Role of eisosome core component Pil1 in mitochondrial dynamics, mitophagy, and cell death in *Saccharomyces cerevisiae*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Role of eisosome core component Pil1 in mitochondrial dynamics, mitophagy and cell death in *Saccharomyces cerevisiae*"** submitted to the University of Hyderabad by **Ms. Amita Pal**, bearing the **Reg. No. 16LBPH01** for the degree of Doctor of Philosophy in Biochemistry, is based on the studies carried out by her under my supervision. To the best of my knowledge, this work has not been submitted earlier for the award or diploma from any other University or Institution, including this University.

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DECLARATION

I, Amita Pal, hereby declare that the work presented in this thesis entitled **"Role of eisosome core component Pil1 in mitochondrial dynamics, mitophagy and cell death in *Saccharomyces cerevisiae*"** is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Prof. Naresh Babu V Sepuri.** I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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This thesis is free of plagiarism and has not previously been submitted in part or whole for the award of degree or diploma from this or any other University or Institution. Furthermore, prior to submitting the thesis/monograph for adjudication, the student had the following publication(s) and proof for it in the form of reprints in the relevant field of this study.

Amita Pal, Arun Kumar Paripati, Pallavi Deolal, Arpan Chatterjee, Pushpa Rani Prasad, Priyanka Adla,, Naresh Babu V Sepuri. **Eisosome protein Pil1 regulates mitochondrial morphology, mitophagy and cell death in *Saccharomyces cerevisiae***. Journal of Biological Chemistry. 2022.

The student has attended the following conferences during her Ph.D program:

1. Presented a poster entitled **"Role of Eisosome Proteins, Pil1 and Lsp1, in mitophagy and cell death in *Saccharomyces cerevisiae*"** in **XI International Conference on Biology of Yeasts and Filamentous Fungi** held on 27th-29th November 2019, organized by University of Hyderabad and Centre for DNA Fingerprinting and Diagnostics, Hyderabad.
2. Presented a poster entitled **"Role of Eisosome Proteins, Pil1 and Lsp1, in mitophagy and cell death in *Saccharomyces cerevisiae*"** in **90th Annual Meeting of SBC(I)**

"Metabolism to Drug Discovery: Where Chemistry and Biology Unite" organized in virtual mode during 16th to 19th December 2021 by Amity Institute of Biotechnology and Amity Institute of Integrative Sciences and Health, Amity University, Haryana (AUH), Gurugram.

3. Oral talk entitled **" Role of Eisosome Proteins, Pil1 and Lsp1, in mitophagy and cell death in *Saccharomyces cerevisiae*"** in **XI International Conference on Biology of Yeasts and Filamentous Fungi** held on 27th-29th November 2019, organized by University of Hyderabad and Centre for DNA Fingerprinting and Diagnostics, Hyderabad.
4. Participated in conference **"International Congress of Cell Biology (ICCB)"** held on 27th to 31st January 2018, organized by Centre for Cellular and Molecular biology, Hyderabad.
5. Participated in the **10th Conference on Yeast Biology** organized by School of Life Sciences, Jawaharlal Nehru University, New Delhi & Amity University, Gurgaon during February 8-11, 2018.

Furthermore, the student has completed the following courses to fulfill the coursework requirement for the Ph.D.

Course Code	Name	Credits	Pass/Fail
BC801	Analytical Techniques	4	Pass
BC802	Research ethics, Data Analysis and Biostatistics	3	Pass
BC803	Lab Seminar and Record	3	Pass

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Amita Pal

Table of Contents

Abbreviations

ACD	Accidental cell death
ATP	Adenosine triphosphate
Atg	Autophagy related
BAR	Bin/Amphiphysin/Rvs
BF	Bright Field
BSA	Bovine Serum Albumin
COX	Cytochrome c oxidase
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
DiOC6(3)	3,3′-dihexyloxacarbocyanine iodide
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic acid
Dnm1	Dynamin- related
Dpm1	Dolichol Phosphate Mannose synthase
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic Reticulum
Fe-S	Iron Sulfur cluster
GFP	Green Fluorescence Protein
Gly	Glycine
GRIM-19	Gene associated with IFN-beta/RA induced cell mortality
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
His	Histidine

HSP	Heat Shock Protein
Hr	Hour(s)
HRP	Horseradish peroxidase
IMM	Inner mitochondrial membranes
IMS	Inter membrane space
KCl	Potassium chloride
KOH	Potassium hydroxide
LB	Luria-Bertani
Leu	Leucine
LiAc	Lithium Acetate
Lsp1	Long chain bases Stimulate Phosphorylation
MAMs	Mitochondria-associated endoplasmic reticulum membranes
MDV	Mitochondrial Derived Vesicles
$MgCl_2$	Magnesium Chloride
MIA	Mitochondrial IMS Assembly
Min	minute(s)
ml	Milliliter
mm	Millimeter
mM	Millimolar
MnSOD	Manganese superoxide dismutase
MOPS	3-(N-morpholino)propanesulfonic acid
MQC	Mitochondrial Quality Control
MTS	Mitochondrial Targeting Signal
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
ng	Nanogram

nm	Nanometer
nM	Nanomolar
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAM	presequence translocase-associated motor
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pdi1	Protein Disulfide Isomerase
PEG	Polyethylene glycol
PI	Propidium iodide
Pil1	Phosphorylation Inhibited by Long chain bases
Pgk1	3-PhosphoGlycerate Kinase
Pma1	Plasma Membrane ATPase
RCD	Regulated cell death
RIPA	Radio immunoprecipitation assay
RNaseA	Ribonuclease
ROS	Reactive oxygen species
rpm	Rotations per minute
SAM	Sorting and Assembly Machinery
SCD	Synthetic Complete Dextrose
SCL	Synthetic Complete Lactate
SDS	Sodium dodecyl sulphate
Ser	Serine
SGD	Saccharomyces Genome Database
SMD	Synthetic Minimal Dextose

SML	Synthetic Minimal Lactate
TCA	Tricarboxylic acid cycle/ Trichloroacetic acid
TGN	Trans-Golgi Network
TIM	Translocase of Inner Membrane
TOM	Translocase of Outer Membrane
TRIS	Tris(hydroxymethyl)aminomethane
Tryp	Tyrptophan
UPR	Unfolded Protein Response
UPS	Ubiquitin-proteasome system
Ura	Uracil
UT	Untreated
VDAC	Voltage-dependent anion channel
WCE	Whole cell extract
YPD	Yeast extract Peptone Dextrose
YPG	Yeast extract Peptone Glycerol
YPL	Yeast extract Peptone Lactate
µg	Micro gram
µl	Micro liter
°C	degree Celcius

CHAPTER 1

Introduction

1.1 Mitochondria

Mitochondria are essential organelles found in almost all eukaryotes which perform diverse yet interconnected functions. They have been well known as powerhouse of cell because they are responsible for producing the basic unit of energy, ATP, through oxidative phosphorylation. Apart from this, they have various functions such as calcium regulation, apoptosis, Fe-S cluster synthesis, lipid and amino acid metabolism. Several years of study has led to the understanding that mitochondria have potential role in many signaling pathways and stress responses. Mitochondria are highly dynamic in nature and their functions are tightly associated with fission and fusion.

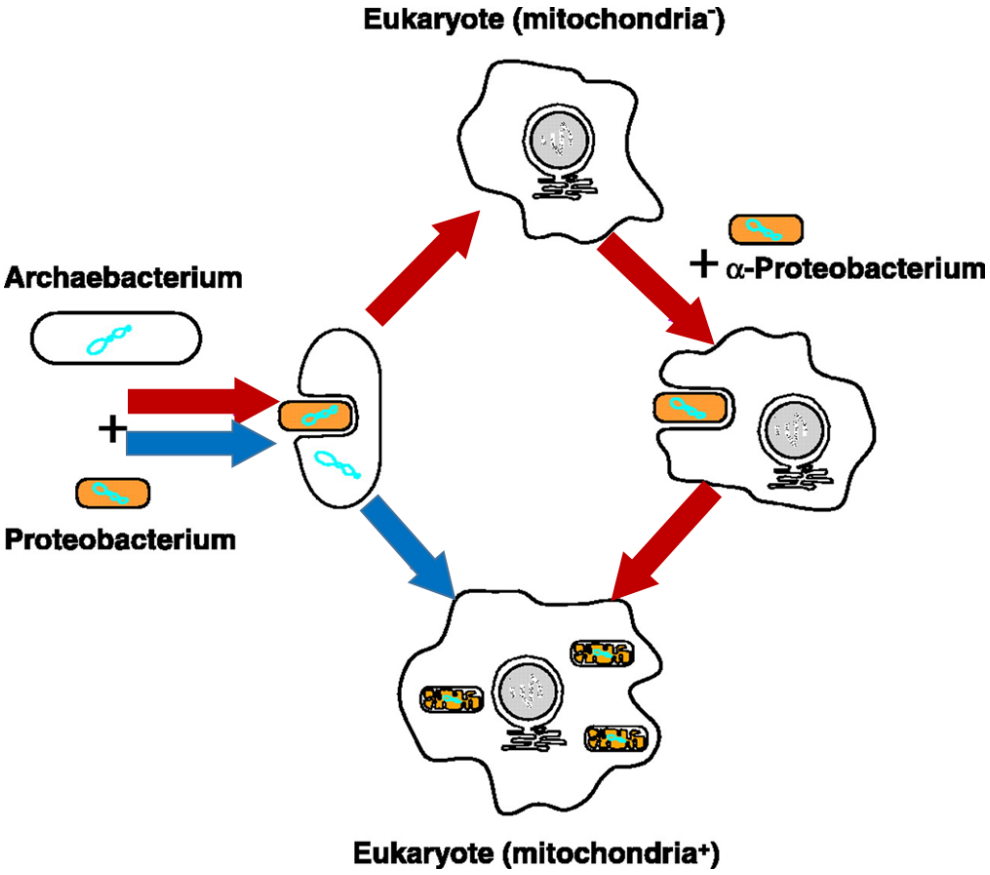
1.2 Evolution of Mitochondria

Studying the mitochondrial genome over past few decades led to the theory of bacterial ancestry (Margulis 1970, Margulis 1981, Sapp 1994). Phylogenetic evidences suggest that mitochondrion originated within the bacterium phylum α-proteobacterium (Yang, Oyaizu et al. 1985, Gillham 1994). The closest known bacterial relatives of mitochondria is intracellular parasites Rickettsia, Ehrlichia, and Anaplasma, the members of the rickettsial subdivision of the α-proteobacterium (Gray, Burger et al. 1999). *Origin of Eukaryotic cells*, book published by Lynn Margulis in 1970 contains an elaborate and substantial details in support of the theory of endosymbiotic origin of mitochondria and plastids. With the advancement of genetic and molecular technologies, several decades of studies support that the origin of mitochondria predates the divergence of eukaryotes, although the details of endosymbiotic theory still remains unclear (Poole and Gribaldo 2014, Pittis and Gabaldon 2016).

Archezoan scenario and symbiogenesis scenario are fundamentally two different themes, the variations of which form the basis for the endosymbiotic models of mitochondrial origin. According to archezoan scenario, a primitive eukaryote lacking mitochondria was host to the proto-mitochondrial endosymbiont. This hypothetical amitochondrial eukaryote was termed as "archezoan". On the other hand, symbiogenesis scenario states that the generation of mitochondria is the result of a single symbiotic event where an archaeal cell engulfed an α-proteobacterium. This was subsequently followed by the compartmentalization of eukaryotic cell and evolution of nucleus (Koonin 2010).

In the course of eukaryotic evolution, though mitochondria maintained their double membrane

structure, their overall composition and form has changed drastically. In the process of acquiring various additional functions, the genetic material of the α- proteobacterium was either lost or largely transferred to the host nuclear genome (Gabaldon and Huynen 2004).



Michael W. Gray, G Burger.1999. Science.

Figure 1.1: Origin of eukaryotic cell.

Blue arrows show fusion of a hydrogen-producing α- proteobacterium (symbiont) with a hydrogen-requiring archaebacterium (host) leading to the origin of eukaryotic mitochondrion and nucleus. Red arrows show the formation of an amitochondriate eukaryote by fusion of a proteobacterium and an archaebacterium, later mitochondrion is acquired through endosymbiosis with an α- proteobacterium.

1.3 Mitochondrial form and structure:

Mitochondria are semi-autonomous organelles ranging from 0.5 to 1.5 µm in diameter. The shape and abundance of mitochondria varies from cell to cell depending on its metabolic state and the energy requirement. For example, a mature human RBC is devoid of mitochondria, whereas a

liver cell contains approximately 2000. The shape usually ranges from round to tubular and they appear like a filament in fused form. They have a double membrane system which separates them from the cytosol and divides it into four compartments - inner mitochondrial membrane (IMM), outer mitochondrial membrane (OMM), intermembrane space (IMS), and matrix. The mitochondrial matrix is the innermost space which is bound by the inner membrane. The presence of various porins on the outer membrane provides permeability for the free diffusion of low molecular weight molecules to the intermembrane space. On the other hand, inner membrane is highly impermeable and requires transporter proteins to transport molecules across the membrane. It extends into the matrix forming invaginations called cristae. In addition to these transporters, inner membrane also contains mitochondrial respiratory complexes. Metabolic pathways like citric acid cycle and beta oxidation of fatty acids are carried out in the matrix.

Human mitochondrial genome is circular and small, approximately 16 kilobases which encodes for 13 proteins. These proteins are core components of respiratory complexes I-IV present in the inner membrane. Mitochondria of *Saccharomyces cerevisiae* has genome of approximately 75 kilobases long encoding for eight proteins. Estimated proteome size of mitochondria in human and yeast is approximately 1500 and 1000, respectively.

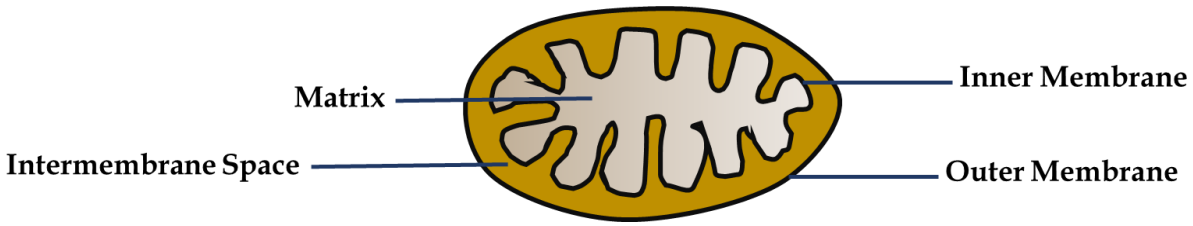


Figure 1.2: Basic structure of the mitochondrion

1.4 Mitochondrial protein import pathways:

Around 99% of mitochondrial proteins are encoded by nuclear genome and synthesized on cytoplasmic ribosomes, which are later imported into the mitochondria through mitochondrial translocase machineries. Based on different types of targeting signal, so far five major protein import pathways have been identified.

Presequence pathway is termed as the classical import pathway. N-terminal presequences are present in most of the matrix proteins and various inner-membrane proteins that act as targeting

signals (Roise, Horvath et al. 1986, Abe, Shodai et al. 2000, Vogtle, Wortelkamp et al. 2009). Translocase of the outer membrane (TOM) are the gate for these proteins to cross the outer membrane. Translocase of the inner membrane (TIM23) imports these proteins through inner membrane (Kiebler, Pfaller et al. 1990, Chacinska, Lind et al. 2005, Mokranjac and Neupert 2015). Finally, they are translocated into matrix by presequence translocase-associated motor (PAM) (Hawlitschek, Schneider et al. 1988, Kang, Ostermann et al. 1990, Horst, Oppliger et al. 1997), where the presequence of the proteins are cleaved off by mitochondrial processing peptidase (MPP) (Hawlitschek, Schneider et al. 1988).

In the other four major pathways of protein import, instead of a cleavable presequence, proteins contain different types of internal targeting signals. However, for both cleavable and non-cleavable precursors, the TOM complex act as the entry gate. The second protein import pathway of mitochondria to be discovered was the carrier pathway. Precursors of metabolite carrier proteins having multispanning hydrophobic domain are imported through TOM, the small TIM chaperones of the intermembrane space, and the carrier translocase channel present in the inner membrane (TIM22) (Sirrenberg, Bauer et al. 1996, Koehler, Jarosch et al. 1998, Sirrenberg, Endres et al. 1998).

β-barrel pathway is the third protein import pathway. β-barrel proteins of the outer membrane are imported via the TOM complex and TIM chaperones. Sorting & assembly machinery (SAM), located on the outer membrane, also called topogenesis of outer membrane β-barrel (TOB) then insert these proteins into the outer membrane (Paschen, Waizenegger et al. 2003, Wiedemann, Kozjak et al. 2003). Many IMS proteins containing specific cysteine motifs are imported through TOM and the mitochondrial import and assembly (MIA) machinery of the IMS, which represents the fourth protein import pathway (Chacinska, Pfannschmidt et al. 2004, Naoe, Ohwa et al. 2004).

Outer-membrane proteins containing α-helical transmembrane segment use the fifth protein import pathway. The mitochondrial import (MIM) complex drives the efficient import of multispanning (polytopic) outer-membrane proteins and outer-membrane proteins possessing a signal-anchor sequence at the N-terminus (Becker, Pfannschmidt et al. 2008, Hulett, Lueder et al. 2008, Popov-Celeketic, Waizenegger et al. 2008).

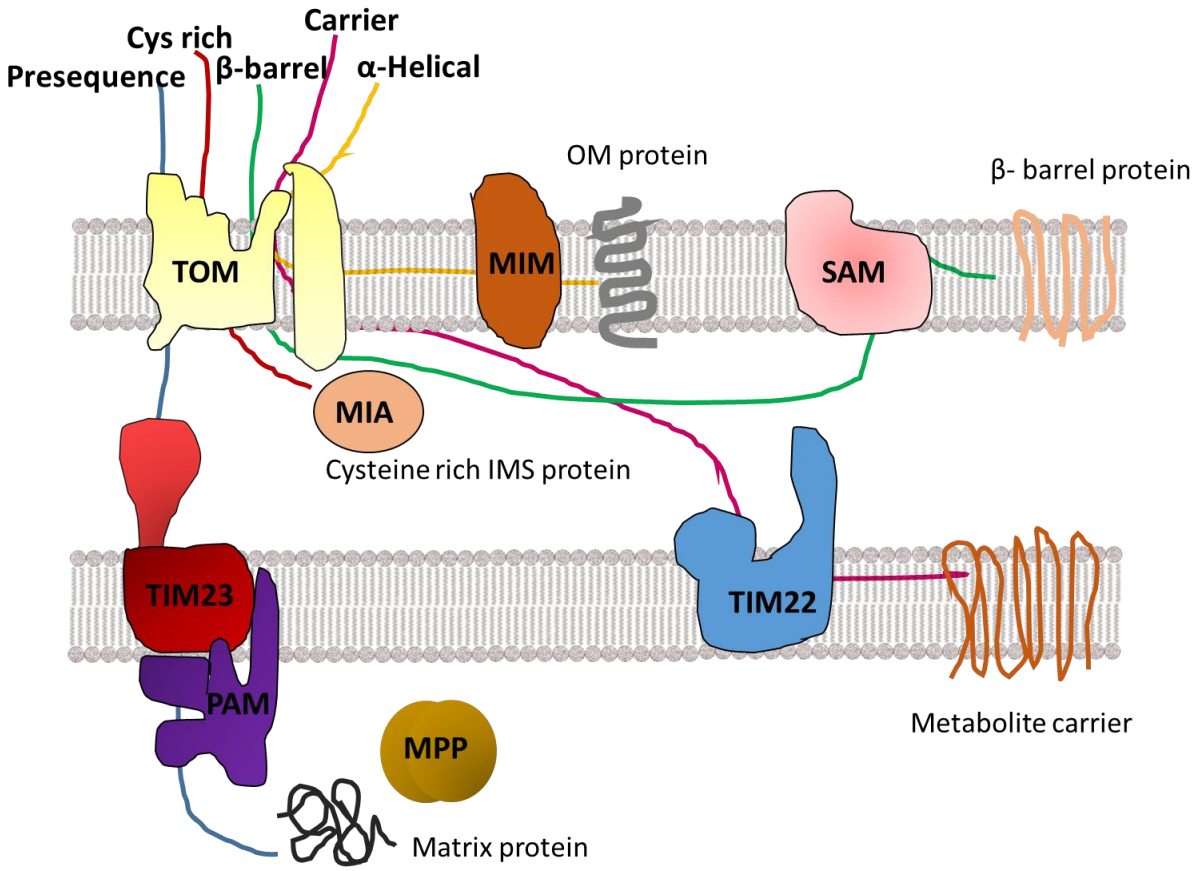


Figure 1.3: Five major pathways of mitochondrial protein import

The translocase of the outer mitochondrial membrane (TOM) and the presequence translocase of the inner mitochondrial membrane (TIM23) import the presequence-carrying pre-proteins. Proteins with a hydrophobic sorting signal are released into the inner membrane (IM), whereas presequence translocase-associated motor (PAM) drives the import of hydrophilic proteins into the matrix where presequences are removed by mitochondrial processing peptidase (MPP). Cysteine-rich proteins of the intermembrane space (IMS) are imported via TOM and by the mitochondrial IMS import and assembly (MIA) system, which inserts disulfide bonds in the proteins. The precursors of β-barrel proteins are transported to the small TIM chaperones of the IMS via TOM and are inserted into the outer membrane (OM) by the sorting and assembly machinery (SAM). α-Helical OM proteins are imported by the mitochondrial import (MIM) complex.

1.5 Mitochondrial quality control (MQC)

As most of the oxygen in the cell is utilized by mitochondria to generate energy, it produces reactive oxygen species (ROS) as a byproduct (Brand 2010) which at low concentrations act as signaling molecules and are useful for the cell but if in excess, they can cause damage to mitochondrial molecules like DNA, proteins, and lipids, and in turn are harmful for the whole

cell. Therefore, the cell has evolved various pathways of quality control systems which act at molecular and organellar level to counteract the damaging effects and to ensure the healthy state of mitochondria (Tatsuta and Langer 2008, Baker and Haynes 2011, Fischer, Hamann et al. 2012, Roca-Portoles and Tait 2021, Song, Herrmann et al. 2021).

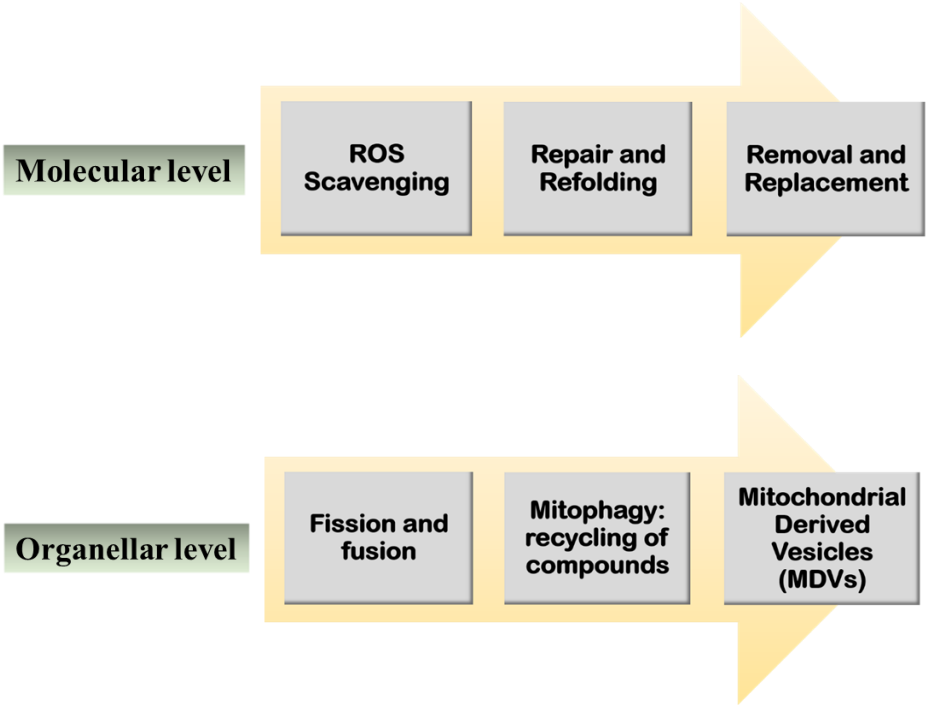


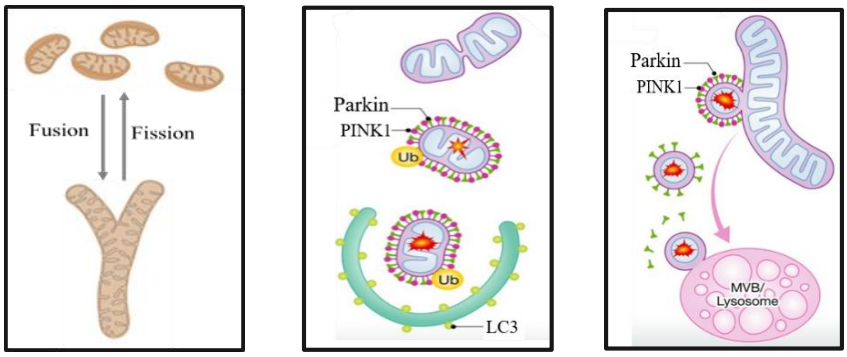
Figure 1.4: Schematic representation of mitochondrial quality control pathways at molecular and organellar levels

ROS scavengers are viewed as a first line of defense in a network of quality control pathways. In mitochondria, ROS scavenging system comprises of enzymes like MnSOD (Superoxide dismutase isoform with manganese in its active centre), glutathione peroxidase, and peroxiredoxin. Molecules like thioredoxin and glutathione also mitigate ROS in mitochondria. Second pathway involves repair and refolding that comes after the damage has happened. These pathways reverse or repair specific modifications and restore the functions of damaged molecules. The methionine sulfide reductase system which consists of MsrA and MsrB, reduces oxidized methionine residues to methionine (Lee, Dikiy et al. 2009). In addition to this, there are chaperones like Hsp22, Hsp60, and Hsp70 that mediate the refolding of misfolded proteins back to their native functional structure (Voos and Rottgers 2002). When the repair and refolding capacity of mitochondria is overwhelmed, mitochondria is capable of inducing mitochondrial

unfolded protein response (mtUPR). This stress response sends signal from mitochondria to nucleus to enhance the expression of nuclear encoded mitochondrial proteins involved in MQC like mtHsp60 and mtHsp70 (Haynes and Ron 2010).

Many proteins cannot be properly refolded or repaired back to their native from. These irreversibly damaged proteins must be efficiently removed by proteolysis. Quality control of proteins that are present on the outer membrane and also those that get imported into the mitochondria is taken care by ubiquitin/26S proteasome system (UPS) of cytosol. (Taylor and Rutter 2011).

Different proteases are active in the inter-membrane space (IMS), the inner mitochondrial membrane (IM) and the mitochondrial matrix (MM) to ensure the quality control inside mitochondria through proteolysis (Koppen and Langer 2007).



Adapted from A.Sugiura et al.2014. The EMBO Journal.

Figure 1.5: Schematic representation of different pathways of mitochondrial quality control at the organellar level.

Mitochondrial fission and fusion is the first pathway of quality control at the organellar level. When mitochondria is damaged severely and cannot be repaired, it undergoes degradation through mitophagy. The damaged portion of mitochondria pinches off the organelle in the form of mitochondrial derived vesicles (MDVs) and gets degraded in the lysosomes.

The first pathway of quality control at the organellar level is fission and fusion of mitochondria that comes into play when the molecular pathways are not sufficient enough to repair the damage. Fission and fusion respond distinctively to different types of stress condition, and maintain mitochondrial function by mixing or separating the mitochondrial contents (Westermann 2010).

When the mitochondria is damaged beyond repair, it is eliminated through the process of

mitophagy where the entire mitochondrion is taken up by autophagosome and gets degraded in the vacuoles/lysosomes (Youle and Narendra 2011).

Lastly, the newly emerging pathway of MQC is where a portion of mitochondria can pinch off and form mitochondria-derived vesicles (MDVs) under stress conditions, carrying the damaged mitochondrial proteins within them, which subsequently fuse with lysosomes/vacuoles for degradation (Sugiura, McLelland et al. 2014).

1.6 Mitochondrial communication with other organelles

Organelles in cell perform essential and distinct functions. Though once they were thought to perform these functions independently, it is now very clear that organelles come in physical contact with each other and these contacts are essential for proper function of organelles and thereby for regulation of cellular homeostasis (Prinz 2014, Eisenberg-Bord, Shai et al. 2016, Gatta and Levine 2017). These contacts are achieved through proteins that tether the organelles directly. Mitochondria make direct contacts with most of the other organelles in the cell, which are of structural and functional relevance. These contacts are mediated through proteins that act as direct tethers between organelles. Hence, such proteins impact on overall mitochondrial homeostasis.

