# Studies on genes that influence NADPH homeostatic levels in *Saccharomyces cerevisiae*

Archi Sharma

*A dissertation submitted for the partial fulfilment of*
*BS-MS dual degree in Science*


**Indian Institute of Science Education and Research (IISER) Mohali**
April 2019

# Certificate of Examination

This is to certify that the dissertation titled "**Studies on genes that influence NADPH homeostatic levels in *Saccharomyces cerevisiae*"** submitted by Archi Sharma (MS14157) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rachna Chaba Dr. Santosh B. Satbhai Prof. Anand K Bachhawat
(Supervisor)

Date: April 26, 2019

# Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Anand Kumar Bachhawat at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Archi Sharma

Dated: April 26, 2019

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand K Bachhawat
(Supervisor)

# Acknowledgement

Firstly, I would like to express my sincere gratitude to Prof. Anand Kumar Bachhawat for providing me the opportunity to be a part of his lab for summer internships as well as for MS-thesis project. I am extremely grateful to him for being an amazing supervisor and for his continuous guidance and motivation. Being a part of his lab has been a wonderful experience for me that helped me to explore and learn many things. I am also thankful to him for giving me the opportunity to work under the guidance of Shambhu Yadav. He has continuously helped me in all my experiments and all the techniques that I have learnt so far were taught by him. He has been very understanding and supportive and along with being a mentor, he was a great friend to me who helped me in tough times. Along with him, Muskan Bhatia has also been a wonderful friend to me and I want to thank her with all the help and support.

I would also like to thank my friends: Avneet, Swati, Anupreet, Varun, Akanksha and Ashwin. Without them, this journey of 5 years would have never been possible. They have always been there for me and I would not have been able to overcome my problems without them.

I would like to thank all lab members of AKB lab as well - Shradha Suyal, Sri Harsha,Prarthna Saraswat, Pratiksha and Banani Chattopadhyay for their help, advice and a good lab atmosphere. I would also like to thank Vidya for helping me and keeping the lab organized.

Lastly, I would like to express my gratitude to God for his blessings and giving me the best family. I have no words to describe the importance of my parents and my sister Prachi in my life. They have always been and will always be the best part of my life.

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# Abstract

The role of secondary redox buffers is masked by the presence of the primary redox buffer (Glutathione) and the genes which can alter the levels of secondary redox buffers are not entirely known. In order to understand the role of NADPH which is a secondary redox buffer, a genetic screen was developed in the lab which can be used to detect changes in NADPH levels. Using this screen, knockout (deletion) and multicopy library approaches were used to identify novel genes that can alter NADPH levels. Through multicopy library approach, ATX2 (antioxidant) was identified that partially suppressed the growth defects of *gsh1*Δ. RTT01 (regulator of Ty1 transposition) gene was also found as a candidate gene which further needs to be confirmed. Through deletion studies, many genes were identified and one among those was FMP40 (Found in Mitochondrial Proteome) which was a putative protein of unknown function (although now, its function has been delineated). In the previous studies on FMP40 in the lab, it was observed that *fmp40*Δ was able to partially suppress the growth defects of cells having deletion of genes involved in glutathione biosynthesis (GSH1: gamma glutamylcysteine synthetase), mitochondrial NADPH generation (POS5: peroxide Sensitive) and iron-sulphur clusters transport (GRX5: glutaredoxin) in mitochondria. To get the further insights of FMP40 function, experiments were performed to confirm the localization, observe the effects of oxidative stress and to determine the potential substrates and interactors. All of these strengthened the hypothesis that FMP40 acts as a consumer of mitochondrial NAPDH which fits well with the delineated function of FMP40 and direct interactions of FMP40 with GRX2, GRX5 and TRX3 are being investigated.

# Chapter 1: Introduction

## 1.1 Glutathione ( Primary redox buffer)

Glutathione (*γ-L-glutamyl-L-cysteinyl-glycine*) is a tripeptide comprising of cysteine, glycine and glutamate which is the most abundant thiol (-SH) containing organic compound synthesized in the cells[1].It is found primarily in almost all eukaryotes and in some gram negative aerobic prokaryotes.

The biosynthesis of glutathione involves two consecutive enzymatic reactions. The first enzymatic reaction involves addition of cysteine to glutamate which is carried out by gamma glutamylcysteine synthetase (GSH1) which acts as the rate limiting enzyme. The next step involves the addition of glycine to cysteine and glutamate which is carried out by glutathione synthetase (GSH2). Both the steps require energy for which ATP is utilized by the cells[2].

Step1: L-γ-glutamate + L-cysteine +ADP $\xrightarrow{\text{GSH1}}$ L-γ-glutamyl-L-cysteine +ATP

Step2: L-γ-glutamyl-L-cysteine + glycine + ATP $\xrightarrow{\text{GSH2}}$ Glutathione + ADP

### 1.1.1 Importance of Glutathione

Glutathione plays various important roles in the cell due to its low redox potential, high reactivity and unique physiological and chemical stability. The main functions of glutathione involve protection against oxidative stress caused by reactive oxygen species (ROS), detoxification of toxic metabolites and xenobiotics, Fe-S protein maturation in mitochondria, protein synthesis, amino acid transport[3] and response to sulphur and nitrogen starvation [1].

### 1.1.2 Role of Glutathione in redox homeostasis

The high concentration of glutathione (1-10 mM) and low redox potential (($E'_o$= - 240mV for thiol disulfide exchange) makes the glutathione a primary redox buffer which plays a critical role in redox homeostasis. Glutathione exists in reduced form (GSH) and oxidized form (GSSG) wherein 2 molecules of glutathione form a disulphide bond and the ratio of these oxidized and reduced forms (GSSG/2GSH) plays an important role in maintaining the redox homeostasis. Glutathione reductase (GLR1) is a major player that maintains this ratio by utilizing NADPH to reduce GSSG to GSH. The cells can also sense the accumulation of GSSG and up-regulate synthesis of GSH to maintain the redox homeostasis[4].

### 1.1.3 Glutathione degradation

Along with the glutathione biosynthesis, the cell also has mechanisms to degrade glutathione which also helps in maintaining the redox homeostasis[5]. There are three pathways known to degrade glutathione. The first pathway involves the degradation of glutathione to glutamate, cysteine and glycine by Ecm38p (extracellular mutant: γ-glutamyl transpeptidase) which is a vacuolar membrane bound enzyme. The second pathway involves degradation of glutathione by GSH degradosomal protein complex comprised of Dug1p, Dug2p and Dug3p (deficient in utilization of glutathione)[6]. The third pathway involves degradation of glutathione by cytoplasmic ChaC-family of enzymes that hydrolysis glutathione to produce 5-oxyproline and cysteinyl glycine. The 5-oxyproline is further cleaved to obtain glutamate and cysteinyl glycine is cleaved to obtain cysteine and glycine [2].

## 1.2 Secondary redox buffers

Along with glutathione which acts as a primary redox buffer, the cells also contain other redox buffers such as thioredoxin, $NADP^+$/NADPH and $NAD^+$/NADH which are present at far lower concentrations (in µM concentrations) as compared to glutathione (in mM concentrations) and acts as secondary redox buffers[7], [8].

### 1.2.1 Thioredoxin

Thioredoxin (Trx) is a protein disulphide reductase which consists of oxidized and reduced thioredoxin ($Trx_{oxd}$/$Trx_{red}$) like glutathione and the equilibrium between the oxidized and reduced thioredoxin is important to maintain the redox homeostasis. The cells utilize thioredoxin reductase (TrxR) which utilizes NADPH as a reducing equivalent for maintaining the ratio of $Trx_{oxd}$/$Trx_{red.}$Thioredoxin also plays various roles in cells such as antioxidant defence, regulation of transcription factors and DNA synthesis & repair[7].

### 1.2.2 Redox couples: $NADP^+$/NADPH and $NAD^+$/NADH

$NADP^+$/NADPH and $NAD^+$/NADH act as secondary redox couples because of their presence in micromolar (µM) concentrations and play distinct roles in signalling and act as cofactors as well as reducing equivalents. The ratio of $NADP^+$/NADPH is kept low as NADPH is utilized as a reducing agent whereas the ratio of $NAD^+$/NADH is kept high because $NAD^+$ is utilized as the oxidizing agent. The maintenance of this ratio is important for the proper functioning of cells and redox homeostasis[8].

## 1.3 Importance of NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH) is a ubiquitous cofactor which is present in all the living organisms and plays a crucial role in the regeneration of reduced glutathione and thioredoxins from their oxidized forms. Along with this, it acts as a reducing equivalent in many biosynthetic reactions and can act as a rate-determining factor in the formation of metabolic products. It also plays a role in macromolecular biosynthesis, detoxification of drugs and superoxide and nitric oxide production[8], [9].

### 1.3.1 Role of ZWF1 in NADPH production in yeast

ZWF1 (zwischenferment: glucose-6-phosphate dehydrogenase) plays a major role in the production in cytosolic NADPH in yeast. This gene in involved in pentose phosphate

pathway and converts glucose-6-phosphate to 6-phosphogluconate with the simultaneous conversion of $NADP^+$ to NADPH. During oxidative stress, the metabolic flux is shifted from glycolysis to pentose phosphate pathway which leads to more NADPH generation and protect the cells from oxidative stress[10].

### 1.3.2 Role of POS5 in NADPH production in yeast

POS5 (peroxide Sensitive) is a NAD kinase present in mitochondria which either converts $NAD^+$ to $NADP^+$ or NADH to NADPH thus acting as a main source of mitochondrial NADPH. Deletion of Pos5 (*pos5*Δ) leads to decrease in the levels of mitochondrial NADPH which affects the growth and functioning of the cells[11].

### 1.3.3 Role of NADPH and GRX5 in the transport of Fe-S clusters

The synthesis of Fe-S clusters is a multi-step process that takes place in mitochondria. It is initiated by Isu1 which acts as a scaffold protein and involves utilization of NADPH as a reducing equivalent. The synthesis is accompanied by the transport of Fe-S clusters which involves glutathione and GRX5 (glutaredoxin). *grx5*Δ leads to the accumulation of Fe-S clusters in mitochondria and causes oxidative stress in the cells[3].

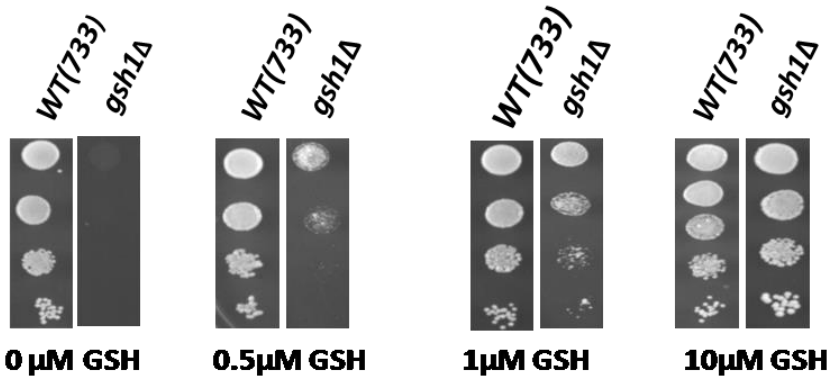
## 1.4 Identification of genes affecting NADPH levels

The role of NADPH is masked by the presence of glutathione since glutathione is present in millimolar (mM) concentrations and NADPH is present in micromolar (µM) concentrations[1], [8]. In order to identify changes in NADPH levels and genes affecting NADPH levels, a screen was developed which involved the deliberate lowering of intracellular glutathione levels to understand the role of NADPH and genes which might affect NADPH levels were identified.

### 1.4.1 Development and validation of screen

In order to unmask the role of NADPH, yeast cells having *gsh1*Δwere considered which leads to glutathione auxotrophy (cells were not able to survive without glutathione) and

different concentrations of glutathione were provided from outside. Very low glutathione concentrations (0.5µM and 1µM) were considered to detect changes in NADPH levels at which the role of NADPH becomes significant.




*Yadav et al, Unpublished*

**Fig.1.1 Growth of WT and *gsh1*Δ on different glutathione concentrations.** ABC733 yeast strain (WT) and ABC1195 yeast strain having *gsh1::kanMX* were grown on minimal media, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

In order to verify that the changes in NADPH levels could be detected at lower glutathione concentrations, deletion and over-expression of ZWF1 gene was carried out which is known to affect NADPH levels.

*zwf1*Δ leads to decrement in the levels of NADPH and ZWF1 over-expression leads to increment in the levels of NADPH which could be recognized at lower glutathione concentrations using the screen. This suggested that indeed, the genetic screen can be utilized to identify changes in NADPH levels.

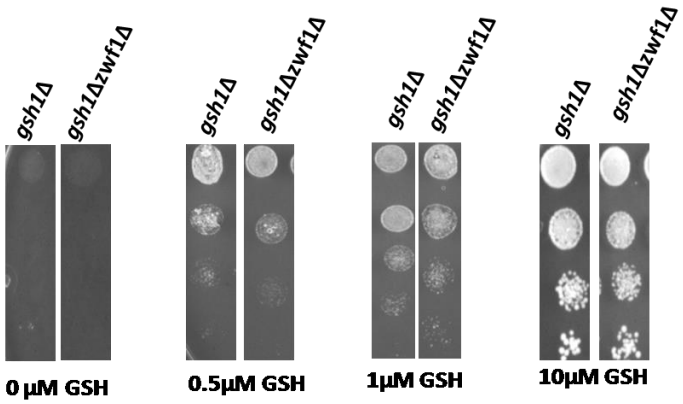



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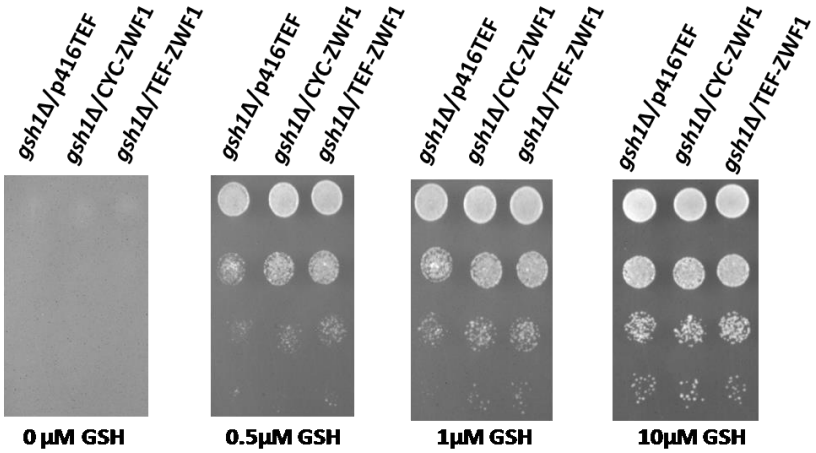
**Fig.1.2 Growth of *gsh1*Δ and *gsh1Δzwf1*Δ on different glutathione concentrations.** ABC5525 yeast strain having *gsh1::*LEU2 and ABC5189 yeast strain having *zwf1::*kanMX4 *gsh1::*LEU2 were grown on minimal media lacking leucine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking leucine and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.



