Chapter 1

INTRODUCTION
AND
REVIEW OF LITERATURE
1.1 Introduction: General properties and functions of glutathione

Glutathione (GSH), a tripeptide (γ-glu-cys-gly), is a major low molecular weight thiol compound present in almost all eukaryotes and in some prokaryotes (Meister and Anderson, 1983). The intra-cellular GSH levels have been found to range between 0.1 to 10mM (Hwang et al., 1992). GSH acts as the principal intra-cellular redox buffer and plays a key role in protection of the cell against various oxidants and toxic chemicals thereby helping the cell to maintain an intra-cellular reducing environment (Meister and Anderson, 1983; Penninckx and Elskens, 1993). GSH has been found to participate in scavenging of free radicals and reactive oxygen species like hydroxyl radical, lipid peroxyl radical and H_2O_2 as well as in detoxifying various electrophiles, physiological metabolites (melanine and estrogen etc) and xenobiotics (bromobenzene and acetaminophen etc) (Fang et al., 2002). It also has a role in protein folding and functions in some enzymatic reactions as a co-enzyme. GSH also acts as a reservoir of sulphur and nitrogen in cell (El skens et al., 1991; Mehdi and Penninckx, 1997).

Studies have revealed that redox homeostasis plays a very important role in cellular and metabolic processes that include DNA synthesis, cell cycle regulation, transcriptional activation of specific genes and apoptosis. GSH, as the principle redox buffer, thus plays very important role in the cell (Arrigo, 1999). In recent years, GSH has also been found to play a role in regulation of cellular processes by the glutathionylation of cysteine residues of many proteins thus regulating the functions of proteins participating in signal transduction, cell proliferation and apoptosis under various cellular physiological conditions. Phosphorylase, creatine kinase, carbonic anhydrase, ras, glutathione transferase, GAPDH and other protein have also been shown to be glutathionylated. S-Nitroso glutathione (GSNO) has been observed to play role in storage and transfer of nitric oxide. Glutathionylation is reversible, and the glutathionylation of proteins is determined by physiological conditions like the intra-cellular redox state. Reversible glutathionylation is also associated with initiation and maintenance of various protein complexes (Sies, 1999; Cotgreave and Gerdes, 1998; Mohr et al., 1999).

GSH deficiency contributes towards the increased oxidative stress, which has been associated with many disease states that include liver diseases, macular eye degeneration, alzheimer’s disease, aging, parkinson’s disease, cancer and HIV infections.
GSH has been found to play crucial role in lipid and glucose utilization. Formaldehyde, a cellular metabolite that acts as a potent carcinogen, is detoxified via GSH dependent pathway. GSH is vital for successful and potent immune response as has been observed in case of activation of T-lymphocyte and polynuclear leukocytes and cytokine production (Wu et al., 2004).

In eukaryotes, GSH is essential for growth. In yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* deletion of the first enzyme in GSH biosynthesis leads to growth stasis (unless supplied with exogenous GSH) even under non-stressed conditions. The strains deficient in *de novo* GSH biosynthesis show sensitivity towards oxidative stress conditions as well (Grant et al., 1996; Chaudhuri et al., 1997). In mice, the deletion of the first enzyme leads to embryonic lethality due to GSH deficiency. The embryos failed to gastrulate, did not form mesoderm and died of apoptosis. It was hypothesized that, as GSH and redox state might be involved in embryonic signaling pathways, so its deficiency led to embryonic lethality (Shi et al. 2000).

Overproduction of the rate-limiting enzyme in GSH biosynthesis has recently been demonstrated to lead to increased longevity in *Drosophila melanogaster*. The over-expression of DmGCLc, catalytic sub-unit of DmGCL (*Drosophila melanogaster* Glutamate-Cysteine Ligase or γ-glutamyl-cysteine synthetase) alone in the tissues of central nervous system extended the mean and maximum life span of *Drosophila melanogaster* by increasing the amount of intra-cellular GSH produced (Orr et al., 2005).

