Glutathione (GSH) is the most abundant thiol compound present in living organisms and it performs numerous functions within the cell. Cells obtain their GSH either by endogenous synthesis or by the uptake of GSH through specific transporters. Very little is known about the genes encoding the GSH transport systems in various organisms. Recently, our laboratory reported the identification, cloning and characterization of a high affinity GSH transporter, Hgt1p from *S. cerevisiae*. Hgt1p encoded by the ORF *YJL212c*, belongs to a novel class of transporters, the Oligo Peptide Family (OPT family), and represents the first high affinity glutathione transporter to be described in any system so far. However, confusions surrounded the understanding of the true function of this protein when a second group reported the same ORF *YJL212c* as an ologopeptide transporter (Opt1p) since it could mediate the uptake of the penta-peptide enkephalin. The primary objective of this work was, therefore to understand this transporter in greater detail and to examine it's role in the regulation of GSH homeostasis in the cell.

Detailed studies were carried out on the regulation of *HGT1*. The studies revealed that *HGT1* expression was repressed by the organic sulphur sources methionine and cysteine, and mildly repressed by GSH. By genetic analysis it was found that cysteine or one of it's metabolites was the key molecule causing this repression. Furthermore, the classical sulphur regulatory network and not the nitrogen regulatory network, was found to regulate *HGT1*. These findings suggested that the physiological function of Hgt1p/Opt1p was GSH transport and not general peptide transport. The Met4p protein, a key transcription activator of the sulphur biosynthesis genes, was found to be responsible for the expression of *HGT1*, while Met28p and Met31p were involved in negative regulation of *HGT1* expression. The involvement of the VDE endonuclease in *HGT1* regulation that was described by a different group did not appear to have any role in the sulphur mediated regulation of *HGT1*. The cis-regulatory elements responsible for conferring sulphur regulation of *HGT1* were investigated. The *HGT1* promoter lacked the known cis elements present in the promoters of the sulphur biosynthetic pathway genes. Investigations into the cis-regulatory elements by using a comparative genomics strategy (phylogenetic footprinting) as well as mutational analysis revealed that the previously described motif, a 9-bp cis-element 'CCGCCACAC', located at the -356 to -364 region of the promoter could in fact be refined to a 7-bp motif 'CGCCACA' that is also repeated at -333 to -340. Moreover, another novel cis

element, 'GCCGTcTGCAAGGCA' conserved in the *HGT1* promoters of the different *Saccharomyces* spp. was observed at position –300 to –285 and was also experimentally found to be involved in the negative regulation of *HGT1* expression. The strong repression at the transport level by glutathione seen in strains constitutively expressing Hgt1p was found to be a consequence of a glutathione-dependant toxicity that resulted from a 5-6 fold increase in total intracellular GSH and could not be abrogated in cells that had a lower GSH/GSSG ratio

Structure function studies of Hgt1p were also initiated. Hgt1p represents the first member of the OPT family that has been subjected to such an analysis. A method commonly used for studying the structure-function related aspects of membrane proteins is the Substituted Cysteine Accessibility Method (SCAM). This cysteine substitution strategy requires a functional cysteine-free protein. With a long-term goal of using the SCAM for Hgt1p structure-function analysis, we sought to generate a cysteine-free-Hgt1p. Wild type Hgt1p has 12 native cysteine residues. Using site directed mutagenesis these 12 cysteines were mutated to generate a cysteine-free Hgt1p. A dual, complementation-cum-toxicity plate assay, based on the complementation of organic sulphur requirements of an auxotrophic yeast strain (*hgt1Δmet15Δ*) that was also lacking in the GSH transporter, was devised to test the functionalities of the mutant GSH transporters. The assay simultaneously evaluated the toxicity of GSH at higher concentration in the presence of a functional overexpressing transporter. Using this plate assay various cys-deficient mutants of Hgt1p were analyzed for function. The plate assay correlated well with direct uptake assays. These studies revealed that the cysteine-free Hgt1p retained only negligible transport capabilities. To obtain a functional cys-free mutant, an intragenic suppressor screen was carried out by *in vitro* mutagenesis on the gene encoding cys-free Hgt1p to identify mutations that can suppress the non-functional phenotype. Several suppressors were obtained, but sequence analysis revealed only a single suppressor class, bearing a serine to cysteine double reversion for 2 of the native cysteines (S622C and S632C). The C622 and C632 of native Hgt1p are the conserved cysteines of the OPT family. Independent mutagenesis experiments yielded the same double mutant (S622C and S632C). Analysis of Hgt1p bearing single mutations at C622S or C632S further confirmed the essential requirement of both residues for proper functionality.

