Glutathione Transferase P1 is Modified by Palmitate

By

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ABSTRACT

The tripeptide glutathione (GSH, γ-Glu-Cys-Gly) is the most abundant non-protein thiol in mammalian cells. The thiol group within the Cys residue forms a nucleophilic center and neutralizes electronegative charges conferring antioxidant activity to GSH. The addition of GSH to electrophiles can be spontaneous but the majority require catalysis from glutathione transferases (GSTs). GSTP1 is mostly known for its role in detoxification. In addition, GSTP1 has been recognized to have non-catalytic activities regulating cell proliferation, apoptosis, and signalling. GSTP1 is involved in a wide range of diseases, suggesting it has multiple functions. Regulated subcellular localization is important for protein function, especially when a protein is involved in multiple cellular processes. GSTP1 has been shown previously to strongly associate with the plasma membrane. Protein palmitoylation increases hydrophobicity and affinity for cell membranes. Palmitate forms a covalent thioester bond with Cys residues in proteins. Palmitoyl transferases (PATs) catalyze the addition of palmitate to protein, and this occurs most commonly at Cys residues in a basic environment (e.g., Cys residues flanked by polybasic amino acids). In this thesis the mechanism by which GSTP1 associates with the plasma membrane was investigated. GSTP1 was identified as a palmitoylated protein. Subcellular localization of GSTP1 was also characterized. Investigation of lipidated proteins can be quite challenging, we mostly utilized bioorthogonal labelling and click chemistry to identify palmitoylated GSTP1 and screen for palmitoylation sites in MCF7 and analyzed by western blotting and immunohistochemistry, the latter was applied in conjunction with proximity ligation assay. Surprisingly, GSTP1 is a palmitoylated protein that is palmitoylated on at least one non-Cys residue and the palmitoylation is resistant to NaOH treatment. Moreover, incorporation of the

fatty acid by GSTP1 requires long metabolic labelling with palmitate, suggesting that modification is not as dynamic as classic palmitoylation of proteins such as, non-receptor tyrosine kinase lck, the Ras proteins and the postsynaptic density protein PSD-95. In addition, we characterized palmitoyl acyl transferase (PAT)-independent palmitoylation of human GSTP1 (purified from *E. coli) in vitro* and identified Cys48, Cys102, and Lys103 as PAT-independent palmitoylation sites using liquid chromatography tandem mass spectrometry (LC-MS/MS). To further characterize the association of GSTP1 with cellular membranes, subcellular fractions of MCF7-GSTP1 cells were analysed. Palmitoylated GSTP1 is present not only in the membrane but also in the cytosol. Moreover, the GSTP1 in the membrane does not conjugate 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. GSTP1 isolated from *E. coli*, and MCF7 cells grown under fatty acid free (FAF) or fetal bovine serum (FBS) conditions were palmitoylated *in vitro* and incubated with plasma membrane-enriched vesicles. GSTP1 associated with cellular membranes without *in vitro* palmitoylation, suggesting that another mechanism might be important for this association. Investigation of the role of membrane association and lipidation of GSTP1 provides new insight into the cell biology of this multifunctional protein.

PREFACE

Chapter 2

This chapter is in preparation for journal submission: "Glutathione Transferase P1 is Post-translationally Modified by Palmitate". The authors are Vanessa Marensi, Megan Yap, Yuhuan Ji, Cheng Lin, Luc G. Berthiaume, and Elaine M. Leslie. I executed all aspects of the experiments shown in Figure 2.1 to 2.5, 2.7 and 2.8C in collaboration with Megan Yap, a technical assistant in Dr. Luc Berthiaume's lab (University of Alberta), who performed the click chemistry. I was completely responsible for Figures 2.8A, B, D and Figure 2.9. Experiments in Figure 2.6 were performed in our collaborator's laboratory (Dr. Cheng Lin, Boston Universtiy School of Medicine) by his former PhD student Dr. Yuhuan Ji. I prepared Figure 2.6C.

Appendix I

Mass spectrometry of GSTP1 purified from MCF7 was performed by myself and Yuhuan Ji. Data analysis was performed by Yuhuan Ji and Cheng Lin. Metabolic labelling and click chemistry was performed by myself and Megan C. Yap.

Appendix II

I performed all experiments except 2-bromo palmitate images in Figure A2.5 were acquired by Anaida Osoria Peréz, former technician in Elaine Leslie's laboratory.

Appendix III

I performed all experiments.

