Glutathione, γ-glutamyl-cysteinyl-glycine, (GSH) is the most abundant non-protein thiol compound present in several prokaryotic and almost all eukaryotic organisms. Intracellular GSH levels have been found to range between 0.1 to 10 mM. GSH acts as the principal intracellular redox buffer in eukaryotic cells. It has a low redox potential (-240mV) and highly reactive thiol group, which causes it to play a significant role in the maintenance of the cellular redox environment and the detoxification of various toxic compounds. It also contributes to the cellular nitrogen and sulphur stores. GSH reservoirs in the cell are maintained by the *de novo* synthesis of GSH as well as uptake from the extracellular medium by specific transporters. Hgt1p was identified as a specific glutathione transporter from the yeast *Saccharomyces cerevisiae*. It is a 799 amino acid, polytropic membrane protein, belonging to the Oligopeptide Transporter (OPT) family, a family of transporters largely restricted to fungi and plants, with a few members in the bacteria, and no apparent homologues in higher animals. None of the other OPTs from either plants or fungi have been demonstrated to function as high affinity transporters for glutathione in a manner similar to Hgt1p.

One of the goals of this thesis was therefore, to identify the role of the OPT family members of *S. pombe* in glutathione transport. The *S. pombe* genome has revealed the presence of three members of the oligopeptide transporter family. These ORFs are *Isp4*$^+$ shown to transport oligopeptides, as well as two other hypothetical ORFs, *SPAC29B12.10c* and *SPCC1840.12*. These three proteins displayed 37%, 53% and 32% identity at the amino acid level respectively, to the *S. cerevisiae* glutathione transporter, Hgt1p. The ORFs *Isp4*$^+$, *SPAC29B12.10c*, and *SPCC1840.12* were cloned but they failed to complement the *S. cerevisiae hgt1Δ* for growth on glutathione. The ORFs were subsequently deleted in a *cys1aΔ* (Cysteine auxotroph) strain of *S. pombe* and examined for the growth on glutathione. Disruption of ORF *SPAC29B12.10c* in a *cys1aΔ* background showed a very clear defect of growth on glutathione, even at high concentration of glutathione. In contrast, disruption of *Isp4*$^+$ or the ORF *SPCC1840.12* did not show any defect of growth on glutathione. Transport experiments further revealed that ORF *SPAC29B12.10c* could mediate glutathione transport. The ORF was named as *Pgt1*$^+$ (Pombe Glutathione Transporter). The biochemical characterization of the transporter revealed that it was localized to the plasma membrane and exhibited a high affinity for glutathione (K_m = 62.9 μM). From

inhibitor studies it was further concluded that *Pgt1*$^+$ is a glutathione-specific transporter being inhibited only by glutathione and its conjugates. Its regulation by sulphur-limitation further underlined the primary physiological function in glutathione transport. Despite the strong similarity between *HGT1* and *Pgt1*$^+$, *Pgt1*$^+$ failed to function in *S. cerevisiae*. Attempts were made to investigate the causes behind this observation. A genetic strategy was undertaken to isolate the functional *Pgt1*$^+$ mutants in *S. cerevisiae*. Several mutants of *Pgt1*$^+$ were isolated that were functional in *S. cerevisiae*. These mutants carried mutations or deletion in the region 300-350 before the 1st transmembrane domain TMD. Comparison of the wild-type *Pgt1*$^+$ with the functional mutants revealed that a loss in protein expression was responsible for lack of functionality of wild type *Pgt1*$^+$ in *S. cerevisiae*. The mRNA levels in wild-type and mutants were comparable, suggesting that the block was in translation. The formation of a strong stem-loop structure appeared to be responsible for inefficient translation in *Pgt1*$^+$ and disruption of these structures in the mutants was probably permitting translation. This was confirmed by making silent mutations in this region of WT *Pgt1*$^+$ which led to their functionality in *S. cerevisiae*.

A second goal of the work was to follow up initial leads on the identification of the substrate translocation channel of the Hgt1p transporter. Very little structural information is available on the OPTs. An earlier study from our lab that had targeted the charged and polar residues in transmembrane domains revealed, TMDs 1,4 and 9 as being important in substrate recognition and translocation. In the present study this was followed up, focusing on TMD9. Alanine scanning mutagenesis of transmembrane domain 9 of Hgt1p was undertaken to obtain insights into the other residues in this TMD involved in substrate translocation. A set of 21 different alanine mutants were analyzed using a plate-based growth assay and radiolabeled glutathione uptake. This analysis identified different mutants which exhibited severe to moderate loss in functional activity. The detailed biochemical characterization of these mutants included their effect on protein expression levels, trafficking to the cell surface, followed by detailed kinetic studies of severely affected mutant. Kinetic analysis of the most severely affected mutants revealed that in addition to the previously identified Q526A, F523A also led to a severe defect in transport while retaining nearly wild-type protein expression levels and trafficking to the cell surface. Kinetic analysis of the F523A mutant exhibited a 4-fold increase in K*m* for GSH and a loss in