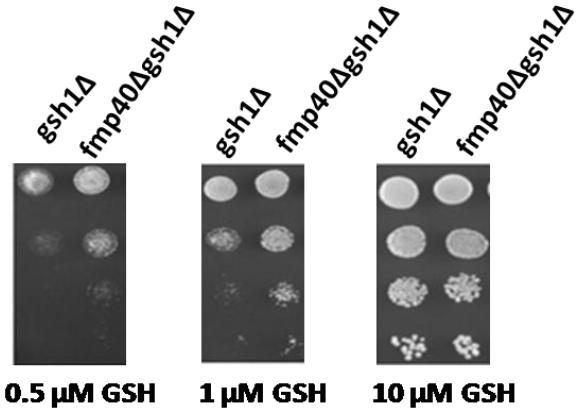

*Yadav el al, Unpublished*

**Fig.1.3. Growth of *gsh1Δ* on different glutathione concentrations transformed with ZWF1 and empty vector (p416TEF).** p416TEF, CYC-ZWF1 and TEF-ZWF1 plasmids were transformed in ABC5525 yeast strain having *gsh1::*LEU2 and were grown on minimal media lacking leucine and uracil, harvested, washed and resuspended in water

and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking leucine and uracil and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

### 1.4.2 Knockout (deletion) approach to identify novel genes

Approximately, 400 mitochondrial associated genes were deleted in *gsh1*Δ background to identify genes whose deletion can partially suppress the growth defects of *gsh1*Δ at low glutathione concentrations. 48 genes were identified that gave the desired phenotype and one among those was FMP40 (Found in Mitochondrial Proteome). This gave an idea that FMP40 might act as a consumer of NADPH and *fmp40*Δ might lead to enhancement in the levels of NADPH because of which *fmp40*Δ was able to suppress the growth defects of *gsh1*Δ.

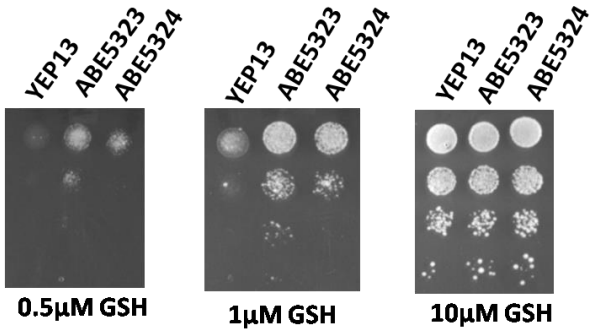



*Yadav et al, Unpublished*

**Fig.1.4 Growth of *gsh1*Δ and *fmp40Δgsh1Δ* on different glutathione (GSH) concentrations.** ABC5525 yeast strain having *gsh1::*LEU2 and ABC4555 having *fmp40::*kanMX4 *gsh1::*LEU2 were grown on minimal media lacking leucine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking leucine and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

### 1.4.3 Multicopy library approach to identify novel genes

Plasmids containing fragments from yeast multicopy library cloned in YEP13 (Yeast episomal vector) were transformed in *gsh1*Δ background to identify the genes which in multicopies can partially suppress the growth defects of *gsh1*Δ.Plasmids containing two fragments named as ABE5323 and ABE5324 gave the desired phenotype.



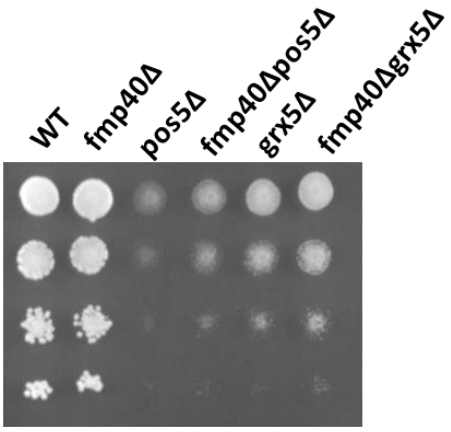

*Yadav et al, Unpublished*

**Fig.1.5 Growth of *gsh1Δ* on different glutathione concentrations transformed with YEP13 vector and plasmids bearing fragments of yeast multicopy library (ABE5323 and ABE5324).**YEP13 vector and plasmids bearing fragments of yeast multicopy library cloned in YEP13 vector (ABE5323 & ABE5324) were transformed in ABC1195yeast strain having *gsh1::kanMX4*andwere grown on minimal media lacking leucine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking leucine and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

## 1.5 *Saccharomyces cerevisiae* FMP40

When studies on FMP40 were initiated in the lab, it was found to be a putative protein of unknown function shown to be present in mitochondria through high-throughput studies (although now, its function has been delineated[12]). It was also known that the homologue of FMP40 is Selenoprotein O which is involved in redox homeostasis. Studies on FMP40 in the lab depicted that *fmp40*Δ was able to partially suppress the growth

defects of *pos5*Δ and *grx5*Δ.An increased growth was seen in *fmp40Δpos5Δ and fmp40Δgrx5Δ* as compared to *pos5*Δ and *grx5*Δstrengthening the hypothesis that FMP40 might act as a consumer of NADPH. (Tejasvinee Mody, MS Thesis: Deciphering the function of *Saccharomyces cervisiae* FMP40)




*Shambhu Yadav & Tejasvinee Mody, Unpublished*

**Fig.1.6 Growth pattern of WT, *pos5*Δ, *fmp40Δpos5Δ*, *grx5*Δ *and fmp40Δgrx5Δ*.** ABC733(WT), ABC4306 having *fmp40::kanMX4*, ABC5163 having *pos5::kanMX4*, ABC4837 having *grx5::kanMX4*, ABC5456 having *pos5::kanMX4fmp40::HIS3* and ABC5457 having *grx5::*kanMX4*fmp40::HIS3* were grown on minimal media, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium. The images were taken after 3 days of incubation at 30˚C.

### 1.5.1 Current understanding of FMP40 function

During the course of this work, the function of FMP40 was described in a detailed work[12]. FMP40 was shown in this study to be a pseudo-kinase which carries out AMPylation of various proteins to protect the cells from oxidative stress. Under oxidative stress, it utilizes NADPH for the reduction of cysteines and breaking of disulfide bonds. In its reduced state, it releases 2 molecules of phosphate to convert the bound ATP to AMP and undergoes auto- AMPylation. After its auto-AMPylation, it transfers the AMP to carry out AMPylation of various proteins. One such protein that is AMPylated in *E.Coli* is GRX A.

GRX A is involved in the deglutathionylation of proteins by breaking the disulphide bond formed between the cysteine moieties of glutathione and protein. When GRX A gets AMPylated, it is not able to carry out deglutathionylation and the proteins remain glutathionylated which protects them from oxidative stress[12].

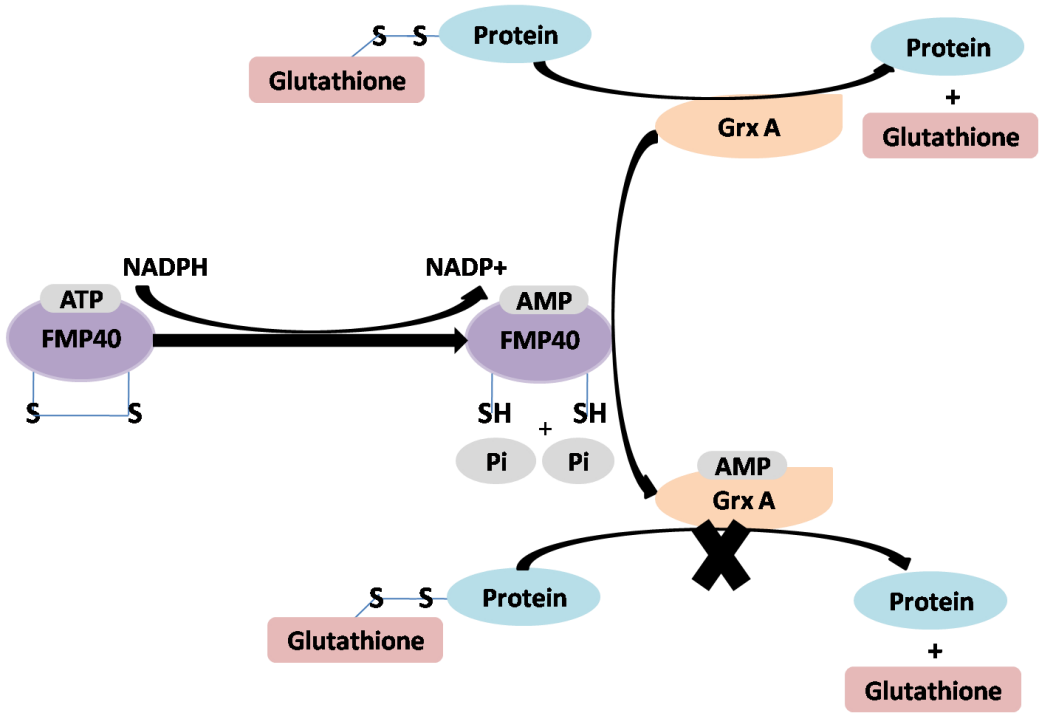



**Fig.1.7 Working mechanism of FMP40**

## 1.6 Sensors for the measurement of NADPH levels

iNAP1 (indicators of NADPH levels) and iNAPc (control) are the genetically encoded fluorescent sensors which can be used for the *in vivo* measurements of NADPH levels.

**iNAP1**: iNAP1 is the chimera of circularly permuted YFP (cpYFP) and NAD(H) binding domain of Rex protein from *Thermus aquaticus* which has been mutated to specifically bind to NADPH. It has two excitation peaks around 420 nm and 500 nm and one emission peak near 515-550 nm. Upon NADPH binding, it shows a 3.5-fold increase and 2.5-fold decrease in fluorescence excited at 420 nm and 485 nm respectively.

**iNAPc** : iNAPc is the mutated iNAP1 having no affinity for NADPH or any other nucleotide which acts as a control[9].

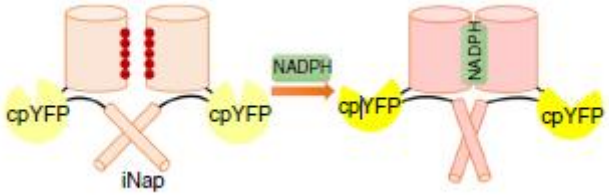



**Fig.1.8 Binding of NADPH to iNAP1 leads to enhancement in fluorescence**[9]

## 1.7 Objectives

(a) To identify the precise genes from the plasmids bearing fragments from yeast multicopy library(ABE5323 & ABE5324)responsible for the suppression of growth defects of *gsh1*Δ

(b) To further investigate the function of FMP40

(i) To confirm the localization of FMP40 protein and to determine if it is exclusively mitochondrial

(ii) To investigate if FMP40 plays a role in oxidative stress (similar to Selenoprotein O) by providing internal oxidative stress (using different carbon – sources) and external oxidative stress (using different menadione concentrations)

(iii) To determine whether NADPH oxidation by FMP40 is linked to the reduction of any metabolic substrates

(iv) To determine the interactors of FMP40 by co-immunoprecipitation

(v) Complementation of *fmp40*Δ*pos5*Δ *and fmp40*Δ*grx5*Δ by transforming FMP40

(vi) Measurement of NADPH levels to confirm that FMP40 acts as NADPH dehydrogenase.

# Chapter 2: Materials and Methods

## SECTION A: Materials

### 2.1 Chemicals and Reagents

All chemicals used were obtained from commercial sources and were of analytical grade. Media components, chemicals and reagents were purchased from Sigma Aldrich (St. Louis, USA), HiMedia (Mumbai, India), Merck India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Snap Gene software and were purchased from Integrated DNA Technologies (IDT) and Sigma-Aldrich. Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP),*Vent* DNA polymerase, *Phusion* High-fidelity DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA ladder and protein molecular weight markers were purchased from New England Biolabs Inc. (Beverly, MA, USA) or Thermo Scientific. Gel-extraction kits and plasmid miniprep columns were obtained fromBioneerand Promega. GSH was obtained from Sigma-Aldrich, USA.PVDF membrane and Western Blotting Detection Reagents were purchased from Millipore(U.S.A.). c-Mycmouse monoclonal antibody, Porin/VDAC1 mouse polyclonal antibody, Act1 mouse polyclonal antibody, ATP6VIA mouse polyclonal antibody and anti-mouse HRP-linked antibody were bought from Abcam and Cell Signaling (Danvers, MA, USA).

### 2.2 Strains and Plasmids

*Escherichia coli* DH5α was used as the cloning host and BL21 (DE3) was used as the expression host. The genotype for *E. coli* and *Saccharomyces cerevisiae* strains used in this study are given in Table 2.1 and Table 2.2 respectively. The lists of various plasmids

used in this study and constructed for this study are given in Table 2.3 and Table 2.4 respectively.