Mitochondria and endoplasmic reticulum contact sites were first visually observed and studied in 1950s and the functional relevance of these contact sites has been extensively studied since 1990s. (Bernhard and Rouiller 1956, Copeland and Dalton 1959, Vance 1990, Rizzuto, Pinton et al. 1998). Over the past two decades, contacts between mitochondria and other organelles have been identified and studied extensively (Figure 6). In addition to ER, mitochondria communicate with vacuoles/lysosomes (Honscher, Mari et al. 2014, John Peter, Herrmann et al. 2017, Wong, Ysselstein et al. 2018), peroxisomes (Fan, Li et al. 2016), lipid droplets (Rambold, Cohen et al. 2015), endosomes (Raiborg, Wenzel et al. 2015), the Golgi (Nagashima, Tabara et al. 2020) , the plasma membrane (Lackner, Ping et al. 2013), melanosomes (Daniele, Hurbain et al. 2014), and nucleus (Eisenberg-Bord, Zung et al. 2021). These contacts vary significantly in structural and functional aspects, and have differential impact on various aspects of mitochondrial function and behavior.

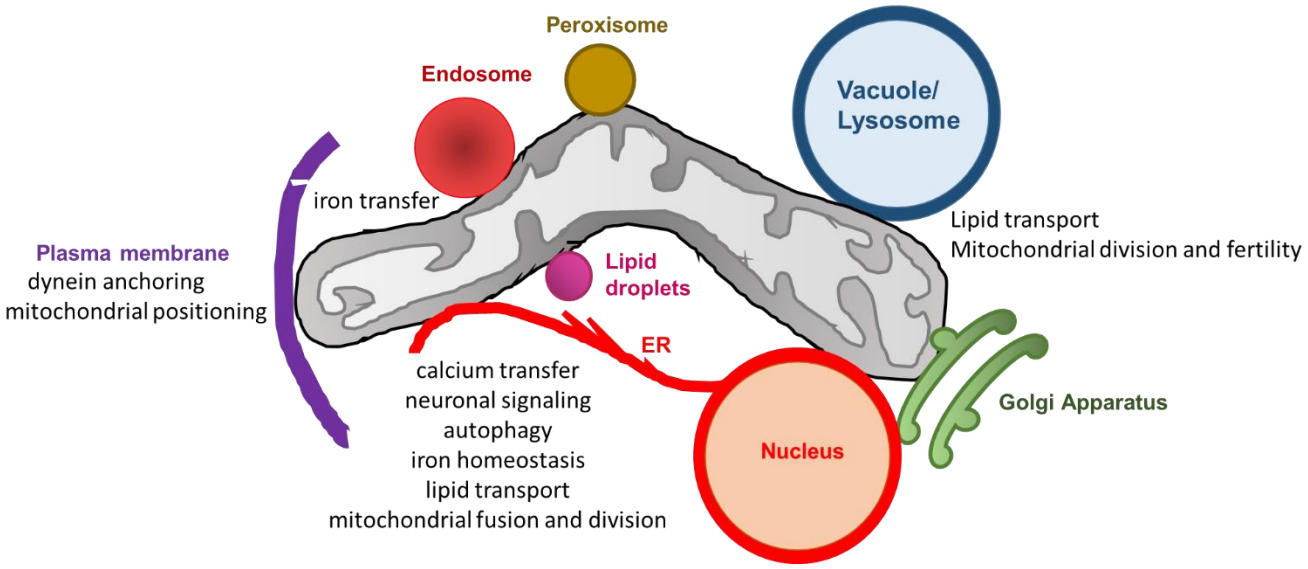


Figure 1.6: Mitochondria communicate with different organelles

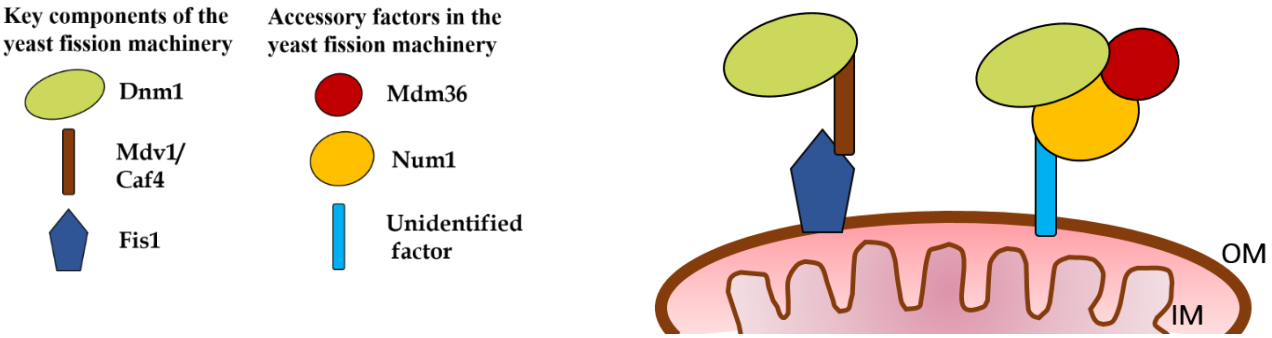
Diagramatic representation of structural and functional relevant mitochondrial contacts with different organelles like nucleus, plasma membrane, vacuole/lysosome, endoplasmic reticulum, lipid droplets, endosomes, peroxisomes and Golgi.

1.7 Mitochondrial Dynamics

In order to maintain their shape, size, and distribution, mitochondria constantly undergo coordinated cycles of fission and fusion, together called as 'mitochondrial dynamics'. These dynamic and transient morphological adaptations of mitochondria are crucial for many cellular processes such as mitochondrial quality control, metabolism, mitophagy, apoptosis, cell cycle, and immunity. Fusion of mitochondria is required to mitigate the damage and non-functionality by mixing components and protecting them from autophagic degradation during starvation (Blackstone and Chang 2011, Gomes, Di Benedetto et al. 2011). Fission helps produce new mitochondria and ensures quality control by removing damaged or unwanted mitochondria through mitophagy (Burman, Pickles et al. 2017). A coordinated balance of fission and fusion is critical for maintaining mitochondrial biology (Scorrano 2007, Chen and Chan 2009). Deregulation in mitochondrial dynamics has been associated with numerous human disorders.

In *Saccharomyces cerevisiae*, Fzo1, Mgm1, and Ugo1 facilitate mitochondrial fusion. Outer membrane-anchored proteins, Fzo1 and Ugo1 carry out the outer membrane fusion of adjacent mitochondria and inner membrane-anchored Mgm1 forms trans-complexes to tether the apposing inner membranes together (Hermann, Thatcher et al. 1998, Wong, Wagner et al. 2000, Sesaki and Jensen 2001). Dnm1, a dynamin-related GTPase, is the major protein in mitochondrial fission

(Otsuga, Keegan et al. 1998, Bleazard, McCaffery et al. 1999). It is predominantly present in the cytosol and recruited to mitochondria via Fis1 (Mozdy, McCaffery et al. 2000). Dnm1 assembles into oligomers which form rings and spirals at the outer membrane of mitochondria. Recruitment of Dnm1 to the mitochondrial surface is mediated through two adaptor proteins, Mdv1 and Caf4 (Tieu and Nunnari 2000, Griffin, Graumann et al. 2005). These four proteins together constitute the core proteins of the mitochondrial fission machinery. A list of proteins regulating the process of mitochondrial fission and fusion in both yeast and mammals have been listed in Table 1.1 and Table 1.2. However, there is a possibility that other unidentified factors still exist that take part in mitochondrial dynamics (Legesse-Miller, Massol et al. 2003). Several studies have shown that any deregulation in mitochondrial dynamics leads to neuronal disorders like Alzheimer's, Parkinson's, and Huntington's (Narendra, Tanaka et al. 2008, Cho, Nakamura et al. 2009, Shirendeb, Calkins et al. 2012).



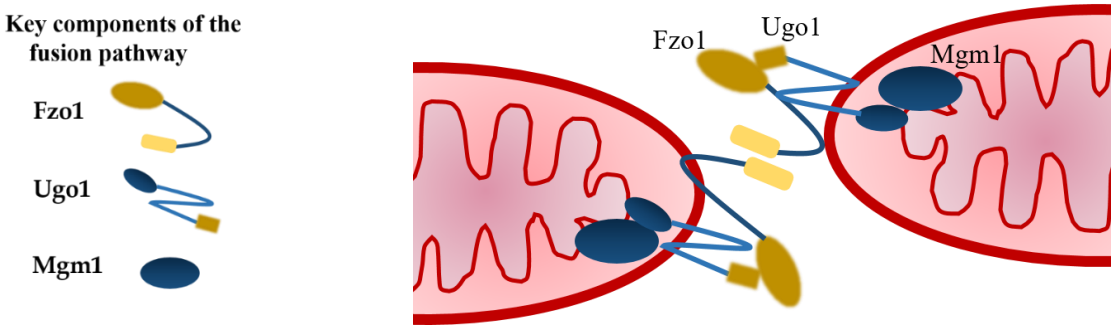


Figure 1.7: Diagrammatic representation of mitochondria fission and fusion in *S.cerevisiae*

In yeast, mitochondrial fission is driven by two major proteins Fis1 and Dnm1. Dnm1 is recruited to mitochondrial outer membrane via Fis1, with the help of two adapter proteins Mdv1 and Caf4. Other accessory proteins like Num1 and Mdm36 also help in mitochondrial divison. Mitochondrial fusion is carried out by three core proteins Fzo1, Mgm1, and Ugo1. Fzo1 and Ugo1 together are repsonsible for the fusion of outer membrane whereas Mgm1 facilitates the fusion of the inner membrane of mitochondria.

Table 1.1- Mitochondrial fission proteins in yeast and mammals

Protein	Yeast	Mammals	Subcellular Localization
Key Players	Dnm1	Drp1	Cytosol and MOM-associated
	Fis1	Fis1	MOM-anchored
	Mdv1	-	Cytosol and MOM-associated
	Caf4	-	Cytosol and MOM-associated
	-	Mff	MOM-anchored
	-	MiD51/MiD49	MOM-anchored
Regulators	Num1p	-	Cell-cortex and MOM-associated
	Mdm36p	-	MOM-associated
	Mdm33p	-	MIM-anchored
	-	Endophilin B1	Cytosol and MOM-associated
	-	GDAP1	MOM-anchored
	-	MTP18	MIM-associated
	-	MTGM	MIM-anchored

Table 1.2- Mitochondrial fusion proteins in yeast and mammals

Protein	Yeast	Mammals	Subcellular Localization
Key Players	Fzo1p	Mfn1/2	MOM-anchored
	Mgm1p	OPA1	MIM-anchored, IMS
	Ugo1p	–	MOM-anchored
	–	MIEF1	MOM-anchored
Regulators	–	MICS1/GHITM	MIM-anchored
	–	MIB/VAT1	Cytosol and MOM-associated
	–	Stoml2/SLP2	IMS/MIM-associated
	–	BAX and BAK	Cytoplasm and MOM-associated
	–	mitoPLD	MOM-anchored
	Mdm38p	LETM1	MIM-anchored

Apart from these proteins, other organelles have also been found at the mitochondrial fission sites. It is well studied that membrane contact sites between mitochondria and ER are essential to drive the initial steps of fission process. Several studies have reported that not only ER, but other organelles like lysosome (Wong, Ysselstein et al. 2018), actin (Korobova, Ramabhadran et al. 2013), and *trans*-Golgi network (TGN) vesicles (Nagashima, Tabara et al. 2020) have important role in mitochondrial division.

Given the complexity of the process, there is possibility that there are still various other factors which are yet to be discovered.

1.8 Eisosomes

What are they?

The fungal plasma membrane is organized in lateral domains with specialized functions like cell

wall synthesis, environmental sensing, nutrient uptake, secretion, and endocytosis (Malinska, Malinsky et al. 2003, Malinsky, Opekarova et al. 2010, Ziolkowska, Christiano et al. 2012). Plasma membrane requires an efficient organization as it performs a vast variety of functions apart from being a protective layer around the cell. The studies over past few years have shown that PM is majorly subdivided into three domains: MCP (membrane compartment of Pma1, the plasma membrane H+- ATPase), MCT (membrane compartment of TORC2) and MCC (membrane compartment of Can1, the arginine permease). MCP is the zone for cell wall synthesis and rapid protein diffusion. It consists of Pma1, which is the most abundant protein of the plasma membrane. MCT domain, as the name suggests, contains TORC2 kinase and is known to regulate cell polarity and sphingolipid synthesis. There are around 50 MCC domains per cell and it has been shown that they are associated with a complex of cytoplasmic proteins called eisosome.

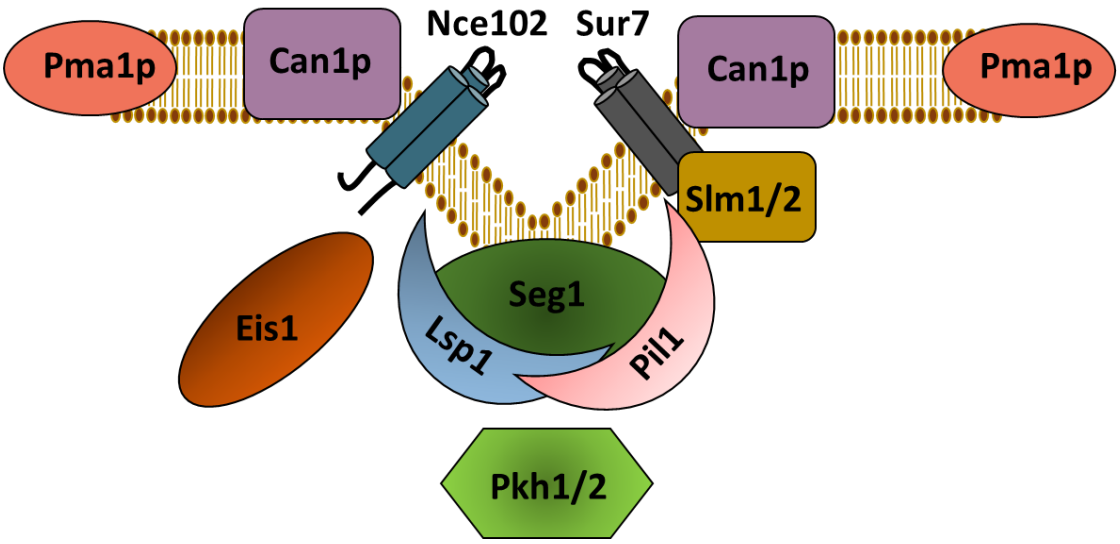


Figure 1.8: Eisosome structure in *S. cerevisiae*

Eisosome is a complex of proteins located peripherally on the plasma membrane . Eisosomes form a furrow like invagination in the plasma membrane. Pil1 and Lsp1 are the core components of this complex.

High resolution electron microscopy of freeze-etched *Saccharomyces cerevisiae* has shown that these domains form furrow like invaginations in the plasma membrane (Moor and Muhlethaler 1963, Malinska, Malinsky et al. 2004, Stradalova, Stahlschmidt et al. 2009). They are known to be found only in a few fungal species, microalgae and lichens (Lee, Heuser et al. 2015). Pil1 and Lsp1 are the major components of eisosomes. Both these proteins contain BAR (Bin/Amphiphysin/Rvs) domains and PIP2-binding PH domains. Around 17 proteins have been

identified so far that form the eisosome complex, and it is possible that many more are are yet to be discovered (Table 1.3).

Table 1.3- List of known eisosome proteins

Eisosome proteins

Name	Domain
Pil1	BAR domain
Lsp1	BAR domain
Pkh1	Ser/Thr protein kinase
Pkh2	Ser/Thr protein kinase
Eis1	Unknown
Slm1	BAR domain and PH domain
Slm2	BAR domain and PH domain
Seg1	Unknown
Mdg1	Unknown
Ygr130c	Unknown
Pst2	Similar to flavodoxin-like proteins
Rfs1	Similar to flavodoxin-like proteins
Ycp4	Similar to flavodoxin-like proteins
Msc3	Protein of unknown function

Why the name eisosome?

In early functional studies, it was suggested that eisosomes coincide with sites of endocytosis. Hence, the structures were named eisosomes after the Greek words *eis* which literally translates to 'entry', and *soma* which means 'body'. However, studies carried out this field later have shown that eisosomes are not sites of endocytosis, but the name has remained.

What is their function?

They have been shown to bind membranes and facilitate the membrane curvature and formation of furrows (Karotki, Huiskonen et al. 2011, Douglas and Konopka 2014). Eisosomes regulate the lipid homeostasis at the plasma membrane and also the recruitment of proteins for subsequently protecting them from endocytosis (Grossmann, Opekarova et al. 2007). Another possible function of eisosome is to maintain membrane reservoir for plasma membrane expansion in response to various stresses (Kabeche, Howard et al. 2015). Recent developments in this area of research has piqued interest particularly due to association of eisosomes with RNP granules, stress granules,

P-bodies and their role in protection of nutrient transporters in response to starvation stress (Grousl, Opekarova et al. 2015, Gournas, Gkionis et al. 2018, Moharir, Gay et al. 2018, Amen and Kaganovich 2020).

1.9 Autophagy

Autophagy, which literally translates to "self-eating" has been extensively studied in past three decades. It is a process of cellular degradation and recycling which is highly conserved in eukaryotes. Though autophagy is cytoprotective, it can be deleterious if in excess. Autophagic dysfunction has been linked with various human pathologies like liver, heart and lung diseases, neurodegenerative disorders, myopathies, ageing, cancer, and diabeties (Wirawan, Vanden Berghe et al. 2012). Though extensive study has been done in this area, there are still many unanswered questions that lie at the regulatory and mechanistic levels, and are yet to be resolved.

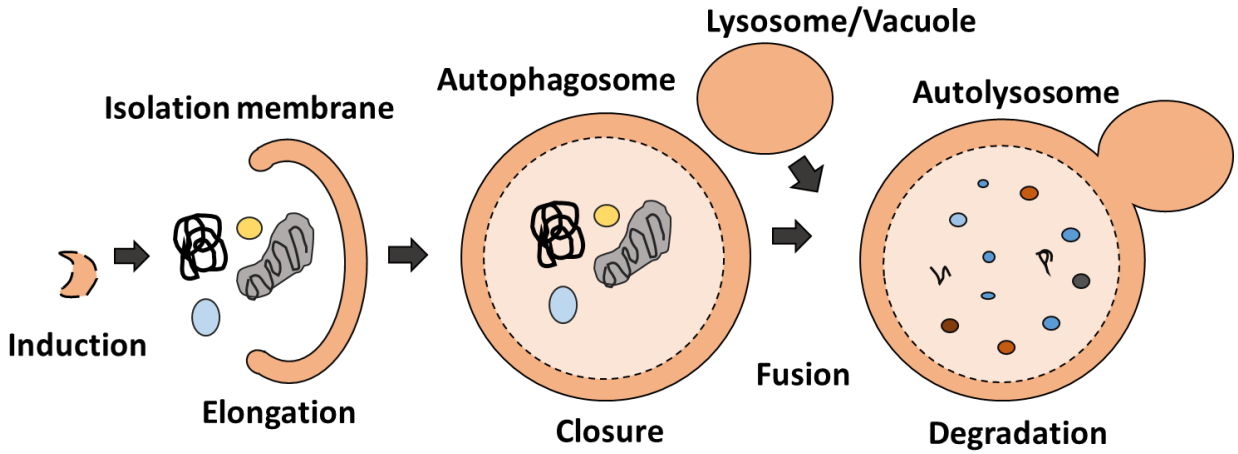


Figure 1.9: General process of Autophagy

Process of autophagy begins with formation of isolation membrane which expands and encloses to form autophagosome. The autophagosome carries the cargo within it and fuses with the lysosome/vacuole where the cargo gets degraded and recycled.

Autophagy can mainly be divided into two types- macroautophagy and microautophagy, both of them consist of selective and non-selective processes (Shintani and Klionsky 2004). In both selective and non-selective autophagy (Suzuki 2013), the morphological hallmark is the formation of isolation membrane which encloses to form the autphagosome that engulfs the cargo in the cytoplasm and fuses with vacuole or lysosome in case of mammals, where the contents undergo

degradation and recycling. Microautophagy refers to the transfer of cytoplasmic components into the lysosome/vacuole by direct invagination and scission to produce intravacuolar vesicles that are degraded subsequently (Kunz, Schwarz et al. 2004) (Uttenweiler and Mayer 2008). Nonselective microautophagy is not very well defined with regard to its physiological role or machinery involved. Selective microautophagy is useful in the turnover of peroxisomes (Dunn, Cregg et al. 2005), mitochondria (Deffieu, Bhatia-Kissova et al. 2009) and parts of nucleus (Krick, Tolstrup et al. 2006). Macroautophagy is the sequestration of the targeted cargo into autophagosomes that subsequently fuse with the vacuole followed by the degradation and recycling of the macromolecules (Eskelinen, Reggiori et al. 2011).

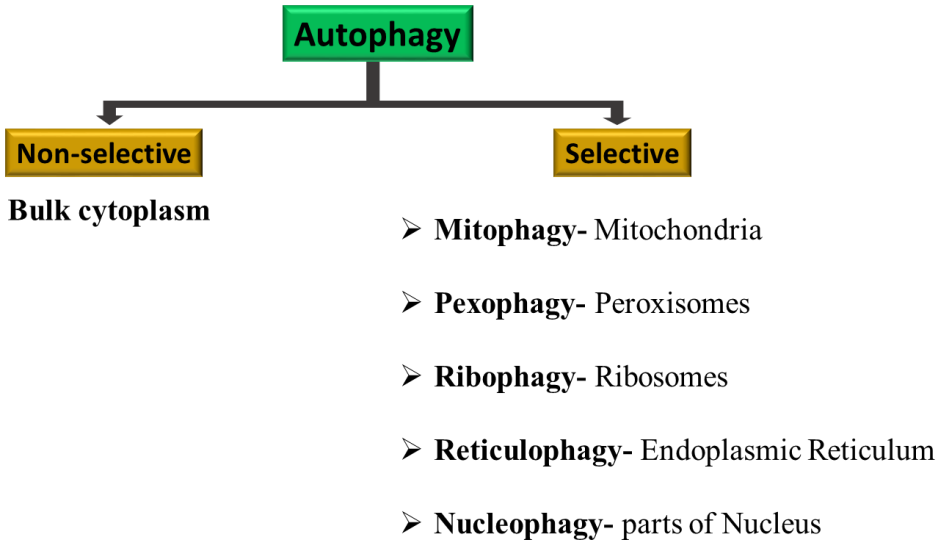


Figure 1.10: Types of Autophagy

Autophagy is mainly divided into selective and non-selective types. Non-selective autophagy involves the random degradation of bulk cytoplasm, whereas selective autophagy targets specific organelles like mitochondria, peroxisomes, ER, or nuclues.

The process of autophagy is carried out by several ATG proteins that form different complexes at the different stages of the autophagic pathway. They were first discovered in yeast and have been studied extensively since past three decades and this work received Nobel Prize in the year 2016. To date, 42 autophagy-related (ATG) genes have been identified. Almost half of them have a clear homolog in higher eukaryotes (Table 1.4). Most of the ATG genes are needed for both non-selective and selective autophagy, but some are specific for specific types of autophagy (Johansen and Lamark 2011).

Table 1.4- List of autophagy genes in yeast and mammals

Yeast ATG gene	Mammalian ATG homolog(s)
ATG1	ULK1
ATG2	ATG2A,ATG2B
ATG3	ATG3
ATG4	ATG4A, ATG4B, ATG4C, ATG4D
ATG5	ATG5
ATG7	ATG7
ATG8	LC3, GABARAP, GABARAPL1, GABARAPL2
ATG9	ATG9
ATG10	ATG10
ATG12	ATG12
ATG13	ATG13
ATG14	ATG14
ATG16	ATG16L1, ATG16L2
ATG17	RB1CC1
ATG18	WIPI1, WIPI2, WIPI3, WIPI4
VPS30/ATG6	BECN1
VPS34	PIK3C3
	VMP1
	EI24
	EPG5
	ATG101

1.10 Mitophagy

Mitochondria perform various important functions in cells apart from its major function of energy generation. During these mitochondrial processes, reactive oxygen species are generated as a by-product, which is a potential threat for cells as they can cause damage to the organelle as well as the entire cell. In order to maintain the healthy population, mitochondria have evolved various pathways to ensure the quality control (section 1.5). Mitochondria when damaged severely and cannot be efficiently repaired by other pathways of quality control, undergo degradation through mitophagy. Mitophagy is a type of selective autophagy where the damaged mitochondria is engulfed by autophagosomes which fuses to vacuoles and gets degraded. Yeast has simple genetics. As fundamental cellular pathways are conserved among eukaryotes, yeast is an excellent model for studying and understanding the process of mitophagy at the molecular and functional level. In budding yeast, Atg32, a mitochondrial outer membrane anchored protein, acts as a receptor for mitochondrial degradation (Okamoto, Kondo-Okamoto et al. 2009). Atg32 interacts with Atg11 and Atg8. Atg11 acts as an adaptor protein for selective autophagy (Kanki and Klionsky 2008). Atg8 is a ubiquitin-like protein which localizes to the isolation membrane and is

essential for formation of autophagosome (Shpilka, Weidberg et al. 2011).

Recent history of studies have revealed that mitochondrial fission and fusion proteins also come into the play in the process of mitophagy (Abeliovich, Zarei et al. 2013, Burman, Pickles et al. 2017). Because autophagy is a complex phenomenon which involves a large number of proteins and various pathways, it is quite possible that there are other factors as well which need to be explored in order to gain better understanding.

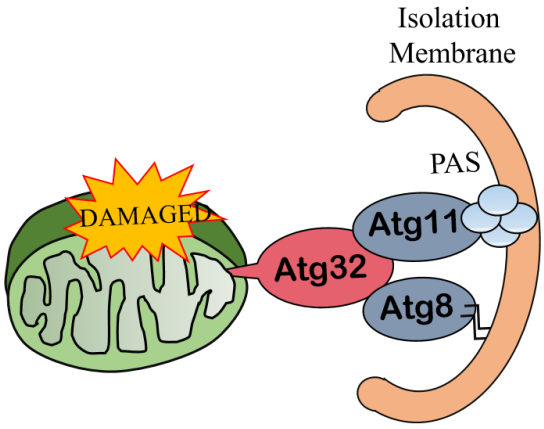


Figure 1.11: Mitophagy in yeast

Atg32, a receptor for mitophagy is present on the outer membrane of mitochondria. Atg32 binds to Atg8 and Atg11, and subsequently the damaged mitochondria are engulfed by autophagosome.

1.11 Reactive Oxygen Species (ROS)

The interest in role of "reactive species" has increased tremendously in the past few decades because they have both beneficial as well as detrimental effects in cell physiology and pathology. In biology and medicine, reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS) are the known reactive species which are basically the chemically reactive molecules of the respective elements. Out of all three, ROS has attracted interest and is the most widely studied reactive species.

The term ROS broadly includes a wide array of molecular oxygen derivatives which includes oxygen free radicals such as hydroxyl (•OH), superoxide anion ($O2^{\bullet-}$) ,and non-radical oxidants such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).

ROS are the natural byproducts of oxidative metabolism in the cells. These are known to play

important roles in the regulation of cell signaling, cell differentiation, inflammation related response, cell survival, and cell death (Mueller, Laude et al. 2005, Touyz 2005).

Reactive Oxygen Species and Mitochondria

Mitochondria are known to be the main source of ROS in cell. ROS production in mitochondria was once considered to be an unwanted by-product of oxidative phosphorylation. But increasing evidence indicates that mitochondrial ROS (mtROS) is also regulated by immune responses and vice versa (Bulua, Simon et al. 2011, West, Shadel et al. 2011). mtROS have also been shown as components of the pathways that regulate stem cell differentiation (Maryanovich and Gross 2013), cellular (Mills and O'Neill 2014, Gurung, Lukens et al. 2015) and tissue level (Mittal, Siddiqui et al. 2014) inflammation, autophagy (Chen, Azad et al. 2009), and apoptosis (Bender and Martinou 2013). ROS and mitochondria have been established as a nexus of cellular homeostasis due to their role in determining the response of cells to cellular physiology.

1.12 Cell death

Cell death is collectively referred to the targeted elimination of irreversibly damaged, potentially harmful, or superfluous cells. There are different genetically encoded mechanisms for a cell to undergo the process of death. It has been now established that regulation of cell death is conserved in yeast (Madeo, Frohlich et al. 1997, Madeo, Carmona-Gutierrez et al. 2009, Carmona-Gutierrez, Eisenberg et al. 2010).

Mainly two types of cell death have been described in yeast (Figure 1.12). One type of cell death in yeast in accidental which occurs when cells are exposed to severely harsh microenvironmental conditions. Another type of cell death is regulated cell death (RCD) which can occur when cells fail to respond to mild stress (Galluzzi, Vitale et al. 2012). While ACD has necrotic characteristics, RCD can exhibit mixed characteristics of regulated necrosis and apoptosis. Though the cytoprotective function of autophagy is well established, what role autophagy has as a cell death pathway still remains unclear. It has been reported in some studies that both cell death and autophagy can be controlled by the same regulators, showing that both the processes are closely connected.