Substrate specificities of Hgt1p were also investigated. The initial studies revealed that the analogues, γ-glu-cys-ser and γ-glu-cys-glu, were able to substitute the requirements of GSH in yeast cells, while a related compound, γ-glu-cys-β ala (Homoglutathione), could not. γ-glu-cys-ser and γ-glu-cys-glu were also toxic to the yeast strains that were overexpressing Hgt1p revealing that Hgt1p could transport these compounds as well. Furthermore, the dipeptidyl derivative of GSH, γ-glu-cys was found to be transported by both Hgt1p as well as by the dipeptide transporter Ptr2p. The Ptr2p mediated transport was weaker compared to that of Hgt1p as seen on plate assays. Transport of cys-gly, on the other hand was seen to be dependent on Ptr2p and not on Hgt1p. These findings suggest that the γ-glu-cys moiety might be an important determinant of substrate specificity for Hgt1p and not for Ptr2p. Investigations into the regulation and GSH transport capacity of Ypr194cp/Opt2p, a close homologue of Hgt1p (encoded by *YPR194c*), could not uncover growth conditions required for Ypr194cp/Opt2p expression. Studies further revealed that the overexpression of Ypr194cp/Opt2p also did not allow plasma membrane GSH transport although an N-terminal deletion of this protein displayed very weak plasma membrane GSH transport (as seen on plate assays).

Studies on the role of GSH and the vacuolar membrane localized glutathione-conjugate pumps of yeast in the detoxification of a widely used mammalian analgesic drug, acetaminophen (or paracetamol) was investigated in this thesis. In humans, acetaminophen is toxic under conditions of overdose or in certain disease conditions, but the mechanism of acetaminophen toxicity is still not entirely understood. To obtain fresh insights into acetaminophen toxicity, we have investigated the toxicity of this compound in yeast. Although a previous report has described that acetaminophen is not toxic in yeast, it was found that this drug was toxic to yeast cells, with *erg* mutants (ergosterol biosynthetic mutants) displaying hypersensitivity. Yeast cells grown in the presence of acetaminophen were found to accumulate intracellular acetaminophen, but no metabolic products of acetaminophen could be detected in these extracts. The toxicity response did not lead to an oxidative stress response, although it did involve Yap1p. The strains overexpressing Yap1p exhibited resistance at high concentration of the drug revealing that this regulator was a mediator of acetaminophen response in yeast. The cytochrome P450 enzymes of yeast, Erg5p and Erg11p, did not appear to participate in this process, unlike the mammalian systems. Furthermore, glutathione depletion or overproduction in yeast strains did not alter the

drug sensitivity, thereby revealing the absence of a central role for glutathione in acetaminophen toxicity. This suggested differences from the mammalian systems in the detoxification pathways. Investigations on the resistance mechanisms revealed that deletion of the glutathione-conjugate pumps, Ycf1p (a target of Yap1p) and Bpt1p, surprisingly, led to acetaminophen resistance, while overexpression of the multidrug resistance pumps, Snq2p and Flr1p (also targets of Yap1p), led to acetaminophen resistance. The Yap1p dependent resistance to acetaminophen was also examined in detail. It was observed that the Yap1p mediated defense required a functional Pdr1p or Pdr3p protein. Although strains lacking the stress regulator Yrr1p were sensitive to acetaminophen, the Yap1p dependent response did not require a functional Yrr1p. In contrast, resistance mediated by Pdr1p/Pdr3p did not require a functional Yap1p, and revealed a distinct hierarchy of transcription factors in the resistance to acetaminophen. These studies revealed several novel, and hitherto unconsidered aspects in the cellular response to acetaminophen.