**Table 2.1: List of bacterial strains used in the study**

| Strain | Genotype | Source |
|---|---|---|
| ABE 460 (DH5α) | F *-gyr A96(Nal) recA1 relA1 endA1 thi-1 hsdR17*($r_k^-$ $m_k^+$) *gln V44 deoR Δ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]* | Lab strain |
| ABE 849 (BL21(DE3)) | *F* $^-$*ompThsd*SB($r_B^-m_B^-$) gal dcm (DE3) | Lab strain |

**Table 2.2: List of yeast strains used in the study**

| Strain | Genotype | Source |
|---|---|---|
| ABC 733 (BY4741) | *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | Lab stock |
| ABC 5525 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gsh1::*LEU2 | Lab strain |
| ABC 4555 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fmp40::*kanMX4 *gsh1::LEU2* | Lab strain |
| ABC 4306 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fmp40::kanMX4* | Euroscarf |
| ABC 4837 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grx5::kanMX4* | Euroscarf |
| ABC 5163 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pos5::kanMX4* | Euroscarf |
| ABC 5165 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 zwf1::kanMX4* | Euroscarf |
| ABC 5410 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fmp40::HIS3;* | Lab strain |
| ABC 5455 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pos5::kanMX4 fmp40::HIS3* | Lab strain |
| ABC 5456 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | Lab strain |

| | | |
|---|---|---|
| | *pos5::kanMX4 fmp40::HIS3* | |
| ABC 5457 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grx5::kanMX4 fmp40::HIS3* | Lab strain |
| ABC 5458 | BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grx5::kanMX4 fmp40::HIS3* | Lab strain |

**Table 2.3: List of plasmids used in the study**

| **Plasmid name** | **Clone no.** | **Description** |
|---|---|---|
| p416TEF | ABE 443 | The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression and Amp$^r$marker for selection in *E.coli*. |
| pRS426 | ABE 618 | Yeast episomal vector with a URA3 marker and MCS derived from pBLUESCRIPT II along with Amp$^r$marker for selection in *E.coli.* |
| pRS316 | ABE 931 | *URA3* marker based yeast centromeric expression vector and Amp$^r$marker for selection in *E.coli.* |
| YEP13 | ABC 260 | *S. cerevisiae*/*E. coli* shuttle vector with a LEU marker derived from 2 micron plasmid B form along with Amp$^r$marker for selection in *E.coli.* |
| pET23a(+) | ABE 991 | This plasmid was procured from Novagen |
| pTEF-FMP40 | ABE 5355 | The plasmid contains FMP40 gene (PCR amplified from genomic DNA) at XbaI and XhoI sites of p416TEF vector; confirmed by restriction digestion and sequencing. |
| pNP-FMP40 | ABE 5356 | FMP40 gene along with 800bp upstream region(Native promoter) was PCR amplified from genomic DNA and cloned at XbaI and XhoI sites of pRS316 vector; confirmed by restriction digestion and sequencing. |
| pNP-FMP40 | ABE 5357 | FMP40 gene along with its 800bp upstream region was PCR amplified from genomic DNA and cloned at XbaI and XhoI sites of pRS316 vector; confirmed by restriction digestion and sequencing. |
| pET23a(+)-6X His-FMP40 | ABE 5369 | FMP40 gene with N-terminal 6X His tag cloned at NdeI and XhoI sites of pET23a(+). The clone was confirmed by |

| | | |
|---|---|---|
| | | restriction digestion and sequencing. |
| pET23a(+)-6X His-truncated FMP40 | ABE 5370 | Truncated FMP40 gene (lacking N-terminal 58 amino acid predicted mitochondrial pre-sequence) with N-terminal 6X His tag cloned at NdeI and XhoI sites of pET23a(+). The clone was confirmed by restriction digestion and sequencing. |

**Table 2.4: List of plasmids constructed and used in the study**

| Plasmid Name | Clone no. | Description |
|---|---|---|
| pNP-ATX2 | ABE 5629 | ATX2 gene along with 800bp upstream region (Native promoter) was PCR amplified from genomic DNA and cloned at XhoI and HindIII sites of pRS426 vector; confirmed by restriction digestion. |
| pNP-ATX2 | ABE 5630 | ATX2 gene along with 800bp upstream region (Native promoter) was PCR amplified from genomic DNA and cloned at XhoI and HindIII sites of pRS426 vector; confirmed by restriction digestion |
| pNP-UBX6 | ABE 5881 | UBX6 gene along with 800bp upstream region was PCR amplified from genomic DNA and cloned at XhoI and BamHI sites of pRS426 vector; confirmed by restriction digestion. |
| pNP-FMP40 FLAG | ABE 5850 | FMP40 gene along with 800bp upstream region and C-terminal FLAG tag cloned at XmaI and XbaI sites of pRS426 vector. The clone was confirmed by restriction digestion. |
| pNP-FMP40 c-Myc | ABE 5851 | FMP40 gene along with 800bp upstream region and C-terminal c-Myc tag cloned at XmaI and XbaI sites of pRS426 vector. The clone was confirmed by restriction digestion. |
| pTEF-FMP40 c-Myc | ABE 5882 | FMP40 gene with C-terminal c-Myc tag cloned at XbaI and XmaI sites of p416TEF vector. The clone was confirmed by restriction digestion and DNA sequencing. |
| ABE5323: Sph1 digested | ABE 5631 | ABE5323cloned in YEP13 vector was re-ligated afterdigestion by Sph1. Genes removed: RTT101 and AIM22; Genes left: MTR4, CHM7& UBX6 |

| | | |
|---|---|---|
| ABE5323:Sph1 digested | ABE 5632 | ABE5323cloned in YEP13 vector was re-ligated after digestion by Sph1.Genes removed: RTT101 and AIM22; Genes left: MTR4, CHM7& UBX6 |
| ABE5323:Nhe1 digested | ABE 5633 | ABE5323 cloned in YEP13 vector was re-ligated after digestion by Nhe1. Genes removed: MTR4, CHM7, UBX6; Genes left: AIM22 and partially RTT101 |
| ABE5323:Nhe1 digested | ABE 5634 | ABE5323 cloned in YEP13 vector was re-ligated after digestion by Nhe1. Genes removed: MTR4, CHM7, UBX6; Genes left: AIM22 and partially RTT101 |
| pTEF-iNAPc | ABE 5841 | iNAPc gene was digested from pcDNA 3.1 and subcloned in p416TEF vector using BamHI and HindIII |
| pTEF-iNAPc | ABE 5842 | iNAPc gene was digested from pcDNA 3.1 and subcloned in p416TEF vector using BamHI and HindIII |
| pTEF-iNAP1 | ABE 5843 | iNAP1 gene was digested from pcDNA 3.1 and subcloned in p416TEF vector using BamHI and HindIII |
| pTEF-iNAP1 | ABE 5844 | iNAP1 gene was digested from pcDNA 3.1 and subcloned in p416TEF vector using BamHI and HindIII |

### 2.3. Oligonucleotides (Primers)

The list of various oligonucleotides (primers) used in this study is given in Table 2.5.

**Table 2.5: List of Oligonucleotides and their sequences used in the study**

| **Oligonucleotide name** | **Oligonucleotide sequence (5' to 3')** |
|---|---|
| iNAP1_for | ATCCGGGATCCATGAACCGGAAGTGGGGCCTGTGC |
| iNAP1_rev | ATCTGGAATTCTTAAACGGGCCCTCTAGAAAGCTTGC |
| TRX3_FP_Nhe1 | ATCGCGCTAGCATGTTGTTCTATAAGCCTGTG |
| TRX3_RP_Xho1_HA | TCGCCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTATA GATCTTTGATTCCCTTC |
| GRX2_FP_Nhe1 | ATCGCGCTAGCATGGAGACCAATTTTTCCTTC |
| GRX2_RP_Xho1_HA | TCGCCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTATT GAAATACCGGCTTCAAT |
| GRX5_FP_Nhe1 | ATCGCGCTAGCATGTTTCTCCCAAAATTCAATCC |
| GRX5_RP_Xho1_HA | TCGCCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTAAC |

| | |
|---|---|
| | GATCTTTGGTTTCTTCT |
| ATX2_For_Xho1 | ATCCGCTCGAGCCAACTAAATCAGTTTTCTTG |
| ATX2_Rev_BamHI | ATCGCGGATCCTTATTCTTCAGAGATGCAGTAC |
| UBX6_For_Xho1 | ATCCGCTCGAGCGACAGCATTTTATTTCC |
| UBX6_Rev_HindIII | ATCCGAAGCTTTTAATTGTTGCCGTTATTATTAG |
| pNP_FMP40_For_XbaI | ATCCGTCTAGAGACCTCATCTGTACCAC |
| pTEF_FMP40_For_XbaI | ATCGCTCTAGAATGGGGGAAAAAAGGACAATAATCAAAGCG |
| FMP40_Rev_Xma1_myc | CAGCCCGGGTTACAGATCCTCTTCAGAGATGAGTTTCTGCTC GCTACTGCAGGAAGCTTC |
| RTT101_For_Xho1 | ATCCGCTCGAGGCAGTACAGTGCCACCTCC |
| RTT101_Rev_HindIII | ATCCGAAGCTTTTAGTACTTGTAAGTTGCTG |

## 2.4 Media

All the media, buffers and stock solutions were prepared using deionized milliporewater unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch$^2$ (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2µm (for heat labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100µg/mL.

### 2.4.1 LB (Luria Broth) Media (per 1000 mL)

| | |
|---|---|
| Yeast Extract | 5g/L |
| Tryptone | 10g/L |
| NaCl | 10g/L |

### 2.4.2 YPD (Yeast extract, peptone and dextrose) media (per 1000 mL)

| | |
|---|---|
| Yeast Extract | 10g/L |
| Peptone | 20g/L |
| Dextrose | 20g/L |

### 2.4.3 SD (Synthetic defined) media (per 1000mL)

| | |
|---|---|
| Yeast Nitrogen Base (w/o amino acids & $(NH_4)_2 SO_4$) | 1.7g/L |
| $(NH_4)_2 SO_4$ | 5g/L |
| Dextrose (Glucose) | 20g/L |
| Amimo acids (as required) | 80mg/mL |

### 2.4.4 Drop out media (per 1000 mL)

| | |
|---|---|
| Adenine hemisulphate | 18mg |
| Alanine | 76mg |
| Arginine hydrochloride | 76mg |
| Asparagine monohydrate | 76mg |
| Aspartic acid | 76mg |
| Cysteine hydrochloride monohydrate | 76mg |
| Glutamic acid monosodium salt | 76mg |
| Glutamine | 76mg |
| Glycine | 76mg |
| Myo- inositol | 76mg |
| Isoleucine | 76mg |
| p – aminobenzoic acid | 80mg |
| Phenylalanine | 76mg |
| Proline | 76mg |
| Serine | 76mg |
| Threonine | 76mg |
| Tryptophan | 76mg |
| Tyrosine disodium salt | 76mg |
| Valine | 76mg |
| Yeast Nitrogen Base (w/o amino acids & $(NH_4)_2 SO_4$) | 1.7g/L |
| $(NH_4)_2 SO_4$ | 5g/L |
| Dextrose (Glucose) | 20g/L |
| Amimo acids (as required) | 80mg/mL |

## 2.5 BUFFERS AND STOCK SOLUTIONS

### 2.5.1 Ampicillin (100 mg/mL)

5g of ampicillin was dissolved in the 50mL (1g for 10 mL) volume of deionized water.It was filter-sterilized using 0.2µm membrane filter and stored at -20°C in aliquots.

### 2.5.2 Glutathione Stock Solution (100mM)

307.32 milligrams of GSH (reduced glutathione) was dissolved in 10 mL of deionized water. It and was filter-sterilized using 0.2µm membrane filter and stored at -20°C in aliquots.

### 2.5.3 50% Glycerol (For preparation of *E. coli* stocks)

50 mL of glycerol is dissolved in 50 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

### 2.5.4 IPTG (Isopropyl β-D-1-thiogalactopyranoside) stock solution (1M)

2.38 g of IPTG is dissolved in 8 mL of distilled $H_2O$. Volume made upto 10mL using molecular biology grade $H_2O$. Filter sterilized using 0.2µm membrane filter and stored at -20°C in aliquots.