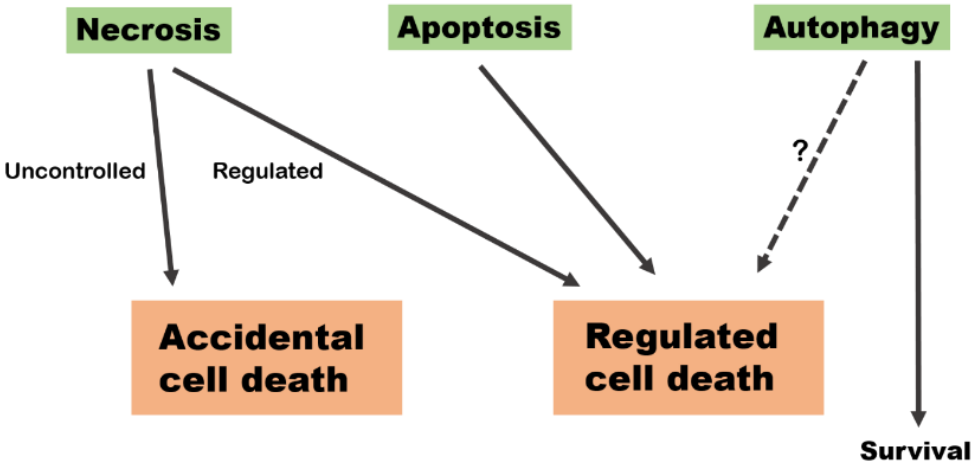


Figure 1.12: Types of cell death in yeast.

An yeast cell can die either via accidental cell death or regulated cell death depending upon the external and internal cues. Uncontrolled necrosis is categorized under ACD while regulated necrosis falls under RCD. Recently yeast has been shown to undergo apoptosis, a type of regulated cell death. Autophagy, though a survival process, can be a regulated cell death process under certain conditions.

1.13 Rationale and Objectives

In this work, we have tried to establish a connection between mitochondria and eisosomal protein Pil1. So far no reports are available that hint towards a plausible connection between these two cellular structures. However, in a study, Nce102, a plasma membrane protein implicated in eisosome formation, has been shown to rescue heme defect in mitochondria (Kim, Jeong et al. 2016). Also, high-throughput study shows the presence of eisosome core proteins Pil1 and Lsp1 in mitochondria (Reinders, Wagner et al. 2007, Ohlmeier, Hiltunen et al. 2010). We tried to understand the topological and functional connection of Pil1 protein with mitochondria based on its known properties and functions. In order to do that, this work was divided in the following four objectives:

1. To study the localization of Pil1 to mitochondria
2. To study the role of Pil1 in maintaining mitochondrial morphology
3. To study the role of Pil1 in autophagy/mitophagy
4. To study the role of Pil1 in oxidative stress and cell death

CHAPTER 2

Pil1 localizes to mitochondrial outer membrane

2.1 BACKGROUND

Though eisosomes were believed to be static structures, a recent study has shown the exchange of Pil1 molecules at the tip of eisosomes indicating their dynamic nature. Also, Pil1 is reversibly phosphorylated at multiple sites which raises the possibility that this protein can be dynamic in nature.

In studies that aimed to characterise the novel role of yeast mitochondrial phosphoproteome, Pil1 has been detected as one of the proteins associated with mitochondria in its phosphorylated form (Reinders, Wagner et al. 2007, Ohlmeier, Hiltunen et al. 2010) . When we tried to search literature to find if any link between mitochondria and any eisosome proteins has been reported already, we did not find any direct link. However, there is a report which says that Nce102 protein, which is a component of MCC, but not a part of eisosome complex, rescues heme defect in mitochondria (Kim, Jeong et al. 2016). Apart from this, when we looked into the interacting partners of Pil1 in *Saccharomyces cerevisiae* genome database (SGD), we found many mitochondrial proteins (Fig 2.1)

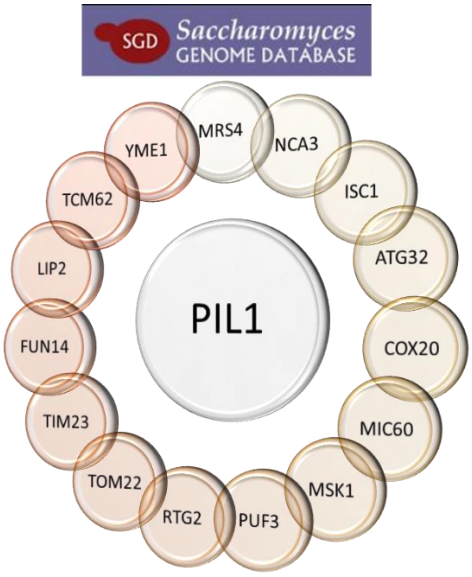


Figure. 2.1- Mitochondrial interacting partners of Pil1 from high-throughput analysis.

Source: *Saccharomyces cerevisiae* genome database (SGD)

Around 60 percent of the proteins that are targeted to mitochondria carry mitochondrial targeting Signal (MTS) which is a short peptide of around 15-70 amino acid long that contains positively charged basic amino acid residues that target the protein to the mitochondria. We did bioinformatical analysis using two different online tools (MitoProt II and PSORT II) to check the possibility of Pil1 being targeted to mitochondria on the basis of the presence of MTS. And we found that probability of Pil1 getting into the mitochondria is very high (Figure 2.2). These evidence provides a possible link between mitochondria and eisosomes and it formed the basis of our study.

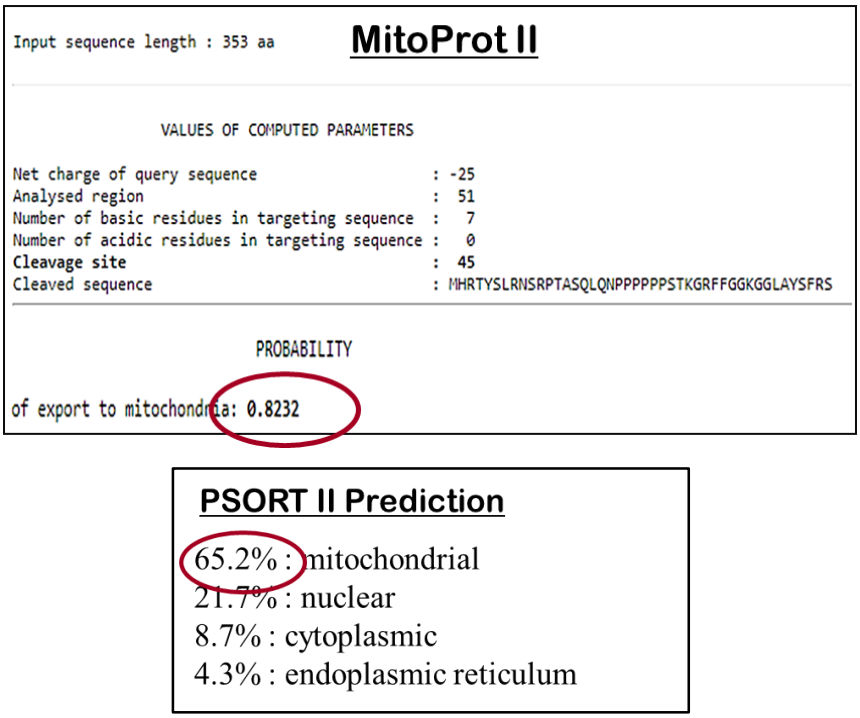


Figure 2.2- Probability of Pil1 getting targeted to mitochondria using MitoProt II and PSORT II online bioinformatics tool

2.2 MATERIALS AND METHODS

2.2.a. Media and growth condition

For mitochondria proliferation, cells were grown in lactate medium (YPL; 1% yeast extract, 2% peptone, and 2% lactate). For fluorescence imaging, strains were grown to log phase (OD_{600} = approximately 1) in synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) as described previously (Sherman 1991). Yeast cells were grown at 30°C.

2.2.b. Yeast strains and plasmids

The yeast strains used in this study are derivatives of BY4741. All strains, plasmids, and primers used in this study are listed in Table 2.1, Table 2.2, and Table 2.3, respectively. For expression of Pil1-GFP under the control of Pil1 promoter, PIL1 was PCR-amplified (approximately 500 bp of 5'UTR before start codon + Pil1 ORF before stop codon) along with its promoter using yeast genomic DNA as template using primers NB879F and NB755R. The PCR product was digested with BamHI and HindIII. GFP sequence was amplified using Mge1-GFP plasmid as template with primers pair NB806F/NB808R and double digested with HindIII/XhoI. PIL1 3'UTR (approximately 500 bp after stop codon) was amplified using primers pair NB797F/ NB798R and double digested with XhoI/ApaI. All the three inserts were ligated simultaneously with BamHI/ApaI digested pRS313 vector. The expression was confirmed with microscopy as well as western blot.

Pil1-Myc strain was a kind gift from Victor J Cid (Complutense University of Madrid, Madrid. pHS12 Mito-mCherry plasmid was a gift from Shirisha Nagotu (IIT Guwahati). For construction of Pil1 deletion strain (YNB 263) used in this study, PCR-based targeted homologous recombination was used to replace complete open reading frame of *PIL1* with KanMX6 cassette (Longtine, McKenzie et al. 1998). This strategy is based on directing the PCR-amplified cassette to the desired chromosomal loci which is achieved due to flanking homologous sequences provided in the PCR-primers. In this method, the PCR primers (NB985/NB986) used have 5'-ends (50 nucleotides) carrying Pil1 gene sequences and 3'-ends (20 nucleotides) that correspond to selectable marker gene (KanMX6 in this case). The amplified DNA is transformed into yeast cells using standard method, and colonies were screened to select those that carry the deleted *PIL1* gene.

Table 2.1. Strains

Strain	Genotype	Reference
YNB 263	BY4741; *PIL1-6XMYC::LEU2*	(Mascaraque, Hernaez et al. 2013)
YNB 474	BY4741; *pil1*Δ::*KanMX*	This study

Table 2.2. Plasmids

Plasmid		Source/Reference
pNB479	pFA6a-KanMX6	(Longtine, McKenzie et al. 1998)
pNB812	pHS12 Mito-mCherry	Addgene
pNB485	pRS313 Pil1-GFP	This study

Table 2.3. Primers

Primer Name	Sequence (5'-3')	Restriction enzyme
NB755	GCCAAGCTTAGCTGTTGTTTGTTGGGGAA	HindIII
NB879	GCCGGATCCACCATGCACAGAACTTACTCTTTAA	BamHI
NB806F	GCCAAGCTTGGAGGTGGAGGTTCTGGT	HindIII
NB808R	GCCCTCGAGTTTGTATAGTTCATCCATGCCATGTG	XhoI
NB797F	GCCCTCGAGTCAAAATAAATCAACAATCTATTAGAAACA	XhoI
NB798R	GCC GGGCCCGAAGGCGGTGAAAACAAAAA	ApaI
NB985	ATGCACAGAACTTACTCTTTAAGAAATTCCAGGGCACCT ACCGCCTCTCACGGATCCCCGGGTTAATTAA	
NB986	TTAAGCTGTTGTTTGTTGGGGAAGAGACTCACTTTGCTGG TGTCCGACTTGAATTCGAGCTCGTTTAAAC	

2.2.c. Yeast transformation

Yeast transformation was done using standard lithium acetate method (Gietz and Woods 2002). Yeast strains were grown overnight in 30°C in 10 mL YPD and were harvested by centrifuging at 3000 rpm for 5 min. Cells were washed with 15 mL of sterile water, supernatant was discarded and the pellet was resuspended in 1 mL of 100 mM lithium acetate (LiAc). Depending on cell density, cells were aliquoted into 1.5 mL tubes for each transformation. Cells were pelleted down and the supernatant was discarded. 350 µL of transformation mixture (216 µL 50% PEG 3000, 36 µL 1M LiAc, 40 µL ssDNA, 58 µL Milli-Q water) was added to each tube. Tubes were vortexed for proper mixing and incubated at 42° C for 40 min. After incubation, cells were spinned down at 7000 rpm for 1 min. and the transformation mix was discarded using pipet. Cells were resuspended in 100 µL of sterile water and plated on respective selection plates. Plates were incubated for 2-3 days at 30°C.

2.2.d. Bacterial transformation

50 µL (for plasmid transformation) or 100 µL (for transformation of ligation product) of ultra-competent DH5α cells were thawed on ice and approximately 100 ng of plasmid to be transformed was added to it. Cells were incubate on ice for 30 min. and then given a heat shock at 42°C for 90 seconds and immediately placed on ice for 3-5 min. 1 mL of autoclaved LB media without any antibiotic was added to the tube and kept in shaker incubator for 1 hour at 37°C. In case of plasmid transformation, 100 µL from 1 mL was taken and for transformation of ligated product, cells were pelleted down, resuspended in 100 µL of LB and then plated on respective antibiotic containing plates. Plates were incubated overnight at 30°C.

2.2.e. Mitochondrial fractionation

Mitochondria isolation was done as described previously (Reinders, Zahedi et al. 2006, Sepuri, Yadav et al. 2007, Sepuri, Gorla et al. 2012). In brief, yeast strains were grown in YPL medium at 30°C, and cells were harvested when the cultures reached OD_{600} of 1 or 2. Cultures were centrifuged at 5,000 rpm for 5 min, and the cell pellets were washed with autoclaved distilled water. The cells were treated with 10 mM dithiothreitol (DTT; Sigma, D0632) in 0.1M Tris-SO4, pH 9.4, buffer for 15 min and centrifuged at 5,000 rpm for 5 min. Cells were converted to spheroplasts by using Zymolyase (20T, USBiologicals, Z1000) in 1.2 M sorbitol/20mM phosphate buffer, pH 7.0. After obtaining 50% lysis of cells (lysis correlated to a decrease in

OD_{600} of 100 µl of cells in 900 µl of water), the resulting spheroplasts were gently washed with 1.2M sorbitol two or three times. The spheroplast pellets were resuspended in SEM buffer (250mM sucrose [Amresco,0335], 1 mM EDTA [Himedia,GRM1195], 10 mM 3-(N-morpholino) propanesulfonic acid [MOPS; Sigma, M1254], 1 mM phenylmethylsulfonyl fluoride [PMSF; Himedia, RM1592], 0.2% bovine serum albumin [BSA; Amresco, 0332], pH 7.0) and homogenized using a Dounce homogenizer (15 times). The homogenates were centrifuged at 3500 rpm. The supernatants were collected and centrifuged at 10,000 rpm for 10 min. The resultant pellets were resuspended in SEM buffer and the supernatant was collected (post-mitochondrial supernatant) and centrifuged at 3500 rpm for 5 min. The supernatants were once again centrifuged at 10,000 rpm for 10 min and pellets were washed three times in SEM buffer. The crude mitochondria were resuspended in SEM buffer (without BSA) and were subjected to 10-15 strokes with a glass-Teflon potter and loaded onto a three-step sucrose gradient (60% 1.5mL, 32% 4mL, 23% 1.5mL ,15% 1.5mL sucrose in EM-buffer) and centrifuged for 1 hr at 1,34,000 g, yielding mitochondrial fraction at the 60-32% sucrose-interface. We repeated the above step to obtain a highly purified mitochondrial fraction.

2.2.f. Hypotonic treatment, Trypsin digestion, and Salt extraction

For making mitoplast, 50 µg of intact mitochondria was taken and the volume of each was made up to 50 µL with Milli-Q water. 950 µL of solubilization buffer (0.6 M sorbitol, 5 mM $MgCl_2$, 50 mM KCl, 0.1 M potassium acetate, 20 mM HEPES-KOH pH 7.4, 1% Triton X-100) was added and incubated on ice for 15 minutes. Mitochondria was centrifuged at 14,000 rpm for 10 minutes at 4°C. Pellets were washed twice with SEM buffer and then dissolved in 1X RIPA buffer and processed for SDS-PAGE.

For trypsin digestion, 50 µg of intact mitochondria were taken in 50 µL of SEM buffer and treated with 1 µg, 2 µg and 5 µg (final concentration) of trypsin (Amresco, 0785) on ice for 15 min. Protease treatment was stopped by adding 5 µg, 10 µg and 25 µg (final concentration) soybean trypsin inhibitor (Amresco, K213), respectively, and incubated on ice for 15 min. Mitochondria were pelleted down at 14,000 g for 10 min at 4°C.

For high salt treatment, 50 µg mitochondria in 50 µl of SEM buffer were incubated with 400 mM KCl on ice for 10 min. Supernatant and pellet were separated by centrifugation at 14,000 rpm for 15 min. For high pH treatment, mitochondria were incubated with 200 mM Na_2CO_3 (pH 11.5) for

15 min followed by centrifugation to separate the pellet and supernatant.

2.2.g. Immunoblotting

After separation in SDS-PAGE, proteins were transferred onto nitrocellulose membranes (PALL, 66485) and blocked with 5% skimmed milk (Himedia,GRM1254) in Tris-buffered saline (TBS). The commercial monoclonal antibody anti-myc (ab9106, Abcam, 1:5000) was used to detect myc epitope-fused protein on immunoblots. Anti-Dpm1 antibody (A-6429) was purchased from Invitrogen. Anti-Por1, Pgk1, Tom70, Tom40, Tim22, and Hsp70 serums were kind gifts from Debkumar Pain (New Jersey Medical School, Rutgers University, Newark, NJ). Anti-Pdi1 antibody (ab4644, Abcam, 1:10000) was a kind gift from Krishnaveni Mishra, University of Hyderabad. Anti-Pma1 antibody (sc-33735) was a kind gift from Rupinder Kaur, Centre for DNA Fingerprinting and Diagnostics, Hyderabad. HRP-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, 111-035-144, 1:25,000) or anti-mouse (Jackson ImmunoResearch Laboratories, 115-035-146, 1:25,000) polyclonal secondary antibodies were used, followed by the detection of signal using ECL reagents (Advansta Western-Bright, K-12045-D20) and imaging in Chemidoc Imaging System (Bio-Rad).

2.3 RESULTS

2.3.a. Pil1 localizes to mitochondria

Previously, in phosphoproteome profiling of yeast mitochondria, it has been reported that Pil1 is present in mitochondria in phosphorylated state (Reinders, Wagner et al. 2007, Ohlmeier, Hiltunen et al. 2010). In order to confirm these findings, we isolated mitochondria from the yeast WT strain where Pil1 is chromosomally tagged with 6XMyc tag at the C-terminus. Whole cell extract, cytosolic fractions (post-mitochondrial supernatant) and sucrose gradient highly purified mitochondrial fractions were processed for SDS-PAGE and probed with antibodies against Myc, Porin, Pgk1, Dpm1, Pdi1 and Pma1. The immunoblotting results show that Pil1 is enriched in mitochondrial fraction as well (Figure 2.3A). Absence of Pgk1 band in mitochondrial fractions shows that it is devoid of any cytosolic contamination. To further check if mitochondrial fraction is contaminated with ER, we used anti-Pdi1 antibody which is ER-lumen specific and anti-Dpm1 antibody which is ER-membrane specific. Though we did not detect Pdi1 band, we could see a

small fraction of Dpm1 in highly purified mitochondrial fraction. As mitochondria are tightly associated with ER membranes, it is highly unlikely to achieve "pure" mitochondria even after sucrose gradient purification. Since eisosome complex is associated with plasma membrane, we wanted to make sure that our mitochondrial extracts do not contain any plasma membrane fractions. Absence of Pma1 band in mitochondrial fraction confirms that it doesn't contain plasma membrane contamination. Additionally, we expressed plasmids carrying Pil1-GFP and mito-mCherry in *pil1*Δ (BY4741) cells. Fluorescence microscopy showed the coincidence of signals that confirmed the mitochondrial localization of Pil1 (Figure 2.3B).

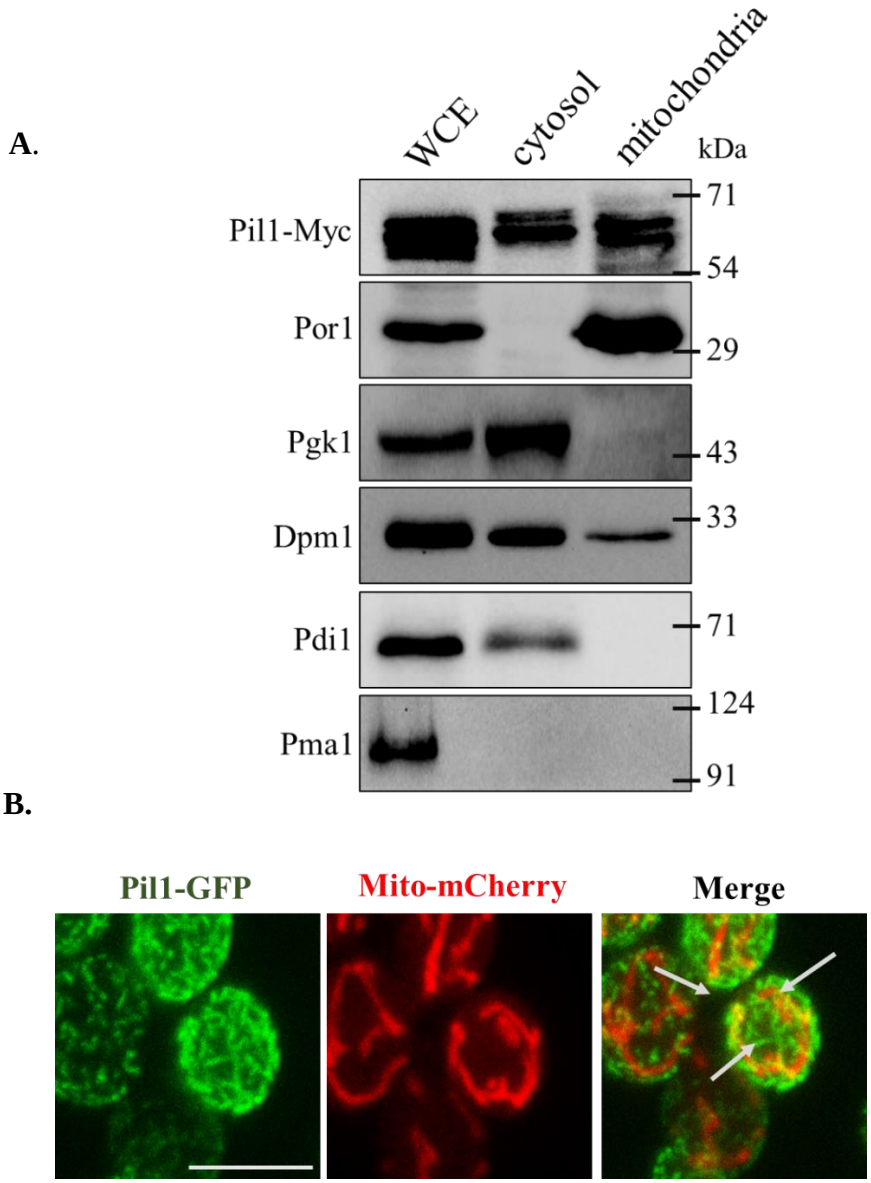


Figure 2.3-Localization of Pil1 to mitochondria**:**

A.Western blot of whole cell extracts, cytosolic fractions (post-mitochondrial fractions) and mitochondrial fractions (50µg each) from Pil1-myc expressing strains. Antibodies against Myc tag, Porin, Pgk1, Dpm1, and Pdi1 were used for the western blot analysis. Antibodies against Por1, Pgk1, and Dpm1/Pdi1, and Pma1 were used as controls for mitochondria, cytosol, ER membrane/ER lumen and plasma membrane, respectively. WCE: Whole Cell Extract. B. Fluorescence microscopy to check the localization of Pil1 to mitochondria. Pil1 tagged with GFP and mitochondria tagged with mCherry were co-expressed in *pil1Δ* strain. Maximum projections of z-stacks are shown in the represented images. Scale bar represents 5 µm.

2.3.b. Pil1 localizes to mitochondrial outer membrane

After confirming the presence of Pil1 in mitochondria, we investigated its exact sub-mitochondrial localization. The mitoplast was prepared by treating the intact mitochondria with hypertonic solution to remove the outer mitochondrial membrane. Pil1 completely disappears in the mitoplast. Tom40, an outer membrane protein is also not detected in the blot. However, Tim22, an inner membrane protein is intact in the mitoplast (Figure 2.4A). This shows that Pil1 is present on the outer membrane. However, we further wanted to see if it is peripherally or integrally bound to the membrane. The intact mitochondria were treated with increasing concentrations of tryspsin followed by treatment with trypsin inhibitor to stop the reaction. The treated and untreated samples were probed against Myc, Tom70 and Tom40 antibodies. Significant amount of Pil1 is digested when treated with trypsin. Tom70, being a peripheral outer membrane protein gets completely digested and Tom40, an integral outer membrane protein remains intact (Figure 2.4B). Our results clearly show that Pil1 is peripherally bound to the outer mitochondrial membrane. To further determine if Pil1 is a soluble protein or associated with the mitochondrial membrane, we carried out protein extraction with potassium chloride (KCl) and sodium carbonate (Na_2CO_3). When subjected to KCl, Pil1 was retrieved in the pellet fraction showing that Pil1 is tightly bound to the mitochondrial membrane. However, after Na_2CO_3 treatment, Pil1 was found in the supernatant fraction (Figure 2.4C). This shows that Pil1, though peripherally and tightly bound to the mitochondrial outer membrane, is a soluble protein. Porin, a membrane protein and Hsp70, a soluble matrix protein were used as controls.

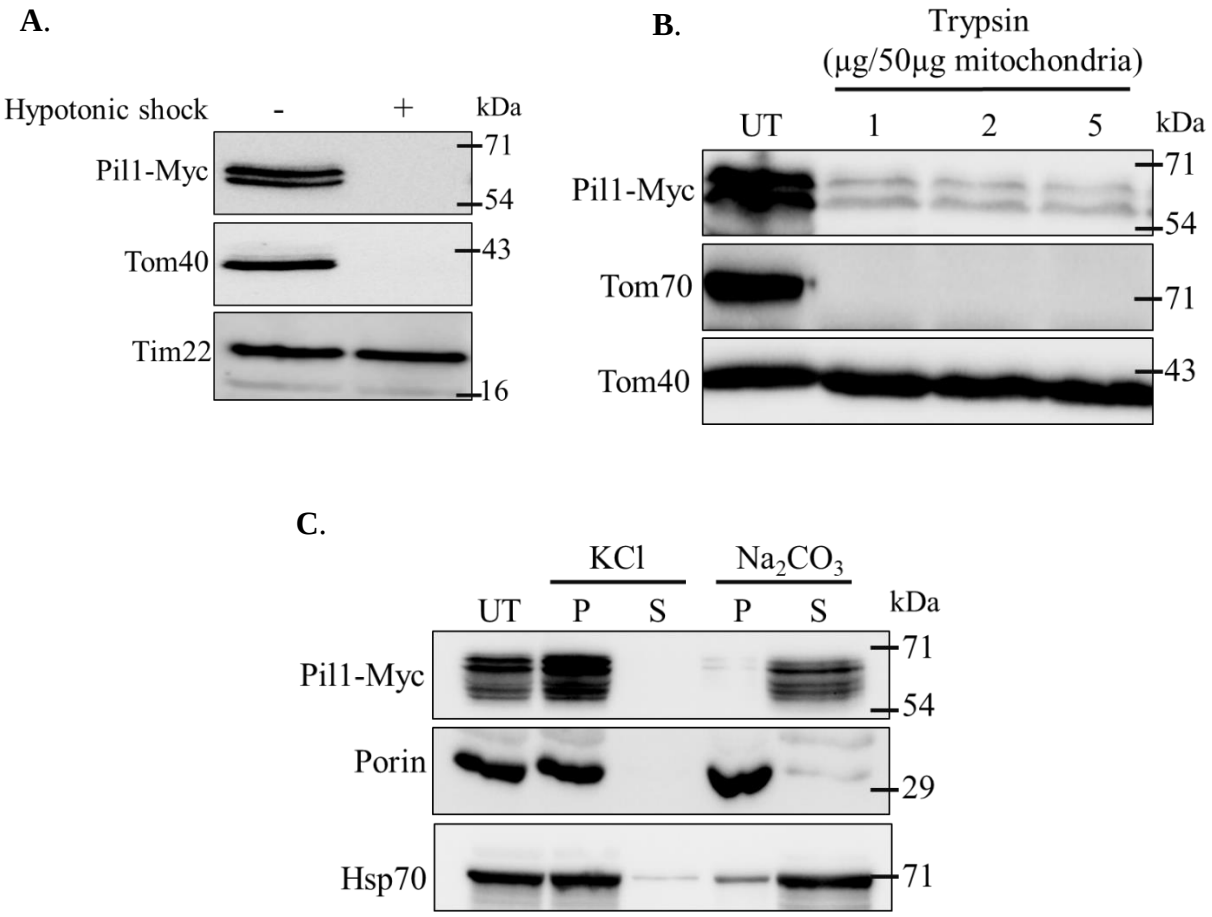


Figure 2.4. Pil1 localizes to outer membrane of mitochondria.

