetrahymena*. The lack of growth defect could be attributed to the compensatory overexpression of the remaining homologs. However, we found that the lack of growth defect in knockout cells is not due to the overexpression of the other homologs as confirmed by semi-quantitative reverse transcription-PCR.

Dephosphorylation of Pah1 by Nem1-Spo7 regulates its PAP activity by promoting its translocation from the cytoplasm into ER, where it converts PA to DA. DAG is used for the synthesis of triacylglycerol; the storage lipid constituting the lipid droplet. Deletion of PAH1 or NEM1 in yeast leads to decrease in lipid droplet number. The reduction in lipid droplet number results due to defect in the TAG production. Deletion of TtPAH1 in *Tetrahymena* also causes severe reduction in lipid droplet count. We establish that TtNem1B regulates lipid droplet biogenesis, whereas neither TtNem1C nor TtNem1D appears to be required for this function since only the loss of TtNem1B results in a reduction in the lipid droplet count. These results suggest that

Tetrahymena NEM1B may be the ortholog of yeast NEM1. The number of lipid droplet is more in the Δ*Ttnem1B* as compared to Δ*Ttpah1* mutants. This suggests that TtPah1 could retain some of its in vivo PAP activity even in the absence of TtNem1B. It is also possible that *Tetrahymena* Nem1A is involved in lipid droplet biogenesis. As discussed earlier, we failed to generate the knock out strain of TtNEM1A in multiple attempts suggesting that TtNEM1A is an essential gene in *Tetrahymena* and is involved in other as yet unknown processes. However, the conserved cascade of lipid homeostasis and membrane biogenesis, comprising Pah1 and Nem1/Spo7, is not essential in any organism studied so far including *Tetrahymena*.

Nem1 regulates lipid homeostasis and membrane biogenesis by dephosphorylating Pah1 (14, 17, 185). The overexpression of TtPAH1 in *ΔTtnem1B* cells partially rescued the lipid droplet biogenesis defect. The rescue may be because of the limiting kinase activity needed to phosphorylate the large pool of overexpressed Pah1. These findings suggest that TtPah1 together with TtNem1B forms a cascade for the regulation of lipid homeostasis and membrane biogenesis in *Tetrahymena*.

Nem1 regulates lipid droplet biogenesis and membrane biogenesis via Pah1. The loss of Nem1 or Pah1 function in yeast results in a defect in ER morphology (284, 285). Interestingly, the loss of TtNem1B does not result in a defect in ER morphology in *Tetrahymena*. This was surprising since TtNem1B functions via Pah1 similar to Nem1 of other organisms, and the loss of TtPAH1 produces a visible defect in the ER morphology (25). Pah1 dephosphorylates PA in the ER membrane to synthesize DAG which acts as the precursor for the synthesis of both TAG and membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (4, 15, 173, 286). In addition to its function in dephosphorylating PA, an independent role of Pah1 as a transcriptional regulator is known in yeast and mammals (202, 217).

Therefore, it is possible that *Tetrahymena* Pah1 also has an additional, Nem1-independent function in regulating the ER morphology. Alternatively, it is possible that in *ΔTtnem1B* cells the heterogeneous phosphorylation state of TtPah1 results in a cohort of protein that is sufficient to regulate the ER morphology but insufficient to stimulate the lipid droplet formation to the wild-type level. Another possible explanation could be the availability of other proteins that could supplement for the loss of TtNem1B function. However, further studies are needed to understand the lack of Nem1 function in regulating the ER morphology in *Tetrahymena*.

PAH1 and NEM1 regulate nuclear membrane expansion in yeast. Deletion of NEM1 in yeast results in accumulation of PA due to loss of phosphatidate phosphatase function of PAH1 thereby leading to the excess synthesis of membrane phospholipids PE and PC via the CDP-DAG pathway. Excess production of membrane phospholipids results in aberrant nuclear expansion. Our results show that TtNEM1B, TtNEM1C or TtNEM1D are not essential for maintaining normal nuclear morphology in *Tetrahymena*. Though *Tetrahymena* possesses the CDP-DAG pathway for phospholipid synthesis, the deletion of NEM1 homologs does not exhibit aberrant expansion of the nucleus.

Our results indicate that a conserved cascade comprising Nem1 and Pah1 functions regulate lipid homeostasis in *Tetrahymena*.

A phylogenetic tree comparing the sequence of *T. thermophila* Nem1 orthologs with yeast and human Nem1 shows that there is a high similarity between TtNEM1B and human Nem1 and yeast Nem1 (Figure 3.22). In summary, *Tetrahymena* possesses multiple Nem1 homologs. Of these, TtNEM1B is functionally related to NEM1 in other organisms.

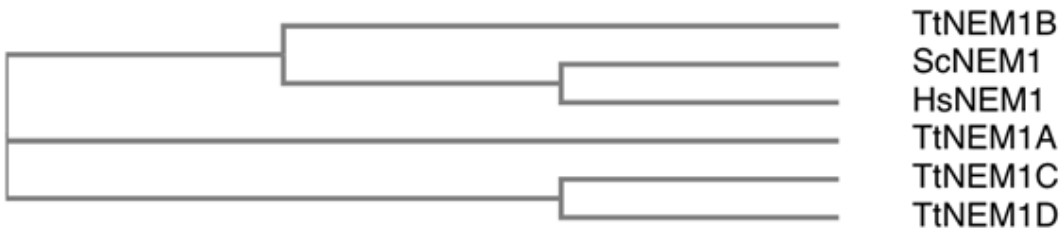


Figure 3.22: Phylogenetic analysis of NEM1 orthologs.
The phylogenetic tree shows the probable relationships analyzed among yeast, Human and *Tetrahymena* NEM1 homologs by Neighbour-joining method. The phylogram was generated using Clustal Omega algorithm on the EBI public https://www.ebi.ac.uk/Tools/msa/clustalo/.

Chapter 4

ANALYSIS OF FUNCTIONAL CONSERVATION OF *TETRAHYMENA* NEM1 HOMOLOGS IN YEAST

4.1 INTRODUCTION

Nem1 (catalytic subunit) and Spo7 (regulatory subunit) constitute a highly conserved membrane-associated protein phosphatase complex, which dephosphorylates and recruits Pah1 to the nuclear/ER membrane (20, 186, 190). The dephosphorylation of Pah1 exclusively by Nem1-Spo7 acts as a rate-limiting step and is essential for its PAP activity. Thus, this cascade is essential in the regulation of lipid metabolism and membrane biogenesis in yeast and mammals (18, 229).

The conserved C-terminal domain of Nem1p is sufficient and essential for binding to Spo7p in yeast (222). Cells lacking either the Nem1 or Spo7 subunits of the complex in yeast, mirror the phenotypes of *pah1Δ* cells including increased phospholipid biosynthesis, aberrant expansion of the nuclear/ER membrane, decreased lipid droplet number, growth inhibition and temperature-sensitivity (28, 145, 174). The *nem1Δ spo7Δ*, double mutants also show phenotypes similar to *pah1Δ* or *nem1Δ* or *spo7Δ*, suggesting that these three proteins work in unison (17, 35, 186). The human CTDNEP1-NEP1R1 complex, mammalian ortholog of NEM1-SPO7, can functionally replace yeast NEM1-SPO7 suggesting that Nem1-Spo7 phosphatase is evolutionarily conserved between yeast and mammals (20). We previously reported that the loss of *Tetrahymena* PAH1 homolog, TtPAH1, causes severe reduction in lipid droplet number and affected the nuclear and endoplasmic reticulum morphology (25). We also found that TtPAH1 functionally complements yeast PAH1 (25).

This chapter discusses the functional conservation of NEM1 proteins between yeast and *Tetrahymena* as assessed by complementation of *nem1Δ* yeast cells with TtNEM1 homologs.

4.2 RESULTS

4.2.1 *Tetrahymena* Nem1 homologs do not functionally replace yeast Nem1

Yeast mutant cells lacking either *PAH1* or *NEM1* exhibit aberrant nuclear expansion and slow growth defect (174, 277). The nucleus is elongated and has projections in the mutants rather than spherical as in wild-type cells (17, 186). Yeast Nem1 can be functionally replaced by its mammalian ortholog, CTDNEP1 (29). Similarly, we previously reported that *Tetrahymena TtPAH1* functionally complements yeast *PAH1* (25).

To investigate if *TtNEM1B* complements the loss of yeast *NEM1*, we overexpressed *TtNEM1B* in the *nem1*Δ yeast cells and assessed its ability to rescue the aberrant nuclear morphology and slow growth phenotypes. A nucleoplasmic marker, PUS-GFP, was expressed to analyze the nuclear morphology in yeast. Surprisingly, *TtNEM1B* failed to rescue either the aberrant nuclear morphology or slow growth phenotypes of the yeast *nem1*Δ mutant cells (Figure 4.1 and Figure 4.2).

To examine if the other *NEM1* homologs of *Tetrahymena* could functionally replace yeast *NEM1*, we overexpressed *TtNEM1A*, *TtNEM1C,* and *TtNEM1D* in *nem1*Δ yeast cells and evaluated the nuclear morphology and growth phenotype. *TtNEM1A, TtNEM1C,* and *TtNEM1D* did not rescue the defects (Figure 4.1 and Figure 4.2).

Interestingly, we found that the growth of *nem1Δ* yeast cells overexpressing TtNEM1A or TtNEM1C is slower than that of *nem1Δ* yeast cells. How TtNEM1A or TtNEM1C affects the growth of *nem1*Δ yeast cells is not known. It could be possible that TtNem1A and TtNem1C have an inhibitory effect on some essential process of yeast. To eliminate the possibility that the absence of rescue is due to the lack of gene expression, we confirmed the expression of *TtNEM1A, TtNEM1B, TtNEM1C,* and *TtNEM1D* homologs in *nem1*Δ yeast cells by reverse transcription PCR analysis.

