Studies on 6-phosphogluconate dehydrogenase, an enzyme of the pentose phosphate pathway of *Saccharomyces cerevisiae*

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Certificate of Examination

This is to certify that the dissertation titled 'Studies on 6-phosphogluconate dehydrogenase, an enzyme of the pentose phosphate pathway of *Saccharomyces cerevisiae*' submitted by Mr. Surendra Yadav (Reg. No. MS15031) for the partial fulfillment 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. Shravan Kumar Mishra Dr. Rachna Chaba Prof. Anand Kumar Bachhawat

(Supervisor)

Dated: April 24, 2020

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.

Surendra Yadav

April 24, 2020

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

Prof. Anand Kumar Bachhawat

(Thesis supervisor)

Acknowledgement

Foremost, I would like to express my sincere gratitude to my master's thesis supervisor Prof. Anand K. Bachhawat for his continuous support, patience, motivation, and enthusiasm. Discussions held with him helped me enormously in diversifying my knowledge. His mentorship has helped me to grow as a young researcher. I doubt that I will ever be able to convey my appreciation fully, but I owe him my eternal gratitude. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Shravan Kumar Mishra, and Dr. Rachna Chaba for their encouragement and insightful comments.

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There are friends, there is family, and then there are friends that become family. The MS thesis year has been a real roller coaster filled with many happy and amazing experiences because of many people around me. I have made friends for life here, and I wish to thank them for being who they are. I am truly grateful to Bhavya, Saurabh, Sahil, Ayush, Amit and Rajesh for being such splendid friends, for their humility and humor and for providing me their unrestricted attention in situations where I needed there help, be it professional or personal ones. I owe a lot of good memories to Rohit, Megha, Nimrat, Raju, Preeti, Jaskaran, Srishti, Raman, and Yateendra without whom my stay and this work would not be complete.

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Surendra Yadav

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Abbreviations

6PGDH	6-phosphogluconate dehydrogenase
$NADP^+$	nicotinamide adenine dinucleotide phosphate
R5P	Ribulose 5-phosphate
PPP	Pentose Phosphate Pathway
G6PD	glucose 6-phosphate dehydrogenase
GSH	Glutathione
GSSG	Oxidized glutathione
ROS	Reactive oxygen species
6-PG	6- Phosphogluconate
MG	Methylglyoxal
MTHFR	Methylene tetrahydrofolate reductase
5-FOA	5-fluoroorotic acid

Contents

Abstract

The pentose phosphate pathway is the major source of NADPH in living cells. The two enzymes in this pathway involved in the generation of NADPH are Glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). In this thesis, studies have been initiated on some aspects of the 6PGDH enzyme of *Saccharomyces cerevisiae*.

6-phosphogluconate dehydrogenase (6PGDH) is the third enzyme in the oxidative Pentose Phosphate Pathway (PPP) that catalyzes the production of Ribulose 5-phosphate from 6-Phosphogluconate [14]. During this process, 6PGDH generates NADPH by using $NADP^+$ as co-factor [14]. In *Saccharomyces cerevisiae*, GND1 gene encodes the major isoform of the protein, which performs 80% of the 6PGDH activity, whereas, GND2 gene encodes the minor isoform, which performs the remaining 20% activity[8].

Multiple sequence alignments of the 6PGDH enzymes across organisms, revealed six conserved cysteines from yeast to humans. As these cysteines did not appear to be in the active site of the enzyme, an important goal of this thesis was to investigate whether these cysteines might have some regulatory roles. We created different cysteine to serine mutants of 6PGDH encoded by GND1. To investigate the functionality of these mutants we carried out experiments to establish an in vivo functional assay. To establish this assay, WT and mutants of deletions of GND1 and GND2 were evaluated on both normal and stress conditions. We also evaluated the ability of NADPH deficient mutants to be complemented by GND1. Finally, as it was not clear from the literature whether the knockout of both GND1 and GND2 would be lethal, as an aid to the in vivo functionality assays, we made attempts to create a double knockout of both GND1 and GND2 using a plasmid shuffling method.

Chapter 1: Introduction

1.1 The Pentose Phosphate Pathway

The pentose phosphate pathway (PPP), also known as hexose monophosphate shunt (HMS), is a central metabolic pathway that runs parallel to glycolysis. Its main role is the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), ribulose 5-phosphate (R5P) and pentoses [14]. There are two distinct phases in the pathway, oxidative phase, and non-oxidative phase. **Oxidative phase or Oxidative PPP** involves the production of NADPH and R5P whereas non-oxidative PPP results in the synthesis of 5-carbon sugars [14]. The PPP occurs in cytosol in most organisms. The primary results of the pathway are as follows:

- Generation of NADPH, which acts as reducing equivalents and is used in several reductive biosynthesis reactions [14].
- Generation of ribulose 5-phosphate (R5P), which is used in the production of nucleotides and nucleic acids [14].
- Generation of erythrose 4-phosphate (E4P), which is used in the synthesis of aromatic amino acids [14].

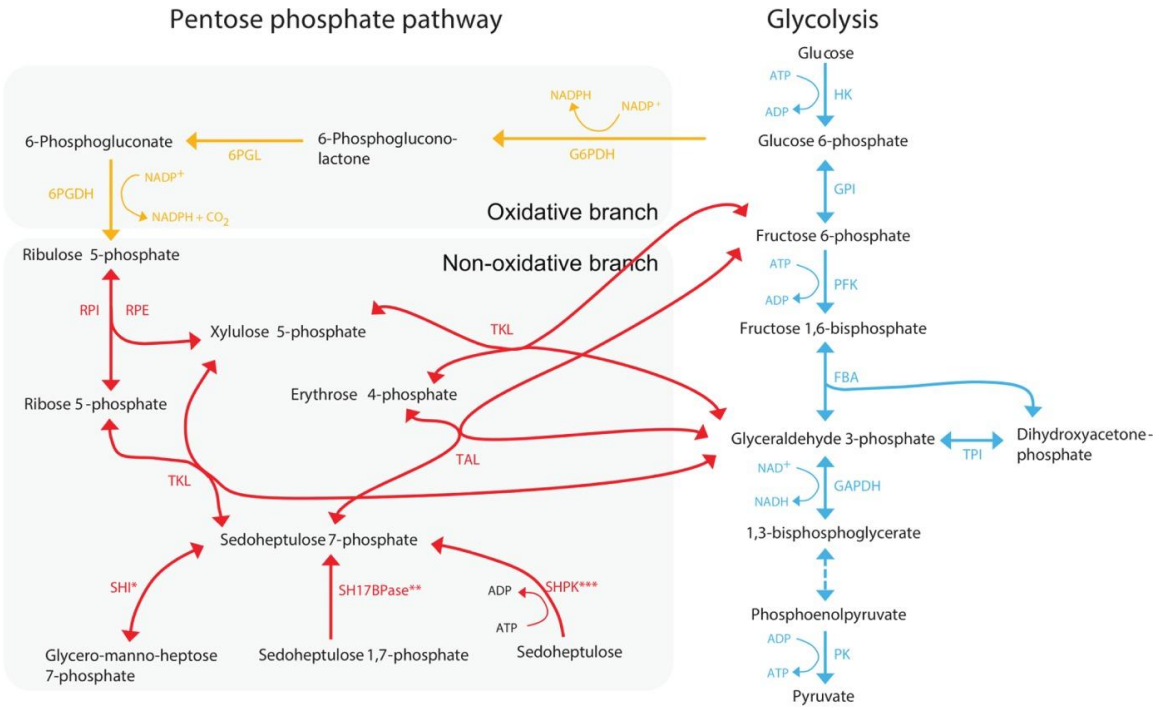

***Figure* 1.1:** The Pentose Phosphate Pathway. It runs parallel to glycolysis [Figure Source: *Stincone, A. et al. 2015*]

1.1.1 Oxidative Phase or Oxidative PPP

The oxidative phase starts with the dehydrogenation of glucose 6-phosphate at carbon 1, this reaction gets catalyzed by *glucose 6-phosphate dehydrogenase* (G6PD) enzyme to produce 6-phosphoglucono-δ-lactone [11][14]. G6PD is strongly specific for $NADP^+$ and during this reaction it generates NADPH from $NADP^+$ [11]. Further, the generated 6-phosphoglucono-δ-lactone gets hydrolyzed by *Gluconolactonase* to produce 6-phosphogluconate [14]. 6-phosphogluconate is then oxidatively decarboxylated by *6-phosphogluconate dehydrogenase* (6PGDH) to yield ribulose 5-phosphate [8][14]. The overall reaction can be written as follows,

$$\text{Glucose 6-phosphate} + 2\ NADP^+ + H_2O \rightarrow \text{ribulose 5-phosphate} + 2\ NADPH + 2\ H^+ + CO_2$$