A. Hypotonic treatment. The mitoplast was prepared by treating the intact mitochondria with hypertonic solution to remove the outer mitochondrial membrane and immunoblotted with Myc, Tom40, and Tim22 antibodies. B. Trypsin digestion assay. Mitochondrial fraction (50 µg) isolated from Pil1-Myc strain was subjected to trypsin treatment and western blotted using antibodies against Myc tag, Tom70, and Tom40. Tom70 and Tom40 are peripheral and integral mitochondrial outer membrane proteins, respectively. C. Salt extractions. Mitochondria (50 µg) from Pil1-Myc cells were treated with 400 mM KCl and 200 mM Na_2CO_3 (pH 11.4), immunoblotted using antibodies against Myc, Porin, and Hsp70. Porin and Hsp70 are used as controls for membrane and soluble mitochondrial proteins, respectively. UT: Untreated, P: Pellet, S: Supernatant.

2.4 CONCLUSION

In this chapter, we conclude that Pil1 localizes to mitochondria through cellular fractionation, which was confirmed using confocal microscopy. Further, hypotonic treatment and trypsin digestion show that Pil1 is peripherally bound to the outer membrane of mitochondria. Salt extraction assay with KCl show that Pil1, though a peripheral protein, is tightly bound to mitochondria. Salt extraction using Na_2CO_3 confirms that Pil1 is a soluble protein, not a membrane protein.

CHAPTER 3

Role of Pil1 in maintaining mitochondrial morphology

3.1 BACKGROUND

Recently, several studies have recognized that mitochondrial morphology is an important factor in regulating cellular functions. As Pil1 contains BAR-domain and as shown in the first chapter, it localizes to mitochondrial outer membrane, we speculated its role in maintaining mitochondrial morphology by modulating the membrane dynamics. The fundamental cellular roles of BAR domain proteins, from unicellular eukaryotes to vertebrates, includes sensing of membrane curvature, binding GTPases, and molding cellular membranes (Habermann 2004, Peter, Kent et al. 2004).

Mitochondrial fission and fusion generally referred to as mitochondrial dynamics, maintain mitochondrial integrity by regulating its size, shape, distribution, and connectivity. Mitochondrial dynamics regulate mitochondrial quality control, metabolism, apoptosis, mitophagy, and other essential processes. A coordinated balance of fission and fusion is critical for maintaining mitochondrial biology, and therefore a plethora of important cellular processes (Youle and van der Bliek 2012, Bockler, Chelius et al. 2017).

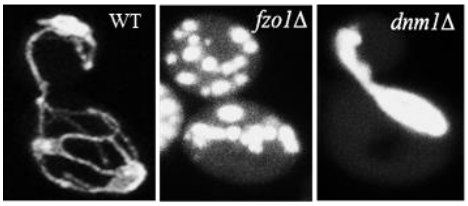


Figure 3.1- Different types of mitochondrial morphologies due to defective fission/fusion.

WT structure of mitochondria is tubular branched, whereas cells lacking fzo1 (defective fusion) show fragmented mitochondria. Cells lacking dnm1 (defective fission) contain hyperfused mitochondria.

Due to experimental advantages, budding yeast *Saccharomces cerevisiae* has been the favorite model to study mitochondrial dynamics (Okamoto and Shaw 2005). Mitochondria have been shown to adopt a variety of shapes in eukaryotic cells, ranging from branched tubular network to multiple small compartments. These different mitochondrial morphologies are maintained by equilibrium between opposing events of fission and fusion of mitochondria. As discussed elaborately in the Introduction (section 1.7), mitochondrial fission in yeast is regulated by four major proteins Dnm1, Fis1, Mdv1, and Caf4. Fzo1, Ugo1, and Mgm1 are the main regulators of

mitochondrial fusion. WT mitochondria forms tubular network, whereas cells lacking any of the core fission proteinsshow hyperfused mitochondria due to defective fission, and the cells lacking any of the core fusion proteins have fragmented mitochondria due to defective fusion process (Figure 3.1).

3.2 MATERIALS AND METHODS

3.2.a. Media and growth conditions

Strains were grown to log phase (OD_{600} = approximately 1) in synthetic complete dextrose media (SCD; 0.8% yeast nitrogen base without amino acids [Becton Dickinson, 291940], 2% dextrose [Himedia, GRM077], pH 5.5) or synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) as described previously (Sherman 1991). Yeast cells were grown at 30°C. Yeast transformations were performed using lithium acetate method as previously described (Gietz and Woods 2002).

3.2.b. Yeast strains and plasmids

All strains, plasmids, and primers used in this study are listed in Table 3.1, Table 3.2, and Table 3.3, respectively. The yeast strains used in this study are derivatives of BY4741 or BY4742. PCR-based targeted homologous recombination was used to replace complete open reading frame of *PIL1* with KanMX6 or HIS3MX6 cassettes, as indicated (Longtine, McKenzie et al. 1998). The method of deletion of *PIL1* has been described in Chapter 2 (section 2.2b). Yeast-two-hybrid strains and plasmids were kind gifts from E.A. Craig (Washington University School of Medicine). Pil1-Myc strain was a gift from Victor J Cid (Complutense University of Madrid, Madrid*).* Deletion strains were kind gifts from Krishnaveni Mishra's yeast deletion library (Euroscarf) collection (Department of Biochemistry, University of Hyderabad). pUG35, pFA6a-HisMX6, and pFA6a-KanMX6 plasmids were provided by Krishnaveni Mishra's lab. Shirisha Nagotu (IIT Guwahati) provided pHS12 Mito-mCherry plasmid.

For the construction of p426TEF Pil1-FLAG, complete coding sequences of yeast *PIL1* was amplified using yeast genomic DNA as a template and the primer pairs NB754/NB755. The PCR amplified product was digested with EcoRI/HindIII and ligated with pNB270 vector as described

previously (Allu, Marada et al. 2015) , to replace *MXR1* gene with *PIL1* to generate pNB441. Pil1-FLAG was subcloned from pNB441 in p425TEF vector at BamHI/XhoI sites to generate pNB656. For yeast two-hybrid vectors pGBD-Pil1 and pGAD-Pil1, the DNA fragment of *PIL1* was PCR amplified from yeast genomic DNA using primers NB754/NB850 with EcoRI and SalI restriction sites. The double digested PCR product was ligated into the EcoRI and SalI sites of pGBD-C1 (pNB415) and pGAD-C1 (pNB422) vectors to generate pNB428 and pNB427 respectively. DNA fragments encoding Fis1, Dnm1, Mdv1, and Caf4 were PCR amplified from yeast genomic DNA with primer pairs NB1149/NB1150 , NB1151/NB1152, NB1153/NB1154, and NB1155/NB1156, respectively. PCR products were digested with BamHI/SalI, and ligated into pGAD-C1 and pGBD-C1 to generate pGAD-Fis1 (pNB526), pGAD-Mdv1 (pNB527), pGAD-Caf4 (pNB528), and pGBD-Dnm1 (pNB525), respectively. The same PCR products of Fis1 and Dnm1 were also ligated into the BamHI and SalI sites of pUG35-GFP vector to generate pUG35 Fis1-GFP (pNB629) and pUG35 Dnm1-GFP (pNB630) constructs. For cloning of pGAD-C1 Mgm1 (pNB530) , pGAD-C1 Fzo1 (pNB813), and pGAD-C1 Ugo1 (pNB531) vectors, *MGM1*, *FZO1*, and *UGO1* were PCR amplified from yeast genomic DNA using primer pairs NB1195/NB1196, NB1197/NB1198, and NB1199/NB1200, respectively. PCR products of *FZO1* and *UGO1* were digested with BamHI/SalI and that of *MGM1* was digested with XmaI/BglII. The double digested inserts were ligated into the respective sites of pGAD-C1.

To clone Pil1 in pET28a vector for purifying Pil1 protein for antibody generation, Pil1 was PCR amplified using forward primer NB879 and reverse primer NB850. PCR-product and pET28a vector were double digested with BamHI and Sal1, and ligated using T4 DNA ligase enzyme to generate pET28a Pil1 construct (pNB547).

Table 3.1 Strains

Strain	Genotype	Reference
YNB105	BY4741; MATa his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB106	BY4742; MATa his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB 339	PJ469-A	(James, Halladay et al. 1996)
YNB 474	BY4741; *pil1*Δ::*KanMX*	This study
YNB 245	BY4742; *pil1*Δ::*KanMX*	Euroscarf

YNB389	BY4741; *fis1*Δ::*KanMX*	Euroscarf
YNB511	BY4742; *fis1*Δ::*KanMX*	Euroscarf
YNB388	BY4741; *dnm1*Δ::*KanMX*	Euroscarf
YNB512	BY4742; *dnm1*Δ::*KanMX*	Euroscarf
YNB 454	BY4741; *fis1*Δ::*KanMX pil1*Δ::*HIS3*	This study
YNB 473	BY4741; *dnm1*Δ::*KanMX pil1*Δ::*HIS3*	This study

Table 3.2. Plasmids

Plasmid		**Source/Reference**
pNB356	p425 TEF	(Mumberg, Muller et al. 1995)
pNB357	p426 TEF	(Mumberg, Muller et al. 1995)
pNB656	p425 TEF Pil1-flag	This study
pNB441	p426 TEF Pil1-flag	This study
pNB547	pET28a Pil1	This study
pNB478	pFA6a-HisMX6	(Longtine, McKenzie et al. 1998)
pNB479	pFA6a-KanMX6	(Longtine, McKenzie et al. 1998)
pNB812	pHS12 Mito-mCherry	Addgene
pNB415	pGBD-C1	(James, Halladay et al. 1996)
pNB422	pGAD-C1	(James, Halladay et al. 1996)
pNB428	pGBD-Pil1	This study
pNB427	pGAD-Pil1	This study
pNB526	pGAD-Fis1	This study
pNB525	pGBD-Dnm1	This study
pNB527	pGAD-Mdv1	This study
pNB528	pGAD-Caf4	This study

pNB530	pGAD-C1 Mgm1	This study
pNB531	pGAD-C1 Ugo1	This study
pNB813	pGAD-C1 Fzo1	This study
pNB636	pUG35	Gueldener U, Hegemann JH. Heinrich Heine University, Germany
pNB629	pUG35 Fis1-GFP	This study
pNB630	pUG35 Dnm1-GFP	This study

Table 3.3 Primers

Primer Name	**Sequence (5'-3')**	**Restriction enzyme**
NB754 PIL1F	GCCGAATTCACCATGCACAGAACTTACTCTTTAA	EcoRI
NB755 PIL1R	GCCAAGCTTAGCTGTTGTTTGTTGGGGAA	HindIII
NB879 PIL1F	GCCGGATCCACCATGCACAGAACTTACTCTTTAA	BamHI
NB850 PIL1R	GCCGTCGACAGCTGTTGTTTGTTGGGGAA	SalI
NB1149 FIS1F	GCCGGATCCACCATGACCAAAGTAGATTTTT	BamHI
NB1150 FIS1R	GCCGTCGACCCTTCTCTTGTTTCTTAAGA	SalI
NB1151 DNM1F	GCCGGATCCACCATGGCTAGTTTAGAAGATCTT	BamHI
NB1152 DNM1R	GCCGTCGACCAGAATATTACTAATAAGGGTTG	SalI
NB1153 MDV1F	GCC GGATCC ACC ATGTCAGTGAACGACCAAAT	BamHI
NB1154 MDV1R	GCC GTCGAC TACGGCCCAAATATTTACGTC	SalI
NB1155 CAF4F	GCC GGATCC ACC ATGGGTTCGGGCGATACTA	BamHI
NB1156 CAF4R	GCC GTCGAC AAGTGTCCATACATTGATGTC	SalI
NB1195 Mgm1F	GCCCCCGGGATGAATGCGAGCCCAGTACGG	XmaI
NB1196 Mgm1R	GCCAGATCTTAAATTTTTGGAGACGCCCTT	BglII
NB1197 Fzo1F	GCCGGATCCATGTCTGAAGGAAAACAACAATT	BamHI
NB1198 Fzo1R	GCCGTCGACATCGATGTCTAAATTTATTTCTT	SalI

NB1199 Ugo1F	GCCGGATCCATGAACAACAATAATGTTACGGA	BamHI
NB1200 Ugo1R	GCCGTCGACGAACTTCTCTTGTTCCATGTTG	SalI

3.2.c. Yeast two-hybrid assay

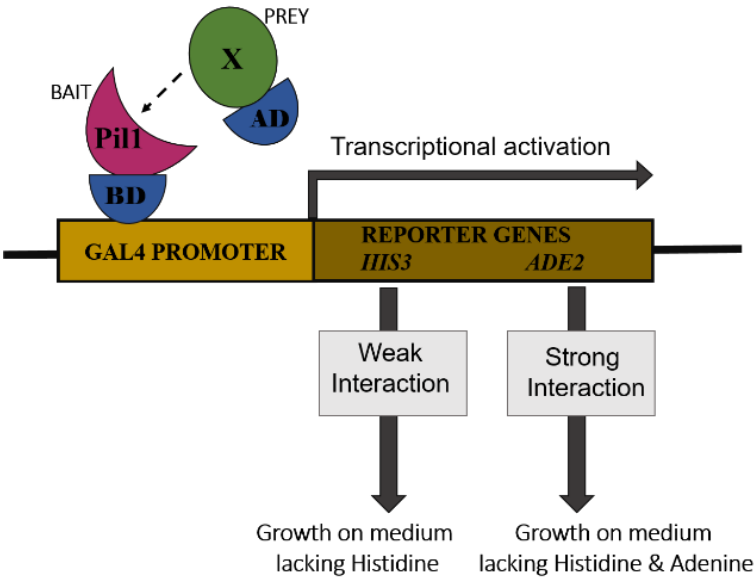


Figure 3.2 Principle of Yeast two-hybrid assay

The basic idea behind two-hybrid assay is to fuse the two proteins of interest X and Y to DNA-binding domain (DBD) and Activation domain (AD) of Gal4, respectively. If there is an interaction between X and Y, it will reconstitute a functional transcription factor which can then drive the expression of reporter genes. Here the reporter genes are *HIS3* which encodes for histidine biosynthesis enzyme and *ADE2* which encodes adenine biosynthesis enzyme.

3.2.d. Growth assay

For performing growth assay on non-fermentable carbon source, indicated strains were freshly streaked on YPD plates and the resultant colonies were grown in YPD medium overnight. These cultures were normalized to $O.D._{600}$= 0.5. 10-fold serial diluted cultures were spotted on YPD plate and YPG (2% glycerol) plate. Images were taken after 2 days of incubation of these plates at 30°C.

3.2.e. Immunoblotting

After separation in SDS-PAGE, proteins were transferred onto nitrocellulose membranes (PALL, 66485) and blocked with 5% skimmed milk (Himedia,GRM1254) in Tris-buffered saline (TBS). The commercial monoclonal antibody anti-GFP (ab183734, Abcam, 1:5000) was used to detect GFP epitope-fused protein on immunoblots. Anti-Pil1 serum (Anti-rabbit, 1:3000) was generated in the laboratory using full-length Pil1-6XHis protein as immunogen. In the procedure of raising antibody, all the required guidelines were followed which were approved by the Institutional Animal Ethics Committee, University of Hyderabad (UH/IAEC/NS/2014-I/25). HRP-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, 111-035-144, 1:25,000) or anti-mouse (Jackson ImmunoResearch Laboratories, 115-035-146, 1:25,000) polyclonal secondary antibodies were used, followed by the detection of signal using ECL reagents (Advansta Western-Bright, K-12045-D20) and imaging in Chemidoc Imaging System (Bio-Rad). Quantification of Western Blot images was done using Fiji software. Graphs and statistical analysis were done using GraphPad Prism8.

3.2.f. Co-immunoprecipitation

For Co-IP assays, cells were collected and lysed with glass beads (Sigma, G8772) in lysis buffer (20 mM Tris-HCL [pH 8.0], 100 mM NaCl [Himeda, GRM031], 1 mM EDTA and 0.5 % Nonidet P-40 [Sigma,I8896], protease inhibitor cocktail [Roche, 04693132001]). After bead-beating, cells were centrifuged at 12,000 rpm for 10 min. Supernatant was collected and incubated with anti-Pil1 serum (and pre-immune serum as control) for 12 hr at 4°C in an end-over-end rotor. 30 µL of protein A/G PLUS-Agarose beads (SCBT, SC-2003)) were added and incubated for 2 hr at 4°C in an end-over-end rotor. Immunocomplexes were then washed three times with lysis buffer and eluted by SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE and immunoblotted with anti-GFP antibody and anti-Pil1 serum.

3.2.g. Fluorescence microscopy

Cells grown till $O.D._{600}$= approximately 1, were harvested by centrifugation and the pellet was washed twice with fresh SMD media. Cells suspended in SMD media were added to slides coated with 0.1% Concanavalin A (Sigma, C2010) solution. The cells were allowed to settle for 2-3 minutes and mounted with coverslips. Images were acquired either on Leica TCS SP8 (HC PL APO CS2 63X/1.40 OIL objective) or Zeiss LSM NLO 710 (63X/1.5 OIL objective) confocal

microscope. Fiji Software was used to process and analyse the microscopy data. Graphs and statistical analysis were done using GraphPad Prism8.

3.2.h Measurement of Mitochondrial Potential

For measurement of mitochondrial potential, cells growing in SMD medium were harvested by centrifugation (1 O.D. cells), washed with PBS twice and then treated with 200 nM 3,3′-dihexyloxacarbocyanine iodide [DiOC6(3); Invitrogen D273) in 500 µL PBS for 30 min at 30°C. Absorbance was measured in a multiplate reader.

3.2.i. Citrate synthase assay

WT, *pil1*Δ, and Pil1 overexpression cells were grown till log phase, cells were harvested and resuspended in 700 µl SEM buffer. Lysates were prepared by bead-beating (3 cycles; 40 seconds ON, 20 seconds OFF). For measuring citrate synthase activity, following components were taken in a 1 mL cuvette: 500 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2%), 300 µl of Milli-Q water, 100 µl of DTNB [5,5′-Dithiobis(2-nitrobenzoic acid) (Sigma, D218200)], 30 µl of Ac CoA [Acetyl CoenzymeA lithium salt ;10 mM; Sigma; A2181]and 20 µg of lysate. MQ water was added to adjusted the volume to 950 µl. Baseline activity was taken at 412 nm for 2 min. 50 µl of 10 mM oxaloacetic acid was added to start the reaction. The reaction solution was mixed by inversion. Increase in absorbance at 412 nm was monitored for 3 min.

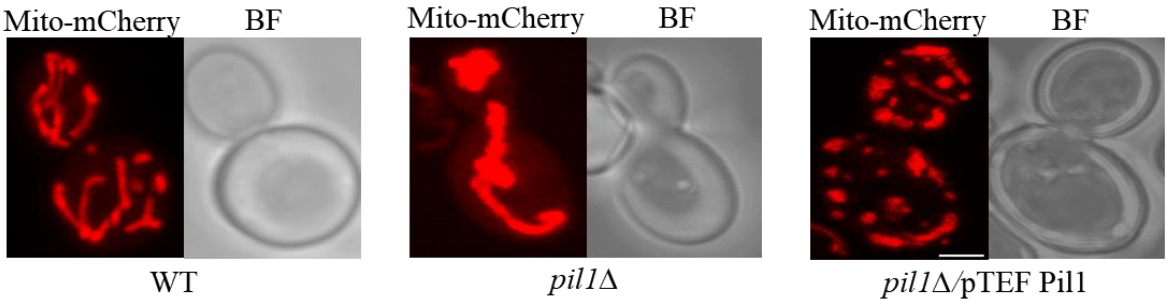
3.3 RESULTS

3.3.a. Absence and abundance of Pil1 leads to abnormal morphology of mitochondrial network:

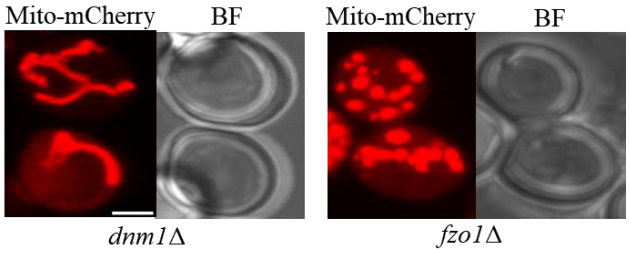
Next, we examined if Pil1 causes any kind of alteration in mitochondrial morphology by using pHS12 Mito-mCherry plasmid which contains Cox4 presequence that targets mCherry to the mitochondrial matrix. We observed the mitochondrial morphology under fluorescence microscope in WT, *pil1*Δ and Pil1 overexpression (*pil1*Δ/pTEF Pil1) cells. The transformed cells were grown to log phase (OD_{600}=1) and mitochondrial morphology was observed under fluorescence microscope. We observed that instead of forming typical tubular structures, cells

lacking Pil1 as well as those with Pil1 overexpression displayed abnormal mitochondrial morphology (Figure 3.2A).

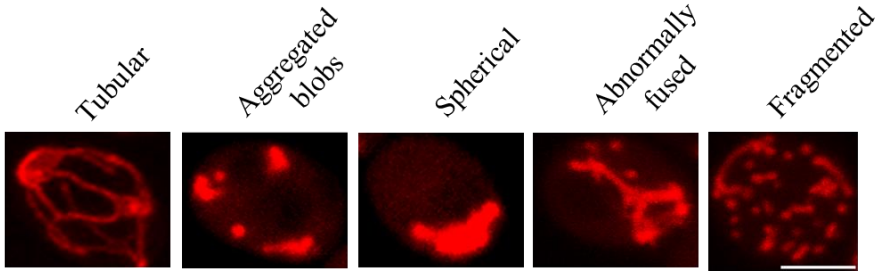
A.



B(i)



B(ii)



WT	98.2	0	0	1.8	0
*pil1*Δ	4.2	20.6	27.9	43	5
*pil1*Δ/ pTEF Pil1	2	0	0	0	98

C.

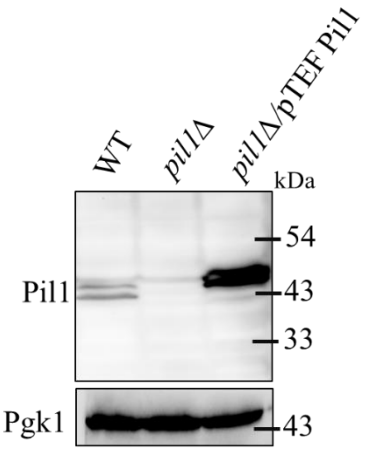


Figure 3.2- Pil1 regulates mitochondrial morphology.

A. Mitochondrial morphology in WT, *pil1*Δ and Pil1 overexpression (*pil1*Δ/pTEF Pil1) was observed under fluorescence microscope. Mito-mCherry plasmid (pNB812) was used to tag mitochondria. Images are maximum projection of z-stacks. B(i). *dnm1*Δ and *fzo1*Δ were used as controls for quantification of mitochondrial morphology. B(ii). Quantification of mitochondrial phenotypes in WT, *pil1*Δ and *pil1*Δ/pTEF Pil1 cells. Mitochondrial morphology shown for each strain was collected from three independent analysis of at least 80 yeast cells. C. Western blot showing the levels of Pil1 protein in WT, *pil1*Δ and *pil1*Δ/pTEF Pil1 cells. WT, *pil1*Δ and *pil1*Δ/pTEF Pil1 cells were lysed, subjected to SDS-PAGE and immunoblotted with anti-Pil1 serum.

Keeping the mitochondrial morphologies in *dnm1*Δ (hyperfused) and *fzo1*Δ (fragmented) as references [Figure 3.2B(i)], we categorized these morphologies as abnormally fused, aggregated blobs, spherical, and fragmented. We observed that 43% of *pil1*Δ cells contained abnormally fused mitochondria while 27.2 % cells displayed a spherical mitochondrial network. To our surprise, we also found that a fraction of *pil1*Δ cells (20.6 %) had aggregated mitochondria [Figure 3.2B(ii)]. On the other hand, when we overexpressed Pil1 by transforming *pil1*Δ cells with TEF-Pil1 plasmid, almost all cells (98%) contained fragmented mitochondria. The levels of Pil1 protein in WT, *pil1*Δ and Pil1 overexpression cells were confirmed by immunoblotting with anti-Pil1 serum (Figure 3.2C). These observations show that Pil1 indeed plays a critical role in regulating mitochondrial morphology.

3.3.b. Pil1 interacts with Fis1 and Dnm1:

As Pil1 induces the change in mitochondrial shape, we were interested to see if it interacts with any of the proteins of mitochondrial fission and fusion machinery. We used yeast two hybrid method to check the interactions. We got the interaction of Pil1 with all four mitochondrial fission proteins (Figure 3.3A).

To confirm the interaction of Pil1 with Fis1 and Dnm1, we expressed Fis1 and Dnm1 in pUG35-GFP vector under *MET25* promoter, and immunoprecipitated with Pil1 antibody. We find that Pil1 specifically pulled down Fis1-GFP and Dnm1-GFP (Figure 3.3B). Based on these data, we conclude that Pil1 physically interacts with major components of mitochondrial fission machinery, Fis1 and Dnm1.

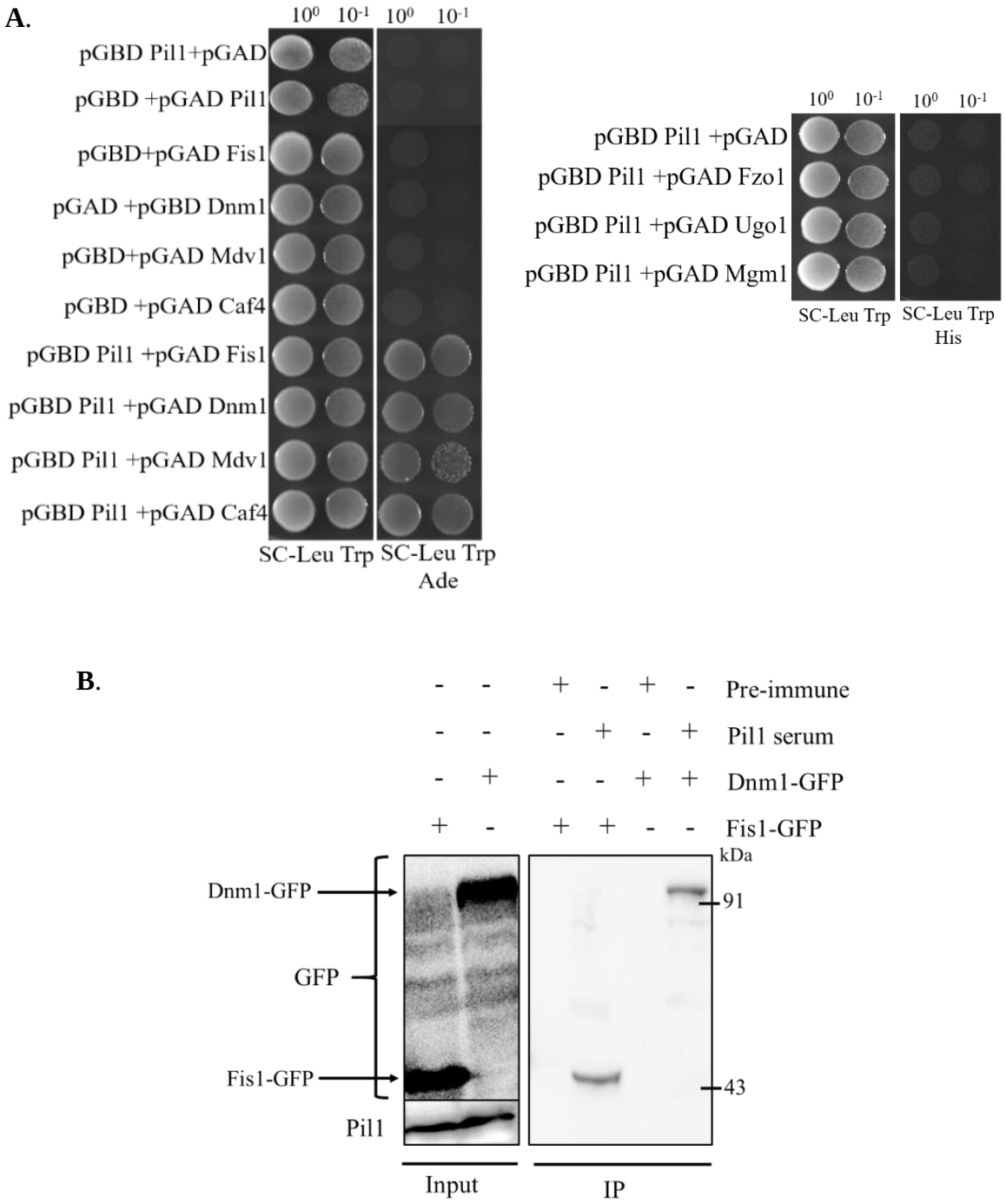


Figure 3.3. Pil1 interacts with mitochondrial fission protein Fis1 and Dnm1

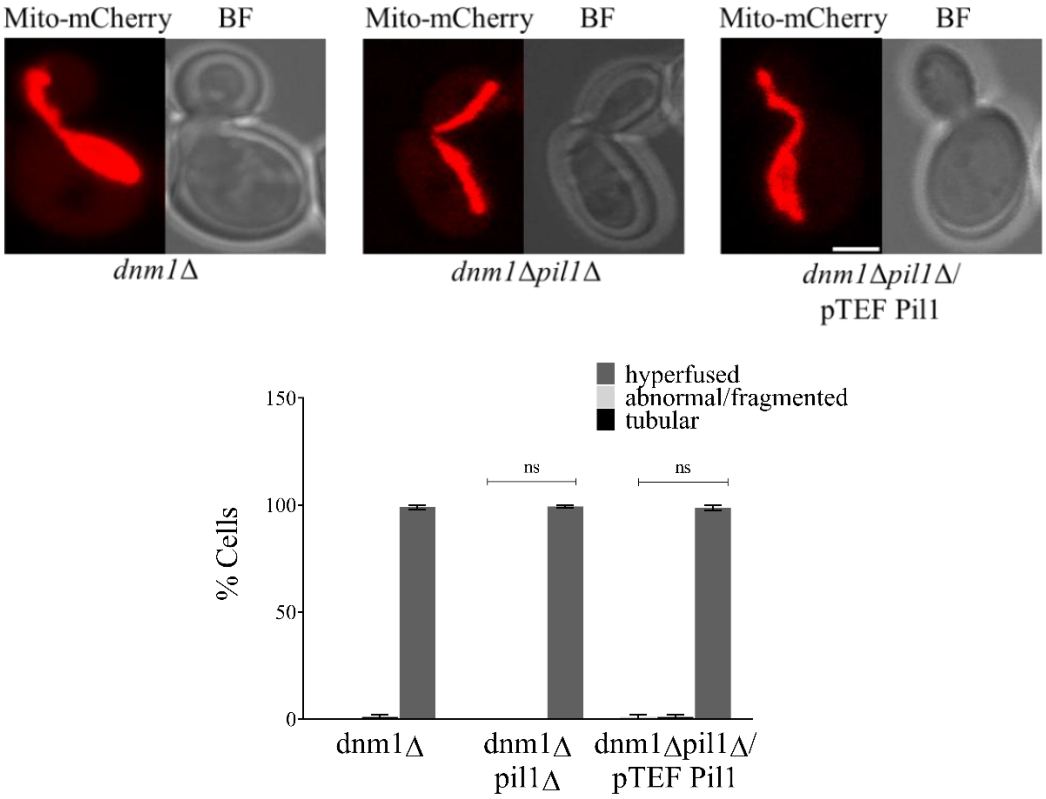
A. Yeast two hybrid analysis between Pil1 and mitochondrial fission and fusion proteins. The PJ69-4A strain was transformed with pGAD-C1 and pGBD-C1 plasmids, which can express the indicated proteins. Cells were grown on +Ade/ –Ade plates or +His/–His at 30°C for 2-3 days. B. Co-immunoprecipitation assays to test the interaction of Pil1 with Fis1-GFP and Dnm1-GFP. *fis1*Δ cells expressing Fis1-GFP and *dnm1*Δ cells expressing Dnm1-GFP were grown in SMD (SCD lacking uracil and methionine) media until log phase. Anti-Pil1 serum was used to precipitate Fis1-GFP and Dnm1-GFP in respective samples. Pre-immune serum was used as negative control. Western

blotting was done using anti-Pil1 and anti-GFP antibodies.