### 2.5.5 Alkaline Lysis Buffers for isolating plasmid DNA preparation from *E. coli*

| Solution 1<br>(Resuspension solution) | Solution 2 (freshly prepared)<br>(Cell lysis solution) | Solution 3<br>(Neutralization solution) |
|---|---|---|
| 50 mM Glucose<br>25 mM Tris-HCl (pH 8.0)<br>10 mM EDTA (pH 8.0)<br>Autoclaved and stored at 4ºC | 0.2N NaOH<br>1% SDS<br>Stored at room temperature | 5M Potassium acetate 60 mL<br>Glacial acetic acid 11.5 mL<br>Deionized water 28.5 mL<br>Stored at 4ºC |

### 2.5.6 Agarose Gel Electrophoresis Reagents

| a) 1xTAE buffer (per 1000ml)<br>(Tris-acetate-EDTA buffer)<br>(prepared from 50× TAE stock) | b) Orange-G dye<br>(Gel loading dye, 6X) | c) Ethidium BromideStock<br>(10 mg/mL) |
|---|---|---|

| | | |
|---|---|---|
| 40 mM Tris-acetate.<br>1mM EDTA (pH 8.0).<br>Autoclaved and stored at room temperature | 0.25% orange-G<br>30% glycerol | 10mg of Ethidium Bromide dissolved in 10mL of deionized water. Final working concentration of 0.5µg/mL is used. |

### 2.5.7 *Saccharomyces cerevisiae* transformation solutions

| 10 X Lithium acetate ( per 1000 mL) | 10 X TE ( Tris- EDTA) ( per 1000 mL) | 50% PEG ( Polyethylene glycol)( per 1000 mL) |
|---|---|---|
| 100.2 g of lithium acetate dissolved in 900mL of deionized water. pH adjusted to7.5 with glacial acetic acid and volume bought upto 1000mL. Autoclaved and stored at room temperature | 15.759 g of Tris-Cl and 2.92g of EDTA added to 800mL of deionized water. pH adjusted to7.5 and volume bought upto 1000mL. Autoclaved and stored at room temperature | 500g of PEG3500 dissolved in 500mL of deionized water by votexing. Volume made upto 1000mlby adding deionized water. Autoclaved and stored at room temperature |

### 2.5.8 Lysis buffer for genomic DNA isolation (from *Saccharomyces cerevisiae*)

| |
|---|
| 10 mM Tris – HCl (pH: 8.0) |
| 1 mM EDTA (pH: 8.0) |
| 100mMNaCl |
| 1% (w/v) SDS |
| 2% (v/v) Triton X-100 |

### 2.5.9 SDS-PAGE reagents and solutions

| | |
|---|---|
| a) 30% Acrylamide Mix | 29.2% (w/v) acrylamide<br>0.8% (w/v) N, N'- methylenebisacrylamide<br>Filtered before use. |
| b) Resolving (Lower) Gel<br>1.5 M Tris-HCl (pH 8.8) | 18.18 g Tris<br>pH adjusted to 8.8 with HCl and volume made upto 100 mL. |
| c) Stacking (Upper) Gel | 6.06 g Tris |

| | |
|---|---|
| 0.5 M Tris-HCl (pH 6.8) | pH adjusted to 6.8 with HCl and volume made upto 100 mL |
| d) Tris-Glycine Gel Running Buffer(pH 8.3) (Laemmli Buffer) | 25 mM Tris base<br>250 mM Glycine (electrophoresis grade)<br>0.1% SDS |
| e) 2X SDS Gel Loading Buffer (pH 6.8) | 100 mM Tris-HCl (pH 6.8)<br>4% (w/v) SDS<br>20% (v/v) Glycerol<br>200 mM β-mercaptoethanol<br>(to be added immediately before use)<br>0.2% (w/v) Bromophenol blue |
| f) Gel Staining Solution | 40% (v/v) Methanol<br>10% (v/v) Glacial Acetic acid<br>50% (v/v) Distilled water<br>0.1% Coomassie Brilliant Blue (R250) |
| g) Gel Destaining Solution | 40% (v/v) Methanol<br>10% (v/v) Glacial Acetic acid<br>50% (v/v) Distilled water |

**Composition of SDS-PAGE Resolving (Lower) Gel (10%) (For 10 mL)**

| | | |
|---|---|---|
| a) | Distilled water | 3.8 mL |
| b) | Resolving Gel 1.5 M Tris-HCl (pH 8.8) | 2.6 mL |
| c) | 30% Acrylamide Mix | 3.4 mL |
| d) | 10% SDS | 100 µL |
| e) | 10% APS (Ammonium persulphate) | 100 µL |
| f) | TEMED ( N, N, N', N'-Tetramethylethylenediamine) | 10 µL |

**Composition of SDS-PAGE Stacking (Upper) Gel (5%) (For 5 mL)**

| | | |
|---|---|---|
| a) | Distilled water | 2.975 mL |
| b) | Stacking Gel 1M Tris-HCl (pH 6.8) | 1.25 mL |
| c) | 30% Acrylamide Mix | 670 µL |
| d) | 10% SDS | 50 µL |
| e) | 10% APS (Ammonium persulphate) | 50 µL |
| f) | TEMED ( N, N, N', N'-Tetramethylethylenediamine) | 5 µL |

### 2.5.10 Western Blotting Reagents

| Transfer Buffer (pH 9.2) | Tris-Buffer Saline (TBS) | TBS-Tween 20 Buffer (TBST) | Blocking Agent |
|---|---|---|---|
| 48 mM Tris base<br>39 mM Glycine<br>0.037% SDS<br>20% Methanol | 150 mM NaCl<br>20 mM Tris-HCl<br>(pH 7.5)<br>(Made as 10× stock and kept at 4°C) | 0.1% Tween 20 in TBS (pH 7.5) | 5% Skim milk in TBST. |

### 2.5.11 Mitochondrial isolation reagents *(from Saccharomyces cerevisiae)*

| | |
|---|---|
| 1.2M Sorbitol<br>( per 1000 mL) | 218.6g of sorbitol added to 800 mL of deionized water and allowed to dissolve. Volume made upto 1000mL and filtered. |
| Zymolase buffer<br>( per 1000 mL) | 980 mL of 1.2M sorbitol<br>20 mL of 1M potassium phosphate (pH 7.4)<br>Filtered and stored at 4°C |
| Homogenization buffer<br>( per 1000 mL) | 500mL of 1.2M sorbitol<br>1.21g Trizma ( Tris) (10mM)<br>0.292g EDTA (1mM)<br>300mL of deionized water<br>Stirred at 50̊ C for 40 minutes. pH adjusted to 7.4 and volume made upto 1000mL. Filtered and stored at 4°C<br>1mM PMSF & 0.2% (w/v) bovine serum albumin<br>( to be added on the day of experiment and need to be filtered) |
| SEM buffer<br>( per 500 mL) | 250 mM sucrose ( 42.8g)<br>1mM EDTA<br>10mM MOPS-KOH (pH7.2)<br>Filtered and stored at 4°C |
| DTT (dithiothreitol) buffer<br>(to be prepared on the day of experiment) | 100mM Tris-H2SO4, pH 9.4<br>10mM DTT (dithiothreitol) |
| Zymolase<br>(to be added on the day of experiment) | 3mg of zymolase to be added to 7mL of zymolase buffer |

### 2.5.12 Lysis buffer for Co-immunoprecipitation

| |
|---|
| 50 mM Tris-Cl ( pH 8.0) |
| 100 mMNaCl |
| 1mM EDTA |
| 5mM magnesium chloride |
| 10% glycerol |
| 1% Triton X- 100 ( to be added on the day of experiment) |
| Protease inhibitor cocktail ( to be added on the day of experiment) |

### 2.5.13 Protein purification reagents

| Lysis Buffer | Washing buffer | Elution Buffer |
|---|---|---|
| 50 mM Tris-Cl (pH 8.0)<br>300mM NaCl<br>1mM PMSF<br>Protease inhibitor<br>( to be added on the day of experiment)<br>Autoclaved water | Lysis buffer<br>10 mM imidazole | Washing buffer<br>300mM imidazole<br>10% glycerol |

### 2.5.14 Assay buffer for NADPH dependant oxidoreductase activity assay

| |
|---|
| 10mM $K_2HPO_4$(pH 7.4) |
| 10 mM $NaH_2PO_4$(pH 7.4) |

## SECTION B: METHODS

### 2.6 Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* DH5αstrains were grown in LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin antibiotic. The *Saccharomyces cerevisiae* strains were maintained on YPD medium and

grown at 30°C. The yeast transformants were selected and maintained on SD medium supplemented with amino acids as per the requirements.

### 2.7 Recombinant DNA methodology (PCR, restriction digestion and ligation)

All the molecular techniques used in the study for manipulation of DNA, proteins, bacteria and yeast were performed according to standard protocols or as per manufacturer's protocol, unless specifically mentioned. DNA sequencing was done using 1st BASE DNA Sequencing Services | Base Asia

### 2.8 Plasmid DNA isolation from *Escherichia coli*

The cells were grown in LB medium having ampicillin antibiotic for 14-16 hrs. Centrifugation was performed at 6000 rpm for 5 minutes and supernatant was discarded. Solution1, solution 2 and solution 3 were added and each step was followed by gentle mixing and incubation for 5 minutes. The cells were centrifuged at 11,000 rpm for 15 minutes and the supernatant was collected. Equal volume of phenol-chloroform-isoamyl was added which was mixed by vortexing for 2 minutes and centrifugation was performed at 11,000 rpm for 10 minutes. Supernatant was collected and equal volume of isopropanol was added followed by incubation at -20$^{o}$C for 15 minutes. Centrifugation was performed at 11,000 rpm for 10 minutes and the pellet was resuspended in 70% ethanol. Centrifugation was performed at 11,000 rpm for 10 minutes and supernatant was discarded followed by incubation at 37$^{o}$C for 30 minutes. The pellet was resuspended in TE-RNAse and the plasmids were stored at -20$^{o}$C.

### 2.9 Genomic DNA isolation from *Saccharomyces cerevisiae*

Yeast cells were grown in YPD medium for 14-16 hours at 30$^{o}$C followed by centrifugation at 5000 rpm for 5 minutes. The supernatant was discarded and lysis buffer, glass beads and PCI (phenol chloroform isoamyl) were added to the cell suspension. The cells were disrupted by vortexing for 3 minutes. Tris-EDTA was added followed by centrifugation at 11,000 rpm for 5 minutes. The aqueous phase was transferred to a new

micro-centrifuge tube and ethanol was added to it. Centrifugation was performed at 11,000 rpm for 5 minutes and the supernatant was discarded followed by washing with 70% ethanol. Centrifugation was performed at 11,000 rpm for 5 minutes followed by incubation at 55$^{o}$C for 10-15 minutes. The pellet was resuspended in TE and TE-RNAse followed by incubation at 37$^{o}$C for 45 minutes and the genomic DNA was stored at -20$^{o}$C.

### 2.10 Bacterial transformation

The competent cells were thawed by keeping them on ice for 20 minutes. The ligated product or plasmid was added to the competent cells followed by incubation on ice for 30 minutes. Heat shock was provided at 42$^{o}$C for 1 minute followed by incubation on ice for 5 minutes. LB medium was added and incubation along with shaking was carried out at 37$^{o}$C for 1 hour. Centrifugation was performed at 8000rpm for 3minutes and supernatant was discarded. The pellet was resuspended in the remaining LB medium and spreading was performed on LB agar plate having ampicillin antibiotic. The plate was kept was incubator at 37$^{o}$C for 12-14 hours.

### 2.11 Yeast transformation

*S. cerevisiae* cells were grown in YPD medium at 30$^{o}$C for 16-24 hours. Reinoculation was carried out by using this culture in YPD at $OD_{600}$ = 0.1 and the cells were grown at 30$^{o}$C until $OD_{600}$ reaches 0.6-0.8. Centrifugation was performed at 6000 rpm for 5 minutes and the supernatant was discarded. The pellet was washed with distilled water and 1X Lithium acetate+ 1X TE followed by centrifugation at 6000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1X Lithium acetate + 1X TE. Single stranded DNA, plasmid and PEG were added to the resuspended pellet followed by vortexing to mix the components. Heat shock was provided to the cells by incubating them at 42$^{o}$C for 30 minutes followed by centrifugation at 6000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with distilled water followed by centrifugation at 6000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in autoclaved water. Spreading was performed on SD medium plates having the desired selection marker. The plates were kept in incubator for 3-4 days.

## 2.12 Growth based spotting assay

*S. cerevisiae* cells were grown in selective minimal medium for growth based spotting assay. Primary cultures were grown at 30$^{o}$C for 16-24 hours and were used to inoculate a secondary culture in selective minimal medium for as long as the $OD_{600}$ reaches between 0.6-0.8 at 30$^{o}$C. Centrifugation was performed at 6000 rpm for 5 minutes and the supernatant was discarded. The cells were washed with distilled water followed by centrifugation and resuspension indistilled water.

Four serial dilutions were made in distilled water at $O.D_{600}$= 0.2, 0.02, 0.002, 0.0002. 10µl
of each of these cell suspensions were spotted on the selective minimal medium plates.
Plates were incubated at 30$^{o}$C for 2-5 days and images were taken.

## 2.13 Preparation of chemically competent *E. coli* cells

Escherichia coli DH5α cells were used as the inoculum and were grown in LB medium for 14-16 hours at 37$^{o}$C. Reinoculation was carried out by using this culture in LB medium at $OD_{600}$ = 0.05 and the cells were grown at 37$^{o}$C until $OD_{600}$ reaches 0.4-0.5.The cultures were kept in ice for 30 minutes followed by centrifugation at 2500 rpm for 5 minutes at 4$^{o}$C. The supernatant was discarded and the pellet was kept in ice followed by resuspension in 0.05 M $MgCl_2$by gentle shaking and incubation on ice for 30 minutes. Centrifugation was performed at 2500 rpm for 30 minutes at 4$^{o}$C and the supernatant was discarded. The pellet was resuspended in 0.1 M $CaCl_2$by gentle shaking followed by the incubation on ice for 30 minutes. Centrifugation was performed at 2500 rpm for 30 minutes at 4$^{o}$C and the supernatant was discarded. The pellet was resuspended in 0.1 M $CaCl_2$and 15% glycerol and the aliquots were stored at -80$^{o}$C.

## 2.14 Recombinant protein expression &purification of His-tagged FMP40 protein

pET23a(+)-6X His-FMP40 was transformed in BL21 strain and transformants were selected on LB+ampicillin plates. The primary culture was grown overnight in LB media

containing ampicillin antibiotic for selection. The secondary culture was inoculated at $O.D_{600}$=0.05 and allowed to grow until $O.D_{600}$ reached 0.6-0.8. The culture was induced using 1mM IPTG and kept at 18ºC for 18 hours. Centrifugation was performed at 6000 rpm for 5 minutes at 4ºC and the supernatant was discarded followed by washing with distilled water and centrifugation. The cells were lysed by sonication using lysis buffer with 15 seconds on and 30 seconds off cycle and 20% amplitude and 10 cycles were performed. The cell lysate was centrifuged at 11,000 rpm for 15 minutes at 4°C. The supernatant obtained was incubated with washed Ni-NTA beads for 45 minutes at 4°C. The supernatant-bead slurry was centrifuged at 1600 rpm at 4°C. The supernatant was aspirated and protein-bound beads were collected which were subjected to three washes with washing buffer followed by centrifugation at 1600 rpm for 5 minutes at 4°C. The specific protein bound to beads was eluted by providing 3 washes with elution buffer followed by centrifugation at 1600 rpm for 5 minutes at 4°C.The supernatant containing the specific protein of interest was collected in a micro-centrifuge tube. The protein fractions obtained were run on 10% SDS-PAGE to check the purity of the protein. Dialysis was performed (using Thermo Scientific 3.5K MWCO dialysis tubing) to remove imidazole by buffer exchange with dialysis buffer (50mM Sodium phosphate monobasic and 300mM NaCl). After dialysis, the protein concentration was estimated using Nano Drop and the protein was flash-frozen by using liquid nitrogen and stored at -80°C.