Studies have also revealed that the GSH levels need to be carefully maintained during growth of yeast, *S. cerevisiae*. Yeast could utilize maximally 90% of the total cellular GSH as a source of nutrients like glutamate etc. There prevails a general mechanism to restrict the use of GSH beyond certain limit. Cells try to maintain optimal intra-cellular concentration of GSH thereby preventing complete depletion of GSH (Mehdi and Penninckx, 1997). The significant decrease in the cell viability was observed during large-scale depletion of intra-cellular GSH pool below the critical level of 1% of the wild type (Kistler et al., 1986). On the other hand, the elevated level of GSH in the cell can also lead to toxicity. The non-specific glutathionylation of cellular proteins and subsequent inhibition of activities could be a possible reason for the observed toxicity (Srikanth et al. 2005).
Chapter 1

GSH has thus been associated with many important cellular processes. To function as a redox buffer, GSH also needs to be stable within the cell. The unusual γ-glutamyl linkage with cysteine provides the necessary stability to GSH, as no peptidases, except for γ-glutamyl transpeptidase (γ-GT), are known to degrade GSH till date. This results in slow or negligible turn over of GSH, possibly providing the necessary stability for GSH to act as redox buffer (Ganguly et al. 2003).

1.2 Glutathione (GSH) metabolism: The γ-glutamyl cycle

Glutathione metabolism proceeds through the γ-glutamyl cycle (Meister and Anderson, 1983) (Fig 1.1). GSH biosynthesis is a two-step process. The γ-glutamyl-cysteine synthetase enzyme, encoded by GSH1 in S. cerevisiae, catalyses the ligation of glutamic acid and cysteine to form γ-glutamyl-cysteine in an ATP dependent mechanism. Subsequently, glutathione synthetase, encoded by GSH2 in S. cerevisiae, catalyses the ligation of γ-glutamyl-cysteine and glycine to form γ-glutamyl-cysteinyl-glycine (GSH or Glutathione) in an ATP dependent process. Biosynthesis of γ-glutamyl-cysteine is the rate-limiting step in GSH biosynthesis (Jackson, 1969).

The γ-glutamyl transpeptidase enzyme (γ-GT) encoded by ECM38 or CJS2 gene in S. cerevisiae catalyzes the first step in the degradation of GSH. The γ–GT cleaves the γ-glutamyl moiety of GSH and transfers it to suitable acceptor amino acids or di-peptides to form γ-glutamyl-amino acids. The cysteinyl-glycine, also formed during the process of first step of GSH degradation, is further degraded into constituent amino acids i.e. cysteine and glycine, by the action of cysteinyl-glycine di-peptidase (CG-DP). The γ-glutamyl-amino acids are subsequently converted into 5-oxoproline and corresponding amino acids respectively by the enzyme γ –Glutamyl cyclotransferase (γ -GCT). 5-Oxoproline is further converted into L-glutamate by the action of 5-Oxoprolinase (5-OP).

In S.cerevisiae, however, there is a lack of evidence for the existence of a complete γ-glutamyl cycle. The γ-GCT and 5-OP enzyme activities in S.cerevisiae have not been detected (Jaspers et al., 1985). Thus it appears that only a truncated version of the γ-glutamyl cycle may operate in S. cerevisiae.
1.3 GSH transport.