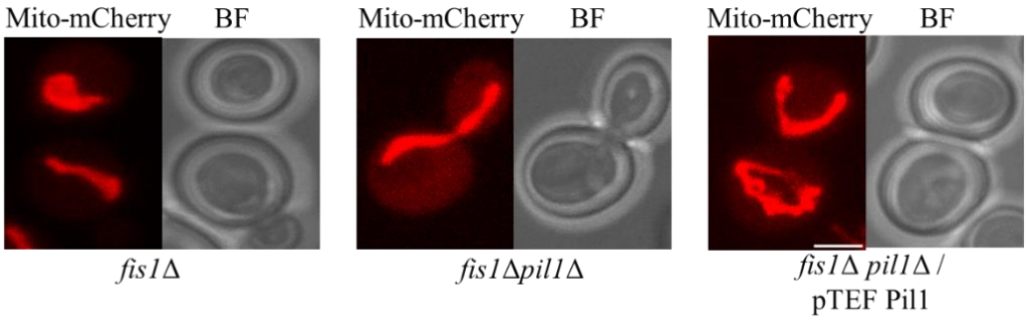
3.3.c. Fis1 and Dnm1 are required for the change in mitochondrial morphology caused due to Pil1:

Next, we wanted to explore whether major mitochondrial fission factors Fis1 and Dnm1 have any role in generating mitochondrial morphology that we observed in the absence and overexpression of Pil1. We analyzed the mitochondrial morphology in *fis1*Δ*pil1*Δ and *dnm1*Δ*pil1*Δ double mutants. Mitochondria remained hyperfused in almost all cells, as reported in *fis1*Δ and *dnm1*Δ single mutants. Additionally, overexpression of Pil1 did not rescue the fission defect in *fis1*Δ*pil1*Δ and *dnm1*Δ*pil1*Δ double mutants (Figure 3.4A&B).

A.



B.



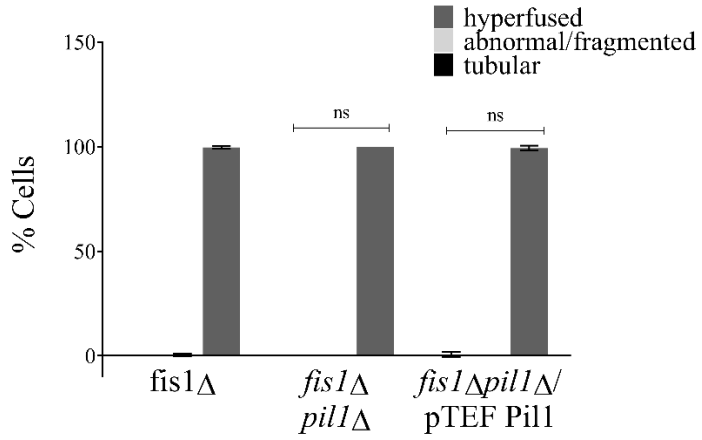


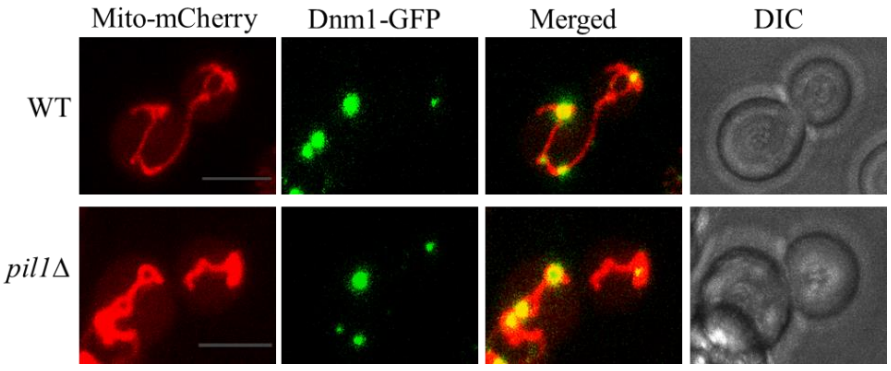
Figure 3.4 Pil1 does not alter mitochondrial morphology without Fis1 and Dnm1

Mitochondrial morphology in *fis1*Δ, *dnm1*Δ, *fis1*Δ*pil1*Δ ,*dnm1*Δ*pil1*Δ and *fis1*Δ*pil1*Δ, *dnm1*Δ*pil1*Δ strains overexpressing Pil1 was observed. Graph represents 3 independent trials of 100 yeast cells. Statistical analysis was done using Multiple Comparisons: Two- way ANOVA. ns:non-significant.

3.3.d. Pil1 does not alter the localization of Fis1 and Dnm1 to mitochondria:

As Pil1 interacts with Fis1 and Dnm1 Section (3.3.b), we speculated that it might also alter the localization of these proteins to mitochondria and thereby leads to change in the mitochondrial dynamics. But we did not see any change in distribution of Dnm1 and Fis1 on mitochondria in the absence of Pil1 (Figure 3.5A&B), which shows that Pil1 does not affect the recruitment of these proteins to mitochondria.

A.



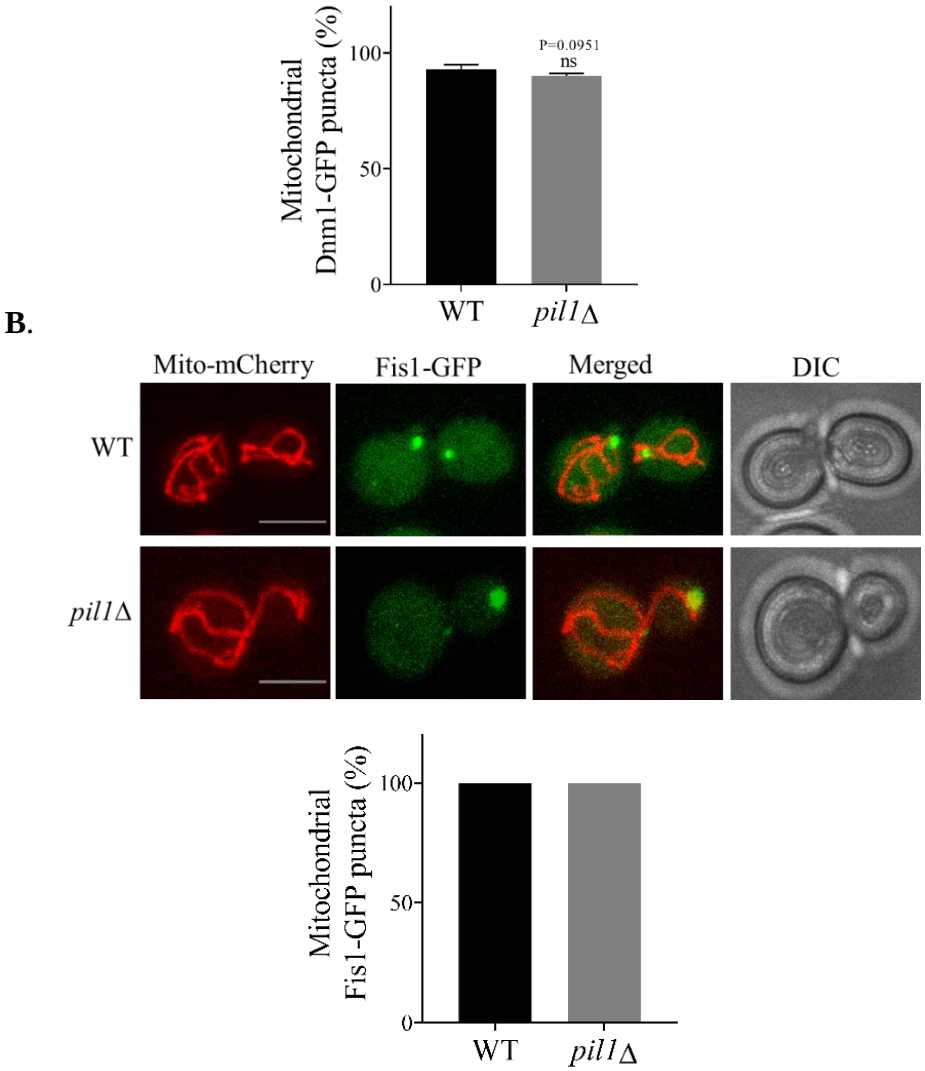


Figure 3.5 Pil1 does not alter the localization of Fis1 and Dnm1 to mitochondria

A. Number of Fis1 foci associated with mitochondria were analysed in WT and *pil1*Δ cells transformed with mito-mCherry and Fis1-GFP plasmids. B. Number of Dnm1 foci associated with mitochondria were analysed in WT and *pil1*Δ cells transformed with mito-mCherry and Dnm1-GFP plasmids. Maximum projections of z-stacks are shown in the represented images. BF: Brightfield. Data shown is the mean ± SD of 3 independent trials. Scale bar represents 2µm. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph). ns:non-significant.

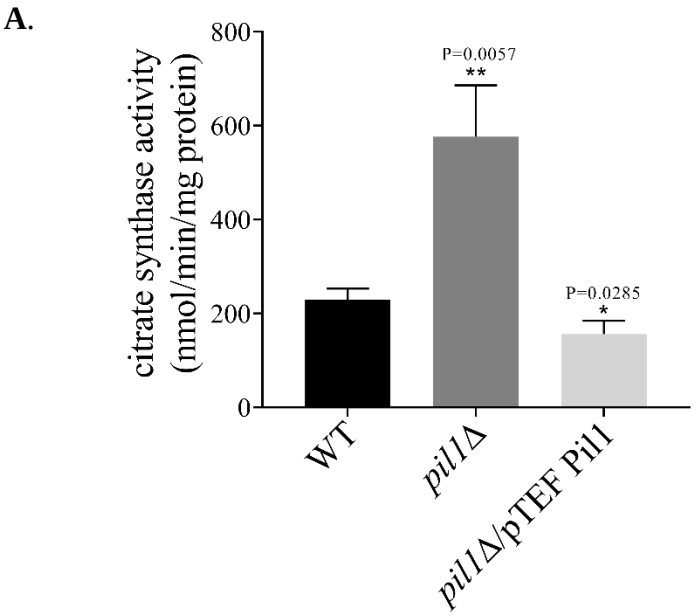
3.3.e. Absence of Pil1 leads to increased mitochondrial mass

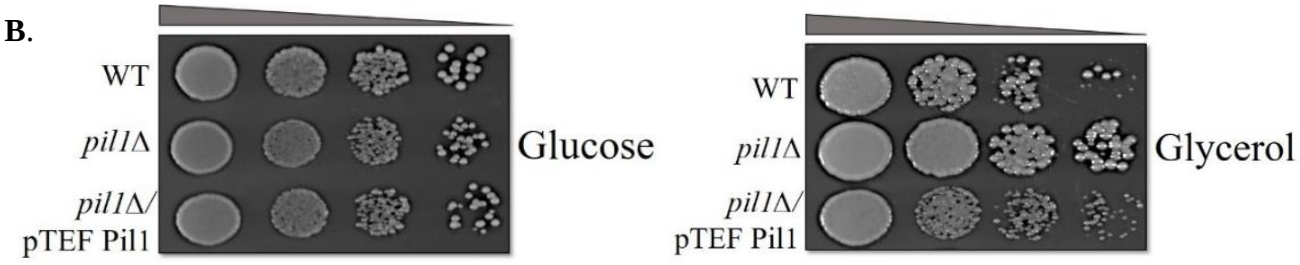
As Pil1 severely affects mitochondrial morphology, we thought that the other aspects like mitochondrial content and its activity might also be getting affected. To check mitochondrial content, we used citrate synthase assay. Citrate synthase is a key enzyme in TCA cycle and its activity is used to measure the mitochondrial content in cells and tissues. We found that *pil1*Δ

cells have higher activity than WT whereas in Pil1 overexpression cells it was slightly reduced (Figure 3.6A). The reason behind this could be more fused mitochondria in *pil1*Δ which tends to be more protected from degradation, and more fragmented mitochondria in Pil1 overexpression which is more prone to degradation.

Also, we checked the growth in glycerol medium. Glycerol is a non-fermentable carbon source, and to utilize the carbon source for energy production, mitochondrial respiration is required. So the change in growth pattern on fermentable vs non-fermentable carbon source is usually an indicative of differential mitochondrial respiration. Compared to WT, *pil1*Δ cells grow better while cells overexpressed with Pil1 seem to grow slower on glycerol medium (Figure 3.6B). This phenotype can also be attributed to the altered mitochondrial content in the cells.

We also measured mitochondrial potential using DiOC6, which is green-fluorescent dye that stains mitochondria depending on its potential, when used at a lower concentration. *pil1*Δ cells show a little higher mitochondrial potential, which again could be due to more fused membranes as more fused mitochondria tend to have a slightly higher potential (Figure 3.6C).





C.

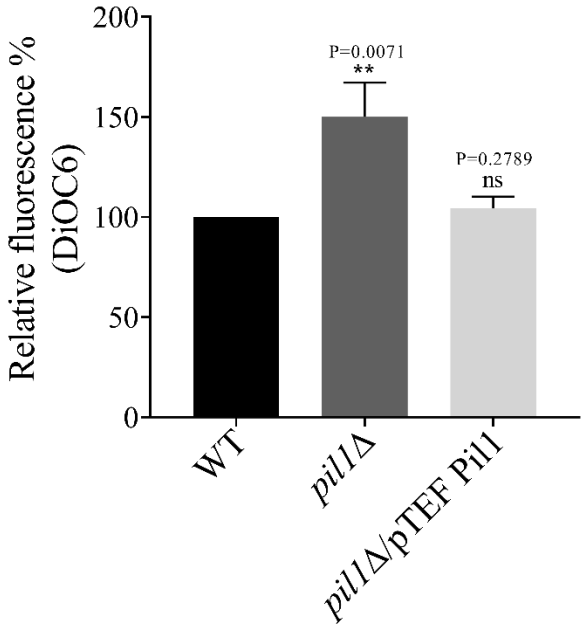


Figure 3.6 Mitochondrial mass increase in the absence of Pil1

A.Citrate synthase activities were determined in whole cell lysates from WT, *pil1Δ*, and Pil1 overexpression cells. Activities of citrate synthase enzyme was measured in nmol/min/mg protein of the whole cell lysate. The amount of lysate used was 20 µg. B. Growth assay. Comparison of growth of WT, *pil1Δ*,, and Pil1 overexpression cells in glucose and glycerol media. Cells were grown till $O.D._{600}$=1, equalized to 0.5 O.D and serially spotted onto the agar plates. C. Measurement of mitochondrial membrane potential using DiOC6(3) dye. Cells were grown till log phase and then incubated with 200 nM of DiOC6(3) dye for 30 min. Readings were taken in a multiplate reader. Data are presented as mean ± SD of 3 independent experiments. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 vs WT.

3.4 CONCLUSION

In this chapter, we have shown that Pil1 alters the dynamics of mitochondria. Its absence leads to abnormally fused mitochondria while its overexpression causes severe fragmentation. Also, we have seen that it interacts with Fis1 and Dnm1, but the functional relevance of this interaction is not known. Change in mitochondrial shape caused due to Pil1 is dependent on Fis1 and Dnm1. Due to more fused mitochondria in the absence of Pil1, we have observed more mitochondrial activity and membrane potential.

CHAPTER 4

Role of Pil1 in autophagy/mitophagy

4.1 BACKGROUND

Autophagy, which literally translates to “self-eating” is a process of cellular degradation and recycling which is highly conserved in eukaryotes. It can be either selective or non-selective. In non-selective autophagy, also called bulk autophagy, the cytoplasmic contents are randomly targeted for degradation, whereas selective autophagy targets specific organelles. In both selective and non-selective autophagy, the morphological hallmark is the formation of isolation membrane which encloses to form the autophagosome that engulfs the cargo in the cytoplasm and fuses with vacuole/lysosome in case of mammals, where the contents undergo degradation and recycling. The process is carried out by several ATG proteins that form different complexes at the different stages of the autophagic pathway.

One reason why we decided to study the role of Pil1 in autophagy was its interaction with ATG proteins in high throughput analysis (Figure 4.1) and another reason was that we saw (Chapter 2) that Pil1 alters mitochondrial morphology which is very well known to regulate mitochondrial autophagy.

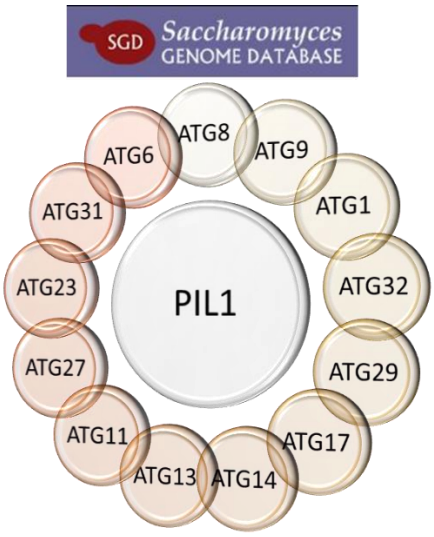


Figure. 4.1-ATG proteins as interacting partners of Pil1 from high-throughput analysis.
Source: Saccharomyces cerevisiae genome database (SGD)

4.2 MATERIALS AND METHODS

4.2.a. Media and growth conditions

Strains were grown to log phase ($O.D._{600}$ = approximately 1) in synthetic complete dextrose media (SCD; 0.8% yeast nitrogen base without amino acids [Becton Dickinson, 291940], 2% dextrose [Himedia, GRM077], pH 5.5) or synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) as described previously (Sherman 1991). For mitochondria proliferation, cells were grown in lactate medium (YPL; 1% yeast extract, 2% peptone, and 2% lactate) or synthetic minimal medium with lactate (SML; 0.67% yeast nitrogen base, 2% lactate, and auxotrophic amino acids and vitamins as needed). Autophagy/mitophagy was induced by shifting the cells to nitrogen starvation medium with glucose (SD-N; 0.17% yeast nitrogen base without ammonium sulfate or amino acids [Becton Dickinson, 233520], and 2% dextrose). Yeast cells were grown at 30°C. Yeast transformations were performed using lithium acetate method as previously described (Gietz and Woods 2002).

4.2.b. Yeast strains and plasmids

All strains, plasmids, and primers used in this study are listed in Table 4.1, Table 4.2, and Table 4.3, respectively. The yeast strains used in this study are derivatives of BY4741 or BY4742. PCR-based targeted homologous recombination was used to replace complete open reading frame of *PIL1* with KanMX6 or HIS3MX6 cassettes, as indicated (Longtine, McKenzie et al. 1998). The method of deletion of *PIL1* has been described in Chapter 1 (section 2.2b). Construction of pTEF Pil1-FLAG (pNB656, pNB441), pGAD-Pil1 (pNB427), and pGBD-Pil1 (pNB428)) have also been described in Chapter 3 (section 3.2b). Yeast-two-hybrid strains and plasmids were kind gifts from E.A. Craig (Washington University School of Medicine). Pil1-Myc strain was a gift from Victor J Cid (Complutense University of Madrid, Madrid). Deletion strains were kind gifts from Krishnaveni Mishra's yeast deletion library (Euroscarf) collection (Department of Biochemistry, University of Hyderabad). pFA6a-HisMX6 and pFA6a-KanMX6 plasmids were provided by Krishnaveni Mishra's lab. Shirisha Nagotu (IIT Guwahati) provided pHS12 Mito-mCherry plasmid. pRS423 GFP-Atg8 plasmid was a kind gift from Ravi Manjithaya (Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore). pRS316 Su9-DHFR-GFP plasmid was a kind gift from Jared Rutter (University of Utah School of Medicine, Salt Lake City). pRS316 Pgk1-GFP was a kind of gift from Michael Thumm (University Medical Center Göttingen,

Germany). pGBD-Atg8 was a gift from Y.Oshumi (Tokyo Institute of Technology, Japan). pGAD-Atg32 and pRS316 Atg32-HA were kind gifts from Koji Okamoto (Osaka University, Japan). pGBDU-Atg11 was a kind gift from Tomotake Kanki (Niigata University, Japan).

For construction of yeast two-hybrid vectors of Atg genes, DNA fragments encoding Atg1, Atg9, Atg27, Atg29, Atg23, Atg13, Atg14, Atg17, Atg31, and Atg24 were PCR amplified from yeast genomic DNA with primer pairs NB1122/NB1123, NB1124/NB1125, NB1126/NB1127, NB1128/NB1129, NB1130/NB1131, NB1132/NB1133, NB1134/NB1135, NB1136/NB1137, NB1138/NB1139, and NB1140/NB1141, respectively. PCR products of Atg1, Atg9, Atg27, Atg23, Atg14, Atg17, Atg31, and Atg24 were digested with BamHI/SalI, Atg29 with EcoRI/SalI, Atg13 with SmaI/SalI and ligated into respective sites of pGAD-C1.

Table 4.1 Strains

Strain	Genotype	Reference
YNB105	BY4741; MATa his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB106	BY4742; MATα his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB 263	BY4741; *PIL1-6XMYC:: LEU2*	(Mascaraque, Hernaez et al. 2013)
YNB 339	PJ469-A	(James, Halladay et al. 1996)
YNB 474	BY4741; *pil1*Δ::*KanMX*	This study
YNB 245	BY4742; *pil1*Δ::*KanMX*	Euroscarf
YNB389	BY4741; *fis1*Δ::*KanMX*	Euroscarf
YNB511	BY4742; *fis1*Δ::*KanMX*	Euroscarf
YNB388	BY4741; *dnm1*Δ::*KanMX*	Euroscarf
YNB512	BY4742; *dnm1*Δ::*KanMX*	Euroscarf
YNB 454	BY4741; *fis1*Δ::*KanMX pil1*Δ::*HIS3*	This study
YNB 473	BY4741; *dnm1*Δ::*KanMX pil1*Δ::*HIS3*	This study
YNB 197	BY4742; *atg32*Δ::*KanMX*	Euroscarf
YNB292	BY4742; *atg1*Δ::*KanMX*	Euroscarf

Table 4.2. Plasmids

Plasmid		Source/Reference
pNB356	p425 TEF	(Mumberg, Muller et al. 1995)
pNB357	p426 TEF	(Mumberg, Muller et al. 1995)
pNB656	p425 TEF Pil1-flag	This study
pNB441	p426 TEF Pil1-flag	This study
pNB478	pFA6a-HisMX6	(Longtine, McKenzie et al. 1998)
pNB479	pFA6a-KanMX6	(Longtine, McKenzie et al. 1998)
pNB812	pHS12 Mito-mCherry	Addgene
pNB415	pGBD-C1	(James, Halladay et al. 1996)
pNB422	pGAD-C1	(James, Halladay et al. 1996)
pNB428	pGBD-Pil1	This study
pNB427	pGAD-Pil1	This study
pNB508	pGAD-Lsp1	This study
pNB510	pGAD-Atg1	This study
pNB511	pGAD-Atg9	This study
pNB512	pGAD-Atg27	This study
pNB513	pGAD-Atg29	This study
pNB514	pGAD-Atg23	This study
pNB515	pGAD-Atg13	This study
pNB516	pGAD-Atg14	This study
pNB517	pGAD-Atg17	This study
pNB518	pGAD-Atg31	This study
pNB519	pGAD-Atg24	This study
pNB433	pGBD-Atg8	Y.Oshumi, Japan

pNB430	pGBDU-Atg11	(Aoki, Kanki et al. 2011)
pNB414	pGAD-Atg32	Koji Okamota, Japan
pNB425	pRS316 Atg32-HA	Koji Okamoto, Japan
pNB362	pRS423 GFP-Atg8	Ravi Manjithaya, JNCASR Bangalore
pNB449	pRS316 Su9-DHFR-GFP	(Heo, Livnat-Levanon et al. 2010)
pNB472	pRS316 Pgk1-GFP	(Welter, Thumm et al. 2010)

Table 4.3. Primers

Primer Name	**Sequence (5'-3')**	**Restriction enzyme**
NB1122 ATG1F	GCCGGATCCACCATGGGAGACATTAAAAATAAAGAT	BamHI
NB1123 ATG1R	GCCGTCGACATTTTGGTGGTTCATCTTCTG	SalI
NB1124 ATG9F	GCCGGATCCACCATGGAGAGAGATGAATACCAGTTA	BamHI
NB1125 ATG9R	GCCGTCGACTCTTCCGACGTCAGACTTCTT	SalI
NB1126 ATG27F	GCCGGATCCACCATGGTATCGAAGACTTGGATAT	BamHI
NB1127 ATG27R	GCCGTCGACAACGGCGCTATAACCGCCT	SalI
NB1128 ATG29F	GCCGAATTCACCATGATTATGAATAGTACAAACACAGT	EcoRI
NB1129 ATG29R	GCCGTCGACGAATTGCAATCTGTCCATTA	SalI
NB1130 ATG23F	GCCGGATCCACCATGGAACTGAATCAGGTTTT	BamHI
NB1131 ATG23R	GCCGTCGACTTCAACTTTTTTTGATATGGCATC	SalI
NB1132 ATG13F	GCCCCCGGGACCATGGTTGCCGAAGAGGACAT	SmaI
NB1133 ATG13R	GCCGTCGACACCTTCTTTAGAAAGGTTCATATCAC	SalI
NB1134 ATG14F	GCCGGATCCACCATGCATTGCCCAATTTGCCAC	BamHI

NB1135 ATG14R	GCCGTCGACGCCTACCACGTACCATCGGT	SalI
NB1136 ATG17F	GCCGGATCCACCATGAACGAAGCAGATGTTAC	BamHI
NB1137 ATG17R	GCCGTCGACAGGATTCTTCACGTTGTAATTT	SalI
NB1138 ATG31F	GCCGGATCCACCATGAATGTTACAGTTACTGTT	BamHI
NB1139 ATG31R	GCCGTCGACTACGGAATTGGAGAGCATTTGT	SalI
NB1140 ATG24F	GCCGGATCCACCATGACAGACAAAGGCAAGAAC	BamHI
NB1141 ATG24R	GCCGTCGACTAGACTATCGTCTACTTTTTCCCATG	SalI

4.2.c. Autophagy and Mitophagy assays

For autophagy, yeast strains to be studied were grown in synthetic minimal (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) media till log phase. Yeast strains to be studied for mitophagy were grown in synthetic minimal (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as required) media. For proliferation of mitochondria, cells were grown in synthetic minimal medium with lactate (SML; 0.67% yeast nitrogen base, 2% lactate, and auxotrophic amino acids and vitamins as needed). Cells equivalent to 3 unit $O.D._{600}$ were collected for zero time point. Autophagy/mitophagy was induced by shifting the same number of cells (3 unit $O.D_{600}$) to nitrogen starvation medium with glucose (SD-N; 0.17% yeast nitrogen base without ammonium sulfate or amino acids, and 2% glucose). To monitor GFP release by immunoblotting, strains transformed with Su9-DHFR-GFP plasmid were used for mitophagy assays and those transformed with pRS423 Atg8-GFP were used for studying autophagy (Figure 4.2). Autophagy was also studied using pRS316 Pgk-GFP as a marker (Welter, Thumm et al. 2010).