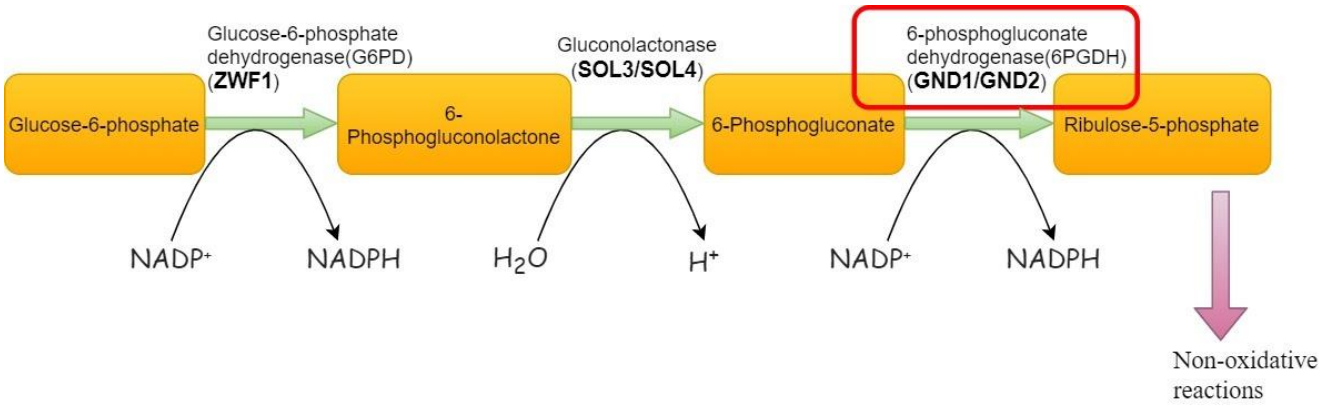

***Figure* 1.2:** Oxidative phase of the pentose phosphate pathway

Reactants	Products	Enzyme
Glucose 6-phosphate + $NADP^+$	6-phosphoglucono-δ-lactone + **NADPH**	Glucose-6-phosphate dehydrogenase (G6PD)
6-phosphoglucono-δ-lactone + H_2O	6-phosphogluconate + H^+	Gluconolactonase
6-phosphogluconate + $NADP^+$	ribulose-5 phosphate + **NADPH** + CO_2	6-phosphogluconate dehydrogenase (6PGDH)

***Table* 1.1:** Reactions in Oxidative PPP

1.1.2 Non-oxidative Phase

Non-oxidative phase catalyzes the interconversion of three-, four-, five-, six-, and seven-carbon sugars and through several non-oxidative reactions it results in the production of five-carbon sugar for nucleotide synthesis [14]. The excess five-carbon sugars are then converted into the intermediates of glycolysis [14].

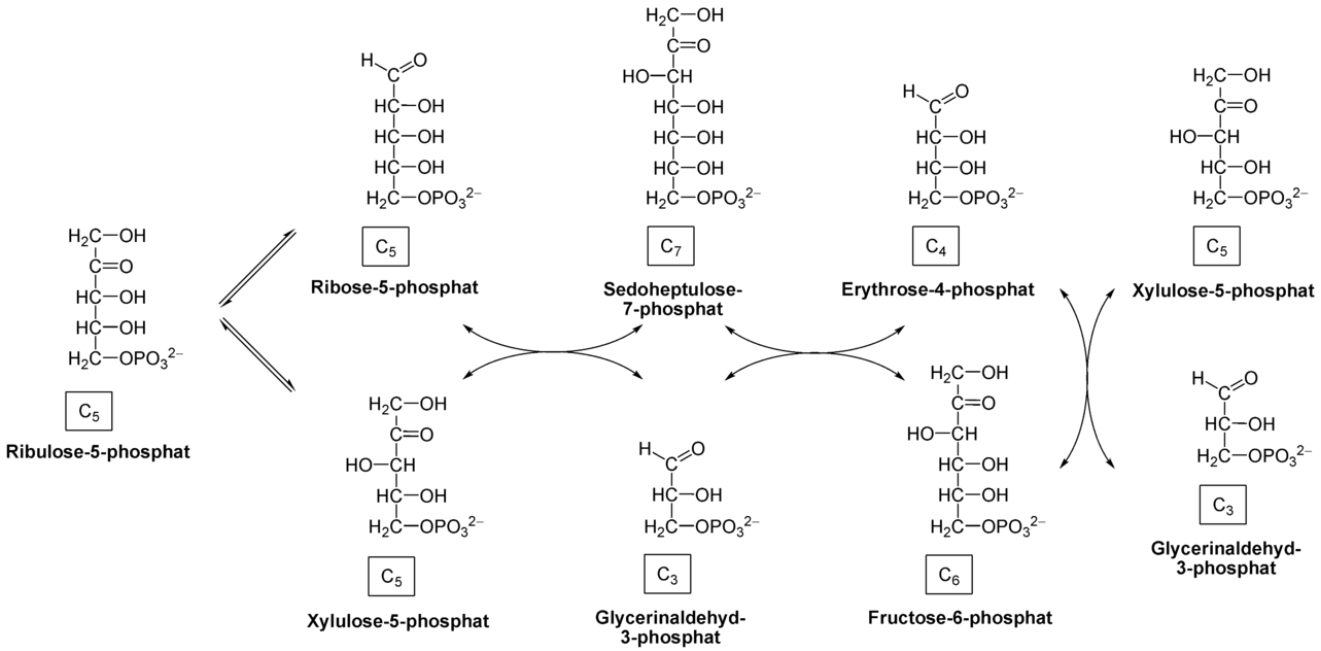

***Figure* 1.3:** Non-oxidative phase of the pentose phosphate pathway. [Figure Source: Berg JM- *Biochemistry*. 5th *edition*]

Reaction	Enzyme
Ribulose 5-phosphate ⇌ ribose 5-phosphate	Phosphopentose isomerase
Ribulose 5-phosphate ⇌ xylulose 5-phosphate	Phosphopentose epimerase
Xylulose 5-phosphate + ribose 5-phosphate ⇌ sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate	Transketolase
Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate ⇌ fructose 6-phosphate + erythrose 4-phosphate	Transaldolase
Xylulose 5-phosphate + erythrose 4-phosphate ⇌ fructose	Transketolase

6-phosphate + glyceraldehyde 3-phosphate	

***Table* 1.2:** Reactions in non-oxidative PPP.

1.2 Introduction to Glucose 6-phosphate dehydrogenase (G6PD)

Glucose 6-phosphate dehydrogenase (G6PD) is the first and the rate limiting enzyme in the oxidative phase of the PPP. G6PD is the most intensively studied enzyme in the oxidative PPP. G6PD produces 6-phosphoglucono-δ-lactone from Glucose 6-phosphate, and it is an $NADP^+$-dependent oxidoreductase that generates NADPH in this reaction [11][14]. In *Saccharomyces cerevisiae*, G6PD is encoded by the gene *ZWF1* and deletion of this gene retains viability, but *Δzwf1* cells are not able to synthesize methionine [6][11]. This methionine auxotrophy is believed to be due to insufficient production of NADPH which is required in the methionine biosynthesis pathway [6]. *Δzwf1* cells require inorganic sulphur to produce organic sulphur, in order to grow. Also, loss of *ZWF1* gene results in increased sensitivity to oxidizing agents which is presumably caused due to reduced levels of NADPH inside the cell [6]. Therefore, G6PD has a major role in NADPH production and redox balancing in yeast.

1.3 Introduction to 6-phosphogluconate dehydrogenase (6PGDH)

6-phosphogluconate dehydrogenase is the third enzyme in the oxidative PPP, which produces ribulose 5-phosphate (R5P) from 6-phosphogluconate, and generates NADPH (using $NADP^+$) in the reaction[14]. As mentioned in Sec 1.2, NADPH is an important reducing power for the cell and plays an important role in combating oxidative stress. In comparison to G6PD, not much research has been carried out to understand the role of 6PGDH in NADPH production and combating oxidative stress. In *S. cerevisiae*, 6PGDH is encoded by two genes, *GND1* and *GND2*. *GND1* encodes the major isoform, which performs 80% of the activity, whereas *GND2* encodes minor isoform which performs the remaining 20% activity [8]. Since, Gnd1p performs majority of the 6PGDH activity, this study primarily focuses on Gnd1p. The size of the coding region of gene *GND1* is 1470 bp and the protein length is 489 amino acids. Gnd1p exists in the form of homodimer, with three domains for each monomer [8]. The enzyme 6PGDH plays an important role in the tolerance to furfural induced stress by converting the toxic furfural to less toxic furfuryl alcohol [5]. *GND1* null mutant and *GND2* null mutant, both are viable but the viability of *Δgnd1Δgnd2* double deletion is not yet tested.

1.4 Glutathione and Oxidative Stress

Glutathione (GSH) or *γ-L-Glutamyl-L-cysteinyl-glycine* is one of the most abundant thiol (-SH) containing organic compounds in several organisms including *S. cerevisiae* [12]. GSH is an antioxidant that is capable of preventing damage to important cellular components caused by reactive oxygen species (ROS) such as peroxides, free radicals, heavy metals and lipid peroxides [12]. Oxidative stress is caused when there is an imbalance between the pro-oxidant and anti-oxidant species inside the cell, i.e. imbalance between free radicals (ROS) and anti-oxidants (GSH), which results in damage to cellular components [10]. Reduced glutathione (GSH) plays an important role in preventing oxidation of critical sulfhydryl groups through a chain of coupled reactions involving glutathione peroxidase, glutathione reductase, and the Pentose phosphate pathway [10]. PPP or more specifically Oxidative phase of PPP, that produces NADPH, has a critical role as it is responsible for providing NADPH which is required by glutathione reductase for the reduction of oxidized glutathione (GSSG) to GSH [10]. Therefore, GSH, together with glutathione reductase and NADPH-producing pathways provides a defense system against oxidative stress [10]. Also, oxidation of GSH during oxidative stress results in increased production of NADPH through oxidative PPP [3]. This regulation of PPP is discussed in detail in Sec 1.5.