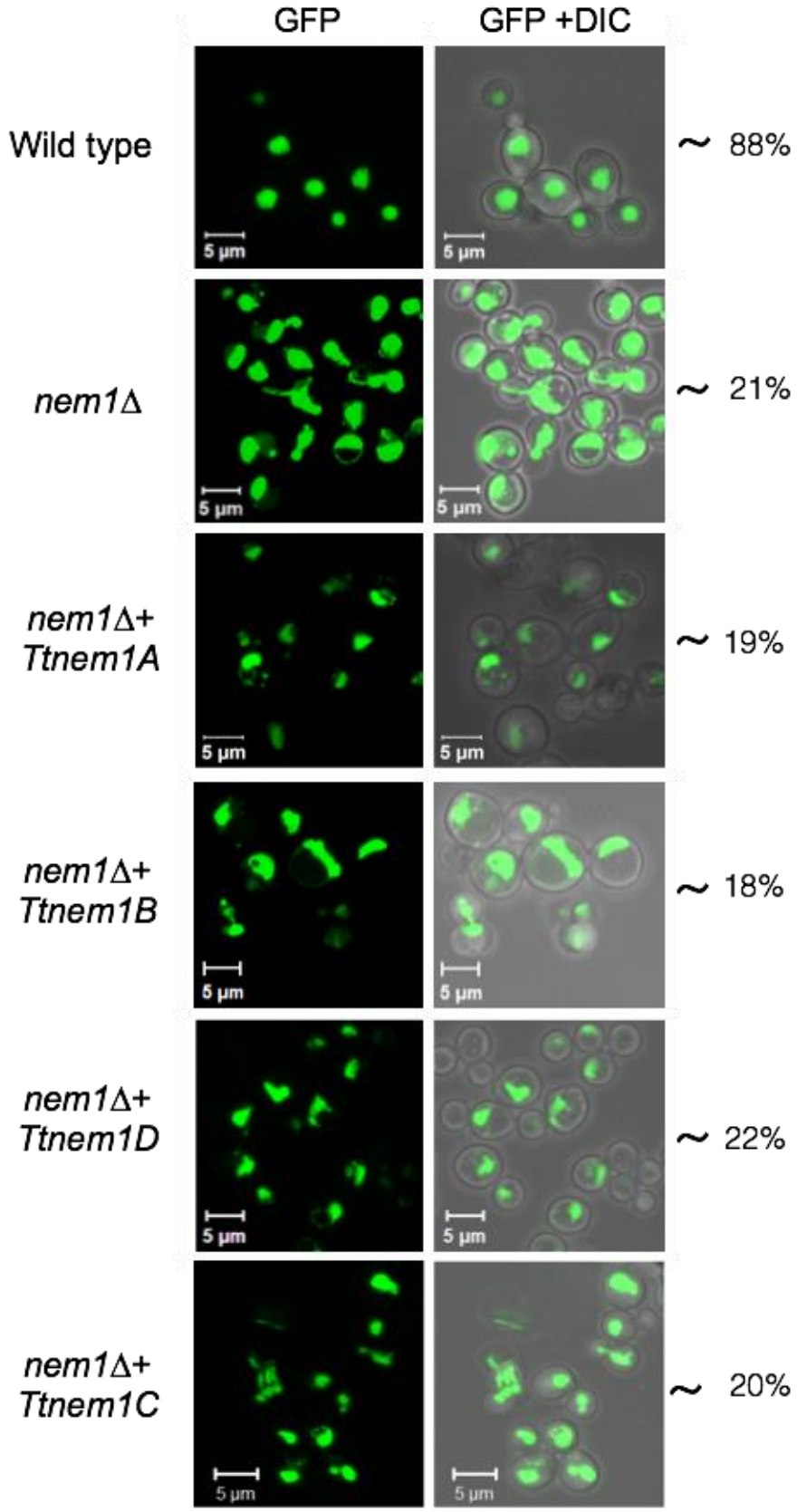


Figure 4.1: *TtNEM1A*, *TtNEM1B*, *TtNEM1C*, and *TtNEM1D* homologs do not rescue the nuclear phenotype of yeast *NEM1*.

Confocal images of *nem1Δ* yeast cells transformed with either empty vector or *TtNEM1A* or *TtNEM1B*, or *TtNEM1D* along with the wild-type cells. PUS1-GFP (a nucleoplasmic marker) was expressed to visualize the nuclei. *TtNEM1* homologs do not restore the aberrant nuclear morphology of the *nem1Δ* yeast cells. Three different transformants per strain were analyzed and the number of cells counted for each transformant was 100 (n= 300). The percentage of cells containing round nucleus is indicated on the right.

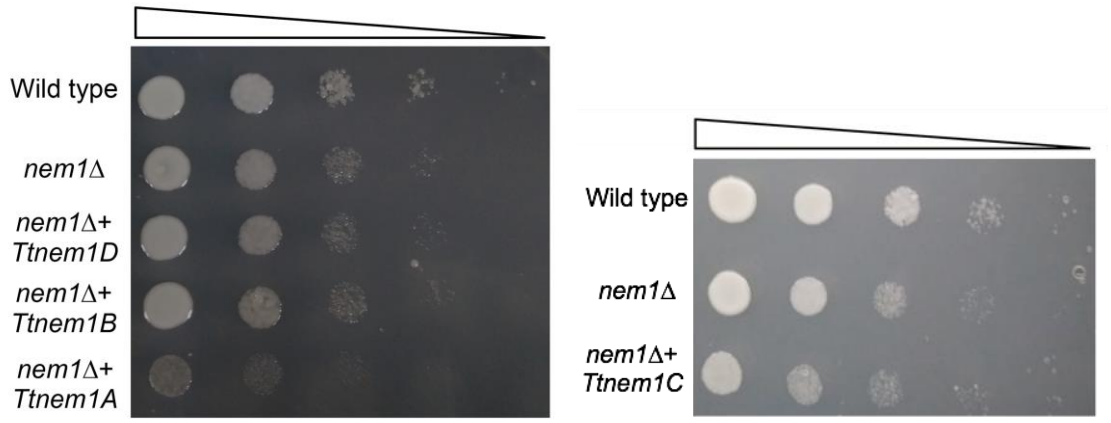


Figure 4.2: *TtNEM1A, TtNEM1B, TtNEM1C,* and *TtNEM1D* homologs do not restore the slow growth and temperature-sensitive phenotype of yeast *NEM1*.
The growth of *nem1Δ* yeast cells transformed with either empty vector or *TtNEM1B,* or *TtNEM1D,* or *TtNEM1A* or *TtNEM1C* along with the wild-type yeast cells grown at 30℃ on SD media lacking leucine and uracil.

4.2.2 Replacing CTD of TtNEM1B with yeast NEM1 does not restore the slow growth, temperature-sensitivity and nuclear defect phenotype of *nem1*Δ yeast cells

Since, *TtNEM1* homologs do not functionally replace yeast NEM1, we hypothesized that the yeast SPO7 (regulatory subunit) does not recognize *Tetrahymena* NEM1. To test this, we replaced the C-terminal domain (CTD) of *TtNEM1B* of *Tetrahymena* with the C-terminal domain of yeast Nem1 since the CTD of Nem1 interacts with Spo7 subunit in yeast. The amino acid sequences of the CTD of yeast Nem1 may be critical for its interaction with Spo7. The replacement of domain was done using Gibson assembly (Figure 4.3) and the construct generated

(TtNEM1B+ScNEM1_CTD) was confirmed by PCR amplification and sequence analysis. The rescue of the slow growth and aberrant nuclear phenotypes of *nem1*Δ yeast cells transformed with TtNEM1B+ScNEM1_CTD were assessed. TtNEM1B+ScNEM1_CTD failed to rescue the slow growth and nuclear phenotypes of *nem1*Δ yeast cells (Figure 4.4 and Figure 4.5). This suggests that either the chimeric protein is not functional or the amino acid in the NTD of yeast NEM1 which is different in TtNEM1B is also important for the function.

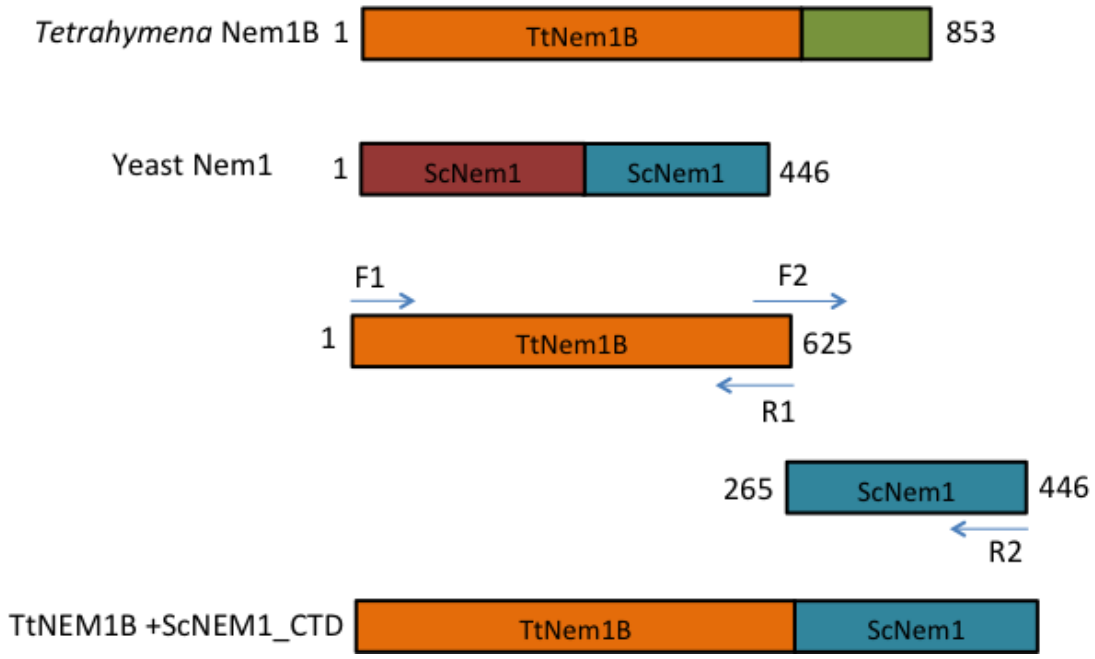


Figure 4.3: Schematic representation of yeast NEM1 and *Tetrahymena* NEM1B. The numbers represent the amino acid position. TtNEM1B+ScNEM1_CTD was generated by replacing the CTD of TtNEM1B with ScNEM1.