## 2.15 NADPH dependent oxidoreductase activity assay

NADPH dehydrogenase activity of His-tagged FMP40 protein was analysedby using a specific assay buffer. 20µg of WT Fmp40p and Fmp40p-MTSΔ (mitochondrial target sequence) protein was used for the assay and different concentrations of potential metabolic substrates were used. The reaction was initiated by adding 0.2mM NADPH. The reaction was monitored at 30°C by following NADPH absorbance at 340nm using Perkin Elmer UV/Vis Lambda 25 Spectrophotometer. The reactions were performed in a 1mL quartz cuvette.

## 2.16 Mitochondrial isolation from *Saccharomyces cerevisiae*

Primary cultures were grown in selective minimal medium at 30$^{o}$C for 16-18 hours and were used to inoculate a secondary culture in YPD medium for as long as the $OD_{600}$ reached between 0.6-0.8 at 30$^{o}$C. Centrifugation was performed at 3000g for 5 minutes and the supernatant was discarded. The cells were washed with distilled water followed by centrifugation and the pellet was stored at -80$^{o}$C. To perform the experiment, the pellet was thawed on ice for 30 minutes and freshly prepared prewarmed DTT buffer was added to the pellet followed by incubation at 30$^{o}$C for 20 minutes. Centrifugation was performed at 3000g for 5 minutes and the pellet was resuspended in zymolase buffer. Centrifugation was performed at 3000g for 5 minutes and the pellet was resuspended in freshly prepared zymolase solution. The $OD_{600}$was taken and the cells were incubated at 30$^{o}$C for as long as the $OD_{600}$ reached 1/3 of the initial $OD_{600}$. The cells were harvested by centrifugation at 3000 g for 5 minutes followed by washing with zymolase buffer and centrifugation. The pellet was resuspended in ice-cold homogenization buffer and the homogenization buffer containing yeast cells mixture was poured to glass Teflon homogenizer kept on ice. 15 strokes were performed using piston B to homogenize the mixture and the mixture was collected in a fresh falcon tube. Fresh homogenization buffer was again added to the homogenizer to rinse it out and the mixture was added to the falcon tube. The homogenate was centrifuged at 1500 g for 5 minutes to pellet the cell debris and nuclei. The supernatant collected was centrifuged at 4000 g for 5 minutes. To isolate the crude mitochondria, the collected supernatant from the previous step was centrifuged at 12000 g for 15 minutes. The supernatant was carefully sucked up using a pipette and the pellet containing the mitochondrial fraction was resuspended in SEM buffer. To isolate the pure mitochondria, the pellet resuspended in SEM buffer was centrifuged at 1, 00,000 g for 1 hour. The supernatant was carefully sucked up and the pellet containing pure mitochondria was resuspended again in SEM buffer. The mitochondrial fraction obtained was flash frozen using liquid nitrogen and stored at -80$^{o}$C.

### 2.17 Western blot analysis of c-Myc tagged Fmp40p to confirm the localization

Equal amount of mitochondrial fraction, pellet and cytoplasmic fraction were loaded on a 10% SDS–polyacrylamide gel along with Thermo-fischer page-ruler prestained protein ladder. The gel was run at 90V for 20 minutes and at 120 V for 1.5 hours. The gel and ion reservoir stack were dipped in transfer buffer and the PVDF membrane (Promega) was

charged with methanol for 3 minutes. Trans – Blot turbo transfer system was used for the transfer to PVDF membrane and the arrangement involved top cassette electrode (cathode) followed by top ion reservoir stack, gel, PVDF membrane, bottom ion reservoir stack and bottom cassette electrode (anode). Immediately after the transfer, the membrane was incubated for 1 hour using 5% skim milk in TBST buffer (blocking buffer). After blocking the membrane, it was probed with mouse anti-c-Myc primary antibody and mouse porin/VDAC1 primary antibody at a dilution of 1:5000 in blocking buffer for overnight at 4$^{o}$C with very slow shaking. After 3×15 minutes washing of the blot with TBST, the membrane blot was incubated for 4 hours in anti-mouse (horseradish peroxidase-conjugated) antibody at a dilution of 1: 8000 in blocking buffer. After 3×15 minutes washing of the blot with TBST, the blot was sprayed with chemiluminescent HRP substrate. The signal was detected in LAS Imaging System. Stripping was performed by using 6M GCl (Guanidinium chloride) followed by blocking with blocking buffer for 1 hour. After blocking, the membrane was probed with mouse Act1 primary antibody and mouse ATP6VIA primary antibody at a dilution of 1:5000 in blocking buffer for overnight at 4$^{o}$C with very slow shaking. . After 3×15 minutes washing of the blot with TBST, the membrane blot was incubated for 4 hours in anti-mouse (horseradish peroxidase-conjugated) antibody at a dilution of 1: 8000 in blocking buffer. After 3×15 minutes washing of the blot with TBST, the blot was sprayed with chemiluminescent HRP substrate. The signal was detected in LAS Imaging System.

### 2.18 Co-immunoprecipitation to determine the interactors

Primary cultures were grown in selective minimal medium at 30$^{o}$C for 16-18 hours and were used to inoculate a secondary culture at $OD_{600}$ = 0.1 in selective minimal medium for as long as the $OD_{600}$ reached between 0.6-0.8 at 30$^{o}$C. After the $OD_{600}$ reached between 0.6-0.8, cells were treated with 2 mM diamide for 1 hour at 30$^{o}$C with shaking. Centrifugation was performed at 7000 rpm for 5 minutes followed by washing with water and the pellet was stored at -80$^{o}$C. To perform the experiment, pellet was thawed on ice for 20 minutes and protease inhibitor cocktail and Triton X-100% were added to the lysis buffer. Glass beads and lysis buffer were added to the pellet and cells were lysed using cell disruptor by agitating 10 cycles of 30 seconds each followed by placing the samples on ice after each cycle and finally 2 cycles of 2 minutes each followed by keeping on ice

(For mitochondria, only lysis buffer was added and the mitochondria was disrupted by gentle pipetting).Centrifugation was performed at 4$^{o}$C for 10 minutes at 10,000 rpm and the supernatant was collected. Total protein concentration of the samples was determined by using Bradford reagent and measuring the $OD_{595}$. Equal amount of protein concentration of each sample was added to the washed Protein-G sepharose beads and c-Myc antibody was added along with it followed by overnight incubation at 4$^{o}$C. Beads were washed 3 times with lysis buffer for 20 seconds and each washing was followed by centrifugation at 800g for 1 minute. Laemmli sample buffer 2X was added to the beads followed by incubation for 10 minutes at 65$^{o}$C. Beads along with Laemmli sample buffer 2X were kept at 99$^{o}$C for 15 minutes and centrifugation was performed at 800g for1 minute. The supernatant was loaded to the gel along with control and the gel was run at 90V for 20 minutes and at 120V for 1.5 hours. Gel was kept in staining solution for 20 minutes and then in destaining solution for 5-6 hours. The gel was observed using white light.

# Chapter 3: Results and discussion

## 3.1 Identification of specific gene in ABE5234 plasmid containing fragment from yeast multicopy library responsible for the suppression of growth defects of *gsh1*Δ

Sequencing of ABE5324 plasmid containing fragment from yeast multicopy library revealed the genes that were present in the plasmid and candidate gene approach was used to identify the specific gene which in multicopies was responsible for the suppression of growth defects of *gsh1*Δby considering the function of the genes involved.

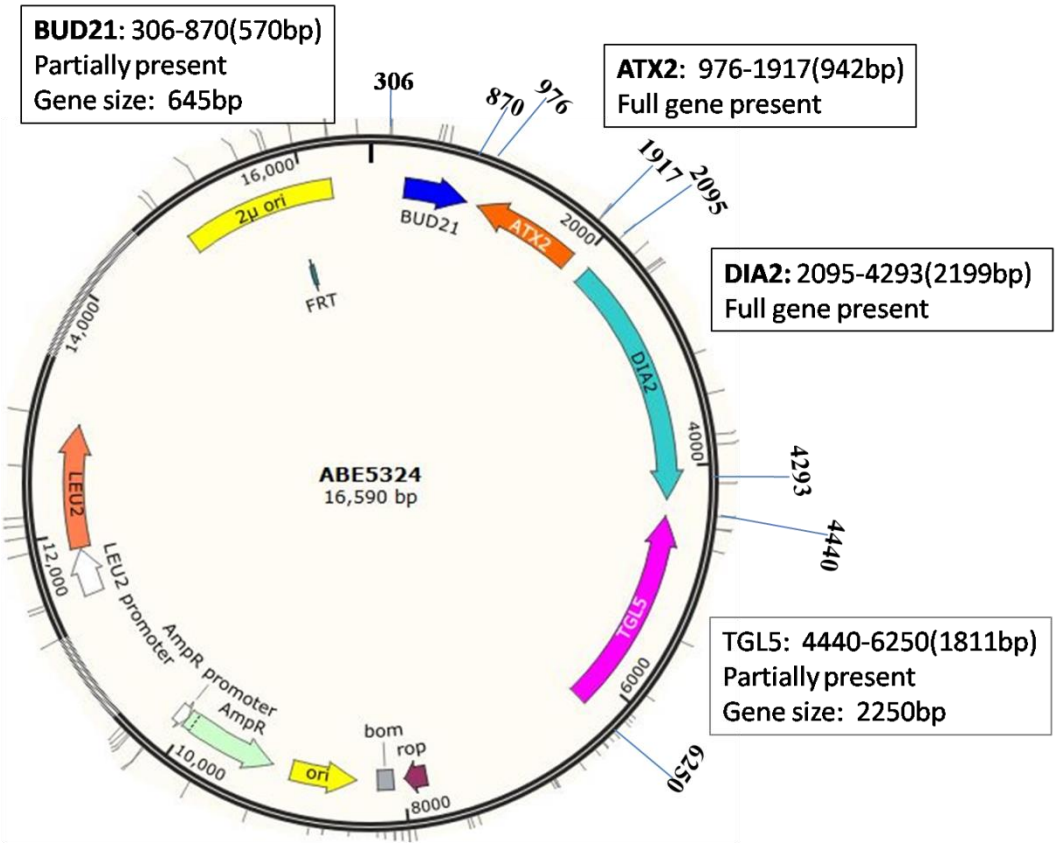



**Fig.3.1 Genes present in ABE5324 plasmid having fragment from yeast multicopy library cloned in YEP13.** ABE5323 plasmid contained BUD21 and TGL5 genes that

were partially present and ATX2 and DIA2 genes that were completely present along with their native promoters

**BUD21 (**BUD site selection): Component of ribosomal subunit processosome involved in modifications of rRNA [13]

**ATX2** (antioxidant): Golgi membrane protein involved in manganese homeostasis whose overexpression can suppress the growth defects of *sod1*Δ (superoxide dismutase1) which to required to protect the cells from reactive oxygen species [14]

**DIA2 (**digs into agar): Origin-binding F-box protein involved in DNA replication and nucleosome positioning [15]

**TGL5 (**triacylglycerol lipase): Bifunctional triacylglycerol lipase involved in the triacylglycerol mobilization [16]

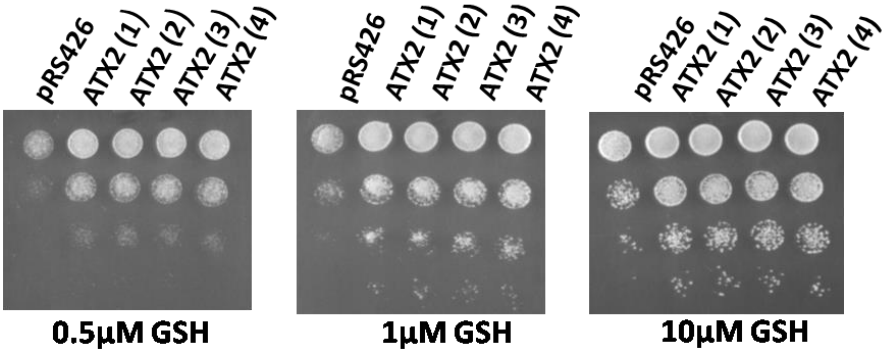



**Fig.3.2 Growth of *gsh1Δ* on different glutathione concentrations transformed with ATX2 gene and empty vector (pRS426).** pRS426 and 4 different clones of NP-ATX2-pRS426 named as (1),(2),(3),(4) were transformed in ABC1195 yeast strain having *gsh1::kanMX4* and were grown on minimal media lacking uracil, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking uracil and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

Based on the function of the genes, ATX2 was considered as the potential candidate. So, it was cloned along with its native promoter (800bp upstream) in pRS426 vector followed by its transformation in *gsh1*Δ and growth based spotting assay. ATX2 showed better growth at low glutathione concentrations as compared to empty vector pRS426 (control) depicting that ATX2 gene in multicopies was responsible for the partial suppression of growth defects of *gsh1*Δ in ABE5324plasmid.

### 3.2 Identification of specific gene in ABE5323 plasmid containing fragment from yeast multicopy library responsible for the suppression of growth defects of *gsh1*Δ

Sequencing of ABE5323 plasmid containing fragment from yeast multicopy library revealed the genes that were present in the plasmid and candidate gene approach was used again to identify the specific gene that led to partial suppression of growth defects of *gsh1*Δby considering the function of the genes involved.