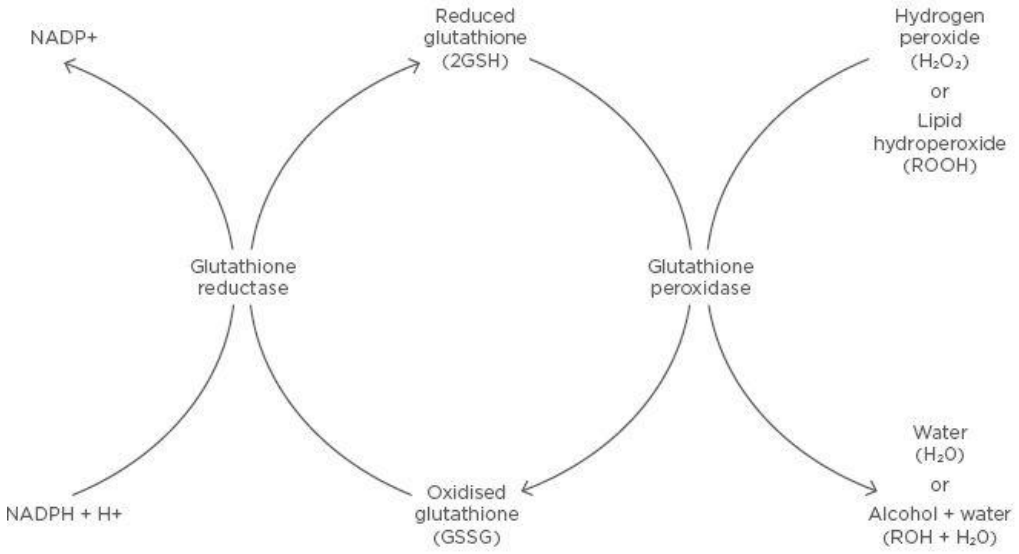

***Figure* 1.4:** *Glutathione Redox Reaction*: Reduced glutathione is converted to oxidized glutathione through a disulfide bridge by glutathione peroxidase in the presence of hydrogen peroxide or lipid hydro peroxide, forming water or an alcohol and water, respectively. Oxidized glutathione is converted to reduced glutathione through glutathione reductase, which requires the oxidation of NADPH to $NADP^+$. [Figure Source: Robinson B. et al. 2018.]

1.5 Regulation of Pentose Phosphate Pathway

The damage caused due to ROS constitutes a severe threat to cell survival. Reduced Glutathione (GSH) plays an important role in protecting the cellular components during oxidative stress by itself getting oxidized to GSSG and converting the toxic ROS to water or an alcohol and water [10]. The regeneration of GSH from GSSG is required in order to combat the oxidative stress continuously. The main component required for this regeneration is NADPH which is majorly produced through oxidative PPP [10]. The first enzyme of the PPP, G6PD, is under a negative feedback loop; NADPH produced by the enzyme inhibits its own activity [3]. NADPH consumption during oxidative stress leads to removal of this inhibition [3]. The PPP is under a strong regulatory mechanism that is described below [3]:

1. Under normal conditions, the lower glycolytic pathway is active and lesser glucose flux is directed towards the PPP.
2. When under normal conditions, G6PD is inhibited, due to abundant NADPH and this results in less production of NADPH.
3. When oxidative stress conditions arise, the lower glycolytic pathway enzymes get deactivated due to ROS and the glucose flux is redirected into the oxidative PPP.
4. Increased amount of NADPH is produced during oxidative stress because of more glucose flux in oxidative PPP, and also due to removal of NADPH inhibition from G6PD, which is a major NADPH producer in cells.

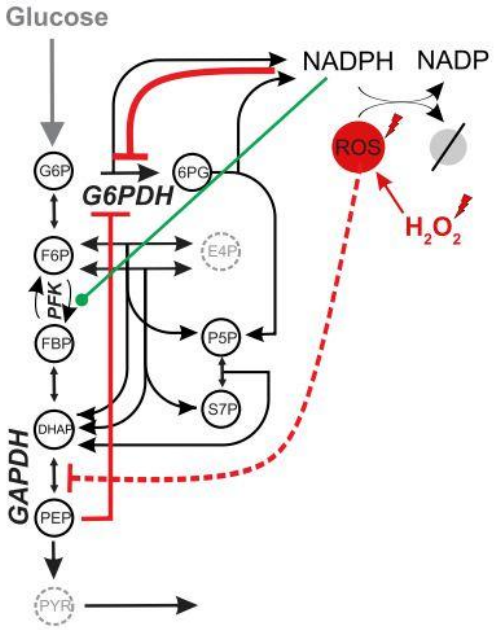

***Figure* 1.5:** Regulation of the Pentose Phosphate Pathway and glycolytic pathway in *S. cerevisiae*. (Green arrow shows up regulation, Red arrows shows inhibition) [Figure Source: Christodoulou et al.2019]

1.6 Structure of 6PGDH (Gnd1p)

Gnd1p is a homodimer with three domains for each monomer, containing an $NADP^+$ binding Rossmann fold domain and a substrate (6PG) binding pocket in each monomer [8]. The enzyme has a *Km* of 50 ± 9 μM for 6-phosphogluconate (6PG) and of 35 ± 6 μM for $NADP^+$ at pH 7.5 [8]. The structure of Gnd1p is similar to 6PGDH of other species [8]. Also, the substrate and coenzyme binding sites show high level of conservation either from the 3D structural superposition or from the primary sequence alignment [8]. The C-terminal domain of the enzyme plays two important functions. First, even though the C-terminal domain is not needed for dimerization, it functions as a hook to tighten the dimer [8]. Second, it controls the binding of substrate and release of product by acting as a lid on the substrate binding pocket [8].

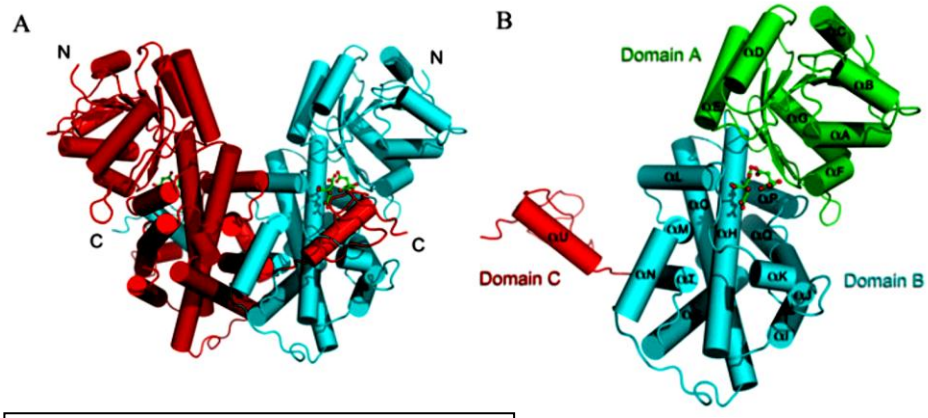

Figure Source: He, W. et al. 2007

***Figure* 1.6: The overall structure and organization of Gnd1**. **(A)** The cartoon representation of Gnd1 homodimer. **(B)** The Gnd1 monomer contains three domains, domain A, B and C coloured in green, cyan and red, respectively.

The conserved cysteine residues observed in Multiple Sequence Alignment of Gnd1p were plotted on its structure and it was found that none of those residues is present at the active site of the enzyme. Therefore, they might have some other critical regulatory function in the enzyme. The marked residues are shown in figure 1.8.

1.7 The role of Cysteine residues in Proteins and their function during oxidative stress

Cysteine is a proteinogenic amino acid with $HO_2CCH(NH_2)CH_2SH$ as its chemical formula [7]. The objective to focus on Cysteine residues in Gnd1p lies in the properties and importance of this residue in various biological functions and especially in Redox signaling.

Cysteine is an uncommonly reactive amino acid because of the presence of nucleophilic sulfur atom which allows it to undergo several chemical modifications [7]. The modifications include redox reactions, lipid acylation, and metal binding motifs that play an important role in protein structure, localization, catalysis and regulation [7]. Though cysteines present at the active site of the enzymes are essential for its functioning, the cysteines at non-active site also play an important role in controlling their biological functions [7][9]. The cysteines at non-active site, as in the case of Gnd1p, might be crucial in regulating the activity of the enzyme through their redox modifications during oxidative stress or other cellular events [9][4]. The thiol (-SH) group in cysteine residues is susceptible to oxidation and leads to formation of disulfide bond, yielding cystine, which has an important structural role in various proteins [4].

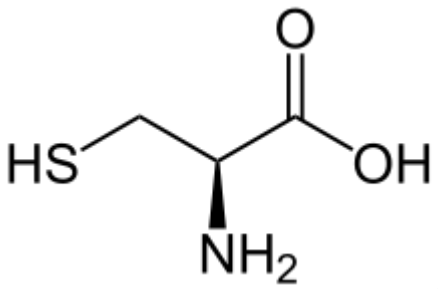

***Figure* 1.7:** Chemical structure of Cysteine

This thesis focuses on understanding the role of conserved cysteine residues in the functioning of the enzyme, Gnd1p, by preparing their mutants and evaluating the NADPH producing capability of Gnd1p and its mutants under normal and oxidative stress conditions. Also, it attempts to create and evaluate *Δgnd1Δgnd2* double knockout phenotype and evaluating in-vivo approaches to determine Gnd1p functionality.

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, fine 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 SnapGene (version 2.4.3) software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Phusion* High-fidelity DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA 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 from BioNEER and Promega. GSH was obtained from Sigma-Aldrich, USA.