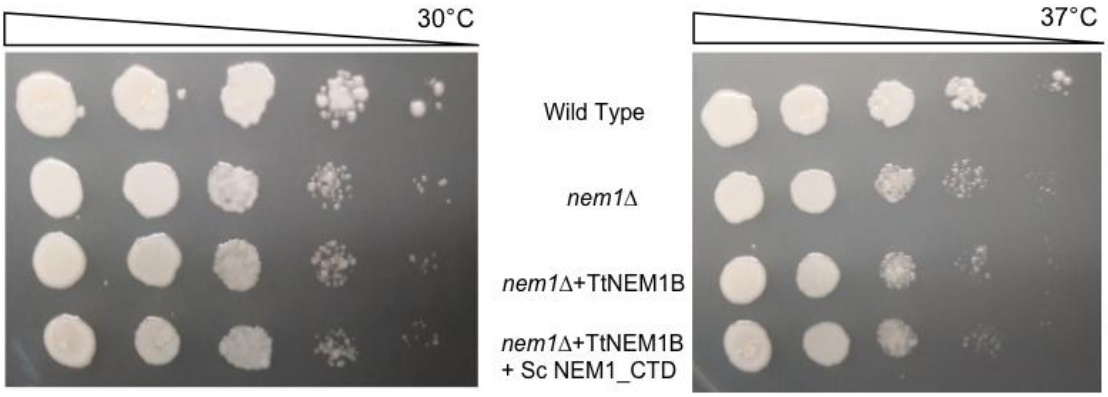


Figure 4.4: TtNEM1B+ScNEM1_CTD does not rescue the slow growth phenotype of *nem1*Δ yeast cells

Cultures of *nem1*Δ yeast cells transformed with empty vector, or TtNEM1B or TtNEM1B+ScNEM1_CTD along with wild-type yeast cells were adjusted to OD_{600}= 1 followed by 10-fold serial dilutions. Five microliters of each were spotted on the SD plates containing glucose but lacking leucine, and uracil and were incubated for 3 days at 30°C or 37°C.

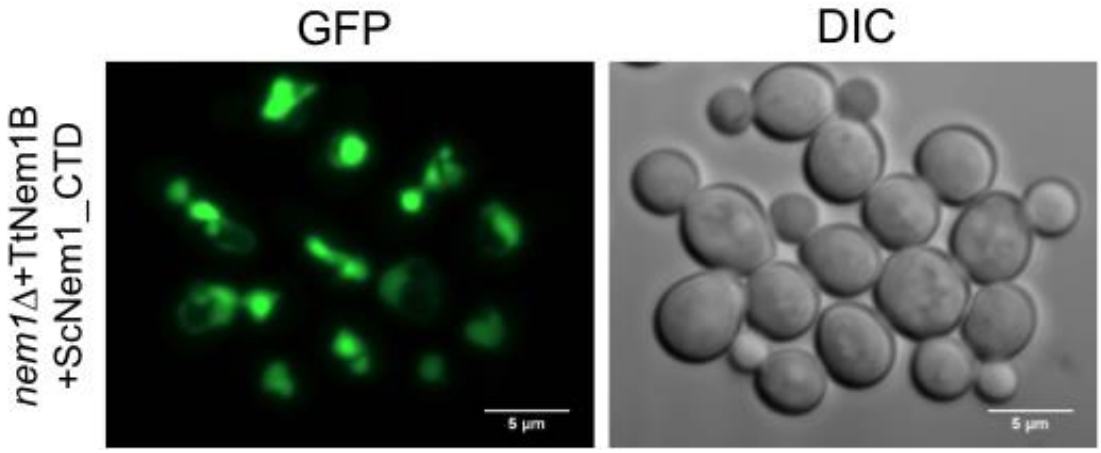


Figure 4.5: TtNEM1B+ScNEM1_CTD does not rescue the nuclear phenotype of *nem1*Δ yeast cells.

Confocal images of PUS1-GFP expressing *nem1*Δ yeast cells transformed with TtNEM1B+ScNEM1_CTD. The aberrant nuclear phenotype of yeast *nem1*Δ cells was not rescued by TtNEM1B+ScNEM1_CTD.

4.3 DISCUSSION

Our earlier study showed that T. thermophila PAH1 can functionally replace yeast PAH1 (25). However, NEM1A, NEM1B, NEM1C or NEM1D did not complement the loss of yeast NEM1. One possible explanation is that Spo7, which directly regulates Nem1 in S. cerevisiae, does not recognize Tetrahymena Nem1 and hence

fails to dephosphorylate yeast Pah1. Consistent with this idea, we could not find any SPO7 homolog in the Tetrahymena genome (Figure 4.6). This suggests that the regulatory subunit of TtNem1 may have diverged drastically from Spo7. Since replacing the CTD of TtNEM1B with CTD of yeast (SPO7 interacting domain) did not complement yeast NEM1, SPO7 interaction though essential is not sufficient for NEM1 function and the amino acids in the NTD of yeast NEM1 which is different in TtNEM1B are also important for yeast NEM1 function. We speculate that the regulatory subunit of TtNEM1B has diverged drastically in its sequence from the SPO7 and further studies are required to identify the regulatory subunit in *Tetrahymena*.

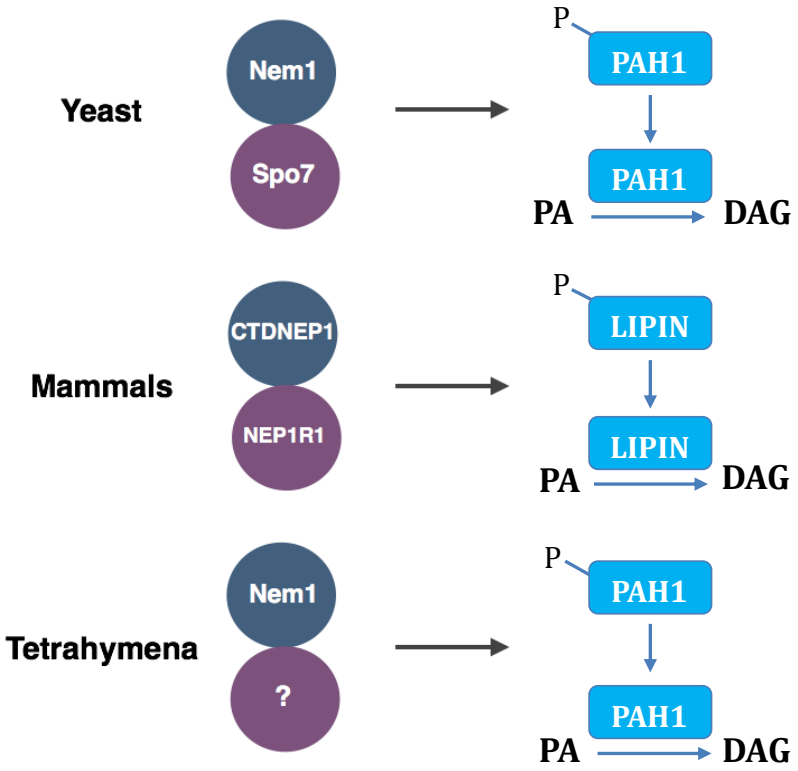


Figure 4.6: Schematic representation of NEM1/SPO7 orthologs in different organisms.

Chapter 5

ANALYSIS OF FUNCTIONAL CONSERVATION OF *TtPAH2*

5.1 INTRODUCTION

Phosphatidate phosphatase enzymes (PAP) are involved in the penultimate step in TAG synthesis, catalyzing the dephosphorylation of phosphatidate (PA) to generate DAG (13, 154, 173). The DAG generated is used for the synthesis of TAG as well as the phospholipids. PAP enzymes are categorized into two classes, the type 1 enzymes are Mg^{2+}-dependent PAP1 enzymes, and the type 2 enzymes are Mg^{2+} independent PAP2 enzymes (13, 175). PAP1 enzymes are essential for lipid synthesis and play transcriptional regulatory roles, whereas PAP2 enzymes are reported to play a role in lipid signaling in yeast and mammals (175). PAP1 and PAP2 enzymes vary in nature of membrane association. PAP1 enzymes are cytosolic or membrane associated proteins whereas PAP2 enzymes are low molecular weight, integral membrane proteins (175). The yeast PAP1 enzyme, encoded by *PAH1* (phosphatidate phosphatase 1), has 862 amino acids. PAP2 enzymes are encoded by *DPP1* (diacylglycerol pyrophosphate phosphatase 1) and *LPP1* (lipid phosphate phosphatase 1) in yeast. Dpp1p is 289 amino acids in length and has a predicted molecular mass of 33.5 kDa and Lpp1p is 274 amino acids in length and has a molecular mass of 31.6 kDa (166, 175). PAP1 enzymes are encoded by high molecular weight protein in other organisms including yeast, mammals, *C.elegans*, *Arabidopsis thaliana*, *Drosophila melanogaster*.