DEDICATION

To my family

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ABE	Acyl biotin exchange
AD	Alzheimer's diseases
AKT	Protein kinase B
ANOVA	Analysis of variance
ANS	8-anilino-1-naphthalene-sulphonate
AP1	Activator protein 1
APT	Acyl protein thioesterases
ARE	Antioxidant response element
$As(GS)_3$	Arsenic triglutathione
ASK-1	Apoptosis signal-regulating kinase 1
Asp-N	Aspartic acid – N
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BICT	Benzyl isothiocyanate
2-BP	2-Bromo palmitate
BSA	Bovine serum albumin
CDDP	Cisplatin
Cdk5	cyclin dependent kinase-5
CDNB	1-chloro-2,4-dinitrobenzene

Cl^-	Chloride ion
CLICs	Chloride intracellular channel
CoA	Coenzyme A
COX	Cyclooxygenase
CT-B	Cholera toxin subunit B
$CuSO_4$	Copper sulfate
DAPI	4′,6-Diamidino-2-phenylindole
DCC	Dextran-coated-charcoal
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNDGIC	Dinitrosyl diglutathionyl iron complexes
DNP-SG	2,4-dinitrophenyl-S-glutathione
DTT	1,4-Dithiothreitol
EA	Ethacrynic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal grow factor
EGFR	Epidermal grow factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FAF	Fatty acid free fetal bovine serum
FAS	Fas cell surface death receptor

FBS	Fetal Bovine Serum
FTMS	Fourier transform mass spectrometry
GOAT	Ghrelin O-acyltransferase
GPI	Glycophosphatidylinositol
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSSG	Glutathione disulfide
GST	Glutathione transferase
GSTA	Glutathione transferase A
GSTK	Glutathione transferase kappa
GSTP1	Glutathione transferase P1
^{3}H	Tritiated
H69AR	Doxorubicin resistant small cell lung carcinoma
HCl	Hydrochloric acid
HCT8	Human colon cancer cell line
HEK293T	Human embryonic kidney 293T
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hh	Hedgehog
Hhat	Hedgehog acyltransferase
HMG	3-hydroxy-3-methyl-glutaryl-CoA
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
HSD	Honest significant difference

IB	Immunoblot
IgG	Immunoglobulin
IP	Immunoprecipitation
IPF	Idiopathic pulmonary fibrosis
JNK	c-Jun N-terminal kinase
K_d	Constant of dissociation
K_2PO4	Dipotassium sulfate
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LiCoA	Lithium CoA
LTC_4	Leukotriene C_4
mAb	Monoclonal antibody
MAPEG	Membrane associated protein in eicosanoid and glutathione
MAPK	Mitogen-activated protein kinase
MBOAT	Membrane bound O-acyl transferase
MCF7	Human breast adenocarcinoma cell line
$MgCl_2$	Magnesium Chloride
MOPS	3-(N-morpholino) propanesulfonic acid
MRP	Multidrug resistance protein
Mrp2	Multidrug resistance protein 2
MS	Mass spectrometry
MuDPIT	Multi-dimensional protein identification technology
M/Z	Mass to charge ratio
NaCl	Sodium chloride

Na_2HPO_4	Sodium phosphate dibasic
NaOH	Sodium hydroxide
NBDHEX	6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol
NEM	N-ethylmalemide
NFE2	Nuclear factor erythroid-2
NF-kB	Nuclear factor kappa B
NH_2	Amino
NH_2OH	Hydroxylamine
NMT	N-myrisotyltransferase
NO	Nitric oxide
NOS	Nitric oxide synthase
4-NQO	4-Nitroquinoline 1-oxide
Nrf2	Nuclear factor erythroid-2-related factor 2
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAT	Palmitoyl acyl transferase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDG_2	Prostaglandin G2
15d-PDJ_2	15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2
PFA	Paraformaldehyde
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C

PLA	Proximity ligation assay
PM	Plasma membrane
PORCN	Protein-serine O-palmitoleoyltransferase porcupine
PSA	Prostate-specific antigen
PVDF	Polyvinylidene difluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
SIM	SUMO interacting motifs
Sirt6	Sirtuin 6
SUMO	Small ubiquitin like modifier
TBS-T	Tris buffered saline and tween 20
TBTA	Tris-(benzyltriazolylmethyl) amine
TCEP	Tris-carboxyethylphosphine
TFA	Trifluoroacetic acid
ThC	Thapsigargin
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor- alpha
TRAF2	Tumour Necrosis Factor receptor-associated factor 2
TRE	12-O-tetradecanoyl-phorbol-acetate (TPA)-response element

TuM	Tunicamycin
XBP	Xanthophyll-binding protein
UDP	Uridine 5’-diphospho
zDHHC	Zinc finger DHHC-type palmitoyl transferase