In addition to de novo biosynthesis of GSH, cells can also transport GSH from the extra-cellular medium. GSH uptake has been experimentally shown in bacteria (Sherrill and Fahey, 1998), plants (Jamai et al., 1996) and human epithelial cells (Iantomasi et al., 1997). The gene for a GSH transporter was reported for the first time from the yeast S. cerevisiae (HGT1) and belongs to a novel class of transporters having homologues in only yeasts and plants (Bourbouloux et al., 2000). HGT1 is under transcriptional regulation by the sulphur regulatory pathway. The strong repression by cysteine and methionine and milder repression by GSH indicates the role of HGT1 in scavenging GSH to meet sulphur requirements (Srikanth et al., 2005). Sulphur mediated transcriptional regulation of the sulphur assimilatory enzymes in yeast is dependent on Met4p and Met28p, which along with Cbf1 interact to form a complex on TCACGTG motif found upstream of several sulphur assimilatory genes (MET3, MET10, MET14 and MET16) (Blaiseau and Thomas, 1998) to activate transcription. Met4p along with Met28p can also interact and form a complex with Met31p/Met32p and bind to a different Cis-regulatory promoter element AAACTGTG (Blaiseau et al., 1997) present upstream of many sulphur assimilatory genes to activate transcription. Surprisingly, in the case of HGT1 regulation, both Met28p and Met31p acted as a repressor of transcription (Srikanth et al., 2005). In addition, a new 9-bp cis-acting motif 5'-CCGCCACAC-3' located at −356 to −364 of the HGT1 promoter was identified (Miyake et al., 2002). Later studies revealed that the 9-bp motif could actually be refined to two copies of a 7-bp motif 5'-CGCCACA-3' located at −357 to −363 and −333 to −340 region of the HGT1 promoter as well. Both the copies of CGCCACA motif were found to be essential for the regulation of HGT1 gene expression (Srikanth et al., 2005).

Homologues of the HGT1 transporter have not been found in bacteria and mammals including human. An alternative family of transporters possibly exists in these organisms. In case of animals and especially human, γ-GT may play the major role in transport of GSH across the membrane (Griffith and Meister, 1979; Schulman et al., 1975).

In E. coli, yliABCD, which is a part of the ybiK operon, has recently been found to encode for a glutathione transporter. The yli A, B, C, D structural genes are present
downstream of *ybiK ORF* in this operon. Detailed sequence analyses have revealed that *YliABCD* belongs to the ATP-binding cassette transporter superfamily, where *YliA* and *B* form the ATP binding component and periplasmic binding protein respectively, and *Yli-C* and *D* are the plasma membrane component with six and 7 transmembrane helices respectively. The homologues of *yliABCD* have been found in *Shigella flexneri*, *Salmonella enterica* serovar typhi and *Salmonella enterica* serovar typhimurium (Suzuki et al., 2005).

1.4 GSH degradation in the γ-glutamyl cycle: γ-glutamyl transpeptidase

GSH degradation proceeds through the γ-glutamyl cycle. The γ-glutamyl transpeptidase is the first enzyme involved in the degradation of glutathione. The γ-glutamyl transpeptidase has been the only enzyme considered to be involved in breakage of γ-glutamyl bond of GSH leading to the release of the cysteinylglycine di-peptide. The cysteinylglycine dipeptide is in turn cleaved by a cysteinylglycine di-peptidase (Meister and Anderson, 1983). In mammals, CGDP activity has been reported in hog kidney membrane fraction (Semenza, 1957). Mammalian alanyl-aminopeptidase (APM) (Rankin et al., 1980) and leucyl-aminopeptidase (Josch et al., 2003) have also been found to exhibit CG-DP activity. In yeast, based on biochemical evidences, CG-DP activity has been described earlier (Jaspers et al., 1985) but the ORF has not been characterized yet.

Intra-cellular turn over of GSH is very minimal under normal condition. In *S. cerevisiae*, when grown in ammonium sulphate medium, GSH half-life was found to be approximately 900min with very little turnover (Jaspers et al., 1985) but in a subsequent study where cells were deprived of sulphate, increased γ-GT activity was found and significant GSH turnover was observed (Elsken et al., 1991). Studies form this lab has also revealed that GSH turnover is minimal under growth conditions with adequate nitrogen or sulphur sources (Kumar et al., 2003b).

*In vitro* characterizations have led us to understand that γ-GT can catalyze three types of reactions, namely (a) Transpeptidation (b) Auto-peptidation and (c) Hydrolysis. In the transpeptidation reaction, the γ-GT cleaves and transfers the γ-glutamyl moiety to an acceptor which may be L-isomers of methionine, cysteine etc. The γ-GT can also transfer the already cleaved γ-glutamyl moiety to GSH following a reaction mechanism,
which is called Auto-transpeptidation. In a third type of reaction, the $\gamma$-GT hydrolyse $\gamma$-glutamyl moiety using water molecule (McIntyre and Curthoys, 1979; Tate and Meister, 1985).