Tetrahymena thermophila possesses two *PAH* homologs, *TtPAH1* and *TtPAH2* (25). Ttpah1 encodes a high molecular mass protein, ~ 96kDa, similar in size to PAP1 proteins of other organisms (25). Conversely, Ttpah2 is a low molecular mass protein comprising 335 amino acids. *TtPAH2* is smaller than the other known PAH homologs and is similar in size to Dpp1 and Lpp1 enzymes belonging to PAP2 family (26).

However, TtPAH2 lacks the three signature motifs of PAP2 proteins and possesses the highly conserved amino-terminal (N-LIP) and carboxyl-terminal (C-LIP) region present in other PAP1 proteins (26). The N-LIP domain of TtPAH2 contains a conserved glycine residue at the 79^{th} position, and the haloacid dehalogenase (HAD)-like domain present in the C-LIP region possesses the conserved DVDGT catalytic motif essential for its PAP function (26). TtPah2 shows 34% identity with human lipin and 22% with yeast Pah1 (26). TtPAH2 exhibits Mg^{+2}-dependent phosphatidic acid phosphatase activity. *TtPAH2* is the smallest PAH protein belonging to the PAP1 class (26). TtPAH2 is localized in both cytoplasm and membranes and is not essential for the normal growth of *Tetrahymena* (26). Unlike TtPAH1, TtPAH2 does not regulate lipid droplet biogenesis and ER morphology in *Tetrahymena* (26). Other lipins/PAHs previously characterized in organisms like yeast, *C.elegans*, mammals, and *Arabidopsis* regulates ER morphology (148, 194, 222). TtPAH2 is the first phosphatidate phosphatase reported which does not regulate lipid droplet number or membrane biogenesis (26). TtPAH1 and TtPAH2 show non-overlapping expression pattern in different cell cycle stages suggesting that TtPAH2 may have a specialized function in *Tetrahymena* (26).

The loss of *PAH1* function in yeast shows phenotypes like reduced lipid droplet number, a hyper proliferation of the nuclear membrane, defect in ER morphology, slow growth, temperature sensitivity, and impaired growth on the non-fermentable carbon sources (respiratory deficiency) (35, 185). The inability of *pah1Δ* yeast cells to grow on the non-fermentable substrate is correlated with a reduction in ATP level (192). The yeast *pah1Δ* cells also exhibit vacuolar fragmentation, increased level of mitochondrial superoxide and decreased tolerance to oxidative stress leading to reduced lifespan (287).

PAH proteins are evolutionarily conserved in human, mice, flies, plants, and worms. Yeast PAH1 is functionally replaceable with PAHs from *Dictyostelium discoideum*, mammals, *Trypanosoma brucei, Tetrahymena thermophila* and *Arabidopsis thaliana* (25, 184, 187). *PAH1* from *Dictyostelium discoideum* rescued growth, respiratory and nuclear phenotypes but did not rescue the temperature sensitivity of *pah1*Δ yeast cells in the complementation assay (25). The *Arabidopsis PAH* homologs, *AtPAH1 and AtPAH2*, rescue the slow growth phenotype of *pah1*Δ yeast cells (184, 197). In addition, *AtPAH2* mitigates the aberrant nuclear morphology of *pah1*Δ yeast cells (25). TtPAH1, the large homolog of *Tetrahymena*, also complements yeast PAH1 (25). Though *TtPAH1* does not regulate nuclear morphology in *Tetrahymena*, it rescues the nuclear phenotype of *pah1*Δ yeast cells (25). The phosphatidic acid phosphatase cascade regulating membrane biogenesis and lipid homeostasis is evolutionarily conserved across the eukaryotic lineages indicating that it was present in common ancestor before the divergence of lineages during eukaryotic evolution (25). In addition to lipid homeostasis and membrane biogenesis, *PAH* has additional roles such as nuclear envelope break down in *C.elegans* and nuclear expansion in yeast, suggesting that the lineage-specific role of *PAH* is adopted after divergence from the common ancestor during evolution. The role of *PAH* in the nuclear expansion is restricted only to yeast. However, *PAH* homologs from all the lineages rescue the aberrant nuclear expansion phenotype of *pah1Δ* yeast cells.

To find out if yeast PAH1 is also functionally replaceable with TtPAH2, *pah1Δ* yeast cells were complemented with *TtPAH2* and rescue of the aberrant nuclear morphology, slow growth, temperature sensitivity, respiratory deficiency and phenotype of *pah1Δ* cells were assessed. In this chapter, we discuss the results obtained with the complementation of *pah1Δ* yeast cells with *TtPAH2*.

5.2 RESULTS

5.2.1 *TtPAH2* does not restore aberrant nuclear morphology of *pah1*Δ yeast cells

In the *pah1*Δ yeast cells, the nuclear membrane organization is altered drastically with the elongated nuclei appearing as two lobes interconnected by a long nuclear membrane extension. The long extensions of the nuclear membrane that extend into the cytoplasm contain nuclear pore complexes and intranuclear content but lack any DNA. The ciliate protein, *TtPAH1,* is not required for regulating the nuclear shape in *Tetrahymena* but functionally complements yeast *PAH1* (25). To examine if *TtPAH2* could rescue the nuclear defect in *pah1*Δ yeast cells, we expressed *TtPAH2* in *pah1*Δ yeast cells and monitored the nuclear morphology using nucleoplasmic marker PUS as a GFP-fusion to assess the nuclear morphology in yeast. The *pah1*Δ yeast cells expressing *TtPAH2* did not rescue the abnormal nuclear phenotype and exhibited long protrusions extending into the cytoplasm as visualized by the expression of PUS-GFP (Figure 5.1). This result suggests that *TtPAH2* does not regulate the nuclear expansion. To exclude the possibility that the absence of rescue is due to lack of expression, the transcript corresponding to *TtPAH2* was confirmed by reverse transcription PCR analysis (Figure 5.2).

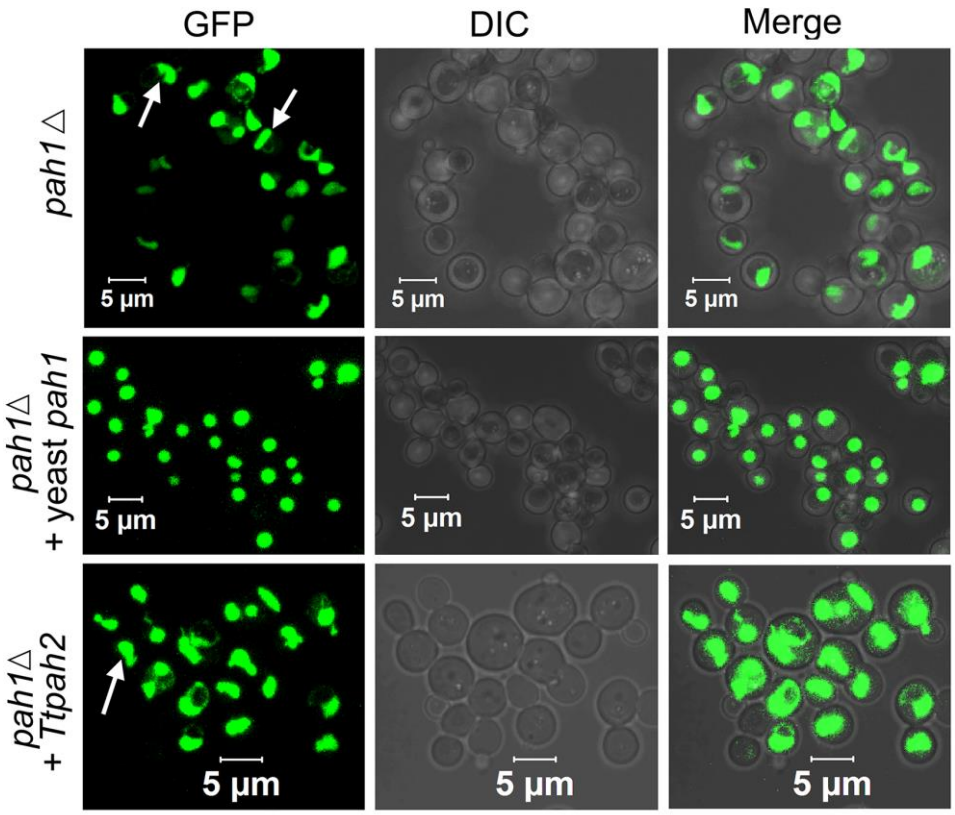


Figure 5.1: TtPAH2 does not restore the defective aberrant nuclei of pah1Δ yeast cells

Confocal and DIC microscopic images of PUS1-GFP expressing *pah1*Δ yeast cells transformed either with empty vector (top), yeast PAH1 (middle) or TtPAH2 (bottom). The aberrant structure (arrows) of the *pah1*Δ nuclei were rescued by yeast PAH1 but not by TtPAH2.