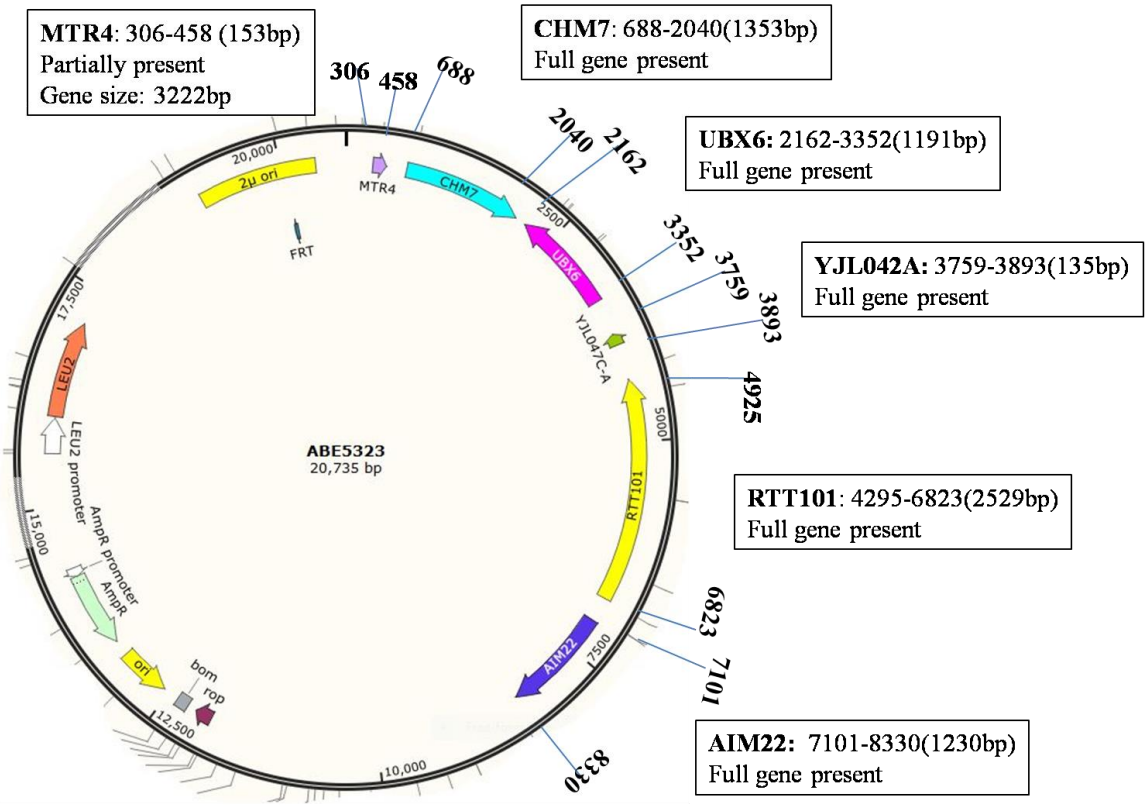


**Fig.3.3 Genes present in ABE5323 plasmid having fragment from yeast multicopy library cloned in YEP13.** ABE5323 plasmid contained MTR4 gene which was partially present along with CHM7, UBX9, YJL042A, RTT101and AIM22 genes which were completely present along with their native promoters.

**MTR4 (**mRNA transport): ATP-dependent 3'-5'RNA helicase involved in nuclear RNA processing and degradation[17].
**CHM7 (**charged multivesicular body protein): Putative protein of unknown function
**UBX6 (**ubiquitin regulatory X): Ubiquitin regulatory X domain containing protein which is found out to interact with genes playing a role in oxidation and reduction[18], [19].
**RTT101 (**regulator of Ty1 transposition): Subunit of E3 ubiquitin ligase complex involved in DNA repair and recovery mechanism[20]
**AIM22 (**altered inheritance rate of mitochondria): Putative lipoate-protein ligase required for lipoylation[21]

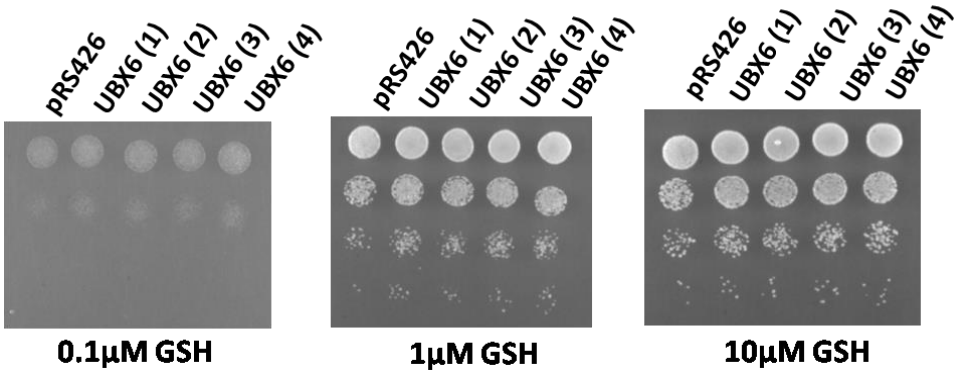



**Fig.3.4 Growth of *gsh1Δ* on different glutathione concentrations transformed with UBX6 gene and empty vector (pRS426).** pRS426 and 4 different clones of NP-UBX6-pRS426 plasmids were transformed in ABC1195 yeast strain having *gsh1::kanMX4* and were grown on minimal media lacking uracil, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking uracil and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

Based on the function of the genes, UBX6 was considered as the potential candidate. So, it was cloned along with its native promoter (800bp upstream) in pRS426 vector followed by its transformation in *gsh1*Δ and growth based spotting assay. However, no change was observed in the growth of UBX6 at low glutathione concentrations as compared to empty vector pRS426 (control) depicting that UBX6 gene was not responsible for the partial suppression of growth defects of gsh1Δ in ABE5323 library.

Since UBX6 did not appear to be responsible, we tried to look for the other possible genes by doing a deletion analysis of genes present in the plasmid. This was done by restriction digestion (NheI/SphI) followed by re-ligation.

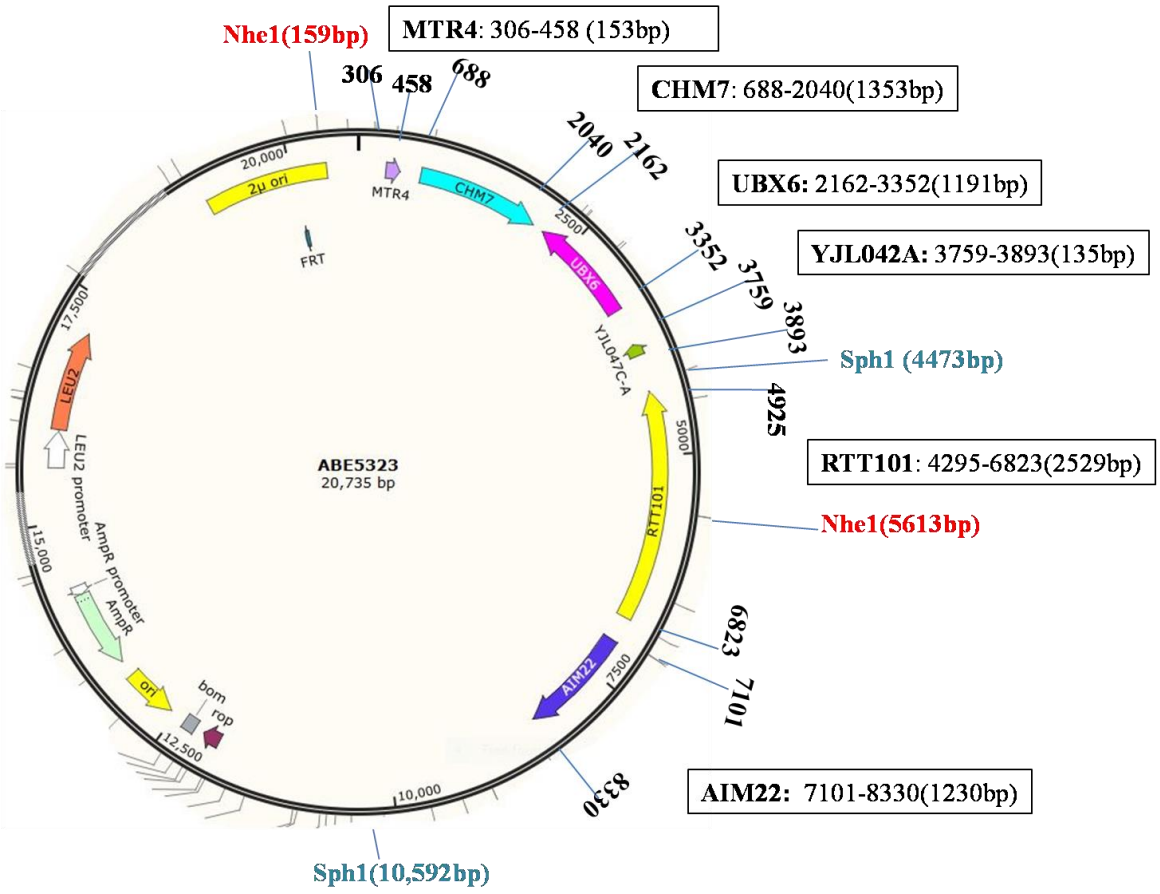



**Fig. 3.5 Genes present in ABE5323 plasmid having fragment from yeast multicopy library cloned in YEP13 along with restriction sites.** ABE5323 plasmid contained two

restriction sites for Nhe1 enzyme at 159 and 5613 base pairs and two restriction sites for Sph1 at 4473 and 10,592 base pairs which were chosen for restriction digestion followed by re-ligation.

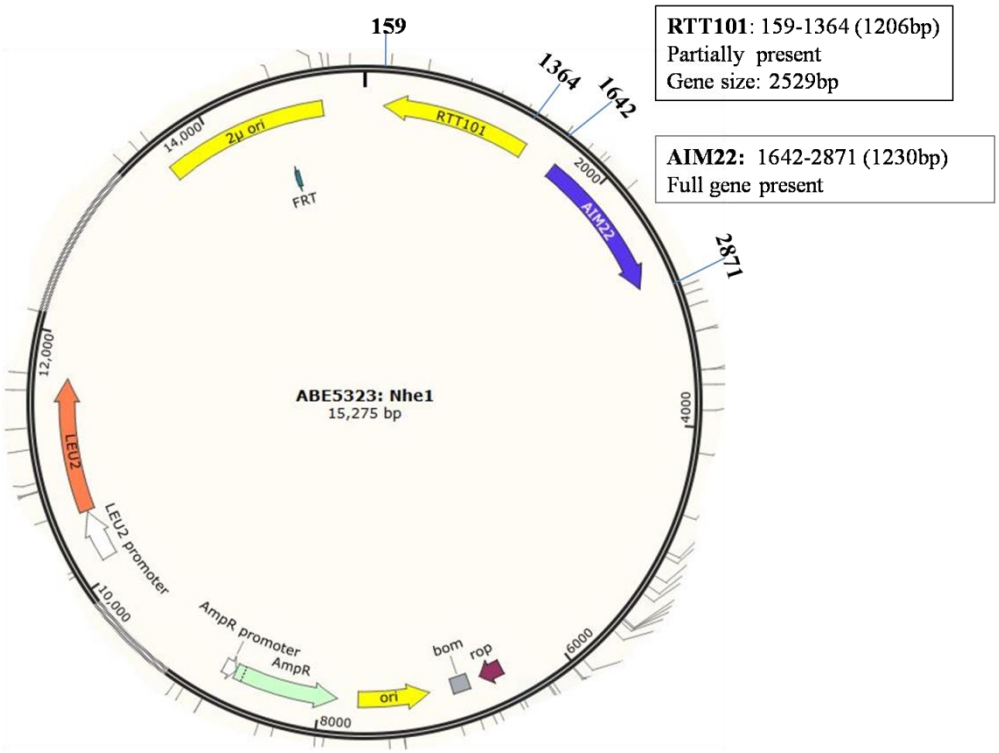



**Fig. 3.6(a) Genes left in ABE5323 plasmid after restriction digestion and re-ligation by Nhe1 (ABE5323-Nhe1).** ABE5323 plasmid after digestion by Nhe1 and re-ligation contained RTT101 gene that was partially present and AIM gene that was completely present along with its native promoter.