V*max* value indicating that it plays a critical role both in substrate recognition and translocation. The residue F523 of Hgt1p was thus a second residue together with Q526 of TMD9 identified as playing an important role in determining the affinity of the transporter towards its substrate. The functional analysis of F523 mutants and second-site suppressors of F523A suggested that both size as well as hydrophobicity of the residue were important at that position rather then aromaticity.

A further goal of the study was also to identify a possible signature motif for glutathione as the substrate in the OPT family and use it to see if we could obtain information on other transporters. Multiple sequence alignments of the TMD9 region of fungal OPTs revealed that the 2 key residues of TMD9 involved in glutathione recognition in Hgt1p, F523 and Q526 were conserved in some OPT members. Other than *S. cerevisiae*, these members include the *S. pombe* glutathione transporter, Pgt1p, as well still uncharacterized homologues in six other yeasts. Functional analysis of the putative orthologues from three of the yeasts of this group, *Kluveromyces lactis, Pichia guillerimondii* and *Schizosachromyces japonicus* revealed that these were indeed coding for glutathione transporters. Kinetic studies were carried out with two of these transporters that were functional in *S. cerevisiae*, *K. lactis* (*Kl-HGT1*) and *S. japonicas* (*Sj-HGT1*). We observed that the transporter *Kl-HGT1* had a K_m of 64.3±8.7 μM while *Sj-HGT1* had a K_m of 204.1±56 μM. The orthologous proteins with F and Q in TMD9 were observed to form a clear cluster in phylogenetic analysis that we refer to as the 'F and Q cluster' or the 'Sc-Hgt1 cluster'. Some members contained E and I at these positions, two changes that were examined in Hgt1p, and found to be acceptable changes in relation to glutathione transport by Hgt1p. *C. neoformans CnHGT1*, belonged to this group and it was found that *CnHGT1* also functioned as a high affinity glutathione transporter K_m = 82.5±15.5μM . Interestingly phylogenetic analysis with members of the OPT family reveals proteins with the 'E and I residues' also cluster together E and I cluster or the "Cn-Hgt1 cluster" In contrast, CaOpt1p of *Candida albicans* which has hydrophobic amino acids at both these positions in TMD9, despite sharing a high overall similarity with Hgt1p, retained a very weak glutathione transport activity. In this study we have thus identified high affinity glutathione transporter clusters in the PT clade of OPT family of transporters and a possible signature motif.

In addition to the structural characterization of the yeast glutathione transporter, a study was also initiated to identify domain–domain interaction in TMD9 of Hgt1p. One face of TMD9 that includes F523 and Q526 are expected to line the substrate translocation channel while the other face is very hydrophobic. The hydrophobic face of TMD9 helix was subjected to charged residue scanning. The residues A509, V513, L517, L520, I524, and I528 were each mutated to lysine, glutamine and glutamic acid by site directed mutagenesis. These mutants were analyzed using the plate-based assay and radiolabeled glutathione uptake. The L517Q, L520Q, L520E, I524E, I524K and I524Q were non functional even at higher concentrations of glutathione. V513K showed a severe effect on functional activity of Hgt1p. The non-functional and severely affected mutants were further subjected to suppressor analysis. Second site-suppressors were obtained only for I524Q and I524K, both of which involved a G202Q, G202K and G202I substitutions in the hydrophilic loop of Hgt1p between TMD3 and TMD4. The suppressors G202K, G202Q and G202I were able to suppress the phenotypic defect caused by I524Q and I524K. To evaluate the role of these suppressors for their role in substrate binding and translocation, the kinetic parameters of I524Q-G202K were determined and *Km* found to be 282.8± 44.5 μM. This suggested that the primary defect in I524Q may be causing a perturbation in the substrate binding site of the protein.

In conclusion, the results described in this thesis have provided important insights into the mechanism of substrate translocation by Hgt1p. In addition to characterizing a glutathione transporter from *S. pombe* and identifying a glutathione transporters in several others yeasts, the study has identified residues important for substrate recognition of Hgt1p in TMD9. The hydrophobic face of the TMD9 helix was also evaluated by a novel strategy and yielded new insight into possible interacting domains. The studies have thus significantly advanced our knowledge of this large family of transporters.