2.2 Strains and Plasmids

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* and *Saccharomyces cerevisiae* strains used in the study are given in Table 2.1. The list of various plasmids used in this study is given in Table 2.2.

Table 2.1: List of bacterial and yeast strains used in the study

Strain	Genotype	Source
Escherichia coli strains		
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

Saccharomyces cerevisiae strains		
ABC 733 (BY4741)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Lab stock
ABC 734 (BY4742)	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Lab stock
ABC 5165	*Δzwf1* in BY4741; *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YNL241C::KanMX4*	Euroscarf
ABC 6011	*Δgnd1* in BY4742; *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; YHR183W::KanMX4*	Euroscarf
ABC 6012	*Δgnd2* in BY4742; *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; YGR256W::KanMX4*	Euroscarf
ABC 2614	*Δmet13* in BY4742; *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; YGL125W::KanMX4*	Lab stock
ABC 6013	*Δsol3* in BY4742; *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; YHR163W::KanMX4*	Euroscarf
ABC 6014	*Δsol4* in BY4742; *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; YGR248W::KanMX4*	Euroscarf

Table 2.2: List of plasmids used in the study

Plasmid name	**Clone no.**	**Description**
Plasmids used in the study		
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*. (Mumberg, et al., 1995)

pRS315TEF	ABE 3488	The CEN-vector bearing LEU2 marker and TEF promoter-MCS- terminator for yeast expression and Amp^r marker for selection in *E. coli*. Vector was constructed by excising TEF promoter and MCS from pRS416TEF using SacI and ApaI site and cloned at pRS315 vector at SacI and ApaI site.
pRS313TEF	ABE 3569	The CEN-vector bearing HIS3 marker and TEF promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli*. Vector was constructed by excising TEF promoter and MCS from pRS416TEF using SacI and ApaI site and cloned at pRS313 vector at SacI and ApaI site.
pRS313TEF-ZWF1	ABE5287	ZWF1 cloned from genomic DNA of *S. cerevisiae* in pRS313TEF between BamHI and EcoRI sites.
pRS416TEF-MET13-R357A	ABE5507	Mutation at R357A in MET13 constructed by SOE PCR and cloned between XbaI and ClaI restriction sites
Plasmids constructed during the study		
pRS313TEF-GND1	ABE5984	GND1 cloned from genomic DNA of *S. cerevisiae* in pRS313TEF between BamHI and XhoI sites (1470 bp)
pRS313TEF-GND1-C29S-6XHIS	ABE6022	GND1-C29S mutant constructed by SOE PCR with C-terminal 6X His tag and cloned under TEF promoter in pRS313 vector between BamHI and XhoI sites
pRS313TEF-GND1-C168S, C169S-6XHIS	ABE6045	GND1-C168S,C169S mutant constructed by SOE PCR with C-terminal 6X His tag and cloned under TEF promoter in pRS313 vector between BamHI and XhoI sites

pRS313TEF-GND1-C197S-6XHIS	ABE6031	GND1-C197S mutant constructed by SOE PCR with C-terminal 6X His tag and cloned under TEF promoter in pRS313 vector between BamHI and XhoI sites
pRS313TEF-GND1-C287S-6XHIS	ABE6032	GND1-C287S mutant constructed by SOE PCR with C-terminal 6X His tag and cloned under TEF promoter in pRS313 vector between BamHI and XhoI sites
pRS313TEF-GND1-C365S-6XHIS	ABE6046	GND1-C365S mutant constructed by SOE PCR with C-terminal 6X His tag and cloned under TEF promoter in pRS313 vector between BamHI and XhoI sites
pRS416TEF-GND1	ABE6047	GND1 cloned from genomic DNA of *S. cerevisiae* in pRS416TEF between BamHI and XhoI sites (1470 bp)

2.3. Oligonucleotides

The list of various oligonucleotide primers used in this study is given in Table 2.3.

Table 2.3: List of Oligonucleotides (and their sequences) used in this study

Oligonucleotide name	**Sequence (5' to 3')**
GND1 For.	ACTCGGATCCATGTCTGCTGATTTCGGTTTG
GND1 Rev.	TACGGTCTCGAGTTAAGCTTGGTATGTAGAGG
GND1-6XHIS Rev.	TACGGTCTCGAGTTAGTGGTGATGGTGATGATGAGCTTGGTATGTAGAGG
GND1-C29S For.	CACGGTTTCACTGTTAGTGCTTACAACAGAAC
GND1-C29S Rev.	GAGTTCTGTTGTAAGCACTAACAGTGAAACCG
GND1-C168S,C169S For.	CCGACGGTGAACCAAGTAGCGAATGGGTTGGC
GND1-C168S,C169S Rev.	TGGGCCAACCCATTCGCTACTTGGTTCACCG

GND1-C197S For.	TGATATGCAATTGATTAGTGAAGCTTATG
GND1-C197S Rev.	TCATAAGCTTCACTAATCAATTGCATATC
GND1-C287S For.	TGTCTTTGCCCGTAGTCTATCTGCTTTG
GND1-C287S Rev.	AAAGCAGATAGACTACGGGCAAAGACAG
GND1-C365S For.	AGAGGTGGTAGTATCATTAGATCTG
GND1-C365S Rev.	ACAGATCTAATGATACTACCACCTCTC
GND2::LEU2 Disruption For	ATGTCAAAGGCAGTAGGTGATTTAGGCTTAGTTGGTT TAGGTGGGAATACTCAGGTATC
GND2::LEU2 Disruption Rev	TTAAGCTTGGTAGGTTGAGGAAGATATATTACCTCCG TTAAGCAAGGATTTTCTTAAC

2.4 Media

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water 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 0.2-0.45 µm (for heat labile compounds). Additional amino acids, nutrients and, oxidative reagents such as Methylglyoxal and Diamide 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 Medium (Luria–Bertani) (per 1000 mL)	Yeast extract 5 g/L Tryptone 10 g/L NaCl 10 g/L [pH was adjusted to 7.0 with 1N NaOH]
2.4.2. YPD Medium (Yeast extract Peptone Dextrose) (per 1000 mL)	Yeast extract 10 g/L Peptone 20 g/L Dextrose 20 g/L
2.4.3 SD Medium (Synthetic Defined) (per 1000 mL)	YNB(Yeast Nitrogen Base) 1.7 g/L (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ 5 g/L Dextrose 20 g/L Amino acids (as required) 80 mg/L [pH was adjusted to 6.0–6.5]

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1 Ampicillin (100 mg/mL) stock solution

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and was filter-sterilized using 0.2μm membrane filter.

2.5.2 Glutathione Stock Solution (100mM)

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

2.5.3 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

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

2.5.4 25% Glycerol (used for preparing –80°C stocks of *S. cerevisiae*)

25 mL glycerol is dissolved in 75 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.5 Alkaline Lysis Buffers (Plasmid DNA preparation from *E. coli*)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	0.2N NaOH (freshly diluted from a 10N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	5M Potassium acetate 60 mL Glacial acetic acid 11.5 mL Deionized water 28.5 mL The resulting solution is 3M with respect to potassium and 5M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) (pH 8.0)	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
e) TE-RNAse (stock 10 mg/mL)	Working stock 20 µg/mL in TE Buffer, pH 8.0.

f) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100mL)	a) Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] b) Chloroform 48 ml c) Isoamylalcohol 2 ml Stored at 4°C in a dark brownbottle.

2.5.6 Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA) Buffer (per 1000 mL) **(prepared from 50× TAE stock)**	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G 30% glycerol
c) 0.7-1% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/mL) Stock	Final working concentration used at 0.5µg/mL.

2.5.7 Yeast Transformation Solutions (*S. cerevisiae*) [31]

a) 10X Lithium acetate (per 1000 ml) (pH7.5)

100.2 g of lithium acetate dissolved in 900mL of deionized water. pH adjusted to7.5 with diluted glacial acetic acid and volume bought up to 1000mL. Autoclaved and stored at room temperature. It is autoclaved and stored at RT.

b) 10X Tris-EDTA(TE) (per 1000 ml) (pH7.5)

15.759 g of Tris-Cl and 2.92g of EDTA added to 800mL of deionized water. pH adjusted to 7.5 and volume bought up to 1000mL.Autoclaved and stored at room temperature. It is autoclaved and stored at RT.

c) 50% PEG (Polyethylene glycol) (per 1000 ml)

500g of PEG3500 dissolved in 500mL of deionized water by vortexing. Volume made up to 1000ml by adding deionized water. It is autoclaved and stored at RT.

2.5.8 Solutions for preparation of chemical competent *E. coli* cells

a) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 mL of water. 10 mL of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 mL and autoclaved. Just before use, 5 mL of filter sterilized 2 M $MgCl_2$ was added.
b) SOC	SOB + 20 mM Glucose
c) 10% glycerol	10 mL glycerol is dissolved in 90 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

SECTION B: METHODS

2.6 Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* DH5α strains were routinely grown in LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *Saccharomyces cerevisiae* strains were regularly maintained on YPD medium and grown at 28-30°C. The yeast transformants were selected and maintained on SD medium with amino acid supplements as per requirements.

2.7 Recombinant DNA methodology (restriction digestion, ligation, transformation of *E. coli.* PCR amplification, etc)

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols or as per manufacturer's protocol, unless specifically mentioned. DNA sequencing was done using agrigenome sequencing services.