In mammalian cells, the $\gamma$-GT enzyme is anchored at the plasma membrane with the active site towards the extra-cellular milieu (Finidori et al., 1984). In *E. coli*, the $\gamma$-GT was observed to be a soluble periplasmic enzyme (Suzuki et al., 1987). In yeast, the $\gamma$-glutamyl transpeptidase was found to be a vacuolar membrane bound enzyme (Jaspers and Penninckx, 1984).

Sequence analyses of $\gamma$-GTs from various organisms have revealed that the polypeptide is translated as an inactive precursor, which undergoes post-translational N-terminal modification to become catalytically active. Thus $\gamma$-GT belongs to the N-terminal nucleophile (Ntn) hydrolase superfamly (Brannigan et al., 1995). The processing is autocatalytic and the oxygen atom of the side chain of Thr-391 (N-terminal residue) acts as the catalytic nucleophile (Suzuki and Kumagai, 2002) that is conserved in all organisms. In mammals, the $\gamma$-GT is produced as a single pro-polypeptide (Curthoys and Hugey, 1979), which subsequently gets cleaved auto-catalytically and matures into hetero-dimeric glycoprotein (Barouki et al., 1984). The yeast $\gamma$-GT enzyme, encoded by the ORF *YLR299w (ECM38/CIS2)* (Mehdi et al., 2001) is also a hetero-dimeric protein.

Surprisingly, the $\gamma$-GT activities were found to be different in two congenic *S. cerevisiae* strains namely BY4742 and YPH499 (both derived from S288c parent strain). Detailed investigations have led to the finding that *ECM38* locus (which encodes for $\gamma$-GT) has several nucleotide polymorphisms in YPH499. Two of the polymorphisms led to the changes in amino acids, H171R and G494D. The G494 is found to be a highly conserved residue in $\gamma$-GT across many organisms and was thought to be responsible for the lack of $\gamma$-GT activity in some strains of *S. cerevisiae* (Kumar et al., 2003a).

Preliminary studies on the regulation of $\gamma$-GT have led to a greater understanding of the role of $\gamma$-GT in *S. cerevisiae*. The $\gamma$-GT was primarily found to be under nitrogen regulation. Growth on glutamate (a non-repressing source of nitrogen) led to increase in promoter activation whereas presence of ammonium represses the transcription. Methionine, cysteine and glutathione showed only mild repression of $\gamma$-GT activity. The expression of genes under nitrogen regulation is controlled by four GATA family
transcription factors, namely two positive regulators (Gln3p and Gat1p) and two negatively regulators (Gln3p and/or Dal80p and Deh1p) (Coffman and Cooper, 1997). These transcription factors has been found to bind to 5′-GATA-3′ motif found in upstream sequence of ORFs whose expression is under the control of nitrogen regulation. Ure2p has also been found to negatively regulate gene expression under nitrogen regulation (Courchesne and Magasanik, 1988). Studies revealed that the γ-GT activity was found to be significantly repressed in gln3Δ strain and constitutively expressed in ure2Δ strains (Kumar et al., 2003b). Earlier biochemical studies had also showed that the γ-GT activity was upregulated under nitrogen starving conditions. The highest γ-GT enzyme activities were found under de-repressing sources of nitrogen like glutamate, proline or urea and the γ-GT enzyme activity was down regulated in presence of ammonium (NH₄⁺) as nitrogen source. In presence of various nitrogen sources like glutamine, arginine, ornithine or citrulline, the γ-GT enzyme activity was found to up-regulated up to intermediate level (Penninckx et al., 1980). During nitrogen starvation growth condition, in yeast S. cerevisiae, GSH pool distribution was observed to undergo changes. Most of the cellular GSH was shifted towards vacuole and subsequently, 90% of the accumulated vacuolar pool of GSH, acting as a source of nitrogen, underwent degradation by the increased activity of γ-GT. Increased de novo biosynthesis of GSH was also found to be associated with nitrogen starvation condition (Mehdi and Penninckx, 1997). GATA zinc-finger transcription factors like Nil1p and Gln3p were observed to play crucial role in up-regulation of γ-GT expression during nitrogen starvation. Gzf3p, another GATA zinc-finger transcription factor, was found to negatively regulate γ-GT expression. During growth on a preferred nitrogen source like NH₄⁺, γ-GT expression was observed to remain repressed through a mechanism involving the Gln3-binding protein Ure2p/GdhCRp (Springael and Penninckx, 2003).