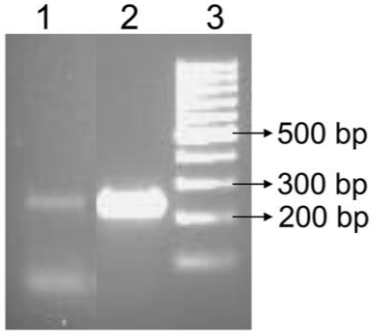


Figure 5.2: The expression of TtPAH2 in *pah1*Δ yeast cells transformed with TtPAH2 was confirmed by RT-PCR analysis

RT-PCR analysis of the total RNA isolated from *pah1*Δ yeast 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. The top band in lane 1and 2 corresponds to TtPAH2 (238 bp).

5.2.2 *TtPAH2* partially rescues the respiratory defect of *pah1*Δ yeast cells

The *pah1*Δ yeast cells exhibit slow growth at 30°C, temperature sensitive growth at 37°C and growth defect (respiratory deficiency) on the non-fermentable carbon source (35, 185). The *pah1*Δ yeast cells were complemented with TtPAH2 to see whether it rescues the mutant phenotypes. The expression of TtPAH2 in *pah1*Δ yeast cells transformed with TtPAH2 was confirmed by RT-PCR analysis (Figure 5.2). Although TtPAH2 was expressed, it failed to restore the growth of *pah1*Δ yeast cells under normal growth conditions (Figure 5.3). TtPAH2 also failed to rescue the temperature-sensitive phenotype of *pah1*Δ yeast cells (Figure 5.7). To evaluate the role of *TtPAH2* in rescuing the respiratory deficiency phenotype, *pah1*Δ yeast cells complemented with TtPAH2 were grown on plates containing glycerol as the non-fermentable carbon source. The *pah1*Δ expressing *TtPAH2* grew faster on glycerol (Figure 5.3) than the control *pah1*Δ cells, suggesting that *TtPAH2* partially restores the respiratory function of the yeast homolog (26). These results suggest that TtPAH2 plays a role in respiration.

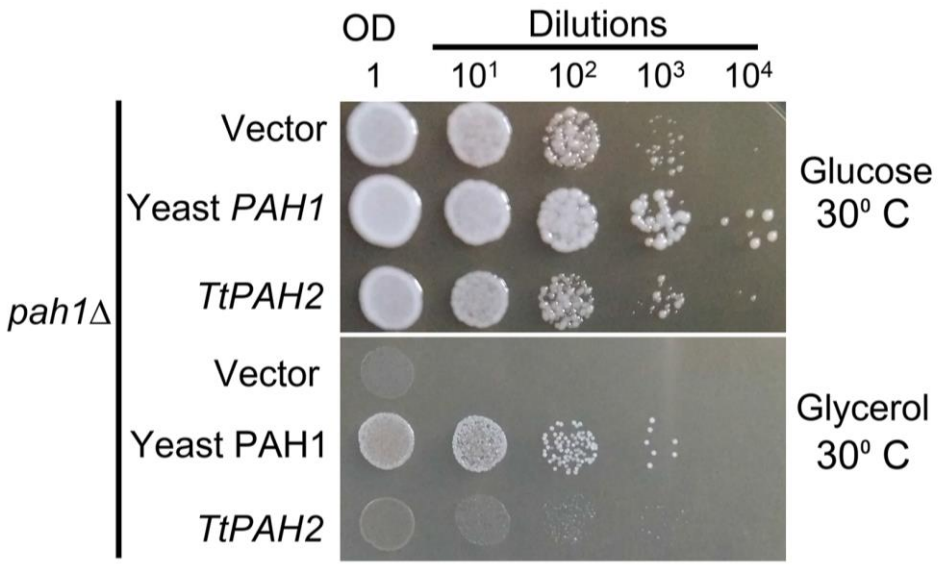


Figure 5.3: TtPAH2 does not rescue the slow growth phenotype but partially rescues the respiratory deficiency phenotype of *pah1*Δ yeast cells

Cultures of *pah1*Δ yeast 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 were spotted on the 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.

5.2.3 Addition of middle domain of TtPAH1 in TtPAH2 does not restore the slow growth, temperature-sensitivity and nuclear nuclear defect phenotypes of *pah1*Δ yeast cells

Unlike *TtPAH2, TtPAH1* complemented the growth phenotype and temperature sensitive phenotype of the yeast *pah1*Δ cells (25, 26). TtPAH1 consists of N-LIP domain, a middle domain, and C-LIP domain whereas the middle domain is absent in TtPAH2 (Figure 5.4). We hypothesized that the amino acid sequences absent in TtPAH2 but present in TtPAH1 may be critical for restoring the growth phenotype and temperature sensitive phenotype of *pah1*Δ yeast cells. Therefore, we incorporated the middle domain fragment of TtPAH1 (1578 bp) in between N-LIP (258 bp) and C-LIP domain (717 bp) of TtPAH2 and cloned it in YCplac111 yeast vector (6103 bp) and used for complementing the yeast PAH1. The construct generated (TtPAH2+midTtPAH1) contained the middle domain of TtPAH1 between the N-LIP

and C-LIP domain of TtPAH2 and was generated by assembling the three fragments by Gibson assembly (Figure 5.5). The construct generated was confirmed by PCR amplification (Figure 5.6). The complementation result shows that TtPAH2+midTtPAH1 failed to rescue the growth phenotype, temperature-sensitive and nuclear phenotype of *pah1*Δ yeast cells (Figure 5.7 and Figure 5.8). This suggests that either chimeric protein is not functional, or the amino acids present in the N-LIP and C-LIP domain of TtPAH1 is important for these functions.

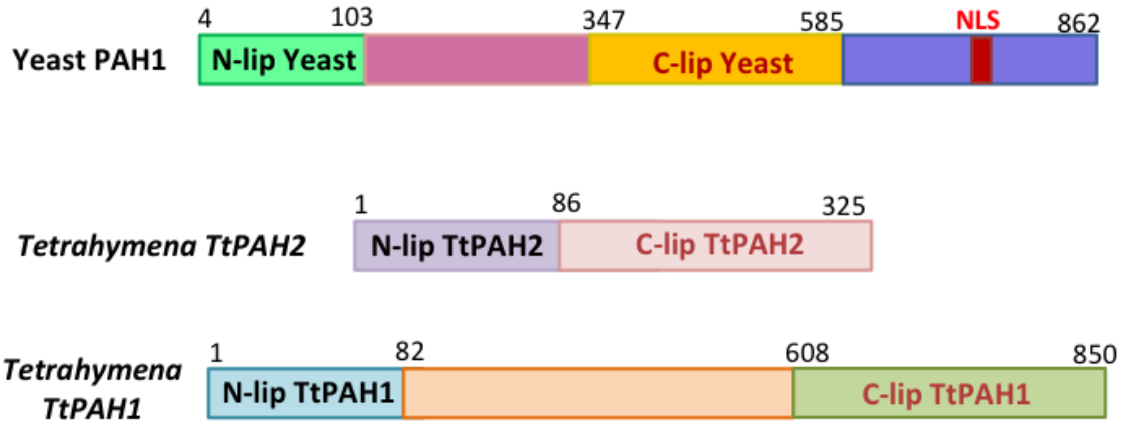


Figure 5.4: Domain organization of yeast PAH1, *Tetrahymena* TtPAH1, and *Tetrahymena* TtPAH2.
Numbers mentioned on the top represents the amino acid position. N-lip and C-lip represent the conserved domain. NLS denotes the nuclear localization signal.

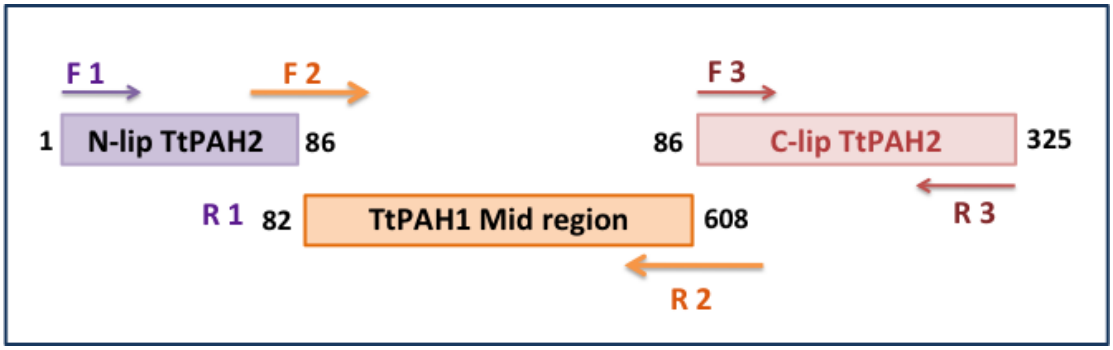


Figure 5.5: Schematic representation of DNA fragments, to be assembled, with the overlapping ends.

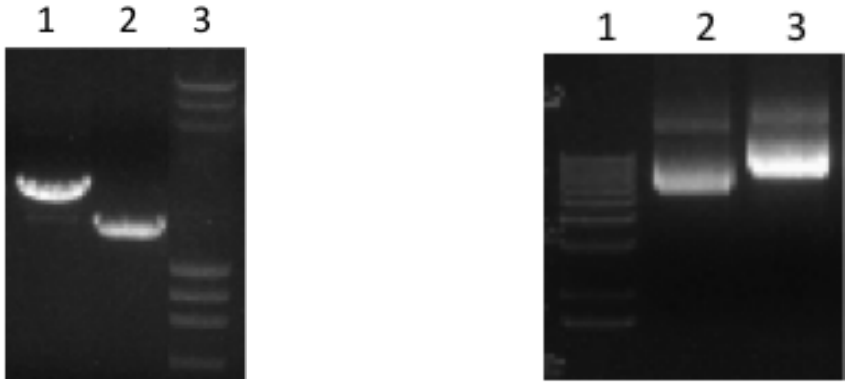


Figure 5.6: Confirmation of TtPAH2+midTtPAH1 construct

Left panel- Lane 1 represents PCR product using YCplac111+TtPAH2+mid TtPAH1 as a template and F1 and R3 as oligos (as shown in figure 5.5) that should give a product of 2613 bp. Lane 2 represents PCR product using YCplac111+TtPAH2+mid TtPAH1 as a template and F1 and R2 as oligos (as shown in figure 5.5) that should give a product of 1836 bp. Lane 3 is molecular weight marker. Right panel- Lane 1 is molecular weight marker. Lane 2 represents YCplac111+TtPAH2 plasmid corresponding to 7138 bp. Lane 3 represents YCplac111+TtPAH2+midTtPAH1 plasmid corresponding to 8656 bp.