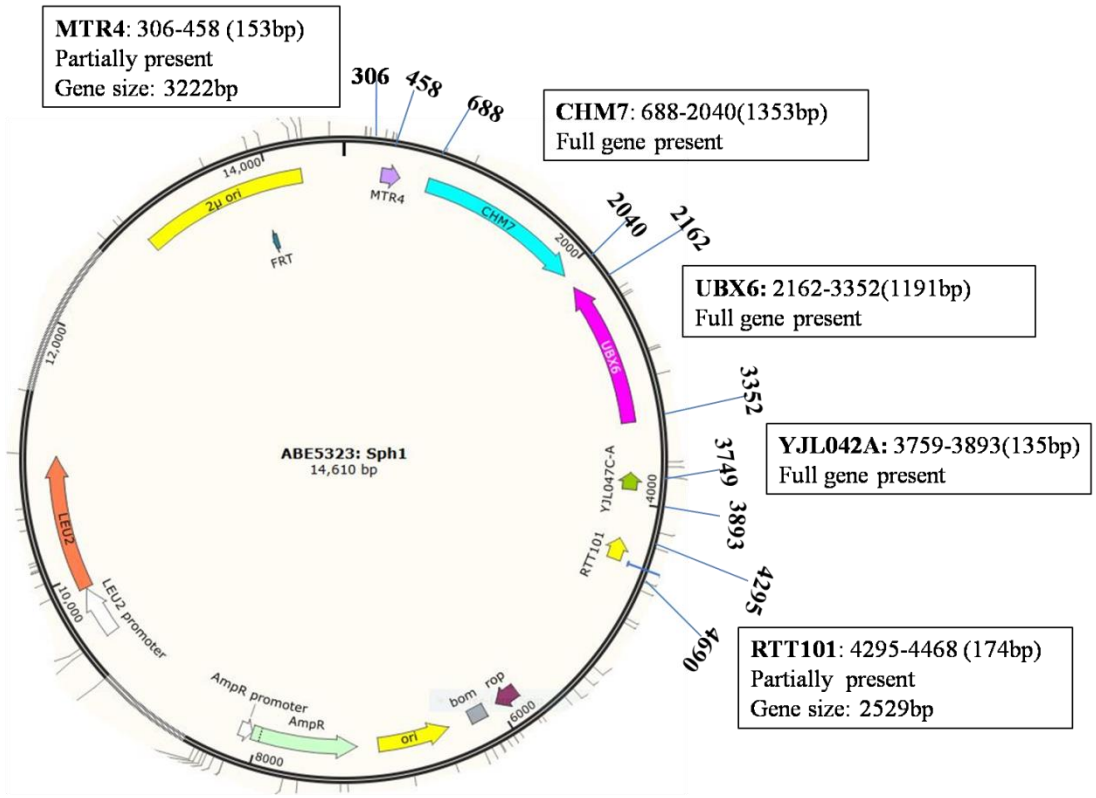



**Fig. 3.6(b) Genes left in ABE5323 plasmid after restriction digestion by Sph1 and re-ligation (ABE5323- Sph1).** ABE5323 plasmid after digestion by Sph1 and re-ligation contained MTR4 and RTT101 genes that were partially present and CHM7, UBX6 and YJL042A genes that were completely present along with their native promoters.


**Fig.3.7 Growth of *gsh1Δ* on low glutathione concentration transformed with YEP13, ABE5323-Nhe1 and ABE5323-Sph1.**YEP13 vector, 3 different clones of ABE5323-Nhe1 and 3 different clones of ABE5323-Sph1 were transformed in ABC1195 yeast

strain having *gsh1::kanMX4* and were grown on minimal media lacking leucine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking leucine and containing 0.2µM of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

ABE5323-Sph1 and ABE5323-Nhe1 did not show any significant change in the growth on low glutathione concentration as compared to the empty vector YEP13 depicting that neither the AIM22 gene that was completely present after digestion by Nhe1and re-ligation nor the CHM7, YJL042A and UBX6 genes that were completely present after digestion by Sph1 and re-ligation were responsible for the partial suppression of growth defects of gsh1Δ in ABE5323 plasmid. The only gene that was not completely present in either of them was RTT101 which might be responsible for the enhanced growth phenotype of ABE5323 plasmid.

To confirm whether RTT101 gene led to partial suppression of growth defects of *gsh1*Δ, RTT101 was cloned along with its native promoter (800 bp upstream region) in pRS426 vector followed by its transformation in *gsh1*Δand growth based spotting assay.

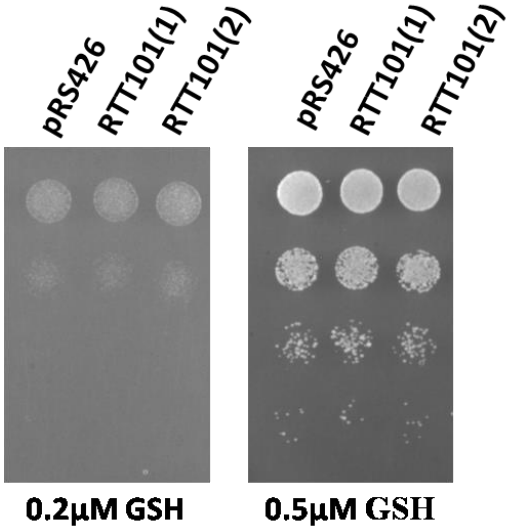



**Fig.3.7(b) Growth of *gsh1Δ* on low glutathione concentrations transformed with RTT101 gene and empty vector (pRS426).** pRS426 and 2 different clones of NP-RTT101-pRS426 were transformed in ABC1195 yeast strain having *gsh1::kanMX4* and were grown on minimal media lacking uracil, harvested, washed and resuspended in

water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking uracil and containing different concentrations of glutathione. The images were taken after 3 days of incubation at 30˚C.

However, no change was observed in the growth of RTT101 at low glutathione concentrations as compared to empty vector pRS426 (control). Through DNA sequencing results, it was found out that the NP-RTT101-pRS426 clones were having mutations which might be the reason because of which no changes in the growth were observed. So, further confirmation needs to be done to find out if RTT101 gene was responsible for the suppression of growth defects of *gsh1*Δ.

### 3.3 Determination of localization of FMP40 protein

FMP40gene was cloned along with its native promoter (800bp upstream region) and c-Myc tag at C-terminus. This clone was transformed in yeast cells having *fmp40*Δ followed by mitochondrial isolation (as explained in methods section2.16) and western blotting to examine in which fraction Fmp40p appears.

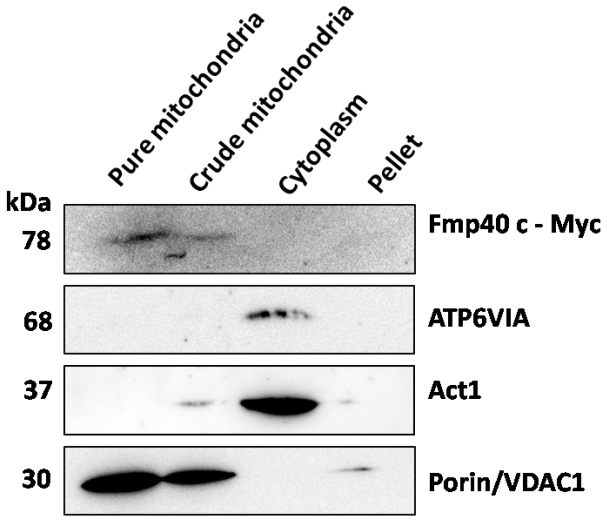



**Fig.3.8 Western blot analysis depicting the localization of Fmp40p along with the purity of various fractions.** Porin/VDAC1 was used as a biomarker for mitochondria.

Act1 was used as a biomarker for cytoplasm and ATP6VIA was used as a biomarker for vacuole.

Antibody was used against c-Myc tag and the expression was only observed in pure mitochondria and crude mitochondria depicting that FMP40 is exclusively a mitochondrial protein.

### 3.4 Complementation of *fmp40*Δ*pos5*Δ & *fmp40*Δ*grx5*Δ by TEF-FMP40

To confirm that the enhanced growth phenotype of *fmp40Δpos5*Δand *fmp40Δgrx5*Δ as compared to *pos5*Δ and *grx5*Δ respectivelywas due to the deletion of FMP40 gene, FMP40 expressed from (i) strong constitutive promoter :TEF (p416TEF-FMP40) and (ii) its native promoter (800bp upstream, NP-FMP40-pRS316) was transformed in cells having *fmp40Δpos5*Δ and *fmp40Δgrx5*Δ along with respective empty vector (control).

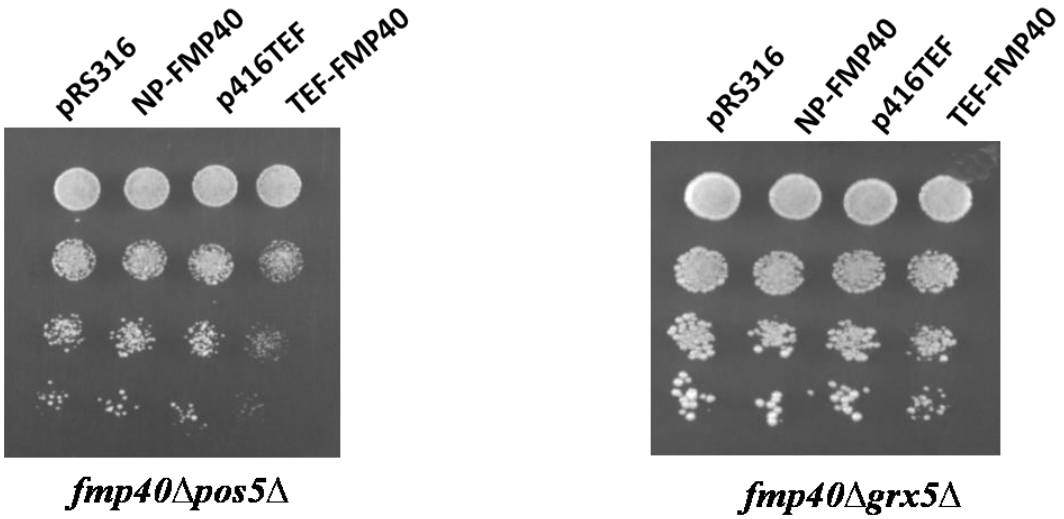



**Fig.3.9 Growth of *fmp40*Δ*pos5*Δ & *fmp40*Δ*grx5*Δtransformed with pRS316, NP-FMP40, p416TEF and TEF-FMP40.** pRS316, p416TEF, NP-FMP40 and FMP40-p416TEF plasmids were transformed in ABC5456 and ABC5457 yeast strains having *pos5::kanMX4fmp40::HIS3* and *grx5::*kanMX4*fmp40::HIS3* and were grown on minimal media lacking histidine and uracil, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium lacking histidine and uracil. The images were taken after 3 days of incubation at 30˚C.

Yeast cells having *fmp40*Δ*pos5*Δ & *fmp40*Δ*grx5*Δ transformed with TEF-FMP40 showed less growth as compared to the empty vector p416TEF depicting that TEF-FMP40 was able to complement the growth of cells having *fmp40*Δ*pos5*Δ & *fmp40*Δ*grx5*, hence validating the phenotype. FMP40 along with its native promoter NP-FMP40-pRS316 was also expected to complement and revert of *fmp40*Δ*pos5*Δ & *fmp40*Δ*grx5*. However, no significant change in the growth was observed.

### 3.5(a) Suppression of the growth defects by *fmp40*Δ during internal oxidative stress provided by using different carbon sources

Low NADPH levels can cause internal oxidative stress in the cells and the levels of NADPH can be altered by providing different carbon sources[22].

Non-fermentable carbon sources were provided (Glycerol, Lactic acid and Acetic acid) which are known to decrease the NADPH levels along with fermentable carbon sources(Glucose and Galactose) to observe if *fmp40*Δ can partially suppress the growth defects of *pos5*Δ and *grx5*Δ during internal oxidative stress provided by different carbon sources.

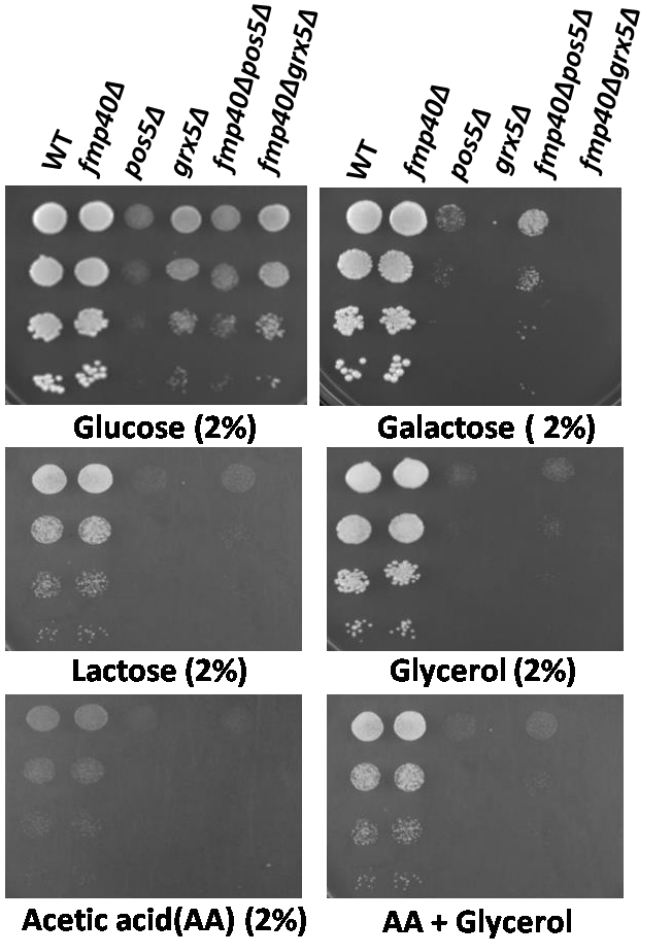



**Fig.3.10 Growth of WT, *fmp40*Δ, *grx5*Δ, *fmp40Δpos5*Δ and *fmp40Δgrx5*Δ during internal oxidative stress provided by different carbon-sources.** ABC733(WT), ABC4306 having *fmp40::kanMX4,* ABC5163 having *pos5::kanMX4,* ABC4837 having *grx5::kanMX4,* ABC5456 having *pos5::kanMX4fmp40::HIS3* and ABC5457 having *grx5::*kanMX4*fmp40::HIS3* were grown on minimal media provided with different carbon sources, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium. The images were taken after 3 days of incubation at 30˚C.

*fmp40*Δ was able to partially suppress the growth defects of *pos5*Δ during internal oxidative stress as well and *grx5*Δ became lethal for cells when non- fermentable carbon sources were provided.

### 3.5(b) Effects on the growth of WT and *fmp40*Δ during external oxidative stress provided by different menadione concentrations

External oxidative stress was provided by treating the cells with different concentrations of menadione which leads to calcium dependent apoptosis and formation of semiquinones which reacts with molecular oxygen to form ROS[23]. The main aim was to observe if *fmp40*Δ can help the cells to overcome external oxidative stress by showing better growth as compared to WT when treated with menadione.