Chapter 1 Introduction

1.1 Oxidative/Nitrosative stress and antioxidant system

1.1.1 Glutathione

Reduced glutathione (GSH) or γ-glutamyl-cysteinyl-glycine is a potent thiol antioxidant, synthesized in the cytosol and present in all eukaryotic cells. The γ-glutamyl bond makes GSH resistant to cleavage by peptidases, preventing its degradation in the cytosol. GSH is replenished constantly by synthesis. Intracellular concentrations range from 0.1 mM to 10 mM, making GSH the major intracellular redox buffer and co-factor for redox balance (Meister, 1988). Within the cell, GSH is available mostly in its reduced form; it becomes oxidized to glutathione disulphide (GSSG) in the presence of oxidants, such as oxygen peroxide and hydroxyl radical. Reduced GSH can be restored enzymatically by glutathione reductases. GSH (as GSH, GSSG, or GS-conjugates) can be transported to the extracellular space through plasma membrane transporters (such as the multidrug resistance proteins ABCC1 and ABCC2) followed by enzymatic degradation beginning with the brush border enzyme γ-glutamyl-transpeptidase (Ballatori et al., 2009). To maintain the redox balance in the cytosol, the physiological ratio of GSH:GSSG is at least 100:1 (Winther & Jakob, 2013). Excessive intracellular GSSG and the resulting imbalance in the GSH:GSSG ratio is indicative of oxidative stress (Meister & Anderson, 1983). Excessive amounts of GSSG in the cytosol can be transported out of the cell by MRPs to restore the GSH:GSSG ratio (Hammond et al., 2007).

1.1.2 Oxidative and nitrosative stress

Maintenance of GSH redox balance is required for cellular homeostasis. The presence of external stimuli, including nutrient stress and toxic xenobiotics, induce production of free

radicals causing redox-stress, and therefore, imbalance of cell homeostasis (Ray et al., 2012; Wellen & Thompson, 2010).

Under basal conditions, reactive oxygen species (ROS) are constantly being produced by the cell, as products of the mitochondrial electron-transport chain (Murphy, 2009). Reactive nitrogen species (RNS) are also produced under basal conditions by NO synthase (NOS) enzymes (Nicotera et al., 1999). These molecules are important for cellular signalling pathways that control cell survival (Sohal & Orr, 2012). However, under chemical or physical stress, cells generate an excessive amount of ROS and RNS, which overwhelm cellular redox balance, leading to damage of cellular membranes, macromolecules, and DNA, which may result in the induction of apoptosis. Consequently, impairment of antioxidant systems may lead to the development of diseases, such as cancer (Jorgenson et al., 2013), vascular diseases (Conklin et al., 2009), aging and degenerative diseases, including neurodegenerative diseases (Kim et al., 2015).

1.2 The glutathione transferase superfamily

Glutathione transferases (GSTs) catalyse the transfer of GSH to cellular products and xenobiotics (Hayes et al., 2005). GSTs have been extensively studied since they were identified in the 1960s, when GST activity was measured in the cytosolic fraction of rat liver (Combes & Stakelum, 1961). Since the 1960s, the major focus of GST research has been the investigation of their critical role in detoxification (Mannervik, 2012). Also, cytosolic GSTs from rat liver were reported to non-covalently bind to bilirubin, steroids, and organic anions, which resulted in the GSTs being referred to as ligandins (Litwack et al., 1971). Lastly, numerous other non-

catalytic functions for GSTs, for example, regulation of cellular signalling pathways, were identified (De Luca et al., 2014; Tew & Townsend, 2011) as described in section 1.3.8.

1.2.1 Characteristics and classification of GSTs

Biotransformation is the chemical modification of compounds that enter the body with the goal of forming products more hydrophilic to facilitate elimination. Phase I biotransformation enzymes, such as cytochrome P450 oxidases, introduce or expose functional groups. The resulting potentially reactive compounds can rapidly undergo conjugation with hydrophilic co-factors, such as uridine 5'-diphospho- (UDP)-glucuronic acid and GSH through Phase II biotransformation, or they can be transported without further modification out of the cell. GSTs are well established phase II biotransformation enzymes, catalysing the conjugation of GSH to exogenous and endogenous molecules. One such GSTs is GSTP1, whose substrates are mostly electrophilic molecules with hydrophobic character (Iyanagi, 2007; Jancova et al., 2010).