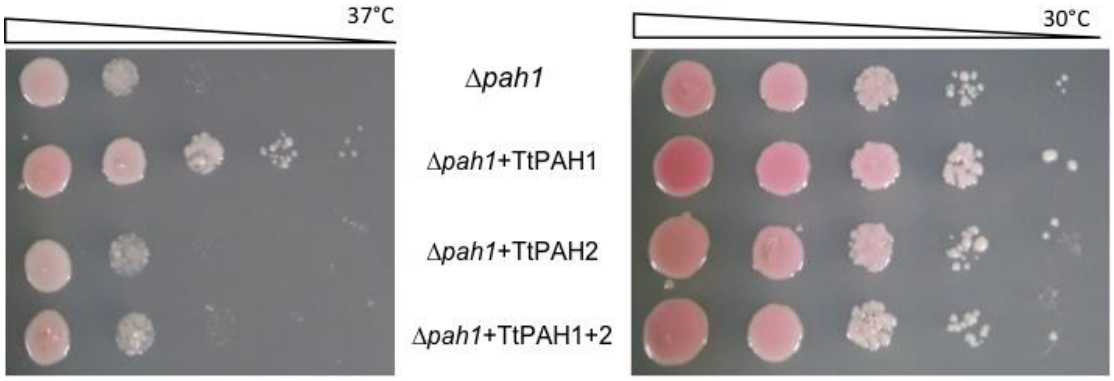


Figure 5.7: TtPAH2+midTtPAH1 does not rescue the slow growth or temperature sensitive phenotype of *pah1*Δ yeast cells

Cultures of *pah1*Δ yeast cells transformed with empty vector, TtPAH1, TtPAH2 or TtPAH2+midTtPAH1 were adjusted to OD_{600}= 1 followed by 10-fold serial dilutions. Five microliters of each were spotted on the SD plates containing glucose but lacking leucine and uracil were incubated for 3 days at 30°C (left panel) or 37°C (right panel).

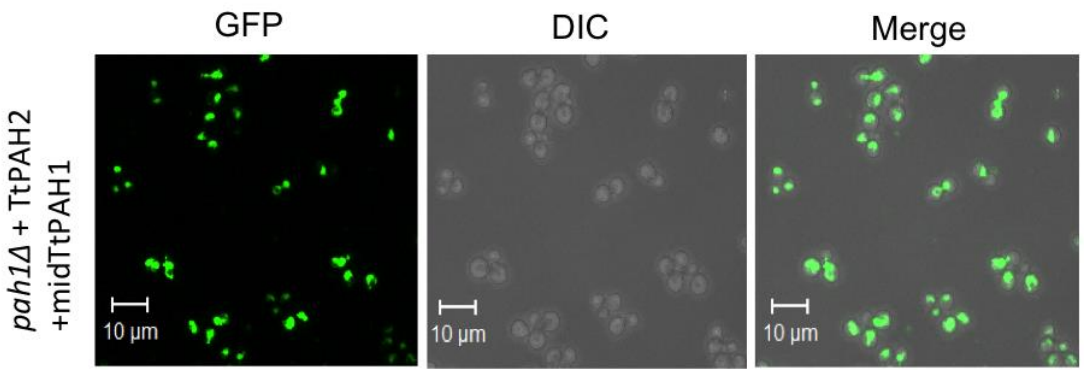


Figure 5.8: TtPAH2+midTtPAH1 does not rescue the nuclear phenotype of *pah1*Δ yeast cells

Confocal images of PUS1-GFP expressing *pah1*Δ yeast cells transformed with TtPAH2+midTtPAH1. The aberrant nuclear phenotype of *pah1*Δ cells was not rescued by TtPAH2+midTtPAH1.

5.2.4 *TtPAH2* does not restore the nuclear or slow growth phenotype of *nem1*Δ and *spo7*Δ yeast cells

Dephosphorylation of Pah1 occurs by a nuclear/ER membrane complex consisting of a catalytic phosphatase subunit nuclear envelope morphology protein 1 (Nem1), and its regulatory subunit, sporulation-specific protein 7 (Spo7). Pah1 is activated upon dephosphorylation and is recruited to the ER membrane, where it acts on its substrate PA (17, 18). Cells lacking either the Nem1 or Spo7 subunits of the complex mirror the phenotypes of yeast *pah1*Δ cells including aberrant expansion of the nuclear/ER membrane, decreased lipid droplet number, and growth inhibition (28, 145, 175). The *nem1*Δ*spo7*Δ, double mutants in yeast, also show phenotypes similar to *pah1*Δ or *nem1*Δ or *spo7*Δ, suggesting that these three proteins work in unison (14, 17, 186). Overexpression of yeast PAH1 in *spo7*Δ, *nem1*Δ, and *nem1*Δ *spo7*Δ yeast mutants suppress their slow growth phenotypes at 37°C (17). To examine if *TtPAH2* could restore the nuclear and slow growth phenotype of *nem1*Δ and *spo7*Δ yeast cells, we overexpressed TtPAH2 and TtPAH2+midTtPAH1 in *nem1*Δ and *spo7*Δ yeast cells and monitored the growth phenotype and nuclear morphology (Figure 5.9, Figure 5.10

and Figure 5.11). TtPAH2, and TtPAH2+midTtPAH1 could not rescue the growth defect or aberrant nuclear morphology of *nem1*Δ and *spo7*Δ yeast cells (Figure 5.9, Figure 5.10 and Figure 5.11).

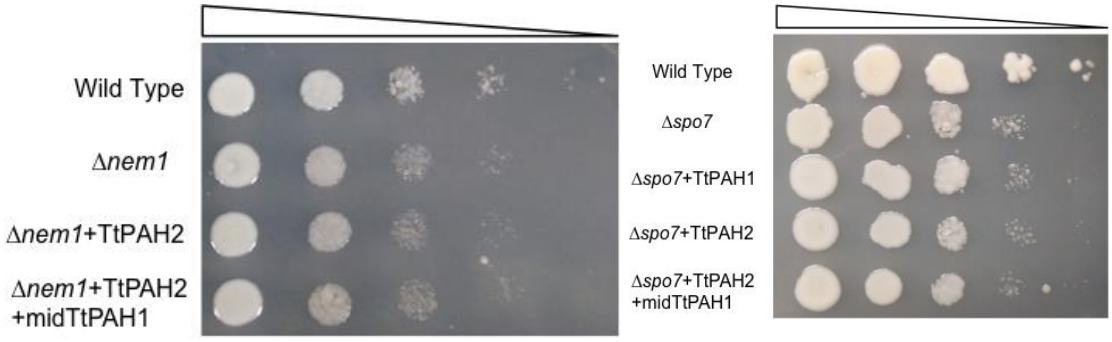


Figure 5.9: *TtPAH2* does not rescue the slow growth phenotype of *nem1*Δ or *spo7*Δ yeast cells

Cultures of *nem1*Δ (Left panel) or *spo7*Δ (Right panel) yeast cells transformed with empty vector, or TtPAH2 or TtPAH2+midTtPAH1 along with wild-type yeast cells were adjusted to OD_{600}= 1 followed by 10-fold serial dilutions. Five microliters of each were spotted on the SD plates containing glucose but lacking leucine and uracil were incubated for 3 days at 30°C.