2.8 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 16-24 hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.1, cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of $1 \cdot 10^9$ cells/mL and divided into 100µL aliquots. Approximately 50 µg (5µL of 10 mg/mL stock solution) of heat denatured, salmon sperm carrier DNA, 0.3µg- 0.7µg of plasmid/DNA fragment and 0.3mL of 50% PEG 3350 (prepared in 0.1M lithium acetate, pH 7.5) were added to each aliquot and whole cell suspension was subjected to heat shock at 42°C for 30 min. The cell suspensions were allowed to cool to room temperature. The cells were centrifuged at 9000 rpm for 5 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

2.9 Construction of *gnd2::*LEU disruption cassette

The *gnd2*::LEU disruption cassette was amplified by PCR with primers from pRS315 followed by agarose gel electrophoresis and elution of the required band.

2.10 Growth assay by dilution spotting

For dilution spotting yeast cells were grown in selective minimal medium. Primary cultures were grown for 12-16 hours at 30°C, with shaking at 200 rpm. Primary cultures were used to inoculate a secondary culture in selective minimal medium for 8 hours or till OD_{600} reaches between 0.6-0.8 at 30°C. Cells were centrifuged , washed with sterile water and resuspended into sterile water at OD_{600}= 0.2.

Four serial dilutions were made in sterile water; $O.D_{600}$= 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°C for 2-5 days and photographs were taken.

2.11 In-vitro Hydroxylamine mutagenesis

Approximately, 10 µg of plasmid DNA was dissolved in 0.5 ml of Hydroxylamine solution (90 mg NaOH, 350 mg hydroxylamine HCl in 5 ml water, pH around 6.5. freshly made up before use). This mixture was incubated at 37°C for 20 hrs, and the DNA was purified using a Qiagen column. Finally, the pool of mutagenized plasmid was directly transformed into the appropriate yeast strain.

2.12 Plasmid isolation from *E.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 the supernatant was discarded. 100 µl Solution 1 was added followed by gentle mixing and incubation in ice for 5 minutes. After that 200µl solution, 2 was added and gently mixed by reverting the microcentrifuge tube and immediately 150µl solution 3 was added by mixing and kept in ice for 10 minutes. The cells were centrifuged at 11,000 rpm for 10 minutes, and the supernatant was collected. An equal volume of phenol-chloroform-isoamyl was added which was mixed by vortexing for 2 minutes, and centrifugation was performed at 13,000 rpm for 5 minutes. The aqueous phase was collected, and an equal volume of isopropanol was added followed by incubation at room temperature for 15 minutes. Centrifugation was performed at 13,000 rpm for 10

minutes, and the pellet was resuspended in 70% ethanol. Centrifugation was performed at 10,000 rpm for 5 minutes, and the supernatant was discarded followed by incubation at 37oC for 30 minutes. The pellet was resuspended in TE-RNAse, and the plasmids were stored at -20°C.

2.13 Transformation of *E.coli*

E. coli competent cells were prepared using the calcium chloride method in accordance with standard protocols [32]. Transformation was carried out by adding plasmid or ligation mixture to the competent cells, incubated, followed by a thermal shock at 42 °C for 1 minute. Transformed cells were incubated in LB at 37 °C for 45 min, and plated on LB medium containing ampicillin.

2.14 Sequence analysis

Protein sequences were retrieved from NCBI and UniProt database and multiple sequence alignment was done using Clustal omega server. JalView software was used for visualization purpose. Protein structures were retrieved from PDB database.

Chapter 3: Results and Discussion

Section A: Results

3.1 Multiple sequence alignment reveals presence of multiple conserved cysteines in 6PGDH

Multiple Sequence Alignment of Gnd1p with its homologs was performed in order to assess sequence conservation of protein domains, and individual amino acids. We found 66% sequence identity between Gnd1p and Human 6PGDH. The central observation was the 85% conservation of cysteine residues among various eukaryotes. Out of 7 cysteines in Gnd1p, 6 were conserved among various eukaryotes as depicted in Fig 1.6.

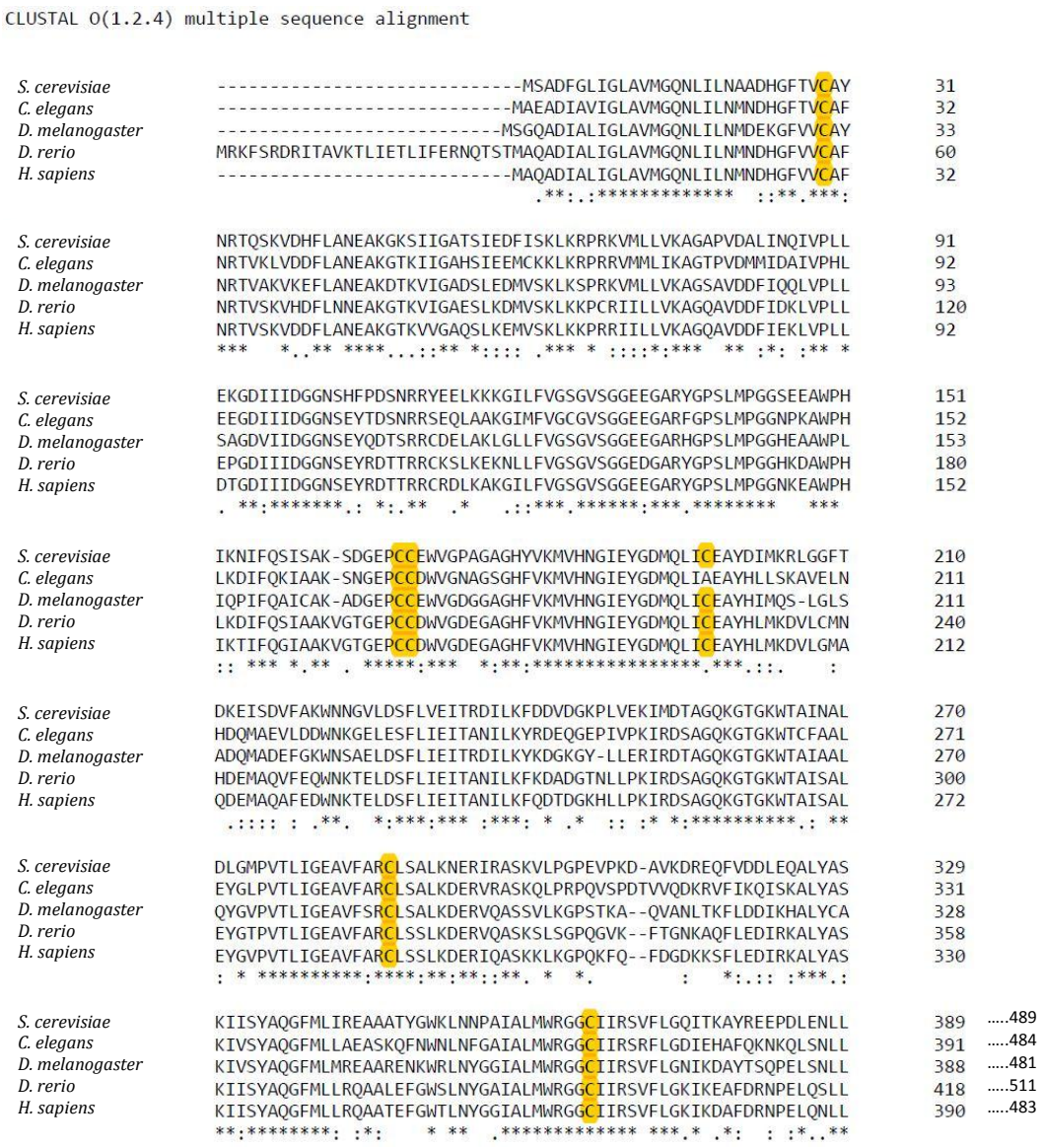

***Figure* 3.1:** Multiple Sequence Alignment of Gnd1p with its eukaryotic homologs.

3.2 Construction of Cysteine to Serine GND1 mutants through Splice overlap extension (SOE)-Site Directed Mutagenesis

The GND1 gene of *S. cerevisiae* was cloned in the pRS313 vector under TEF promoter and the clone was verified by sequencing. The conserved Cysteine to Serine mutants were constructed through SOE-Site directed mutagenesis for functional evaluation [17]. Six conserved cysteine residues (Cys29, Cys168, Cys169, Cys197, Cys287, and Cys365) were mutated to serine residues. In the case of Cys168 and Cys169, they were mutated together and five clones were made. The mutation was done to Serine because of the close structural similarity between Cysteine and Serine residues, although functionally they are different as one of the oxygen atoms is replaced by sulfur in Cysteine.