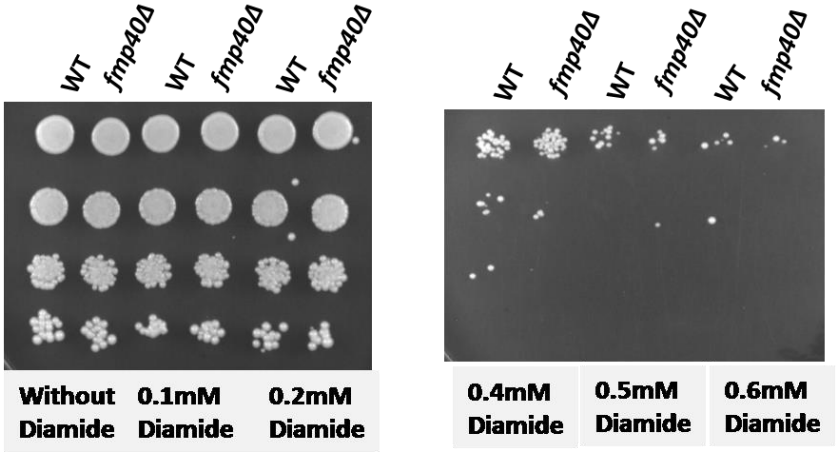



**Fig.3.11 Growth of WT and *fmp40*Δ during external oxidative stress provided by different menadione concentrations.** ABC733(WT) and ABC4306 having *fmp40:: kanMX4* were grown on drop-out media, treated with different menadione concentrations, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on drop out media. The images were taken after 3 days of incubation at 30˚C.

However, no significant changes were observed in the growth of WT and *fmp40*Δ when treated with different menadione concentrations**.**

### 3.5(c) Suppression of the growth defects by *fmp40*Δ during external oxidative stress provided by menadione

External oxidative stress was provided using menadione to observe if *fmp40*Δ can partially suppress the growth defects of *pos5*Δ and *grx5*Δ during external oxidative stress.

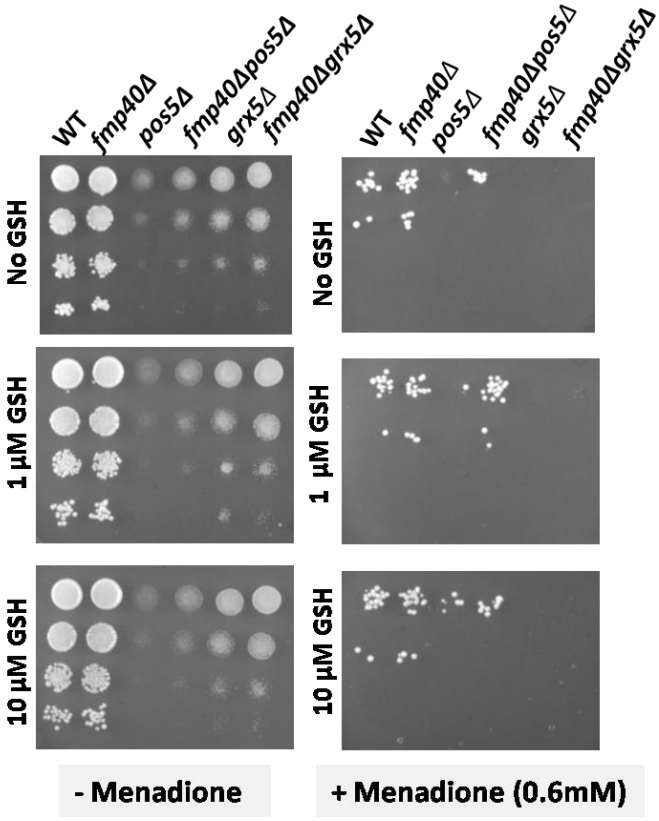



**Fig.3.12. Growth of WT, *fmp40*Δ, *grx5*Δ, *fmp40Δpos5*Δ and *fmp40Δgrx5*Δ during external oxidative stress provided by menadione.** ABC733(WT), ABC4306 having *fmp40::kanMX4*, ABC5163 having *pos5::kanMX4*, ABC4837 having *grx5::kanMX4*, ABC5456 having *pos5::kanMX4fmp40::HIS3* and ABC5457 having *grx5::*kanMX4 *fmp40::HIS3* were grown on minimal media, treated with 0.6mM menadione, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 $OD_{600}$ of cells. 10µL of these dilutions were spotted on SD medium having different glutathione concentrations. The images were taken after 3 days of incubation at 30˚C.

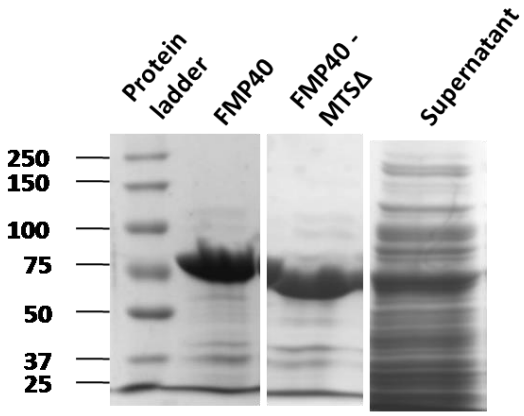
*fmp40*Δ was able to partially suppress the growth defects of *pos5*Δ during external oxidative stress provided by menadione and *grx5*Δ becomes lethal for cells during

external oxidative stress as well. Even high glutathione concentrations were not able to significantly enhance the growth of cells during external oxidative stress.

### 3.6 Attempts to study whether FMP40 dependent utilization of NADPH is linked to metabolic substrates reduction

In order to determine if the metabolic substrates are reduced by Fmp40p, an assay was performed based on the hypothesis that FMP40 acts a NADPH dehydrogenase which converts NADPH to NADP+ and carries out the simultaneous reduction of a metabolic substrate.

When Fmp40p will be incubated with the metabolic substrate that is reduced by it, NADPH (which absorbs at 340nm) will be converted to NADP+ (which does not absorb at 340nm) and there will be decrease in the absorbance of NADPH which can be used to identify the potential substrates that are reduced by Fmp40 protein.



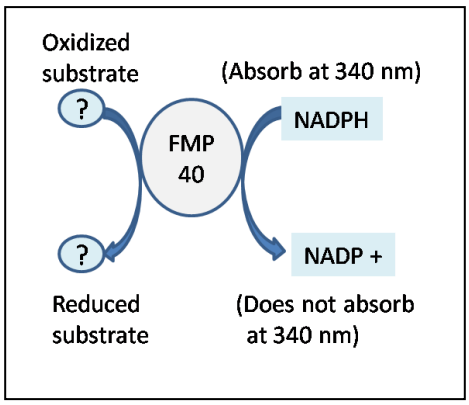




**Fig.3.13(a) SDS-PAGE gel image depicting purified Fmp40p and Fmp40p lacking mitochondrial target sequence (MTS)**

**Fig.3.13(b) Hypothetical model for oxidoreductase activity of Fmp40p**

**Metabolic substrates used**: Hydroxypyruvate, glyoxylate, reduced glutathione, oxidized glutathione and arginine

Fmp40p and Fmp40p lacking mitochondrial target sequence (MTS) were purified (as explained in section 2.14) and incubated with the metabolic substrates mentioned above

along with NADPH. However, no significant change in the absorbance of NADPH was observed suggesting that these substrates were not reduced during NADPH oxidation by Fmp40p.Subsequent work[12] has revealed that FMP40 carries out reduction of its own cysteines rather than reducing any metabolic substrate along with the conversion of NADPH to $NADP^{+}$.

### 3.7 Co-immunoprecipitation to determine the interactors was Fmp40p

FMP40 was cloned along with c-Myc tag and strong constitutive promoter: TEF. FMP40-myc-p416TEF and NP-FMP40-myc-pRS426 were transformed in yeast cells followed by mitochondrial isolation (as explained in section 2.14) and co-immunoprecipitation (as explained in section 2.16) was performed in order to determine the interactors. Fmp40p without any tag was used as a control and cells were also treated with 2mM diamide for one hour to provide oxidative stress which enhances the activity of Fmp40 protein[12].

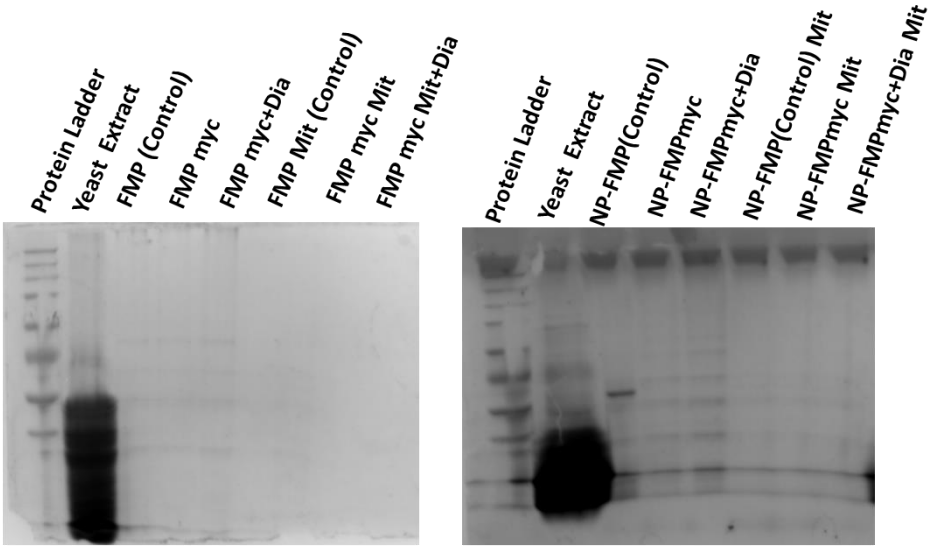



**Fig.3.14 SDS-PAGE gel image taken after co-immunoprecipitation**

No interactors were observed because c-Myc antibody was not working which was confirmed by western blotting.

The delineated function of Fmp40 depicted Fmp40p interacts with GrxA in *Escherichia coli*[12]. Similar interactions are being investigated in *Saccharomyces cerevisiae* with Grx2, Trx3and Grx5 protein.

### 3.8 Standardization of iNAP1 and iNAPc sensors for NADPH detection

iNAP1 (indicators of NADPH) andiNAPc (control) were sub-cloned from pcDNA3.1 in yeast p416TEF vectorand transformed in WT yeast cells. These sensors have only been used in mammalian cells and not in yeast cells to detect changes in NADPH levels[9]. Hence,confocal microscopy was performed after transforming iNAP1-p416TEF and iNAPc-p416TEF in WT yeast cells to detect if these sensors are able to detect changes in NADPH levels.

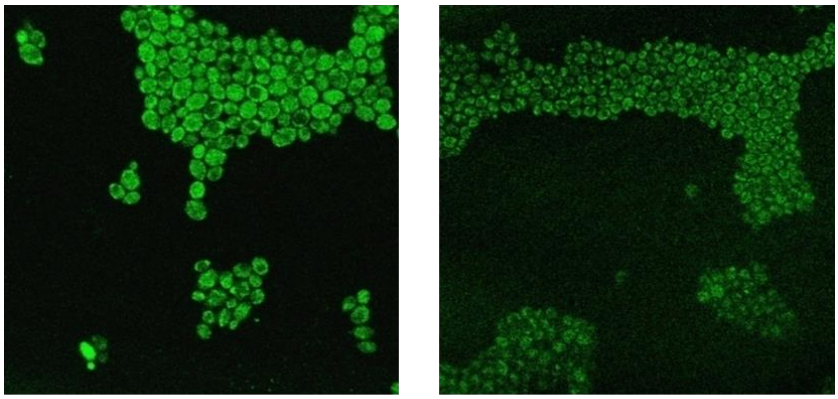



**Fig.3.15 Images of confocal microscopy depicting fluorescence of iNAP1 and iNAPc in WT yeast cells**

iNAP1 showed enhanced fluorescence as compared to iNAPc (control) depicting that it was able to sense NADPH levels of WT yeast cells. iNAPc (control) has no affinity for any nucleotide and depicted auto–fluorescence of the cells. Standardization of these sensors is in progress for NADPH detection.

# Summary and Conclusion

A genetic screen was previously developed and validated in the lab to identify changes in NADPH levels. In this screen, very low concentrations of glutathione were provided externally after blocking the glutathione biosynthesis pathway in the cells by deletion of GSH1 gene. The aim was to identify genes which can partially suppress the growth defects of *gsh1*Δ on low glutathione concentrations either by deletion or over-expressionof genes which can indirectly indicate the enhancement in NADPH levels. It was observed that two genes in multicopy: ATX2 and possibly RTT101 were able to partially suppress the growth defects of *gsh1*Δ.The mechanism of suppression may be due to enhancement of NADPH levels that needs to be investigated. In a previous study, *fmp40*Δ also showed suppression of the growth defects of *gsh1*Δ giving the idea that FMP40 might act as a consumer of NADPH. Fmp40 was a putative protein was unknown function which was also able to partially suppress the growth defects of *pos5*Δ and *grx5*Δ which were also linked to NADPH. To get the insights of FMP40, internal and external oxidative stress was provided and it was observed that *fmp40*Δ was able to partially suppress the growth defects of *pos5*Δ during oxidative stress as well. An attempt was made to find the metabolic substrates that are reduced by FMP40 when NADPH is oxidized, however, very recenyly, it was found out that FMP40 oxidizes NADPH for its own reduction rather than the reduction of any metabolic substrate through the delineated function of FMP40. Our results are consistent withthe delineated function of FMP40[12]that it acts as a consumer of NADPH and protect the cells from oxidative stress by carrying out AMPylation of various proteins.

Attempts are on-going to determine the interactors of Fmp40pby co-immunoprecipitation and direct interactions with Grx2, Grx5 and Trx3 proteins are being investigated. iNAP1 and iNAPc sensors have also been sub-cloned in yeast vector and standardization of these sensors is in progress in order to detect changes in NADPH levels.

# References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