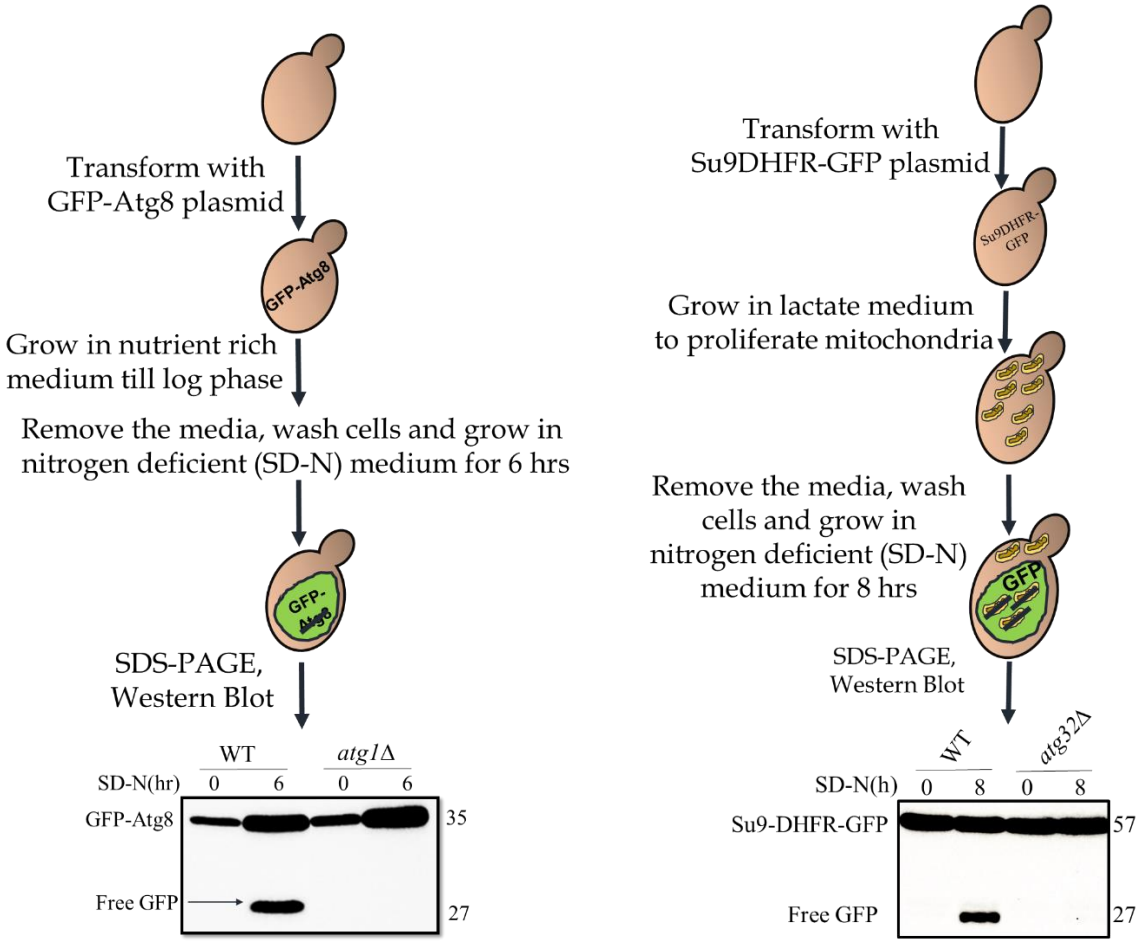


Figure 4.2 Schematic representation of methodology for autophagy and mitophagy assays

4.2.d. Mitochondria isolation

Crude mitochondria isolation was done as mentioned in Chapter 2 (section 2.2e). Sucrose-gradient purification of mitochondria was not done for this study.

4.2.e.Yeast two hybrid

Yeast two-hybrid was performed as described in Chapter 3 (section 3.2c).

4.2.f. Co-immunoprecipitation

For Co-IP assay, cells were collected and lysed with glass beads (Sigma, G8772) in lysis buffer (20 mM Tris-HCL [pH 8.0], 100 mM NaCl [Himeda, GRM031], 1 mM EDTA and 0.5 % Nonidet P-40 [Sigma,I8896], protease inhibitor cocktail [Roche, 04693132001]. After bead-beating, cells

were centrifuged at 12,000 rpm for 10 min. Supernatant was collected and incubated with anti-HA (Anti-mouse, Abcam, ab130275) antibody for 12 hr at 4°C in an end-over-end rotor. 30 µL of protein A/G PLUS-Agarose beads (SCBT, SC-2003)) were added and incubated for 2 h at at 4°C in an end-over-end rotor. Immunocomplexes were then washed three times with lysis buffer and eluted by SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE and immunoblotted with anti-Myc antibody.

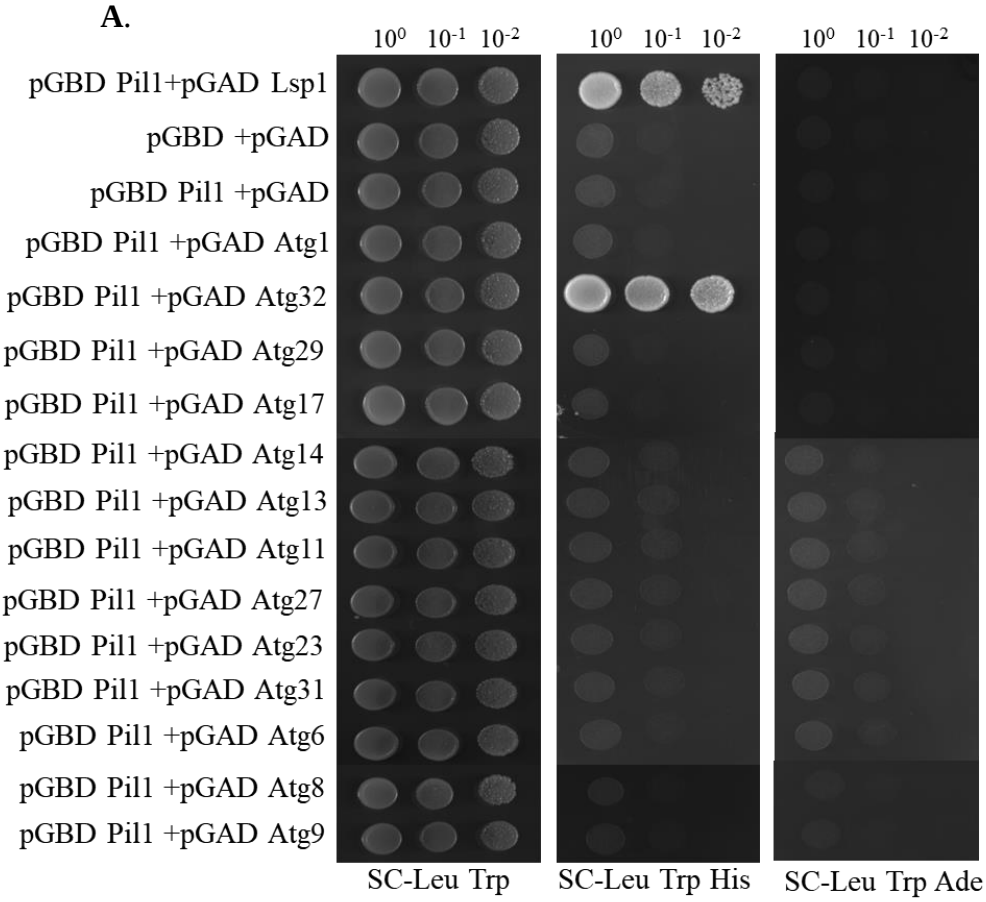
4.2.g. Whole cell extraction and Immunoblotting

Yeast cells (equivalent to A600 2-4) collected for the analysis were resuspended in 1 ml of water, spinned down at 13,000 rpm for 1 min, and the supernatant was discarded. Cells were resuspended in 160 µl of freshly prepared 1.85 M NaOH [Himedia, MB095], 7.4% β-mercaptoethanol [Sigma, M3148], mixed, and incubated on ice for 10 min. 160 µl of 50% TCA [Himedia,GRM6274] was added, mixed, and allowed to incubate on ice for 10 min. Cells were centrifuged at 13,000 rpm for 2 min, and the pellet was washed (not resuspended) with 500 µl of 1 M Tris base (not pH adjusted) (Sigma, T6066). The pellet was resuspended in 50 µl of SDS-PAGE loading buffer and heated at 95°C for 5 min. After cooling down, cells were centrifuged at 13,000 rpm for 5 min, and the supernatant was collected. Ten microliter of the supernatant was loaded into SDS-PAGE gel (10–12%). After separation in SDS-PAGE, proteins were transferred onto nitrocellulose membranes (PALL, 66485) and blocked with 5% skimmed milk (Himedia, GRM1254) in Trisbuffered saline. The commercial monoclonal antibodies anti-GFP (ab183734, Abcam, 1:5000), anti-Myc (ab9106, Abcam, 1:5000) and anti-HA (ab130275, Abcam) were used to detect GFP, Myc, or HA epitope-fused protein on immunoblots. Anti-Por1 and Pgk1 serums were kind gifts from Debkumar Pain (New Jersey Medical School, Rutgers University). HRP-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, 111-035-144, 1:25,000) or anti-mouse (Jackson ImmunoResearch Laboratories, 115-035-146, 1:25,000) polyclonal secondary antibodies were used, followed by the detection of signal using ECL reagents (Advansta Western-Bright, K-12045-D20) and imaging in Chemidoc Imaging System (Bio-Rad). Quantification of Western Blot images was done using Fiji software. Graphs and statistical analysis were done using GraphPad Prism8.

4.3 RESULTS

4.3. a. Yeast two hybrid analysis to screen the Atg proteins as interacting partners of Pil1

To screen the Atg proteins that interact with Pil1, we used yeast two-hybrid analysis and found a weak interaction of Pil1 with Atg32 (Figure 4.3A). Atg32 is mitophagy-specific receptor present on the outer membrane of mitochondria which is known to be indispensable for mitophagy. It is known to sequester the mitochondria to the autophagosomes. To confirm this, we performed co-immunoprecipitation study. pRS316 Atg32-HA or empty pRS316 (as control) were transformed in Pil1-Myc strain. Co-IP was performed as described in the materials and methods section. We found that significant amount of Pil1-Myc was pulled down with Atg32-HA, which shows that both the proteins interact physically (Figure 4.3B).



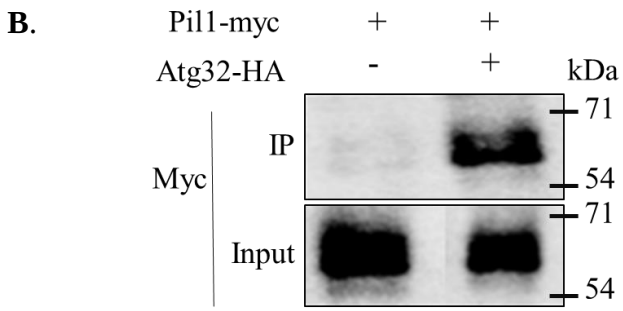


Figure 4.3 Pil1 interacts with Atg32.

A. Yeast two-hybrid analysis. Interaction of Pil1 with the indicated Atg proteins was checked through Y-2-H. The PJ69-4A strain was transformed with pGAD-C1 and pGBD-C1 plasmids, which can express the indicated proteins. Cells were grown on +Ade/ –Ade plates or +His/–His at 30°C for 2-3 days. Pil1 interacts weakly with Atg32. B. Co-immunoprecipitation assay to test the interaction of Pil1-Myc with Atg32-HA. Cells expressing Pil1-Myc were transformed with Atg32-HA plasmid and empty plasmid for control. Transformed cells were grown in SMD (SCD lacking uracil) media until log phase. Anti-HA antibody was used to precipitate Pil1-Myc. Western blotting was done using anti-Myc antibody.

4.3.b. Pil1 is involved not only in mitophagy, but also non-selective autophagy:

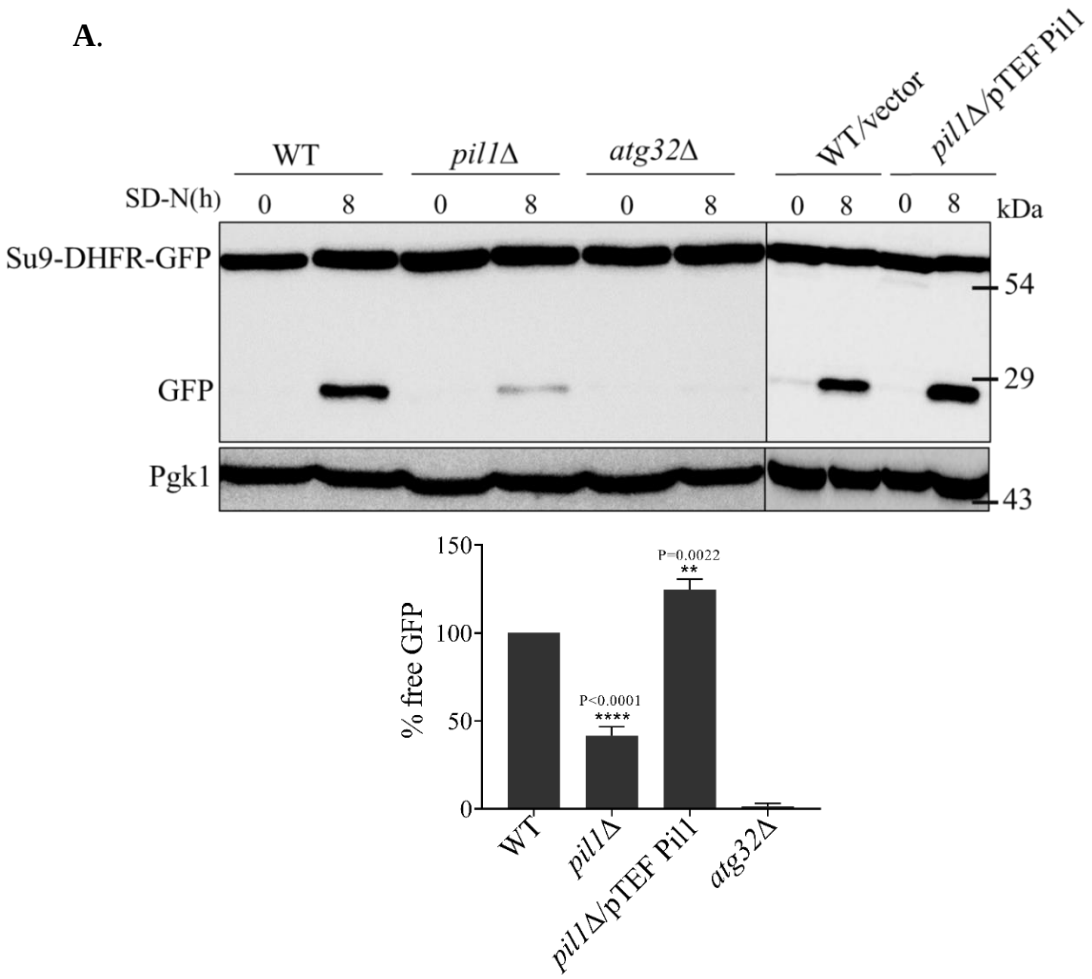
Though there is growing evidence that mitochondrial pro-fission factors play a role in mitophagy, there is still a lack of clarity behind the exact mechanism. In mammalian cells, it has been shown that mitophagy is impaired when mitochondrial fission is inhibited, which leads to accumulation of damaged mitochondria (Twig, Elorza et al. 2008). Nevertheless, we decided to check whether Pil1 regulates the fission factors mediated mitophagy or itself alters mitophagy. To measure mitophagy, we used Su9-DHFR-GFP processing assay. This plasmid has DHFR gene with Su9 presequence at the N-terminal and GFP tag at the C-terminal. Su9 presequence targets DHFR-GFP to mitochondria. During mitophagy, mitochondria are sequestered by autophagosomes and targeted to the vacuole where DHFR gets degraded, and GFP moiety, relatively resistant to proteolysis, remains intact. The amount of free GFP by immunoblotting is used as a measure to monitor mitophagy. After 8 h of nitrogen starvation to induce mitophagy, we found that free GFP release was significantly less in *pil1*Δ than WT and more in overexpressed Pil1 (Figure 4.4A). No GFP release was seen in *atg32*Δ as it is essential for mitophagy (Kanki, Wang et al. 2009, Okamoto, Kondo-Okamoto et al. 2009). Our data indicate that Pil1 is required for mitophagy.

We sought to find out if the function of Pil1 in mitophagy is dependent on Fis1 and Dnm1. For

this, we monitored the mitophagy levels in *fis1*Δ, *dnm1*Δ, *fis1*Δ*pil1*Δ and *dnm1*Δ*pil1*Δ strains. To our surprise, we found that *fis1*Δ and *fis1*Δ*pil1*Δ cells were defective in mitophagy (Figure 4.4B, lanes 7,8 & 9,10). On the other hand, *dnm1*Δ cells showed almost normal mitophagy levels (lanes 13, 14), which were completely inhibited upon Pil1 depletion, i.e., in *dnm1*Δ*pil1*Δ cells (lanes 15, 16). To get further clarification, we checked mitophagy after overexpressing Pil1 in *fis1*Δ*pil1*Δ and *dnm1*Δ*pil1*Δ cells and found that it significantly increased (lanes 11,12 & 17,18). Interestingly, overexpression of Pil1 can complement the mitophagy defect in *fis1*Δ cells. Altogether, our data suggest that the role of Pil1 in mitophagy is independent of Fis1 and Dnm1.

Considering that Pil1 has a role in mitophagy, we next wanted to check if role of Pil1 is limited to mitophagy or it regulates non-selective (bulk) autophagy as well. We utilized the GFP-Atg8 processing assay to monitor autophagy. Atg8 is considered as autophagosome marker, and is known to present on both the sides of the phagophore, the precursor to the autophagosome. After completion of autophagosome formation, Atg8 present on the surface is removed, whereas Atg8 present on the inner side is carried to the vacuole and degraded after lysis of the autophagic body. Hence, monitoring of free GFP processed from GFP-Atg8 fusion directly measures the level of autophagy (Shintani and Klionsky 2004). After 8 hr of autophagy induction in nitrogen starvation medium, we could see that free GFP band was much less intense in *pil1*Δ. In contrast, it was more intense in Pil1 overexpression cells when compared to WT (Figure 4.4C). *atg1*Δ is used as a control. Similar results were obtained when GFP release from Pgk1-GFP was monitored to measure autophagy levels (Figure 4.4D). These results suggest that Pil1 is also required for bulk autophagy. However, the mechanism behind its role in the processes of selective and non-selective autophagy needs to be studied in more detail. However, the mechanism behind its role in the processes of selective and non-selective autophagy needs to be studied in more detail.

A.


WT
pil1Δ
atg32Δ
WT/vector
pil1Δ/pTEF Pil1
SD-N(h)
0 8 0 8 0 8 0 8 0 8
kDa
Su9-DHFR-GFP
GFP
Pgk1
54
29
43
% free GFP
150
100
50
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P=0.0022
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WT
pil1Δ
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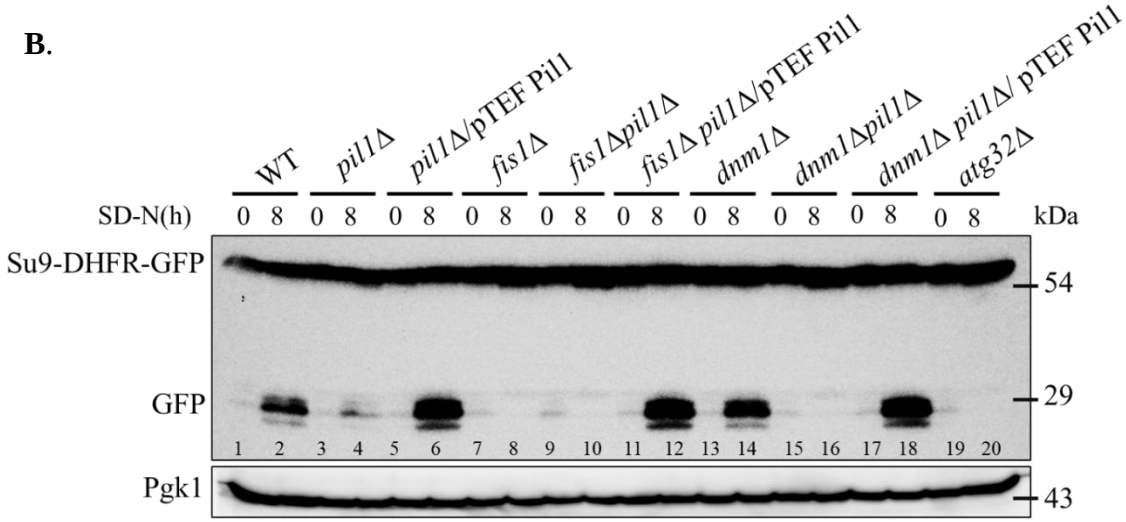
atg32Δ

B.


WT
pil1Δ
pil1Δ/pTEF Pil1
fis1Δ
fis1Δpil1Δ
fis1Δ pil1Δ/pTEF Pil1
dnm1Δ
dnm1Δpil1Δ
dnm1Δ pil1Δ/ pTEF Pil1
atg32Δ
SD-N(h)
0 8 0 8 0 8 0 8 0 8 0 8 0 8 0 8 0 8 0 8
kDa
Su9-DHFR-GFP
GFP
Pgk1
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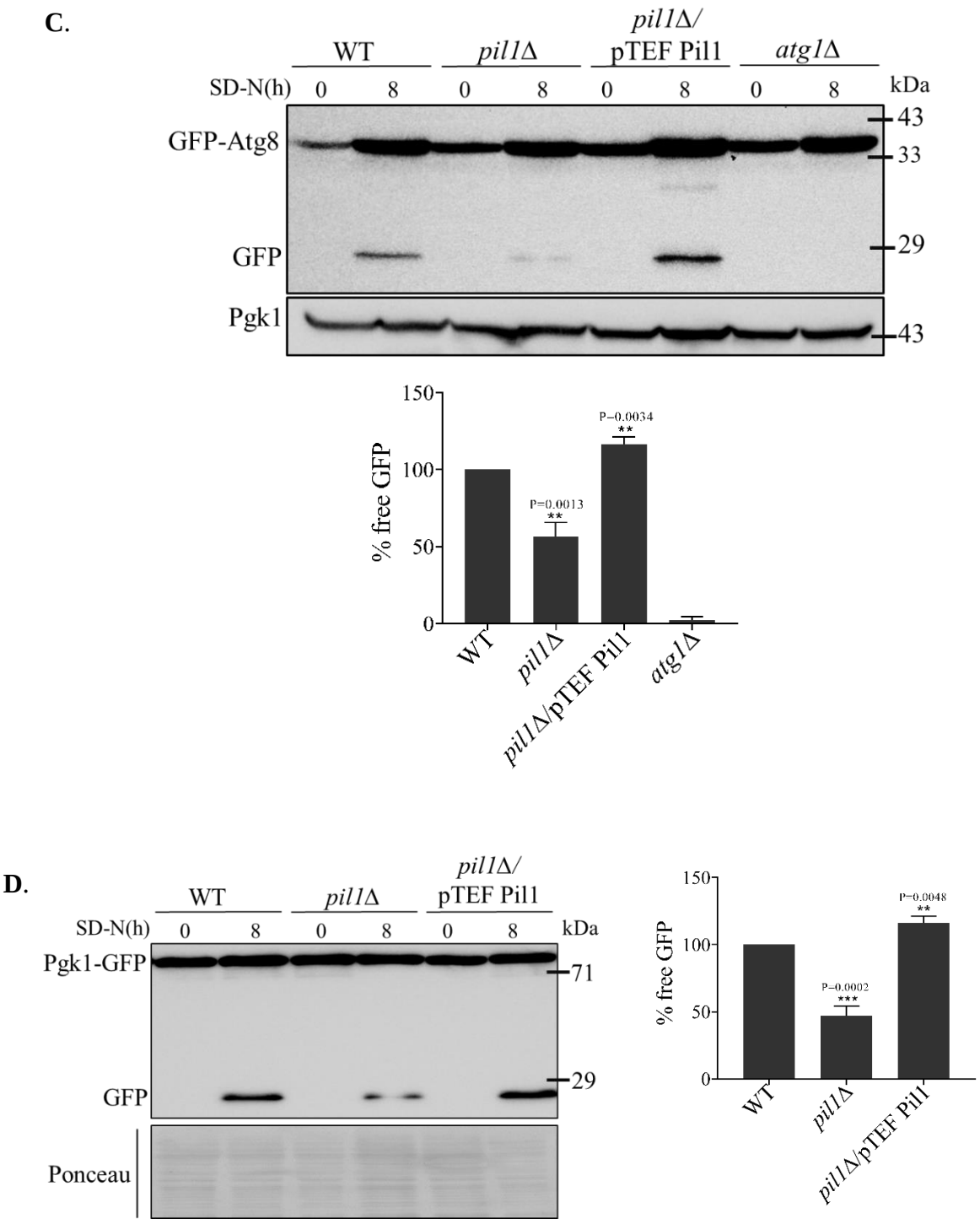


Figure 4.4. Pil1 regulates mitophagy and bulk autophagy

A and B. Mitophagy assay. Indicated strains transformed with Su9-DHFR-GFP plasmid were grown in SML media (SCL lacking leucine and uracil) to proliferate mitochondria. Cells were then shifted to SD-N media for 8 hrs. Samples were collected before and after nitrogen starvation. Protein extracts were immunoblotted with anti-GFP antibody. Positions of full length Su9-DHFR-

GFP and free GFP are indicated. Anti-Pgk1 serum was used as loading control. Quantification of mitophagy after 8 hr of nitrogen starvation is represented as a percentage of the ratio free GFP to total GFP signal. Data are presented as mean ± SD of 3 independent assays. C. Autophagy assay. Indicated strains carrying pRS423 GFP-Atg8 plasmid were grown in SMD media (SCD lacking histidine and leucine) and then shifted to SD-N media for 8 hr. Cells were collected before and after nitrogen starvation. Immunoblotting was done with anti-GFP, and positions of full length GFP-Atg8 and free GFP are indicated. Anti-Pgk1 was used as a loading control. Quantification of autophagy is the percentage of free GFP/total GFP signal. D. Pgk1-GFP was used to monitor bulk autophagy. Ponceau is shown as loading control. Indicated strains carrying pRS316 Pgk1-GFP plasmid were grown in SMD media (SCD lacking histidine and leucine) and then shifted to SD-N media for 8 h. Cells were collected before and after nitrogen starvation. Quantification of autophagy is the percentage of free GFP/total GFP signal. Data are presented as mean ± SD of 3 independent experiments. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 vs WT.

4.3.c. Expression and localization of Pil1 to mitochondria are enhanced during starvation

The levels of autophagy regulating proteins are known to increase by many folds when autophagy is induced by starvation. So we decided to check the levels of Pil1 in the starvation condition and found that it is almost 2-folds higher after 8 hr of N-starvation. We checked the expression levels in both autophagy inducing condition (N-starvation after growing in glucose media; Figure 4.5A) as well as in mitophagy inducing condition (N-starvation after growing in lactate media; Figure 4.5B).

As Pil1 localizes to mitochondria and regulates mitophagy, we were interested to see if the mitochondrial pool of Pil1 also increases when we induce mitophagy. So after growing cells in lactate medium we shifted them to SD-N medium for 2 hr and then separated the mitochondrial and cytosolic fractions. There is a clear increase in Pil1 protein in the mitochondrial fraction as well (Figure 4.5C). Together, these data substantiates the role of Pil1 in autophagy.