Oxidative stress conditions have been found to induce the expression of mammalian γ-GT gene. Under these conditions, intra-cellular GSH concentration depletes thereby making cell sensitive to oxidants. These conditions induce an antioxidant response, which subsequently induces γ-GT gene expression (Hamilton et al., 1985). The transport and degradation of extra-cellular GSH by the γ-GT located at the plasma membrane ultimately produces cysteine, which is a limiting amino acid in intra-
cellular GSH synthesis and re-synthesized into GSH in the cytosol. This in turn helps the cell to maintain the internal redox buffer homeostasis and also xenobiotic detoxification capacity (Meister, 1984; Hanigan and Ricketts, 1993).

1.5 Alternative Pathway of GSH Degradation: Genetic Evidence

To explore if γ-GT was the only enzyme involved in GSH degradation, genetic experiments were carried out in this laboratory by previous workers (Kumar et al., 2003b). *S. cerevisiae* contains a single γ-GT, encoded by ECM38 which was knocked out in the a metl5L1 background to yield a metl5L1ecm38Δ mutant. Strains defective in MET15 are organic sulphur auxotrophs as they lack the enzyme required for the reduction of sulphate (Thomas et al., 1992) (Fig 1.2). The metl5A strain cannot utilize inorganic sulphur sources and are strict organic sulphur auxotrophs. They can utilize methionine, cysteine and GSH as sole source of sulphur. The growth of metl5A and metl5Aecm38 strains was analyzed on medium containing methionine, cysteine or glutathione as sole source of sulphur. If γ-GT was indispensable for utilization of glutathione as sulphur source then metl5Aecm38 strains would not grow on medium supplemented with glutathione as source of sulphur. Surprisingly, both on plates and in broth, GSH could be effectively utilized as a sulphur source in both metl5A and metl5Aecm38 strains when grown in medium supplemented glutathione as sole source of sulphur (Kumar et al., 2003). These observations proved the presence of an alternative pathway of GSH degradation independent of γ-GT.

1.6 Alternative Pathway of GSH degradation: pathway via cysteine formation.

Efforts were made to analyze, genetically, the possible route of GSH degradation and to examine if the degradation be proceeding via intermediates like homocysteine, cystathionine, methionine, cysteine, sulphate or some unknown intermediate (Thomas and Surdin-Kerjan, 1997). It is known that the STR2 gene encodes cystathionine-γ-synthase, which converts cysteine to cystathionine. The str2Δ strain cannot utilize cysteine as a sole source of sulphur as the routes to homocysteine and methionine are blocked (Hansen and Johannsen, 2000) (Fig 1.2). The possibility of cysteine being the intermediate was most likely and was examined by checking the ability of str2Δ strain to
cellular GSH synthesis and re-synthesized into GSH in the cytosol. This in turn helps the cell to maintain the internal redox buffer homeostasis and also xenobiotic detoxification capacity (Meister, 1984; Hanigan and Ricketts, 1993).