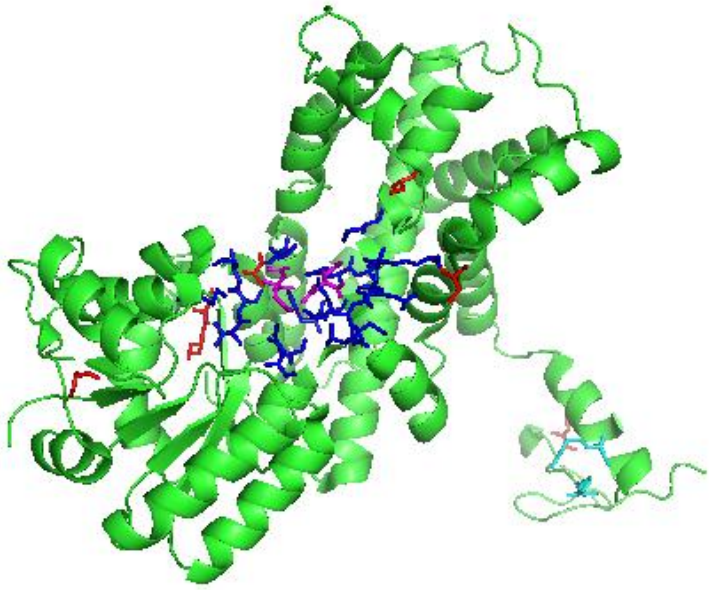

***Figure* 3.2:** The Gnd1 monomer with marked residues. Red: Cysteine, Blue: Active site pocket, Magenta: Substrate. [Source: PDB, PDB ID: 2P4Q]

We were interested in making cysteine to serine mutations in GND1 protein because six of the cysteine residues were highly conserved among the GND1 homologs (from yeast to man) and therefore it seemed likely that they might play some important functions in the protein. Such highly conserved cysteines in cytoplasmic proteins are often seen when the cysteines have a functional role, either in catalysis or in redox regulation. However, as the cysteines in Gnd1p were not in the catalytic site, there was a strong possibility that they may be under redox regulation. Redox regulation could be either through transient oxidation or transient disulfide formation or even as a target of glutathionylation. Given the observation that the ZWF1 protein, the other NADPH producing enzyme

in the same pathway was under post-translational regulation during oxidative stress, there was a strong possibility that some kind of post-translational regulation might be seen in GND1 protein as well. This was precisely what we were interested in investigating. Hence, based on these reasons we took a mutational approach of converting these cysteines to serines, with the intention of evaluating the functional consequences

The five mutants constructed for functional evaluation are as follows:

1. pRS313TEF- GND1-C29S-6XHIS
2. pRS 313TEF-GND1-C168S,C169S-6XHIS
3. pRS 313TEF- GND1-C197S-6XHIS
4. pRS 313TEF- GND1-C287S-6XHIS
5. pRS 313TEF- GND1-C365S-6XHIS

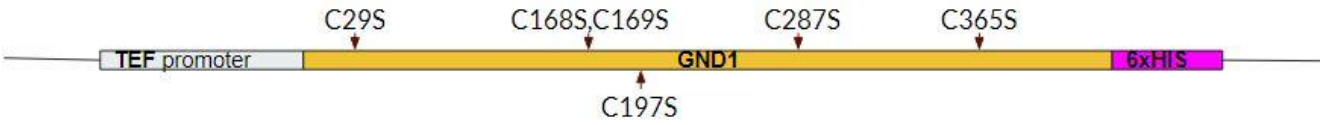

***Figure* 3.3:** A diagrammatic representation of five constructed mutants of GND1. Although they are depicted on the same figure each was a separate clone.

3.3 Characterizing effects of *Δgnd1* and *Δgnd2* under normal and oxidative stress conditions

In order to better understand the role of GND1 and GND2 genes in the cellular metabolism, we procured the *Δgnd1* and *Δgnd2* strains from European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF), respectively, and performed a growth assay by dilution spotting under normal and two different stress conditions to assess the effect on the growth of cells when the gene GND1 or GND2 is deleted. From previous literature it is known that, exposure to reagents such as, Diamide and Methyglyoxal, results in oxidative stress in yeast cells [1][13]. Therefore, these two reagents are used as a source of oxidative stress in this study.

Diamide is an oxidative stress-inducing agent that is used in biochemistry to oxidize thiols in proteins to disulfides [13]. Among the selected oxidative stress-inducing reagents, diamide is a thiol-oxidizing agent that results in oxidation of GSH to GSSG resulting in GSH/GSSG redox imbalance [13].

Methylglyoxal (MG) is a very reactive dicarbonyl compound that is mainly formed as a by-product of glycolysis. MG is extremely toxic to the cells as it results in the modification of proteins by the formation of advanced glycation end-products (AGEs) on them which diminish their function [1]. Methyglyoxal detoxification pathway, also known as Glyoxalase Pathway, is important to get rid of MG from the cells [1]. The pathway utilizes GSH and converts it to oxidized GSSG, to detoxify MG, therefore resulting in lowering the concentration of antioxidant species inside the cells and hence causing oxidative stress [1]. The PPP enzymes are linked to MG detoxification via the formation of NADPH, which is required to recycle back GSH from its oxidized form, GSSG, through the action of Glutathione reductase [1].

The results of the growth assay by dilution spotting on these two reagents are shown in fig. 3.4. After standardization, 8mM concentration of MG was used, whereas 1mM was used for Diamide. It was observed that the *Δgnd1* cells showed lesser growth as compared to *Δgnd2*, and WT strain (BY4742) during normal, as well as, oxidative stress conditions caused by diamide and methyglyoxal. The most probable explanation for this observation can be the decrease in the total NADPH pool inside the cell which is required to recycle the antioxidant GSH, and also the slower rate of production of ribulose 5-phospate (R5P) through non-oxidative PPP, which is required in the production of nucleic acids.

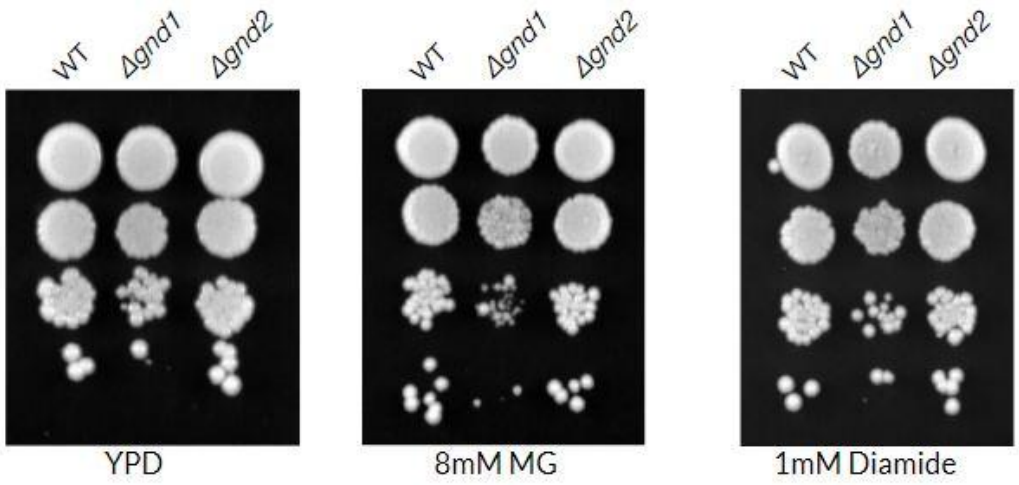

***Figure* 3.4:** Growth of *Δgnd1* and *Δgnd2* under normal and oxidative stress conditions. *Δgnd1* cells show lesser growth as compared to *Δgnd2*, and WT strain (BY4742) during normal, as well as, oxidative stress conditions caused by addition of diamide and methyglyoxal (MG) to the YPD medium. The strains were grown in YPD medium, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 5 µl of each

dilution was spotted onto YPD, YPD containing 8 mM MG, and YPD containing 1 mM Diamide media.

Next, in order to check the effect of deletions of other oxidative PPP enzymes, we did a growth assay by dilution spotting for each of the PPP enzyme deletion. Methionine was added in SD medium as *Δzwf1* is a methionine auxotroph. The results of the growth assay are shown in fig. 3.5. It was observed that *Δzwf1* and *Δgnd1* strains showed growth defects, whereas other strains carrying deletions in the same pathway (*Δgnd2, Δsol3*, and *Δsol4*) did not show any growth defect Again, the most probable explanation for this is the same as given above, that is, the growth defect is due to decrease in the total NADPH pool inside the cell and since the other three enzymes are not much involved in NADPH production, therefore their deletion had little or no effect on the growth of cells. GND2 is involved in NADPH production but its activity levels are not much significant as compared to ZWF1 and GND1. It performs only 20 % of 6PGDH activity.

We also performed growth assay for the strains that were transformed with the plasmids pRS313TEF-GND1 and pRS313TEF-Vector, respectively (Fig. 3.6). We expected that *Δgnd1* strain transformed with pRS313TEF-Vector would show growth defect in both normal condition, as well as, oxidative stress condition caused by MG. However, surprisingly, we observed that the *Δgnd1* strain transformed with pRS313TEF-Vector showed resistance to the oxidative stress caused by MG. The same experiment was repeated two more times and we got the same observation. It appeared that the presence of the HIS3 marker might be making this difference but this is not evaluated yet.

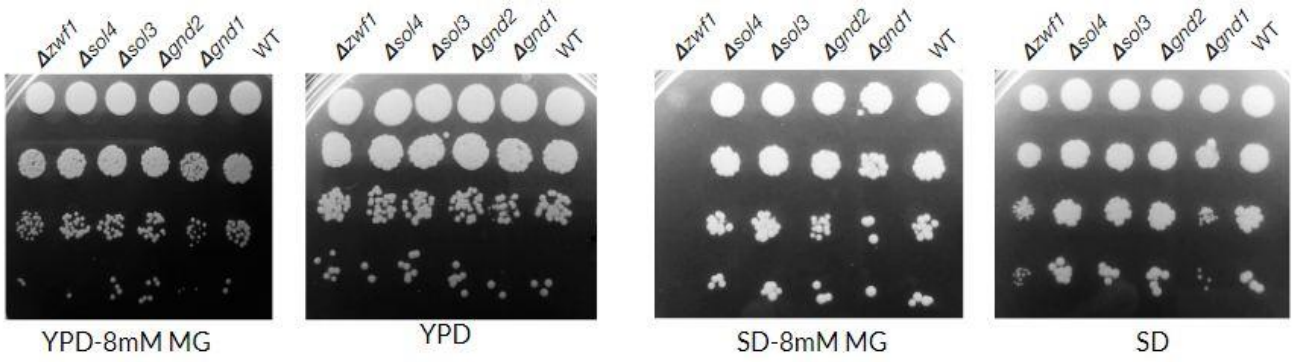

***Figure* 3.5:** Effects of different oxidative PPP enzyme deletions, that is, *Δzwf1, Δgnd1, Δgnd2, Δsol3, and Δsol4*, under normal and oxidative stress condition caused by addition of methyl glyoxal (MG) to the YPD, and SD medium. Methionine was added in SD medium as *Δzwf1* is a methionine auxotroph. *Δzwf1* and *Δgnd1* show growth defect in both normal, as well as, stress conditions. However, no

significant growth defect was observed on normal YPD media. The strains were grown in YPD medium, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 5 µl of each dilution was spotted onto YPD, YPD containing 8mM MG, SD, and SD containing 8mM MG media.