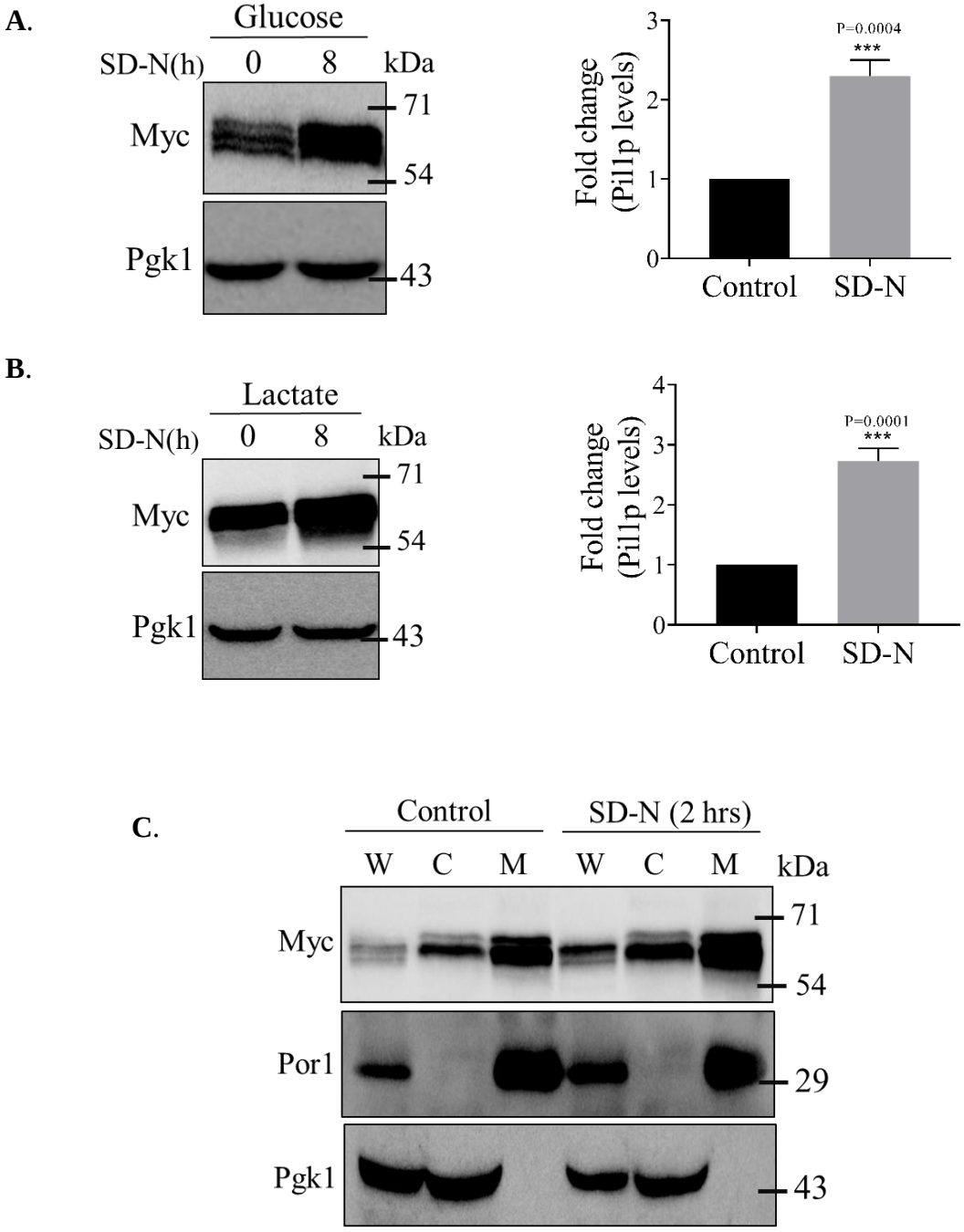


Figure 4.5 Levels of Pil1 and its localization to mitochondria increases after nitrogen starvation

A and B. Pil1-Myc strain was grown in YPD (in A) and YPL media (in B) till log phase. Cells were then shifted to SD-N media for 8 hr. Samples were collected before and after nitrogen starvation. Protein extracts were immunoblotted with anti-Myc antibody. Anti-Pgk1 serum was used as loading control. Quantification of Pil1 levels are presented as mean ± SD of 3 independent assays. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph) ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ vs WT. C. Pil1-Myc strain was grown in YPL media and mitochondria was isolated before and after 2 hr of N-starvation. All

three fractions were collected and immunoblotted with Anti-Myc antibody. Anti-Porin and Anti-Pgk1 were used as controls. W: Whole cell extract, C: Cytosolic fraction, M: Mitochondrial fraction.

4.4 CONCLUSION

In this chapter, we found that Pil1 regulates bulk autophagy as well as selective autophagy (mitophagy). However, its role in mitophagy is independent of mitochondrial fission proteins Fis1 and Dnm1. Also, the expression of Pil1 increases during autophagy/mitophagy conditions. Localization of Pil1 to mitochondria was also found to be more after inducing mitophagy indicating that Pil1 might be getting recruited to mitochondria under stress conditions.

CHAPTER 5

Pil1 in oxidative stress and cell death

5.1 BACKGROUND

We have observed that absence of Pil1 leads to drastically reduced autophagy and mitophagy. Aberrations in autophagy have been linked with accumulation of ROS and protein aggregates which can be detrimental for the cell. Protein aggregates in *S. cerevisiae* has been widely studied using Hsp104-GFP (a disaggregase protein that dissolves protein aggregates) as a marker (Parsell, Kowal et al. 1994). Big and bright puncta of Hsp104-GFP are observed whenever there is accumulation of protein aggregates in cells (Figure 5.1).

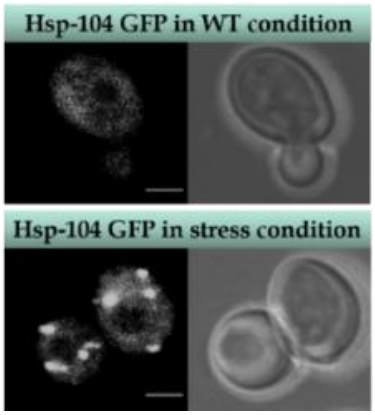


Figure 5.1 Fluorescence images of Hsp104-GFP in *S.cerevisiae* in WT and stress condition.

Mitochondrial dysfunction, ROS and protein aggregates naturally lead to ageing in cells. There are two main approaches to study ageing in yeast. Replicative lifespan of yeast is the number of times a cell can divide before it dies. It is measured by counting the number of buds that a mother cell produces. Chronological lifespan is the time a cell can survive in a non-dividing state, which is measured by monitoring the decrease in survival (ability to form colony) in a stationary phase culture.

Apart from the role of fission proteins in mitophagy, there is a study which has established the role of Fis1 and Dnm1 in programmed cell death in yeast where Fis1 was shown to have protective role whereas Dnm1 was shown to induce apoptotic cell death (Fannjiang, Cheng et al. 2004). As Pil1 interacts with mitophagy protein (Atg32) as well as fission proteins (Fis1 and Dnm1) and regulates both the pathways, we speculated that Pil1 also might have some role in regulating cell death.

5.2 MATERIALS AND METHODS

5.2.a. Media and growth condition

Strains were grown to log phase ($O.D._{600}$ = approximately 1) in synthetic complete dextrose media (SCD; 0.8% yeast nitrogen base without amino acids [Becton Dickinson, 291940], 2% dextrose [Himedia, GRM077], pH 5.5) or synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) as described previously (Sherman 1991). Yeast cells were grown at 30°C. Yeast transformations were performed using lithium acetate method as previously described (Gietz and Woods 2002).

5.2.b. Yeast strains and plasmids

All strains, plasmids, and primers used in this study are listed in Table 5.1, Table 5.2, and Table 5.3, respectively. The yeast strains used in this study are derivatives of BY4741 or BY4742. PCR-based targeted homologous recombination was used to replace complete open reading frame of *PIL1* with KanMX6 or HIS3MX6 cassettes, as indicated (Longtine, McKenzie et al. 1998). The method of deletion of *PIL1* has been described in Chapter 1 (section 2.2b).

For p426TEF Bcl-xL (pNB673) construct, coding region of human Bcl-xL was PCR amplified from HeLa cDNA using primer pair NB838/NB839 with EcoRI and XhoI restriction sites and ligated into p426TEF vector.

Table 5.1 Strains

Strain	Genotype	Reference
YNB105	BY4741; MATa his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB106	BY4742; MATα his3Δ1; leu2Δ0; met15Δ0; ura3Δ	Euroscarf
YNB 474	BY4741; *pil1*Δ::*KanMX*	This study
YNB 245	BY4742; *pil1*Δ::*KanMX*	Euroscarf
YNB437	BY4741; HSP104-GFP::*HIS3*	(Song, Yang et al. 2014)
YNB438	BY4741; HSP104-GFP::*HIS3 pil1*Δ*::KanMX*	This study

Table 5.2 Plasmids

Plasmid		Source/Reference
pNB356	p425 TEF	(Mumberg, Muller et al. 1995)
pNB357	p426 TEF	(Mumberg, Muller et al. 1995)
pNB656	p425 TEF Pil1-flag	This study
pNB441	p426 TEF Pil1-flag	This study
pNB478	pFA6a-HisMX6	(Longtine, McKenzie et al. 1998)
pNB479	pFA6a-KanMX6	(Longtine, McKenzie et al. 1998)
pNB812	pHS12 Mito-mCherry	Addgene

Table 5.3. Primers

Primer Name	Sequence (5'-3')	Restriction enzyme
NB754 PIL1F	GCCGAATTCACCATGCACAGAACTTACTCTTTAA	EcoRI
NB755 PIL1R	GCCAAGCTTAGCTGTTGTTTGTTGGGGAA	HindIII
NB838 bclxlF	GCC GAATTC ATGTCTCAGAGCAACCGGGA	EcoRI
NB839 bclxlR	GCC CTCGAG TTTCCGACTGAAGAGTGAG	XhoI

5.2.c. Fluorescence microscopy

Cells grown till OD_{600}= approximately 1, were harvested by centrifugation and the pellet was washed twice with fresh SMD media. Cells suspended in SMD media were added to slides coated with 0.1% Concanavalin A (Sigma, C2010) solution. The cells were allowed to settle for 2-3 min and mounted with coverslips. Images were acquired either on Leica TCS SP8 (HC PL APO CS2 63X/1.40 OIL objective) or Zeiss LSM NLO 710 (63X/1.5 OIL objective) confocal microscope. Fiji Software was used to process and analyse the microscopy data. Graphs and statistical analysis were done using GraphPad Prism8.

5.2.d. Measurement of ROS

For visualization of cellular ROS via fluorescence microscopy, cells growing in SMD medium were harvested by centrifugation (1 unit OD_{600} cells), washed with PBS twice and then treated with 10 mM 5(6)-carboxy-2'7'-dichlorofluorescin diacetate (CM-H2DCFDA; Invitrogen, C6827) in 500 µL PBS for 30 min at 30°C. DCFDA is a non fluorescent compound which gets oxidized by ROS in the cells into fluorescent compound DCF Similarly, for measurement of mitochondrial ROS, cells (1 unit OD_{600} cells) were treated with 5 µM MitoSOX™ Red (Invitrogen, M36008) for 10 min at 30°C in 500 µL of PBS. MitoSOX dye specifically stains mitochondria with high amount of superoxide ions.

5.2.e. Chronological ageing

WT and *pil1*Δ cells were grown overnight till stationary phase. Cells were harvested and equal number of cells (1 unit $O.D._{600}$) were taken and added in fresh medium. This was counted as day one. 5 µl of culture was plated onto solid YPD medium every one week and number of colonies were counted until 8 weeks. Percent survival was calculated keeping number of colonies on day 1 as control.

5.2.f. Cell viability assays

Cells were grown in SMD media until OD_{600} reached approximately 1. For colony-forming unit (CFU) assays, cells were normalized to OD_{600} 0.5/mL and 200 µl of each culture was collected into 96-well plates. Serial dilutions were done using multichannel pipettor and 10 µL were plated onto SMD solid media. Plates were incubated for 2 days at 30°C and number of colonies were counted. Annexin V/PI co-staining assays using ApoAlert Annexin V-FITC Apoptosis kit (Takara, 630109) were performed as described previously (Buttner, Eisenberg et al. 2007). For trypan blue staining, 10 µL of cell suspension in PBS were mixed with 0.4% trypan blue (Gibco,15250061) for 2-3 min at room temperature and number of cell stained with the dye were counted.

5.3 RESULTS

5.3.a. Absence of Pil1 leads to elevated ROS levels and protein aggregation:

Because altered mitochondrial network and autophagy dysregulation are linked with abnormal ROS levels and protein aggregation in the cell (Tal, Sasai et al. 2009, Filomeni, De Zio et al. 2015, Bhatti, Bhatti et al. 2017), we next investigated if Pil1 regulates ROS levels and protein aggregation. To measure ROS levels, we used CM-H2DCFDA, a fluorescent ROS indicator. We found that ROS levels were significantly high in *pil1*Δ cells compared to WT (Figure 5.2A). In order to study protein aggregation, we used Hsp104, endogenously tagged at the C-terminus with GFP. Hsp104 is a hexameric AAA+ ATPase based chaperone in yeast, a component of disaggregase machinery which is known to bind with protein aggregates, and cause their dissolution (Parsell, Kowal et al. 1994, Glover and Lindquist 1998, Bosl, Grimminger et al. 2006, Doyle and Wickner 2009, Nillegoda and Bukau 2015). Brightness intensity, size, and number of Hsp104-GFP foci increase under the abundance of protein aggregates. Based on the images obtained using confocal microscopy, we quantified the number of Hsp104-GFP aggregates. Surprisingly, we found that Hsp104-GFP foci are much more abundant in *pil1*Δ, whereas it was negligible in WT cells (Figure 5.2B). However, cells with Pil1 overexpression also showed moderate elevation in Hsp-104 aggregates and ROS levels compared to WT, which could be attributed to the fragmented mitochondrial network upon Pil1 overexpression (Yu, Robotham et al. 2006, Shenouda, Widlansky et al. 2011).

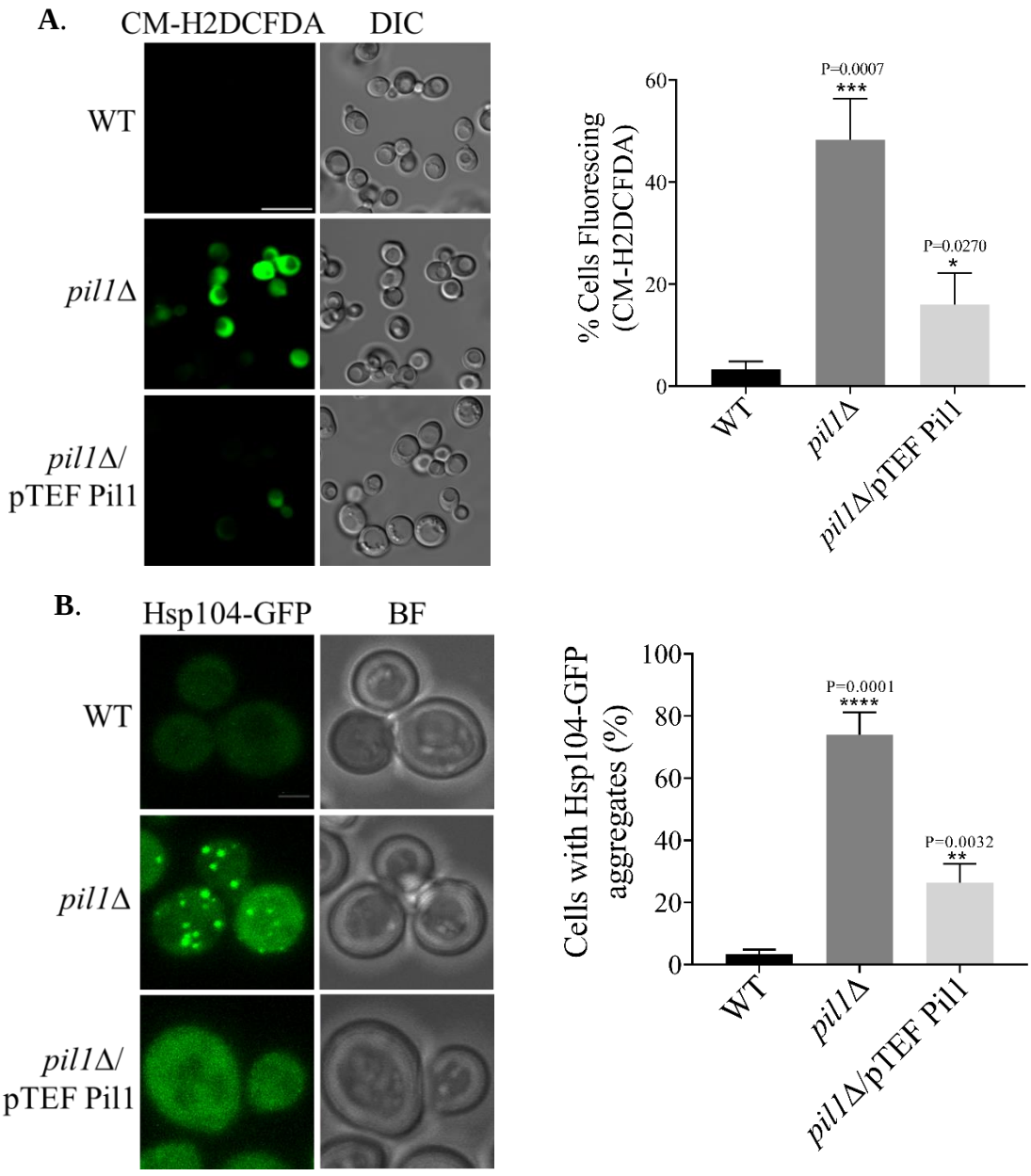


Figure 5.2 Absence of Pil1 leads to elevated ROS and protein aggregates.

A. Measurement of cellular ROS. Indicated strains were grown to log phase in SMD media (SCD lacking uracil) and 1 OD cells were treated with 10mM CM-H2DCFDA for 30 min at 30°C . Imaging by fluorescence microscopy was performed as described in Materials and Methods. Quantification represents the percentage of DCF-positive fluorescing cells, where a minimum of 100 cells for each strain were counted. Scale bar represents 10µm. B. Analysis of Hsp104-GFP aggregates. Indicated strains endogenously expressing HSP104-GFP were grown till log phase and imaging was performed by fluorescence microscopy as described in Materials and Methods. Represented images are maximum projections of z-stacks. Cells containing at least two Hsp104-GFP foci were counted in each strain. Minimum of 100 cells were in three independent trials were counted and data are presented as mean ± SD. Scale bar represents 5µm. Imaging was performed by confocal fluorescence microscopy and represented images are flattened z-stacks.

Scale bar represents 5µm. Ratio of Hsp104-GFP foci localized with mitochondria to total Hsp104-GFP foci was calculated as percentage (31%). BF: Brightfield. DIC: Differential Interference Contrast. Data are presented as mean ± SD of 3 independent experiments. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 vs WT.

5.3.b. Absence of Pil1 leads to increased chronological ageing:

Mitochondrial dysfunction, ROS, and protein aggregates naturally lead to ageing in cells. We studied chronological ageing in WT and *pil1*Δ cells. After the cell reached stationary phase, we monitored the number of viable colonies by plating the culture on solid media after every one week for 8 weeks and counting the number of colonies. *pil1Δ* started showing decreased survival that means enhanced ageing after 3rd week (Figure 5.3). This data suggest that *pil1*Δ shows enhanced ageing. Accumulation of ROS and protein aggregates might be the reason for enhanced ageing in *pil1*Δ cells.

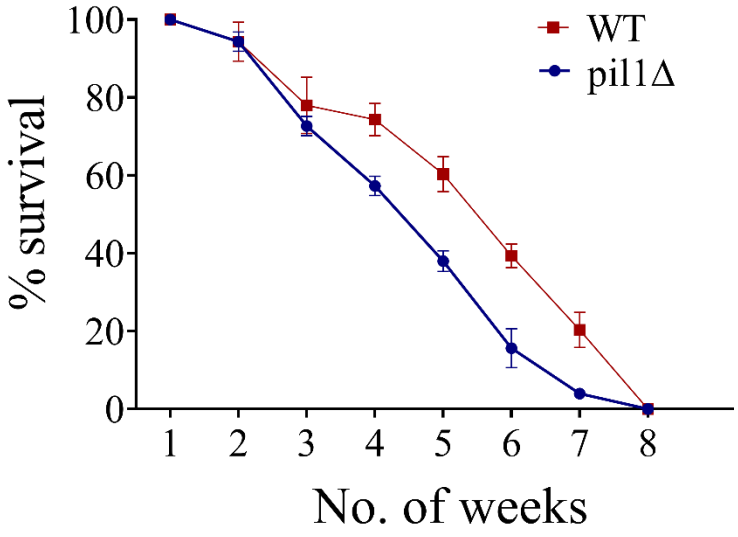
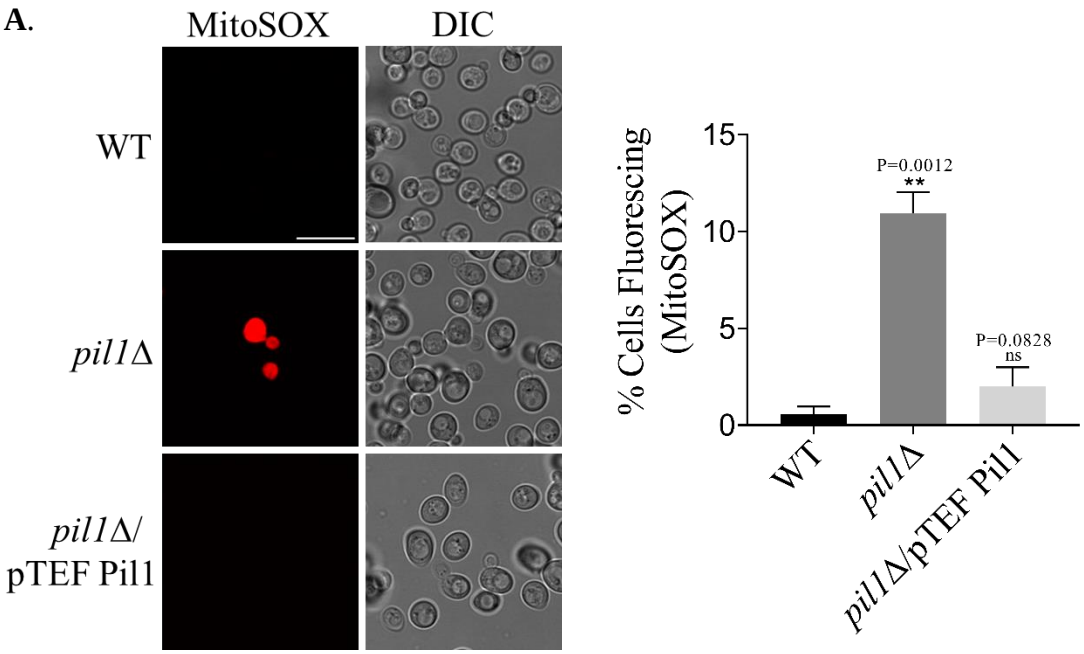


Figure 5.3 Chronological ageing in *pil1*Δ

WT and *pil1Δ* cells were grown overnight till stationary phase for two days. Equal number of cells (1 unit $O.D._{600}$) were inoculated in fresh medium and number of colonies were counted every week for 8 weeks. Survival percentage is shown in the graph. Colonies from three trials were counted and data are presented as mean ± SD.

5.3.c. ROS and protein aggregtates caused due to absence of Pil1 are majorly cytosolic

Mitochondria are the major source of ROS production in cells. Because we found that the absence of Pil1 leads to abnormal mitochondrial morphology and reduced mitophagy, we decided to check if ROS and protein aggregates that we have observed in *pil1*Δ are associated with mitochondria. Using MitoSOX red dye to measure mitochondrial ROS (mtROS), we found a small but statistically significant increase (10.9%) in mtROS between WT and *pil1*Δ (Figure 5.4A) which could be due to the defective mitochondrial morphology and mitophagy in *pil1*Δ. Using confocal microscopy, we analyzed the number of Hsp104-GFP aggregates associated with mitochondria in *pil1*Δ cells. We found that mitochondrial associated aggregates (33%) in *pil1*Δ cells are much less than those of cytosolic aggregates (67%) (Figure 5.4B). Our data suggest that Pil1-depleted cells have higher levels of ROS and protein aggregates, which might be a result of decreased mitophagy/autophagy.



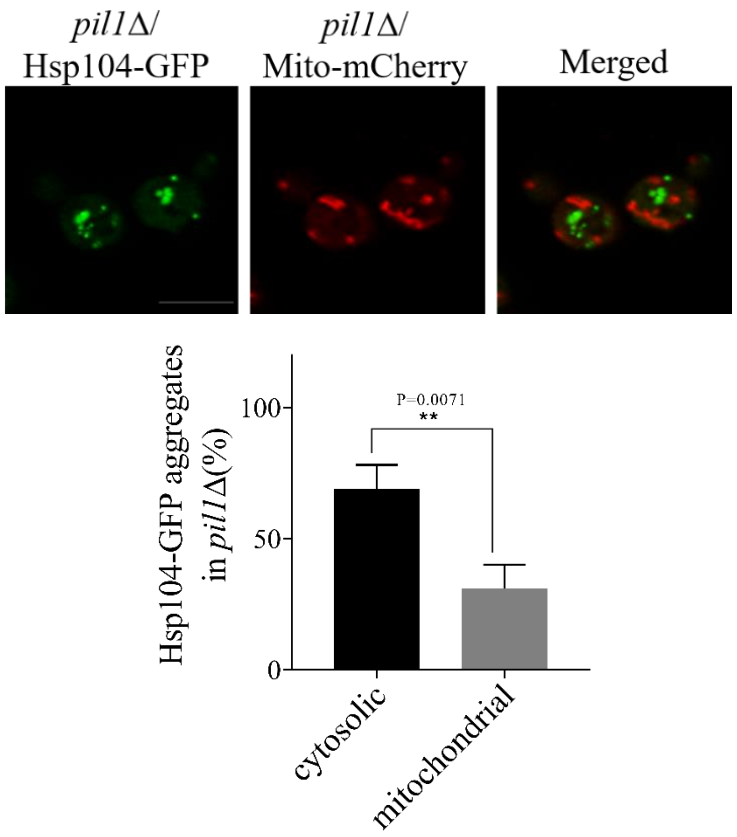


Figure 5.4 ROS and protein aggregtates in *pil1*Δ are majorly cytosol-associated

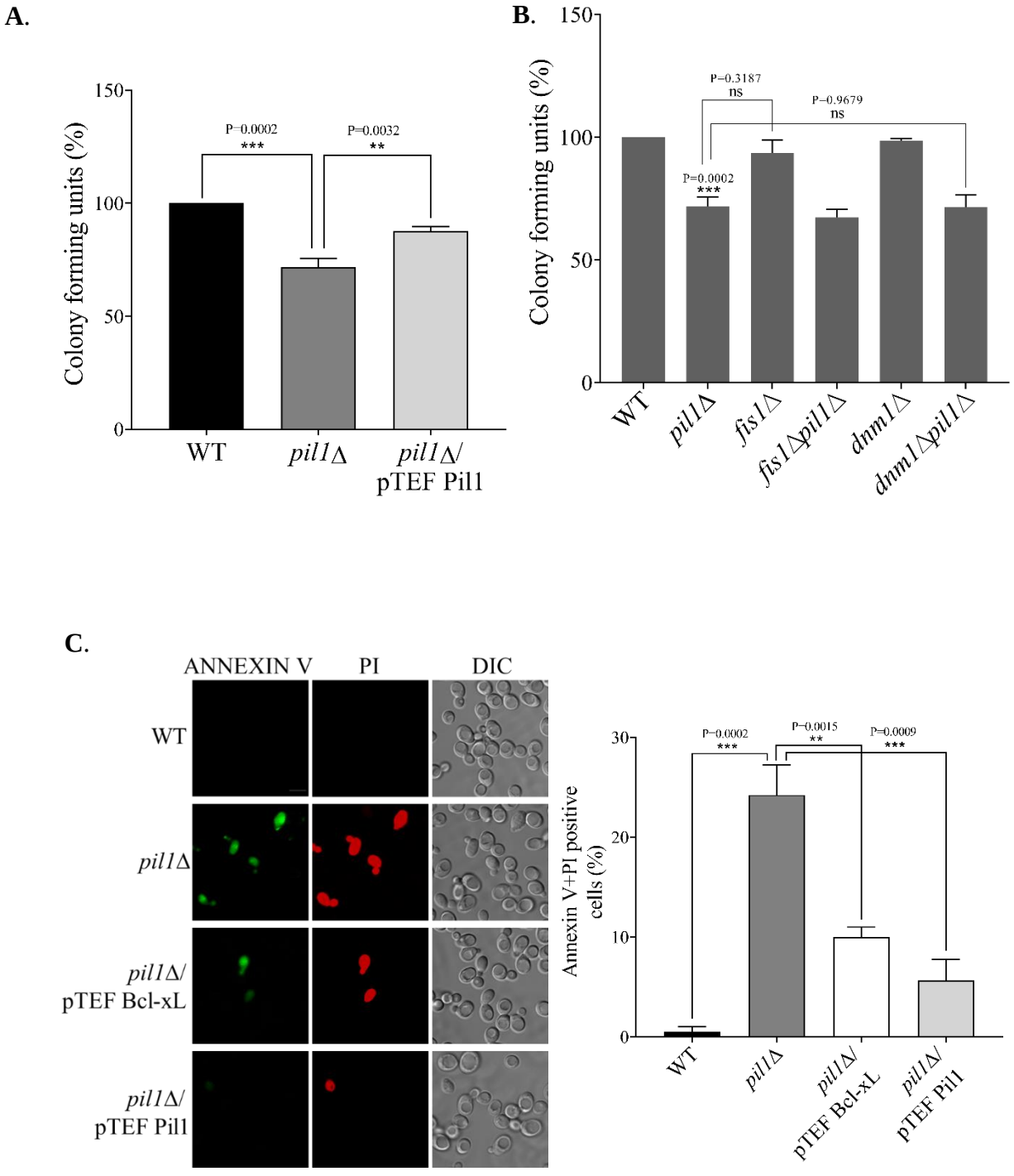
A. Measurement of mitochondrial ROS (mtROS). Indicated strains were grown to log phase in SMD media (lacking uracil) and treated with 5 µM MitoSOX Red for 10 min at 30°C before imaging by fluorescence microscopy. Quantification represents the percentage of MitoSOX-positive fluorescing cells, where a minimum of 100 cells for each strain were counted. Data are presented as mean ± SD of 3 independent experiments. Scale bar represents 10µm. B. Analysis of mitochondrial associated Hsp104-GFP aggregates in pil1Δ. Hsp104-GFP *pil1*Δ strain transformed with mito-mCherry and grown till log phase in SMD media. Imaging was performed by confocal fluorescence microscopy and represented images are flattened z-stacks. Scale bar represents 5 µm. Ratio of Hsp104-GFP foci localized with mitochondria to total Hsp104-GFP foci was calculated as percentage (33%). BF: Brightfield. DIC: Differential Interference Contrast. Data are presented as mean ± SD of 3 independent experiments. Statistical analysis was done using unpaired Student's t-test (p-value is indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 vs WT.