1.5 Alternative Pathway of GSH Degradation: Genetic Evidence

To explore if γ-GT was the only enzyme involved in GSH degradation, genetic experiments were carried out in this laboratory by previous workers (Kumar et al., 2003b). *S. cerevisiae* contains a single γ-GT, encoded by *ECM38* which was knocked out in the a met15Δ background to yield a met15Δecm38Δ mutant. Strains defective in MET15 are organic sulphur auxotrophs as they lack the enzyme required for the reduction of sulphate (Thomas et al., 1992) (Fig 1.2). The met15Δ strain cannot utilize inorganic sulphur sources and are strict organic sulphur auxotrophs. They can utilize methionine, cysteine and GSH as sole source of sulphur. The growth of met15Δ and met15Δecm38 strains was analyzed on medium containing methionine, cysteine or glutathione as sole source of sulphur. If γ-GT was indispensable for utilization of glutathione as sulphur source then met15Δecm38 strains would not grow on medium supplemented with glutathione as source of sulphur. Surprisingly, both on plates and in broth, GSH could be effectively utilized as a sulphur source in both met15Δ and met15Δecm38 strains when grown in medium supplemented glutathione as sole source of sulphur (Kumar et al., 2003). These observations proved the presence of an alternative pathway of GSH degradation independent of γ-GT.

1.6 Alternative Pathway of GSH degradation: pathway via cysteine formation.

Efforts were made to analyze, genetically, the possible route of GSH degradation and to examine if the degradation be proceeding via intermediates like homocysteine, cystathionine, methionine, cysteine, sulphate or some unknown intermediate (Thomas and Surdin-Kerjan, 1997). It is known that the STR2 gene encodes cystathionine-γ-synthase, which converts cysteine to cystathionine. The str2Δ strain cannot utilize cysteine as a sole source of sulphur as the routes to homocysteine and methionine are blocked (Hansen and Johannsen, 2000) (Fig 1.2). The possibility of cysteine being the intermediate was most likely and was examined by checking the ability of str2Δ strain to
Fig 1.2 Schematic representation of the sulfur assimilation pathway in *S. cerevisiae*. Met3, 14, 16, 1, 5, 8, 10: sulphate-reducing pathway; *MET15*: O-acetylmethionine sulphydrylase; *MET6*: homocysteine methyl-transferase; *SAM1*, *SAM2*: S-adenosylmethionine synthase; *SAH1*: S-adenosylhomocysteine hydrolase; *STR4*: cystathionine β-synthase; *STR1*: cystathionine γ-lyase; *GSH1*: γ-glutamylcysteine synthetase; *GSH2*: GSH synthetase; *ECM38*: γ-glutamyl transpeptidase; *CGase*: cysteinylglycine dipeptidase; *STR2*: cystathionine γ-synthase; *STR3*: cystathionine β-lyase; APS: 5'-adenylylsulphate; PAPS: 3'-phospho-5'-adenylylsulphate.
utilize GSH as sole source of sulphur. The studies revealed that \textit{str2A} strain could not
grow on cysteine or GSH as the sole source of sulphur. This suggested that utilization of
GSH as sole source of sulphur by the alternative pathway essentially proceeds via

Efforts were also made to determine the possible role of various known peptidases
of \textit{S. cerevisiae} in the GSH degradation by this alternative pathway. The major
aminopeptidases, carboxypeptidases and dipeptidases of \textit{S. cerevisiae} were not found to
play any role in the alternative pathway of GSH degradation. Proteases, which play role
in vacuolar biogenesis and vacuolar autophagy, were not found to play any role in GSH
degradation by the alternative pathway of GSH degradation as well. It was evident that
GSH degradation was not taking place inside the vacuole. Furthermore, the proteosome
was also not found to be involved in this pathway of GSH degradation since proteasomal
sub-unit mutants grew well on GSH as sole source of sulphur (Kumar C, Ph.D. thesis,
2005).