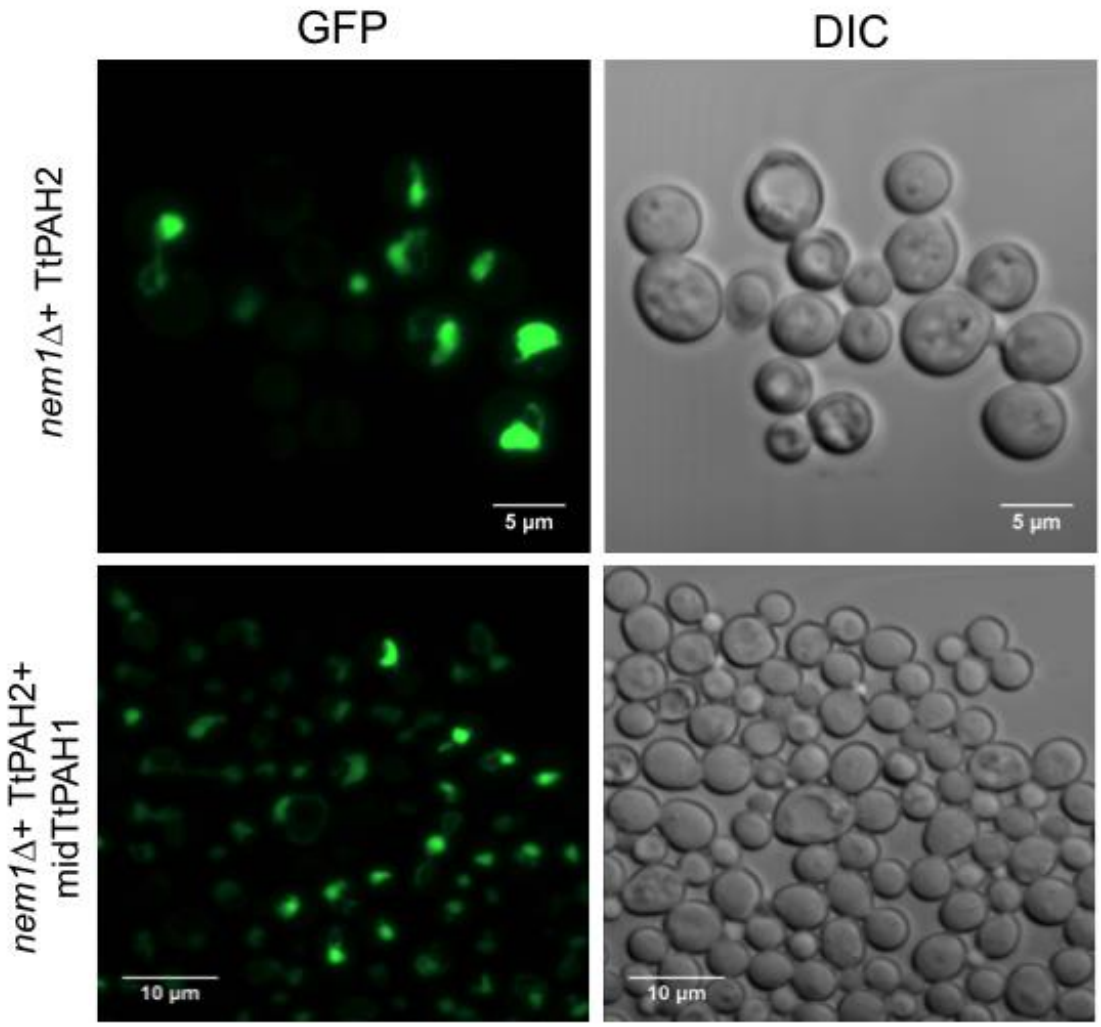


Figure 5.10: TtPAH2 does not rescue the nuclear phenotype of *nem1*Δ yeast cells. Confocal images of PUS1-GFP expressing *nem1*Δ yeast cells transformed with TtPAH2 or TtPAH2+midTtPAH1. The aberrant nuclear phenotype of *nem1*Δ cells could not be rescued by TtPAH2 or TtPAH2+midTtPAH1.

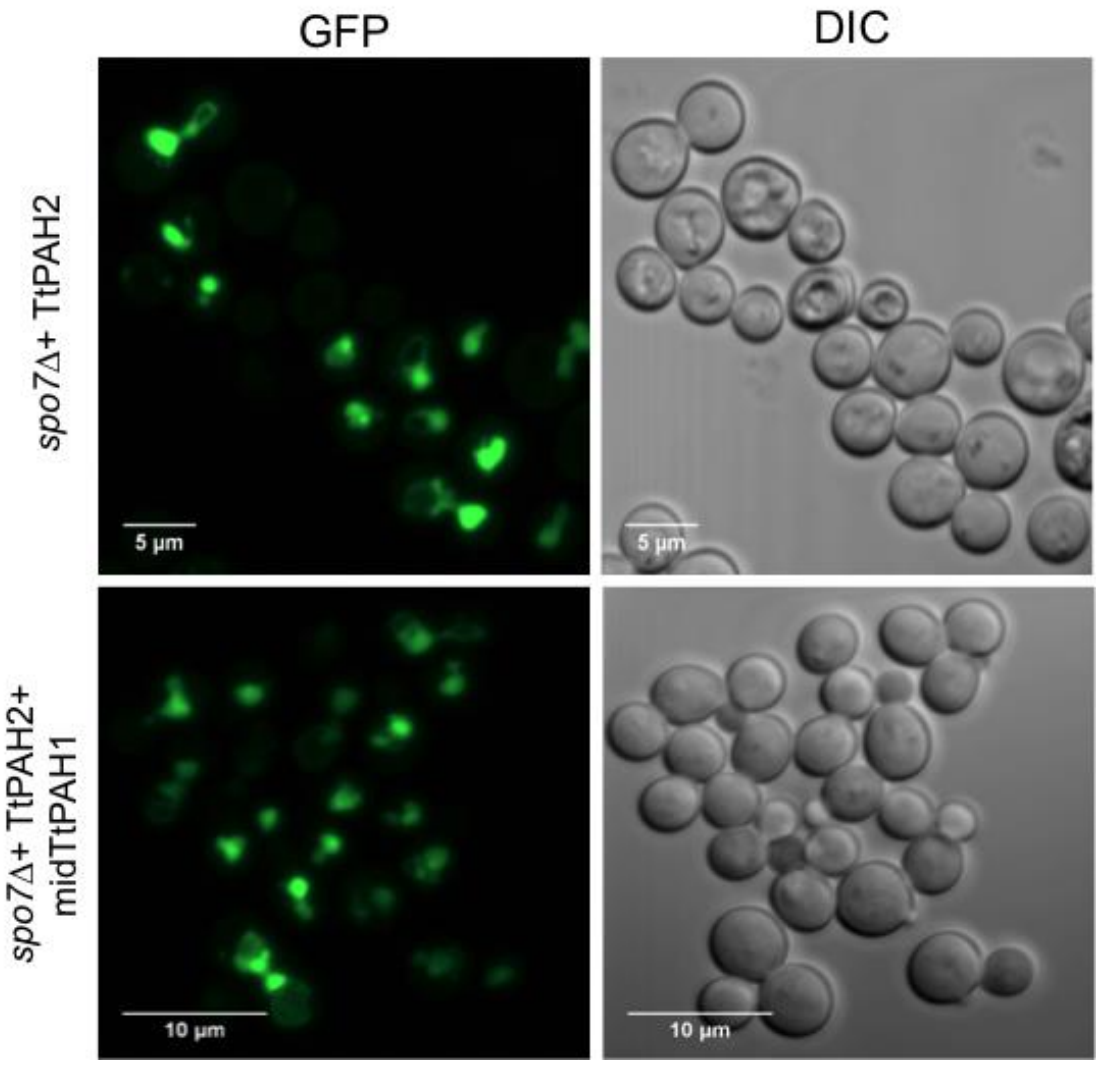


Figure 5.11: TtPAH2 does not rescue the nuclear phenotype of *spo7*Δ yeast cells. Confocal images of PUS1-GFP expressing *spo7*Δ yeast cells transformed with TtPAH2 or TtPAH2+midTtPAH1. The aberrant nuclear phenotype of *spo7*Δ cells could not be rescued by TtPAH2 or TtPAH2+midTtPAH1.

5.3 DISCUSSION

Previously characterized Mg^{2+}-dependent phosphatidate phosphatases are relatively large proteins, close to 100 kDa and low molecular mass proteins exhibiting phosphatidate phosphatase activity are usually Mg^{2+}-independent. Interestingly, Ttpah2, a *PAH* homolog of *Tetrahymena*, is a low molecular weight (37 kDa) protein displaying Mg^{2+}-dependent PAP activity (26). Previous study has shown that *Tetrahymena* homolog, *TtPAH1*, complements the growth phenotype, nuclear defect

and temperature sensitive phenotype of the yeast *pah1*Δ cells (25). *TtPAH1* does not regulate the nuclear morphology in *Tetrahymena,* but surprisingly it rescues the aberrant nuclear morphology of *pah1*Δ yeast cells (25). In contrast, *TtPAH2* does not rescue the aberrant nuclear morphology, growth defect and temperature sensitivity of *pah1*Δ yeast cells (26). However, TtPAH2 rescues the respiratory defect of *pah1*Δ yeast cells suggesting that TtPAH2 plays a specific role in respiration (26). Our results indicate that TtPAH2 possesses the minimal function of PAH protein family in respiration. Unlike TtPAH2, TtPAH1 contains a middle domain other than the conserved N-LIP domain and C-LIP domain. Thus, we speculated that the amino acid sequences absent in TtPAH2 but present in TtPAH1 may be critical for restoring the growth phenotype and temperature sensitive phenotype of *pah1*Δ yeast cells. However, TtPAH2+midTtPAH1, consisting of N-LIP and C-LIP domains of TtPAH2 and middle domain of TtPAH1, failed to rescue the growth phenotype and temperature sensitive phenotype of *pah1*Δ yeast cells. These results suggest that the amino acids in N-LIP and C-LIP domains of TtPAH1, which have diverged in TtPAH2, are critical for performing these cellular functions.

The function of PAH1 in yeast is more prominent during respiration since the loss of PAH1 leads to severe growth inhibition in the non-fermentable substrate as compared to its growth in the fermentable substrate. The exact role of PAH1 in respiration is not understood. Previous studies have reported a two-fold reduction in the ATP levels of *pah1*Δ yeast cells compared to the wild-type cells (192). Therefore, it is conceivable that PAH1 is required for ATP production via TCA cycle since the cell is highly dependent on TCA cycle for energy in the non-fermentable substrates.

Herein, we discuss a possible link between PAH1 and ATP production. The loss of PAH1 results in accumulation of PA (13). In *Arabidopsis* and mammals, it is reported

that accumulation of PA leads to inhibition of the PP2C family of protein phosphatases (170). Interestingly, Ptc5/Ptc6 in yeast, a PP2C family member, activates pyruvate dehydrogenase by dephosphorylation (288). Therefore, we speculate that loss of PAH1, resulting in accumulation of PA, could potentially inactivate Ptc5/Ptc6 leading to the inhibition of pyruvate dehydrogenase (Figure 5.12). Pyruvate dehydrogenase is the first enzyme that links glycolysis and the TCA cycle and is required for the ATP production via TCA cycle. This is in accordance with the previous observations that the loss of PAH1 causes a severe growth defect in the non-fermentable substrate where ATP production by TCA cycle is the primary source of energy (26).