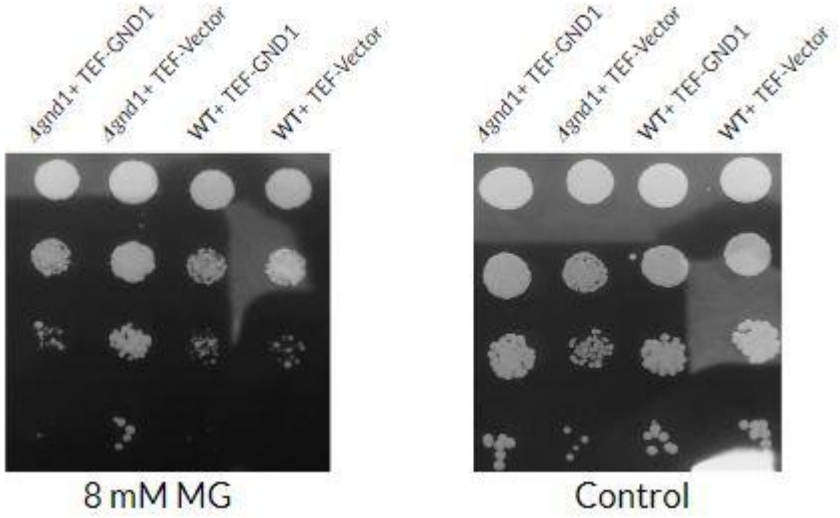

***Figure* 3.6:** *Δgnd1* strain transformed with pRS313TEF-Vector show resistance to the oxidative stress caused by MG. The transformants were grown in SD medium, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 5 µl of each dilution was spotted onto SD, and SD containing 8mM MG media.

3.4 Evaluating *in vivo* approaches to determine Gnd1p functionality

We were interested in comparing the functionality of the different Gnd1p mutants that we created. However, instead of carrying out *in vitro* assays we attempted to standardize *in vivo* assays.

(A) Complementing Methionine Auxotrophy in *Δzwf1*

Methionine auxotrophy is the inability of the cell to synthesize methionine that is required for its growth. *Δzwf1* strain shows Methionine auxotrophy because the cells are not able to synthesize methionine due to the deficiency of NADPH, which is required in the conversion of 5- 10 Methylene Terahydrofolate (THF) to 5- Methyl THF by the enzyme Methylenetetrahydrofolate reductase (MTHFR) in the Methionine biosynthesis pathway (Fig. 3.7) [11][6]. MTHFR is encoded by the gene MET13.

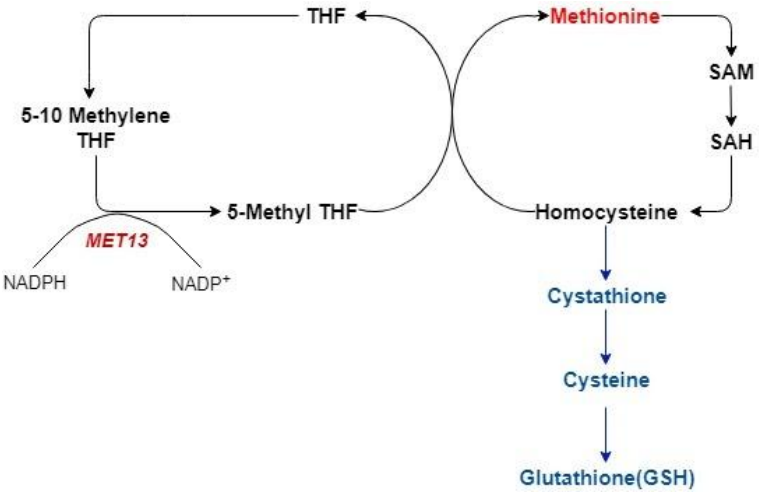

***Figure* 3.7:** The simplified methionine biosynthesis pathway. MET13 converts 5- 10 Methylene THF to 5- Methyl THF, consuming NADPH in the process.

In order to check if GND1 overexpression can complement the Methionine auxotrophy of a *Δzwf1* strain, we overexpressed the GND1 gene in *Δzwf1* strain and performed the growth assay to observe the growth phenotype. We found that the overexpression of GND1 was not able to complement the methionine auxotrophy, as shown in fig. 3.8. The most probable reason is the lack of substrate for the Gnd1p to act upon and produce NADPH. Since, the first enzyme of the oxidative Pentose phosphate pathway is absent, no further products are synthesized in the PPP and hence there is a lack of 6PG for Gnd1p to act upon.

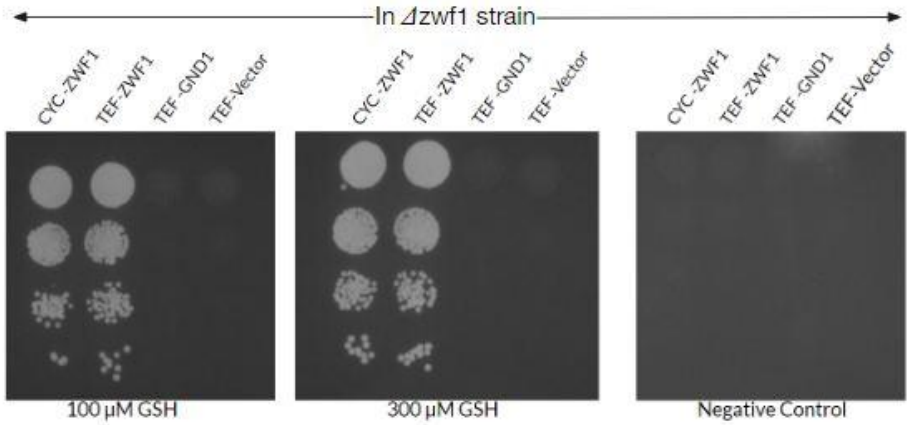

***Figure* 3.8:** Complementing methionine auxotrophy in *Δzwf1*. Overexpression of GND1 was not able to complement the methionine auxotrophy in *Δzwf1*. Micro molar GSH was provided in the media as a sulfur source. The transformants were grown in SD medium containing methionine, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 5 µl of each dilution was spotted onto SD, SD containing 100 µM GSH, and SD containing 300 µM GSH media.

(B) Evaluating a NADPH deficient strain created by deregulated MTHFR (MET13)

MET13 encodes MTHFR, which consumes NADPH to carry out the reaction shown in fig. 3.7. It was found that a mutant of MET13 (MET13-R357A) shows increased consumption of NADPH that results in NADPH deficiency inside the cell to carry out other cellular processes and hence causes growth defect in the cells [*M. Bhatia and A.K. Bachhawat* unpublished results]. In the deregulated mutant 2-3 fold reduction of total NADPH/NADP+ pools has been observed. Previous experiments from the lab showed that this NADPH deficiency can be rescued with the overexpression of ZWF1 as it is the major producer of NADPH inside the cells [*M. Bhatia and A.K. Bachhawat* unpublished results]. Hence, we used this system as an assay for identifying the NADPH producing role of GND1.

In order to compare the NADPH producing capability of ZWF1 and GND1, we overexpressed GND1 in the cells containing only the MET13-R357A mutant without the wild type MET13 [fig. 3.9(A)]. In the other experiment, we overexpressed GND1 in the cells containing both the Wild type MET13, as well as, MET13-R357A mutant [fig. 3.9(B)]. As shown in fig. 3.9, the results of both the experiments were not consistent with the previous observations, where overexpression of ZWF1 rescued the growth defect, and the cells transformed with empty Vector showed growth defect. Considering these observations, we need to check the integrity of the MET13-R357A plasmid construct and if it's present in its original form then the experiment has to be repeated with different or stringent conditions.