\textbf{1.7 Alternative pathway of GSH degradation: Genetic studies reveal three
complementation groups.}

As the known peptidases of \textit{S. cerevisiae} were not found to be involved in
degradation and subsequent utilization of GSH as sulphur source in the alternative
pathway, so attempts were made to isolate mutants defective in utilization of glutathione
as sole sulphur source in a \textit{met15Aecm38A} strain background (Kumar C, Ph.D. thesis,
2005). This strain background was chosen to ensure no participation of \textit{\gamma}-GT in the entire
study in any possible way. Mutant colonies that grew on methionine and cysteine but
failed to grow on glutathione were selected for further analyses. Upon detailed
complementation analyses, mutants could be classified into 4 complementation groups,
which were named as \textit{dug1, dug2, dug3} and \textit{dug4} (Kumar C, Ph.D. thesis, 2005) (Fig
1.3). A failure to transport GSH could also lead to defect in utilization of GSH as sole
source of sulphur, so it was necessary to screen out mutants, which were defective in
GSH transport. Previous studies from our lab have led to the identification of \textit{S.
cerevisiae} Hgt1p, the only high affinity glutathione transporter discovered so far from
ABC1083 (met15::ecm38Δ)

mutagenized by EMS (90% killing)

40,000 colonies screened on SD+GSH, SD+methionine, SD+cysteine

45 mutants defective in growth on GSH

12 revertants  33 stable mutants

Complementation analysis

$\text{dug}1$  \hspace{1cm} $\text{dug}2$  \hspace{1cm} $\text{dug}3$  \hspace{1cm} $\text{dug}4$

(2) \hspace{1cm} (3) \hspace{1cm} (5) \hspace{1cm} (23)

$\text{DUG1}$ cloned by library Complementation

Identical to $\text{HGT1}$ complementation group (GSH transport)

$\text{DUG1} = \text{YFR044c}$ (M20A peptidase family)

Fig 1.3 Alternative Pathway: Mutant isolation and cloning of $\text{DUG1}$ gene (Kumar C., Ph.D. Thesis, 2005)
any organism (Bourbouloux et al., 2000). Complementation analyses revealed that \textit{dug4} and \textit{hgt1} fell under the same complementation group (Kumar C, Ph.D. thesis, 2005).

\textbf{1.8 The Duglp belongs to M20A peptidase family: Zn$^{2+}$ metallohydrolase enzyme}

The gene corresponding to \textit{dug1} mutant complementation group was identified by transforming \textit{dug1} with \textit{LEU2} based yeast genomic library on a multi-copy vector. Transformants were selected on medium supplemented with GSH as sole sulphur source. Further analyses of \textit{dug1} mutants revealed that the \textit{YFR044c} gene corresponds to the WT gene of the \textit{dug1} (Kumar C, Ph.D. thesis, 2005).

The \textit{Yfr044cp} sequence analyses revealed that it belongs to metallohydrolase of the MEROPS MH clan of metallopeptidase (Rawling et al., 2006). This clan contains 6 families namely M18, M20, M25, M28, M40 and M42 with enzymes of known activities such as carboxypeptidases or aminopeptidases. Further sequence analyses of \textit{Yfr044cp} and it's homologues revealed that \textit{Yfr044cp} belongs to M20 family and M20A subfamily of Zn$^{2+}$ dependent metallohydrolases (aminoacylase-1 family). Various other homologues namely, glutamate carboxypeptidase, peptidaseV, cytosolic non-specific dipeptidase, carnosinase, aminoacylase, acetyl ornithine deacetylase belong to M20A subfamily of metallohydrolases.

However, the mechanism by which Duglp was involved in GSH degradation could not be ascertained in this study.

\textbf{1.9 Aim and objective of the work:}

As described in the introduction, earlier work done in this laboratory clearly established the presence of an alternative pathway of glutathione degradation in \textit{S. cerevisiae}.

Genetic studies identified three mutant complementation groups involved in the degradation of which the wild type gene for one of the mutant \textit{dug1} was cloned and identified, but its mechanism of action was not ascertained.

With this background, the objectives of the present work were defined as follows:
Chapter 1

(1) To identify and characterize the genes belonging to \textit{dug2} and \textit{dug3} mutant complementation groups.

(2) Functional characterization of \textit{DUG} genes

(3) To decipher the mechanism by which Dug1p, Dug2p and Dug3p function in GSH degradation.