5.3.d. *pil1*Δ cells are more prone to cell death:

Abnormalities in mitochondria and ROS have been directly linked with cell death. We wanted to see if the higher ROS levels that we observed in *pil1*Δ cells make them more prone to cell death. To study viability, we applied the survival assay which is based on the ability of viable cells to form a colony (clonogenicity) on a nutrient-rich solid medium. We found a 29% reduction in

colony formation in *pil1*Δ compared to WT which was rescued to some extent by overexpressing Pil1 (13%) (Figure 5.5A). A study has well established the role of Fis1 and Dnm1 in programmed cell death induced by different kinds of death stimuli (Fannjiang, Cheng et al. 2004). However, our aim is to check if role of Pil1 in cell death under normal conditions is dependent on Dnm1 and Fis1. To this end, we applied colony survival assay to *fis1*Δ, *dnm1*Δ, *fis1*Δ*pil1*Δ, and *dnm1*Δ*pil1*Δ cells in the absence of any death stimuli. We found that there was a similar pattern of reduction in colony forming units in *fis1*Δ*pil1*Δ (31%) and *dnm1*Δ*pil1*Δ (30.2%) cells as in *pil1*Δ, indicating that they are more prone to cell death compared to WT (Figure 5.5B).

To determine the mode of cell death, we performed Annexin V/PI co-staining. Externalization of phosphatidylserine on the surface of plasma membrane is one of the characteristics of cells which are in the early to mid-stage of apoptosis. Annexin V binds to the exposed phosphatidylserine on the surface of apoptotic cells, whereas PI stains necrotic cells which have lost the plasma membrane integrity. Cells that are co-stained with Annexin V and PI are considered either in late apoptosis or early necrosis stage. We found that 24.7 % of *pil1*Δ cells were Annexin V^+/PI^+ which was significantly reduced Pil1 overexpression (5%). Also, there was a significant reduction in cell death when anti-apoptotic human Bcl-xL protein was overexpressed (Figure 5.5C). Alternatively, we used trypan blue exclusion method. This dye penetrates the plasma membrane and stains the dead cells blue. The results that we obtained were consistent with the Annexin V/PI staining assay (Figure 5.5D). Thus, the absence of Pil1 triggers probably necrotic cell death. However, the role of Bcl-xL in rescuing necrotic cell death in yeast has not been clearly established. Taken together, our data suggest that absence of Pil1 leads to more cell-death but the pathway could be distinct from Fis1 and Dnm1-mediated cell-death pathways.


A.
Colony forming units (%)
150
100
50
0
P=0.0002
***
P=0.0032
**
WT
pil1Δ
pil1Δ/
pTEF Pil1
B.
Colony forming units (%)
150
100
50
0
P=0.3187
ns
P=0.9679
ns
P=0.0002
***
WT
pil1Δ
fis1Δ
fis1Δpil1Δ
dnm1Δ
dnm1Δpil1Δ
C.
ANNEXIN V
PI
DIC
WT
pil1Δ
pil1Δ/
pTEF Bcl-xL
pil1Δ/
pTEF Pil1
Annexin V+PI positive
cells (%)
30
20
10
0
P=0.0002
***
P=0.0015
**
P=0.0009
***
WT
pil1Δ
pil1Δ/
pTEF Bcl-xL
pil1Δ/
pTEF Pil1

D.

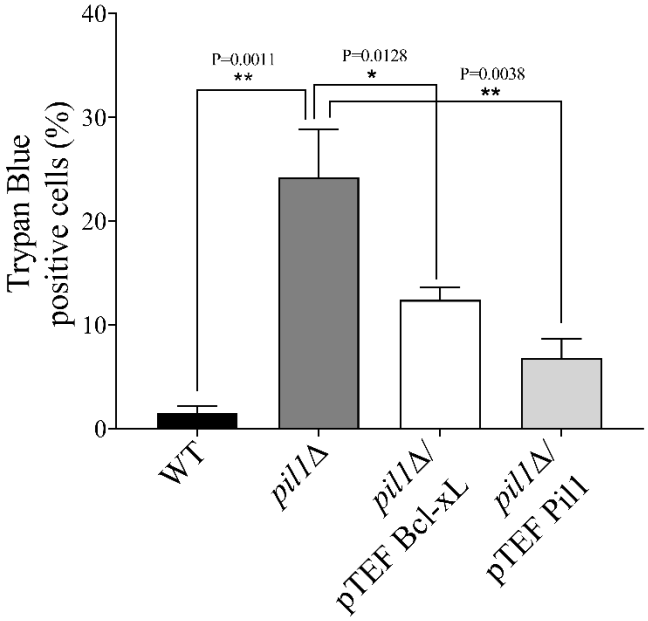


Figure 5.5 Pil1 regulates cell death.

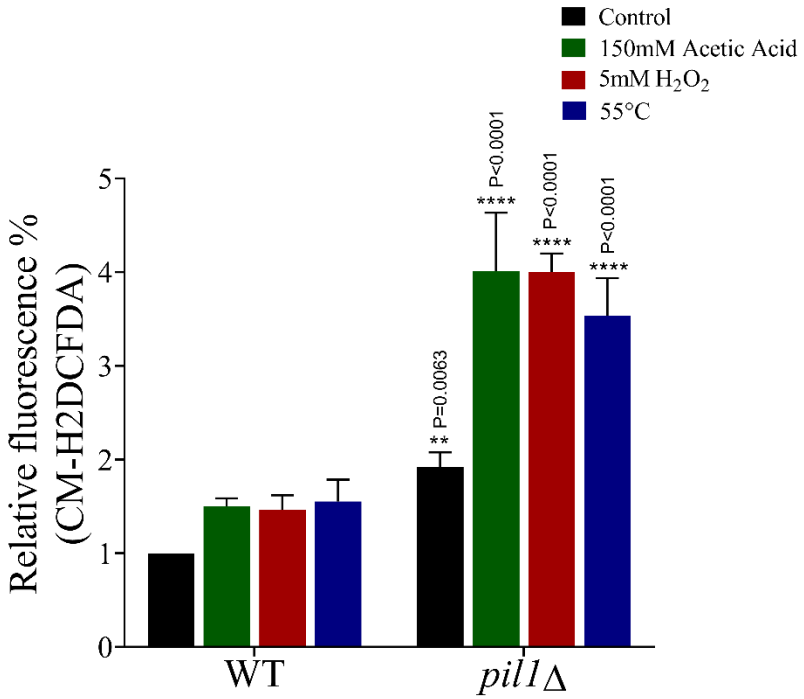
A and B. Indicated strains were grown till log phase in SMD (in A) or SCD (in D), OD_{600} 0.5/mL, serially diluted and then spotted onto agar plates. Plates were incubated at 30°C for 2 days and colonies were counted. C. Viability was assessed by Annexin V/PI co-staining. Annexin V+/PI+ yeast cells referred to as late apoptotic or early necrotic cells were counted and presented as mean ± SD for approximately 100 cells per sample in two biological replicates. Scale bar represents 10 µm. D. Cell viability assay with Trypan Blue. Indicated cells were grown till log phase in SMD media and 10 µL cells were mixed with 10 µL 0.4 % Trypan Blue solution and number of cells trypan blue positive cells were counted. DIC: Differential Interference Contrast. Data are presented as mean ± SD of 3 independent assays. Student's t-test was used to calculate p-values (as indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05.

5.3.e. Absence of Pil1 induces cell death under stress conditions

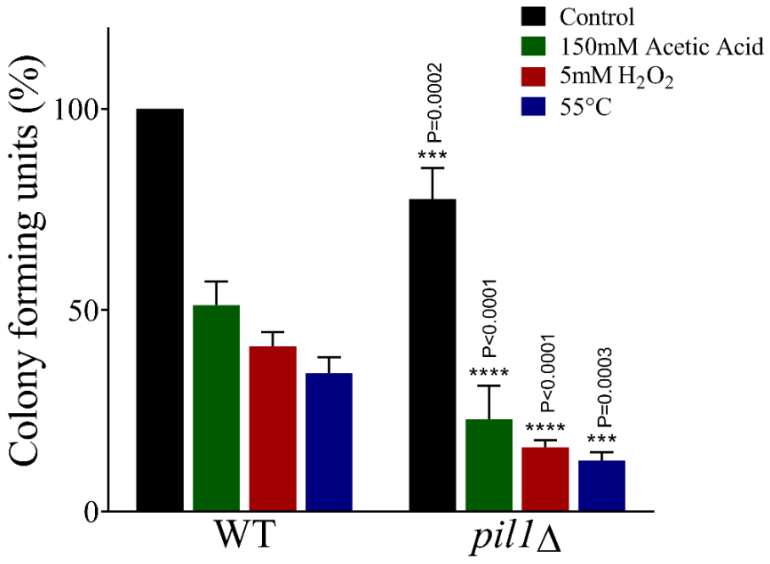
So far we have seen that absence of Pil1 leads to accumulated ROS and more cell death in normal condition, without any stress. So we wanted to see what happens when we induce cell death by different stress stimuli. In yeast, there are specific stress conditions like acetic acid, H_2O_2 and heat shock that are used to induce cell death. We used 150 mM acetic acid, 5 mM H_2O_2 for 1 hr and 55°C heat shock for 15 min. Compared to ROS levels and the cell death rate in *pil1*Δ without stress, we saw a much dramatic increase in ROS (Figure 5.6A) as well as cell death (Figure 5.6B) after exposing them to the stress conditions. This shows that Pil1 regulates cell death in both uninduced and stress-induced conditions.

As mitochondria is the central player in the regulation of cell death, we have checked the fate of mitochondria in *pil1*Δ after treating the cells with acetic acid (100 mM) for different time points, and monitored the levels of mitochondrial proteins Cox4 and Porin (Figure 5.6C). In WT, the levels decrease whereas in *pil1*Δ, the mitochondrial proteins are accumulated. The reason behind this could be due to more fused mitochondria and decreased mitophagy which protects mitochondria from degradation and hence causes the accumulation of bad mitochondria when stress is induced, which is harmful for the cell.

A.



B.



C.

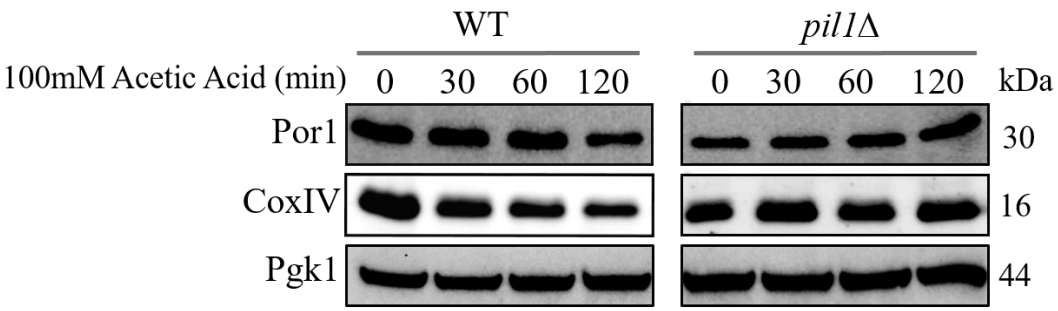


Figure 5.6 Pil1 regulates stress induced cell death.

A. Measurement of ROS. Indicated strains were grown to log phase in YPD media and exposed to indicated stress conditions. 1 OD cells were collected and treated with 10 mM CM-H2DCFDA for 30 min at 30°C . Imaging by fluorescence microscopy was performed as described in Materials and Methods. Quantification represents the percentage of DCF-positive fluorescing cells, where a minimum of 100 cells for each strain were counted. B. Indicated strains were grown till log phase in YPD, induced to stress conditions. Cells were normalized to OD 0.5/mL, serially diluted and then spotted onto agar plates. Plates were incubated at 30°C for 2 days and colonies were counted. Data are presented as mean ± SD of 3 independent assays. Student's t-test was used to calculate p-values (as indicated on the graph) ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. C. WT and *pil1*Δ cells were grown in YPD till log phase and then treated with 100 mM acetic acid for indicated time points. Cells were lysed, subjected to immunoblotting using antibodies against Por1, CoxIV, and Pgk1.

5.4 CONCLUSION

In this chapter, we found absence of Pil1 leads to accumulation of ROS and protein aggregates which inturn leads to increased chronological ageing. Additionally, we found that accumulation of ROS and protein aggregates could lead to increased cell death which can be rescued by overexpressing human anti-apoptotic protein Bcl-xL. Pil1 not only regulates cell death in normal condition, but also in stress-induced conditions.

CHAPTER 6

Discussion

Mitochondrial homeostasis is known to be maintained in the cell with the help of various other organelles. Several studies have shown that mitochondrial dynamics and morphology is known to be sustained through communication with other cellular structures like endoplasmic reticulum (Friedman, Lackner et al. 2011), lysosomes (Wong, Ysselstein et al. 2018), golgi apparatus (Nagashima, Tabara et al. 2020) and actin cytoskeleton (Korobova, Ramabhadran et al. 2013, Ji, Hatch et al. 2015, Li, Xu et al. 2015, Manor, Bartholomew et al. 2015, Lee, Westrate et al. 2016, Moore, Wong et al. 2016, Chen, Cheng et al. 2019). Mitochondrial fission in yeast is known to be executed by four major proteins- Dnm1, Fis1, Mdv1, and Caf4. Other accessory components that have also been shown to be involved in mitochondrial fission (Cerveny, Studer et al. 2007, Hammermeister, Schodel et al. 2010). Given the complexity of the process, there is no denying the fact that unknown factors in the mitochondrial fission machinery are yet to be explored. Recent advances in the study of eisosome functions show that Pil1, a major eisosome complex protein, might be associated with cellular stress responses. There is also a possibility that Pil1 is dynamic in nature and does not remain confined to eisosomes (Lacy, Baddeley et al. 2017). In this study, we have shown the evidence that Pil1 is present on mitochondrial outer membrane and maintains the mitochondrial shape. We have observed a drastic modification in mitochondrial morphology in both Pil1-deletion and Pil1-overexpression. *pil1*Δ cells have abnormally fused or spherical or, aggregated mitochondria, while cells with Pil1 overexpression have fragmented mitochondria. However, change in mitochondrial morphology in Pil1 overexpression and deletion cannot be caused in the absence of Fis1 or Dnm1 which strengthens the fact that both these proteins are the major player in mitochondrial fission.

It is clear from emerging evidences that mitochondrial dynamics and mitophagy are integrated processes that ensure the quality control of mitochondria. However, the role of canonical fission machinery in mitophagy still remains ambiguous. Though mitochondrial dynamics was shown to modulate mitophagy in some previous reports (Abeliovich, Zarei et al. 2013, Mao, Wang et al. 2013, Bernhardt, Muller et al. 2015), there are studies which show that mitophagy could be independent of these proteins (Mendl, Occhipinti et al. 2011, Graef 2016). According to our data, *fis1*Δ is defective in mitophagy while *dnm1*Δ is not, which contrasts with some of the previous findings. Though the exact reason behind these discrepancies are not known, there is a possibility that the change in phenotype that we observed could be due to differential expression of suppressors in the yeast deletion strains (Hawthorne and Leupold 1974). Another possibility that we speculate is that the role of Fis1 and Dnm1 in mitophagy could be growth-phase dependent,

hence there is an inconsistency in the phenotype in different studies. We found that absence of Pil1 reduces mitophagy significantly and overexpression enhances it irrespective of the presence or absence of Fis1/Dnm1. Our results also show that overexpression of Pil1 complements the defective mitophagy in *fis1*Δ cells. This study clearly shows that Pil1 regulates mitophagy pathway independent of Fis1 and Dnm1.

Mitochondrial dysfunction and defective autophagy are responsible for various irregularities in cell, mainly the accumulation of ROS and protein aggregates. Absence of Pil1 leads to accumulation of ROS and protein aggregates which are majorly cytosolic. However, we cannot completely rule out the possibility that mitochondria could be contributing to the elevated ROS levels that we observed in *pil1*Δ because the link between ROS and mitochondrial dysfunctions is a rather complex phenomenon. Mitochondrial dysfunctions need not necessarily increase ROS in the organelle itself but can trigger the ROS production in other organelles (Leadsham, Sanders et al. 2013, Murphy 2013). ROS release from mitochondria in order to maintain its health has also been reported (Zorov, Juhaszova et al. 2014). Though Pil1 interacts with Fis1 and Dnm1, the change in mitochondrial morphology caused by Pil1 are not seen in the absence of these proteins. On the other hand, Pil1 induces mitophagy independent of Fis1 and Dnm1. Also, there are irregularities in the shape of mitochondria when Pil1 is absent. It could be possible that aberrant mitophagy/autophagy leads to increased levels of cellular ROS and altered mitochondrial morphology.

More than nearly two decades ago, *Saccharomyces cerevisiae* was shown to undergo apoptosis which opened the possibility to study this mode of cell death in a model organism that has a combined technical and logistic simplicity (Madeo, Frohlich et al. 1997, Madeo, Frohlich et al. 1999). Our data show that cells undergo necrotic cell death in the absence of Pil1, even without any death stimuli which can be attributed to accumulated cellular ROS and protein aggregates. Also, the cell death pathway induced in the absence of Pil1 is clearly not dependent on already reported Fis1-mediated cell survival or Dnm1-mediated programmed cell death.

Several studies have established a functional link between mitochondrial fission, mitophagy, and cell death. However there is a lack of sufficient study regarding the regulation of these processes, and our study depicts that Pil1 could be a potential regulator of these processes. Though in our study we have reported the novel functions of eisosome protein Pil1 in mitophagy and cell death, further studies are required to explore how eisosome components interact with other cellular

structures.

Limitations of the study

We show that Pil1 localizes to mitochondria, but we do not know if it is consistently present in mitochondria or it is triggered to change its localization under specific conditions. Apart from Pil1, we have found that Lsp1 also localizes to mitochondria (data not part of the thesis), but whether other components of eisosome are also present in mitochondria has not been studied in this work. We found that Pil1 regulates mitochondrial dynamics, mitophagy, and cell death, but we could not decipher the mechanisms behind these processes. Pil1 interacts with mitophagy protein Atg32 and mitochondrial fission protein Fis1 and Dnm1, but how it regulates these proteins is not clear from our study. In our study we show that role of Pil1 in maintaining mitochondrial shape is dependent on Fis1 and Dnm1, but its role in mitophagy is independent of both the proteins, but we lack the understanding of how Pil1 distinctly regulates mitochondrial dynamics and mitophagy.

Significance of the study

Eisosomes are present only in a few fungal species and Pil1 is obviously not conserved beyond fungal kingdom. However, its ability to bind $PI_{4,5}P_2$ and promote membrane curvature are in common with those of mammalian BAR-domain family proteins (Zimmerberg and McLaughlin 2004, Olivera-Couto, Grana et al. 2011). Interestingly, recent studies have suggested potential similarities between MCC/eisosome domains and caveolae, a type of membrane invagination present in mammalian cells (Moreira, Schuck et al. 2012, Kabeche, Howard et al. 2015). Role of BAR-domain proteins in modulating mitochondrial membrane dynamics is largely unknown. Presence of BAR-domain protein Pil1 on mitochondrial outer membrane and its potential role in mitophagy and maintaining mitochondrial shape opens up the possibility that counterpart of Pil1 is present in mammals with functional similarities.

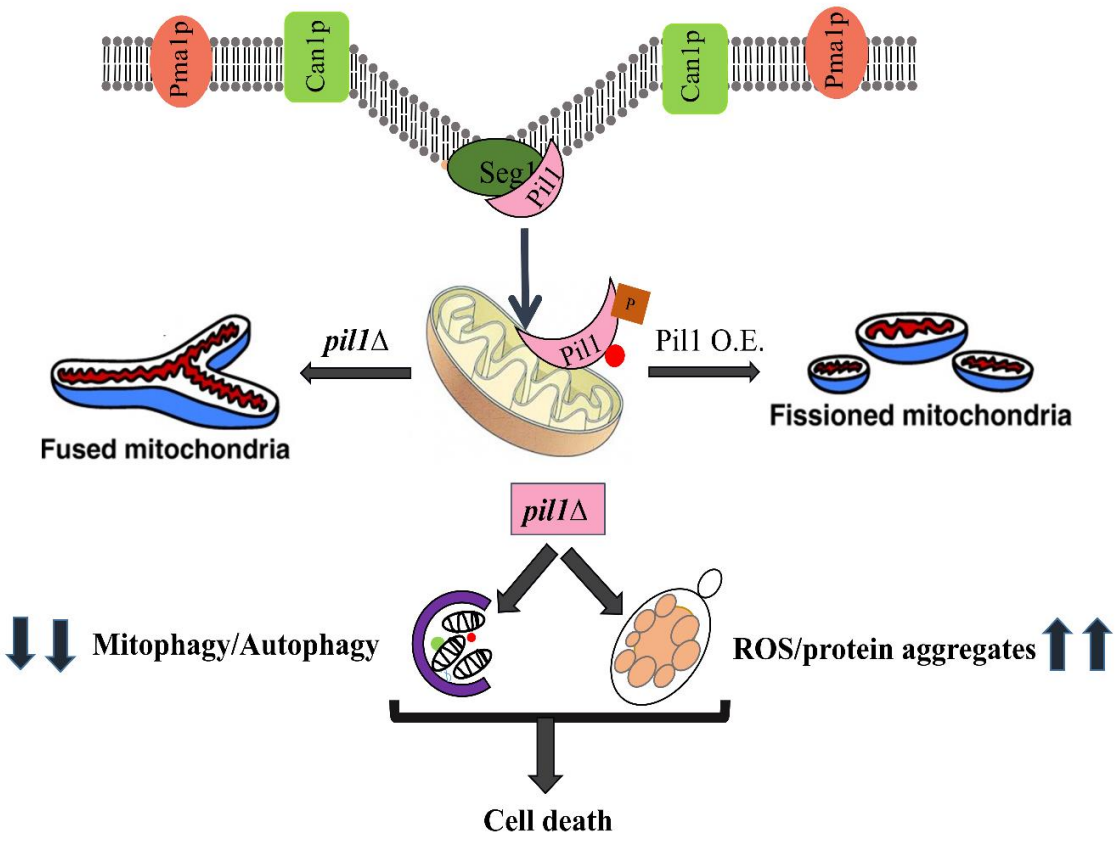


Figure 6.1 Summary model of the work

Pil1, a core component of eisosome complex on the plasma membrane can localize to mitochondria under uknown conditions. It can modulate mitochondrial dynamics. Absence of Pil1 leads to enhanced mitophagy/autophagy and accumulation of ROS & protein aggregates leading to increased cell death.

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Mitochondrial morphology and dynamics maintain mitochondrial integrity by regulating its size, shape, distribution, and connectivity, thereby modulating various cellular processes. Several studies have established a functional link between mitochondrial dynamics, mitophagy, and cell death, but further investigation is needed to identify specific proteins involved in mitochondrial dynamics. Any alteration in the integrity of mitochondria has severe ramifications that include disorders like cancer and neurodegeneration. In this study, we used budding yeast as a model organism and found that Pil1, the major component of the eisosome complex, also localizes to the periphery of mitochondria. Interestingly, the absence of Pil1 causes the branched tubular morphology of mitochondria to be abnormally fused or aggregated, whereas its overexpression leads to mitochondrial fragmentation. Most importantly, *pil1*Δ cells are defective in mitophagy and bulk autophagy, resulting in elevated levels of reactive oxygen species and protein aggregates. In addition, we show that *pil1*Δ cells are more prone to cell death. Yeast two-hybrid analysis and co-immunoprecipitations show the interaction of Pil1 with two major proteins in mitochondrial fission, Fis1 and Dnm1. Additionally, our data suggest that the role of Pil1 in maintaining mitochondrial shape is dependent on Fis1 and Dnm1, but it functions independently in mitophagy and cell death pathways. Together, our data suggest that Pil1, an eisosome protein, is a novel regulator of mitochondrial morphology, mitophagy, and cell death.

Organelles have evolved a variety of stress response processes to maintain their proteostasis and cellular homeostasis. Mitochondrion, one of the essential organelles that carry out several important cellular processes, ensures its quality control through various pathways at the molecular, organellar, and cellular levels (1). Mitochondrial fission and fusion, generally referred to as mitochondrial dynamics, maintain mitochondrial integrity by regulating its size, shape, distribution, and connectivity. Mitochondrial dynamics regulate mitochondrial quality control, metabolism, apoptosis, mitophagy, and other essential processes. Fusion of mitochondria is required to mitigate the damage and nonfunctionality by mixing components and protecting them from autophagic degradation during starvation (2, 3). Fission helps produce new mitochondria and ensures quality control by removing damaged or unwanted mitochondria through mitophagy (4). A coordinated balance of fission and fusion is critical for maintaining mitochondrial biology and therefore a plethora of important cellular processes (5, 6). In *Saccharomyces cerevisiae*, Fzo1, Mgm1, and Ugo1 facilitate mitochondrial fusion. Outer membrane–anchored proteins, Fzo1p and Ugo1p, carry out the outer membrane fusion of adjacent mitochondria and inner membrane–anchored Mgm1 forms transcomplexes to tether the apposing inner membranes together (7–9). Dnm1, a dynamin-related GTPase, is the major protein in mitochondrial fission (10, 11). It is predominantly present in the cytosol and recruited to mitochondria *via* Fis1 (12). Dnm1 assembles into oligomers which form rings and spirals at the outer membrane of mitochondria. Recruitment of Dnm1 to the mitochondrial surface is mediated through two adaptor proteins, Mdv1 and Caf4 (13, 14). These four proteins together constitute the core proteins of the mitochondrial fission machinery. However, there is a possibility that other unidentified factors still exist that take part in mitochondrial dynamics (15). Several studies have shown that any dysregulation in mitochondrial dynamics leads to neuronal disorders like Alzheimer's, Parkinson's, and Huntington's (16–18). When the damage is beyond repair, mitochondria undergo mitophagy. Mitophagy is a selective autophagy where autophagosomes engulf the entire mitochondria and deliver them to the vacuole for their degradation (19–21). Mitophagy is required to eliminate the bad mitochondria from cells. Any kind of aberration in mitochondrial dynamics or mitophagy is harmful to cells. Mitochondrial dysfunction has also been linked with protein aggregation and reactive oxygen species (ROS) generation in cells which in turn leads to cell death.

Cellular organelles communicate with each other in order to cope with stress. Since the plasma membrane is positioned at the frontline to combat the external stress stimuli, it requires high degree of organization as it carries out a diverse array of functions and forms the protective barrier around the cell. The fungal plasma membrane is organized in lateral domains with specialized functions like cell wall synthesis, environmental sensing, nutrient uptake, secretion, and endocytosis (22–24). High-resolution electron microscopy of freeze-etched

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Role of Eisosome Core Component Pil1 in Mitochondrial Dynamics, Mitophagy and Cell Death

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