(A) (B)

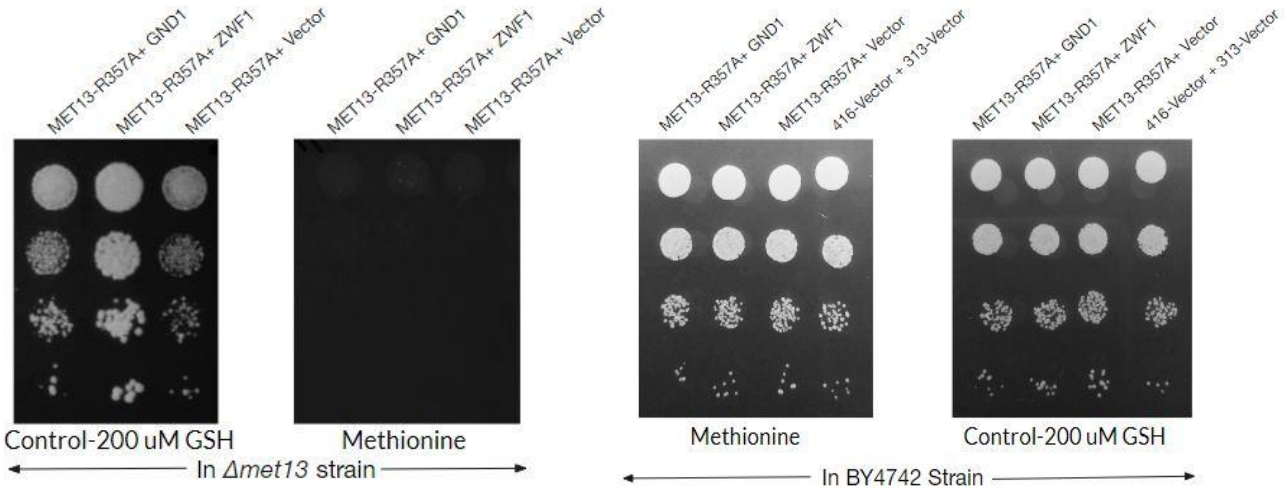

***Figure* 3.9: Evaluating a NADPH deficient strain created by deregulated MTHFR (MET13-R357A).** The transformants were grown in SD medium containing GSH, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 5 µl of each dilution was spotted onto SD containing 200µM GSH, and SD containing Methionine media. (A) MET13-R357A construct was transformed with other gene of interest in *Δmet13* background, resulting in only mutant version of MET13 present in the cells. We did not observe any growth even when ZWF1 was overexpressed, which is not consistent with the previous observations where growth defect was rescued when ZWF1 was overexpressed. (B) MET13-R357A construct was transformed with other gene of interest in BY4742 (WT) background, resulting in the presence of both mutant, as well as, wild type MET13 in the cells. We observed equal growth in all transformants which is contrary to the previous findings where transformants with empty vector along with MET13 mutant showed minimal growth as compared to ZWF1 overexpressed transformants.

3.5 Attempt to create a *Δgnd1Δgnd2* double knockout strain

From the previous literature it is known that, both *Δgnd1* and *Δgnd2* strains are viable but the *Δgnd1Δgnd2* double deletion viability is not yet tested [16]. Some previous findings suggested that *Δgnd1Δgnd2* double deletion can result into a lethal phenotype and the cells can lose viability but this has not been evaluated [16].

However since deletion of the first enzyme in the Pentose phosphate pathway, ZWF1, leads only to methionine auxotrophy, and not lethality, we wanted to understand if *Δgnd1Δgnd2* double deletion is really a lethal phenotype or not [14]. The following experiment was designed to check the viability of the double deletion strain (shown in fig. 3.10).

We have been able to create a *Δgnd2* using *gnd2::LEU* disruption cassette in Wild type strain (BY4742). However, all attempts to create a double deletion of *Δgnd1Δgnd2* in a strain bearing the GND1 on a URA3 plasmid have so far been unsuccessful. It is possible that the experiment has to be repeated with different conditions.

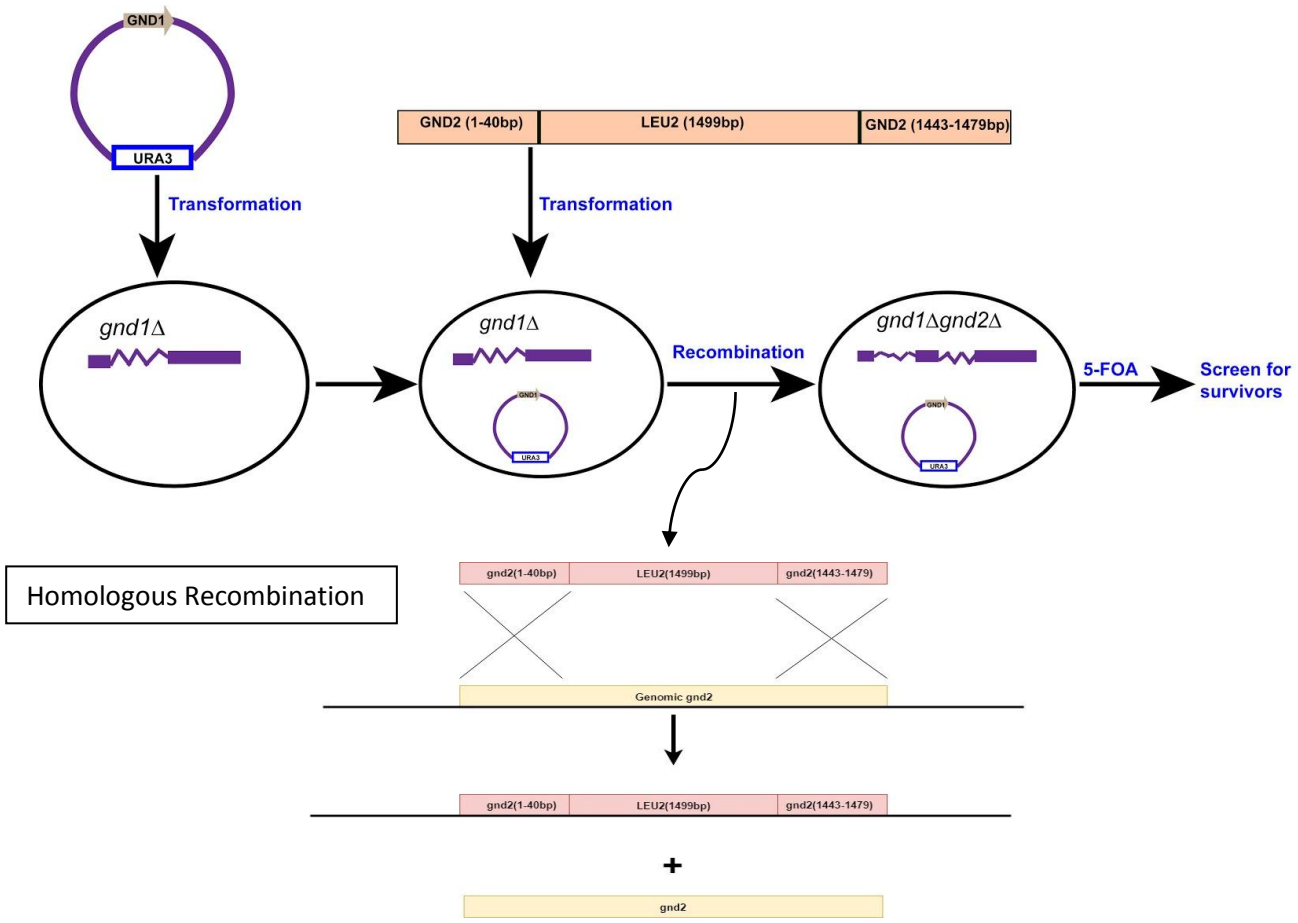

***Figure* 3.10: Evaluating the *Δgnd1Δgnd2* double deletion viability.** A GND1 expressing construct with URA3 marker is transformed into a *Δgnd1* background and the transformants are again transformed with *gnd2::LEU* disruption cassette which disrupts the GND2 gene through homologous recombination. At this stage, the cells lack both genomic GND1 and GND2 gene with a copy of GND1 plasmid construct on a URA3 vector. In order to check the double deletion viability, the cells are now plated on 5-fluoroorotic acid (5-FOA) containing medium. URA3 encodes for the enzyme that performs the decarboxylation of 5-FOA to 5-fluorouracil which is a toxic metabolite for the yeast cells [18]. Therefore, the cells lose URA3 plasmid to save themselves from the toxic metabolite and hence the cells are now *Δgnd1Δgnd2* double deleted. The screening for any survivors is done thereafter and the double deletion is verified using PCR.

Discussion

In this thesis, efforts were initiated to investigate the pentose phosphate enzyme, 6PGDH, in *S. cerevisiae*. We were specifically interested in examining if the protein might be regulated post-translationally since multiple sequence alignments revealed several highly conserved cysteines in the protein.

We constructed cysteine to serine mutants of the GND1 protein. However, we needed a simple method to evaluate their functionality, and tried to discover some simple growth based assays for GND1 functionality. We tried to examine the growth in normal medium as well as in medium containing oxidative stress inducing agents. Although the initial results were encouraging, when we finally transformed these cells with a plasmid vector we observed a surprisingly opposite phenotype. These are interesting findings, but need more detailed investigations. We need to evaluate whether the presence of HIS3 marker in plasmid is making the difference in the results.

We also attempted to create a double deletion of *Δgnd1Δgnd2* as this would be a valuable resource in studies on GND1. We expected that this would be viable, although it could be auxotrophic like the deletion of the first enzyme (ZWF1) of the pathway. However, we have been unsuccessful in our attempts to make the disruption, and perhaps more efforts are needed. It will be interesting to see what would be the phenotype of the double mutant.

Finally, in addition to the in vivo assays, eventually the mutants that we have purified need to be assayed in vitro for their activity and the possible role of these residues in the functionality of the mutants. Since cysteine mutants can have drastic effects on protein folding and stability, therefore, the protein blots need to be done for the mutants.

With the studies that have been initiated, it is hoped that in future the lab would be able to make some significant findings with this enzyme once the assays are carried out. The role of these cysteine residues in oxidative stress would also be interesting to determine.

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