Catalytic activity of GSTs was first described in 1961 (Armstrong, 1997; Booth et al., 1961). The structure of other GSTs was solved in the 1990s. One of the first to be solved was GSTP1 isolated from human placenta (Reinemer et al., 1992; Reinemer et al., 1991). GST ancestors are thioredoxin-like proteins. Common structural characteristics between GSTs include a conserved N-terminus which resembles the structure of the thioredoxin-fold (four-stranded β-sheet and three flanking α-helices), and interacts with the γ-glutatmyl amino acid of the GSH (Armstrong, 1997; Board et al., 2000; Hayes et al., 2005; Martin, 1995). The C-terminal portion of GSTs contain the substrate binding domain, which is distinct between the different GST isoforms, and creates the substrate selectivity. The N-terminal region is commonly called the active site or G-site (where G stands for GSH) and the C-terminus is the

binding site or H-site (where H stands for hydrophobic) (Bhat et al., 2010). GSTs can form homo and/or heterodimers, the dimer interface is located between the N-terminus of one monomer and the C-terminus of the other monomer.

1.2.2 Glutathione transferase classification and function

GSTs are found in many species, from prokaryotes to higher mammals (Allocati et al., 2009). The mammalian GSTs are divided into three major classes, the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), mitochondrial, and cytosolic (Hayes et al., 2005). There are six MAPEG isoenzymes and they were identified in microsomal membranes (fragmented ER obtained by centrifugation). The MAPEGs include the prostaglandin-E synthase from bovine vesicular glands, GSH-dependent microsomal leukotriene-C_4 synthase, 5-lipoxygenase activating protein, and three microsomal proteins with GST and GSH peroxidase activity [microsomal glutathione S-transferase (MGST1, MGST2 and MGST3)] (Jakobsson et al., 1999; Pettersson et al., 2005).

The human cytosolic GSTs are divided into 7 subclasses, the alpha, zeta, theta, mu, pi, sigma, and omega. Overall, evolutionary classification of GSTs is based on structure (including their tertiary structure), primary sequence, and function. Nomenclature review of the cytosolic isoforms lead to the substitution of the Greek symbols with the latin A-, Z-, T-, M-, P-, S-, and O. In addition to these cytosolic forms, there is a mitochondrial GST, denominated GSTK1 which is found in the mitochondria and peroxisomes, but not the cytosol (Hayes et al., 2005; Hayes et al., 2010; Mannervik et al., 1992).

The chloride intracellular ion channel (CLICs) are unique GST family members, sharing similar tertiary structure with cytosolic GSTs. CLICs are water soluble proteins able to insert

into intracellular membranes to form oligomeric Cl^- channels (Cromer et al., 2002; Littler et al., 2010).

Different GST isoforms can co-exist in the same tissues, however, some isoforms predominate in certain tissues, for example; GSTA1 and GSTA2 are the prevalent forms in the adult human liver, whereas GSTP1 predominates in the retina, lungs, erythrocytes, and placenta (Bhosale et al., 2004; Howie et al., 1988). Interestingly, GSTP1 is present in fetal but not adult human liver (Kashiwada et al., 1991).

1.3 Glutathione transferase P1

1.3.1 Human glutathione transferase P1

The *GSTP1* gene is localized on chromosome 11q13 (Board et al., 1989; Eaton & Bammler, 1999). In humans and most other species, GSTP1 is the only P isoform. Mouse is an exception, with two isoforms, Gstp1 and Gstp2 (Pajaud et al., 2015). GSTP1 was first identified in human placenta where it accounts for 75% of total GSTs. Therefore, the P from GSTP1 stands for placenta (Kano et al., 1987). GSTP1 is also expressed in most other tissues, including retina (Bhosale et al., 2004), kidney (Singh et al., 1987), erythrocyte and lung (Howie et al., 1988).

Three missense single nucleotide polymorphisms (SNPs) of the *GSTP1* gene that generates four variants have been identified and characterized (www.ncbi.nlm.nih.gov/snp) (Sharma et al., 2014). The most common allele (designated GSTP1*A) has a nucleotide sequence resulting in the amino acids Ile at position 105 and Ala at position 114 (Ile^{105}/Ala^{114}) (Table 1.1). The SNP variants GSTP1*B have (Val^{105}/Ala^{114}), GSTP1*D (Ile^{105}/Val^{114}) and the undesignated Gly169Asp (Table 1.1) (Ali-Osman, Akande, et al., 1997; Kitteringham et al., 2007; Sharma et al., 2014; Spiteri et al., 2000). A fourth “SNP” designated as GSTP1*C

(Val^{105}/Val^{114}) is the result of two of these missense mutations (*B and *D) occurring simultaneously.

GSTP1 is found in the cytosol as a dimer. Each monomer is composed of 210 amino acids, including the initiator methionine. The NH_2-terminus is formed by four beta-sheets and two alpha-helices, containing the G-site, which as described above, is important for the stabilization and activation of GSH. The COOH-terminus starts at amino acid 81 and contains five alpha helices, including the H-site (Reinemer et al., 1991).