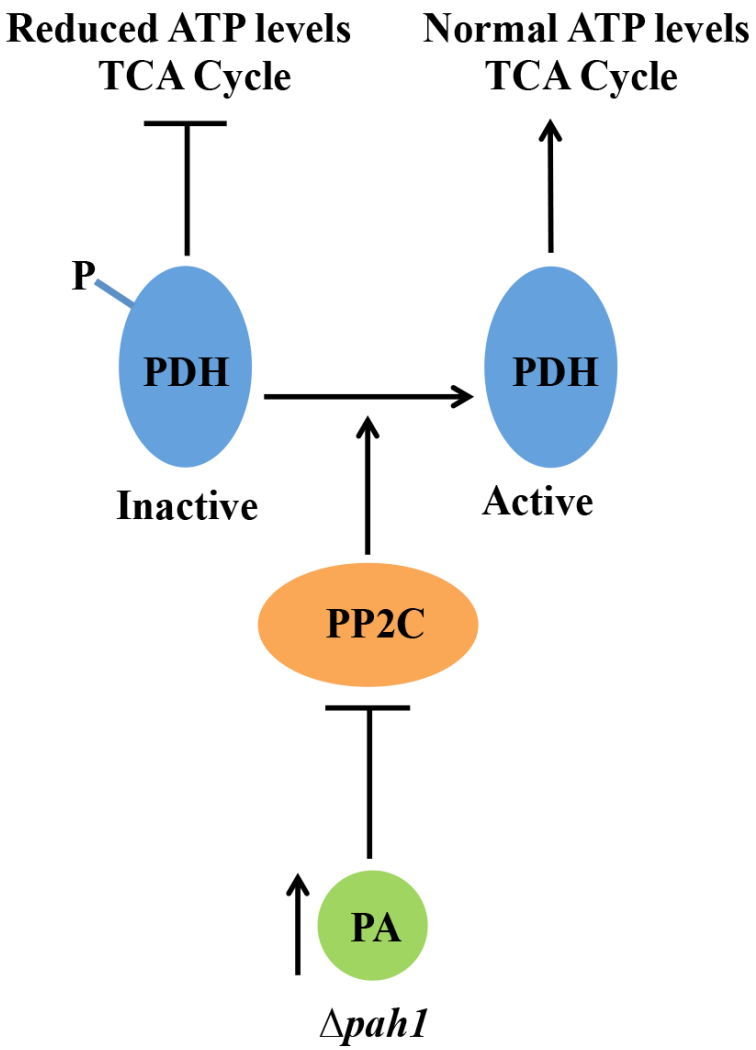


Figure 5.12: Model for regulation of TCA cycle by PAH1
PDH converts pyruvate to acetyl CoA linking glycolysis with TCA cycle. PDH is activated by dephosphorylation by PP2C. PP2C is inhibited by elevated PA in yeast *pah1*Δ cells. This results in inhibition of dephosphorylation of PDH making it inactive and causes reduced levels of cellular ATP as a result of inhibition of TCA cycle. PDH - pyruvate dehydrogenase, PP2C – protein phosphatase 2C, ATP – Adenosine triphosphate.

Over all our results show that TtPAH2 is a protein that possesses the minimal respiratory function of the PAH family of proteins and the extra amino acids present in other homologs are perhaps acquired to perform additional cellular functions (26).

Chapter 6

SUMMARY AND CONCLUSION

6.1 Summary

Phosphatidate phosphatase (PAH) catalyzes the penultimate step in triglyceride synthesis, the dephosphorylation of phosphatidic acid to generate DAG that is converted to TAG and phospholipids (13, 173). A cell cycle-regulated protein phosphatase complex consisting of a catalytic phosphatase subunit NEM1, and its regulatory subunit, SPO7, dephosphorylates PAH1 and regulates its PAP activity (14, 17, 20). Lipid homeostasis and membrane biogenesis are regulated by a cascade comprising Pah1 and Nem1/Spo7 complex in yeast and a similar cascade is also reported in other organisms including plants, mammals, worms, and flies (17, 181, 184, 195, 199, 239, 275). Loss of *NEM1* results in growth defect, ER/nuclear membrane defect and reduced lipid droplet number in yeast and metazoans (17, 181, 184, 195, 199, 239, 275). Thus, Nem1 proteins play a crucial role in lipid homeostasis, membrane biogenesis, and are evolutionarily conserved. The Nem1 proteins are well studied in Opisthokonta (fungi, nematode, flies, and animals) clade (17, 181, 200, 275). Contrarily, the Nem1-Spo7 phosphatase complex and the cascades in which they participate are not reported in Alveolates. In the present study, we investigated the role of four putative homologs of *NEM1 (TtNem1A*, *TtNem1B, TtNem1C,* and *TtNem1D)* in *Tetrahymena thermophila*. *Tetrahymena* is a unicellular ciliate belonging to Alveolata clade, which displays nuclear dimorphism and exhibits membrane complexity comparable to that of the higher organisms (22). The complex nuclear remodeling exhibited by *Tetrahymena* requires membrane biogenesis, therefore, making it a good model system to study membrane biogenesis. Our earlier study reported the presence of two homologs of phosphatidic acid phosphohydrolase (*TtPAH1* and *TtPAH2*) in *Tetrahymena* (25, 26). In this study, we report that TtNem1B, TtNem1C, and TtNem1D are not essential for cell viability unlike the

growth defect reported in yeast upon the loss of *NEM1* (14, 20). The deletion of *TtNEM1B* in *Tetrahymena* resulted in a reduction in lipid droplet number. TtNem1B regulates lipid droplet biogenesis, while neither TtNem1C nor TtNem1D appears to be required for this function. Although not definitive, these results suggest that *Tetrahymena NEM1B* may be the ortholog of yeast *NEM1*. It is also possible that *Tetrahymena* Nem1A is involved in lipid droplet biogenesis. As mentioned earlier, we failed to generate knock out strains of *TtNEM1A* in multiple attempts suggesting that *TtNEM1A* is an essential gene. It should be noted that the cascade of lipid homeostasis and membrane biogenesis (comprising Pah1 and Nem1/Spo7 in yeast) is not essential in any organism studied so far including *Tetrahymena*. Therefore, the essentiality of *NEM1A* suggests that this *Tetrahymena* gene is involved in other as yet unknown processes.

Overexpression of *TtPAH1* in *ΔTtnem1B* cells partially rescued the defect in lipid droplet biogenesis. The rescue may be due to limiting kinase activity needed to phosphorylate the large pool of overexpressed Pah1. These results indicate that TtPah1 together with TtNem1B forms a cascade for regulation of lipid homeostasis in *Tetrahymena*.

The loss of Nem1 or Pah1 function results in a defect in nuclear and ER morphology in yeast (11, 36). Interestingly, TtNem1B was not required to maintain normal ER morphology in *Tetrahymena*. This was surprising given that TtNem1B, like Nem1 in other organisms, functions via Pah1, and loss of TtPAH1 produces a clear defect in ER morphology (25). In addition to the dephosphorylation of PA, an independent role of Pah1 as a transcriptional regulator is known in yeast and mammals (14, 17, 199, 201). Therefore, it is possible that Pah1 in *Tetrahymena* also has an additional, Nem1-independent function in regulating ER morphology. Alternatively, it is possible that in

ΔTtnem1B cells the heterogeneous phosphorylation state of TtPah1 results in a cohort of protein that is sufficient to regulate ER morphology but insufficient to stimulate wild-type lipid droplet formation. Deletion of TtNEM1 homologs did not show a visible defect in size or shape of the nucleus.

Our earlier study showed that *T. thermophila PAH1* can functionally replace yeast *PAH1* (25). However, *NEM1A, NEM1B, NEM1C,* or *NEM1D* did not complement the loss of yeast *NEM1*. One possible explanation is that Spo7, which directly regulates Nem1 in yeast, does not recognize *Tetrahymena* Nem1 and hence fails to dephosphorylate yeast Pah1. Consistent with this idea, we could not find any *SPO7* homolog in the *Tetrahymena* genome, suggesting that the regulatory subunit of TtNem1 may have diverged drastically from Spo7.

We also show that TtPAH2 homolog of *Tetrahymena* does not functionally replace yeast PAH1, however, we have previously shown that TtPAH1 complements yeast PAH1. Our results demonstrate that TtPAH2 has the minimal function of PAH proteins in respiration.

6.2 Conclusion

In this study, we show that *Tetrahymena* possesses multiple *Nem1* homologs. Of these, *TtNEM1B* is shown to be functionally related to *NEM1* in other organisms. Our results indicate that a conserved cascade comprising Nem1 and Pah1 functions in *Tetrahymena* to regulate lipid homeostasis and membrane biogenesis. We have made significant progress in elucidating the role of TtNem1B in lipid biogenesis and established that PAH1 in *Tetrahymena* is also regulated by cell cycle regulated protein phosphatase complex comprising NEM1. However, it is important to identify Spo7p equivalent in *Tetrahymena* to establish the cascade present in other organisms.

Moreover, the role of other NEM1 homologs such as TtNEM1A, TtNEM1C and TtNEM1D are not known and it would be very important to understand their roles in *Tetrahymena* since other organisms do not harbor additional NEM1 homologs.

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