The Tyr8 of GSTP1 is necessary for catalysis by inducing the activation of GSH when it is bound to the active site. The hydroxyl group of Tyr8 donates a hydrogen bond and lowers the pKa of the thiol group of GSH resulting in the formation of a thiolate anion, which can then conjugate with the substrate. Tyr8 is seen as an evolutionary mark among cytosolic GSTs. The GSTT class is thought to be the precursor of GSTA, GSTP, GSTM, and GSTS and uses a Ser rather than a Tyr. The GSTO is an exception, it has a Ser at position 8 and a Cys residue at position 32 causes GSH activation (Board et al., 2000; K. H. Kong et al., 1992; Manoharan et al., 1992; Reinemer et al., 1991; Stenberg et al., 1991).

Structural studies of mouse liver Gstp1 shows that *S*-carboxymethylation of Cys48 decreases catalytic activity. Moreover, modification of Cys48 stabilizes the active site, otherwise disordered in the absence of GSH, GSH-conjugate, or inhibitor (Vega et al., 1998).

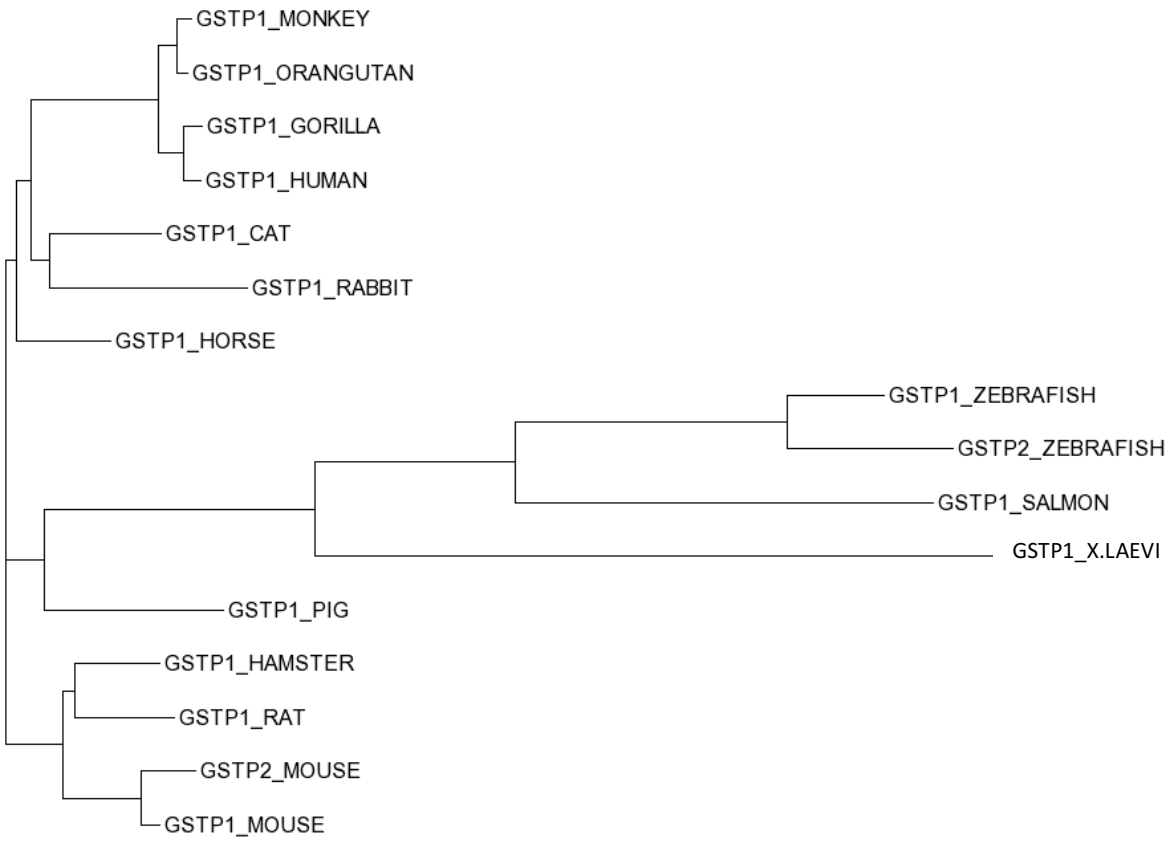


Figure 1.1 Phylogenetic tree of P1 class GSTs from selected species. The dendrogram represents the hierarchical clustering of the relationship between these species. The tree was built using ClustalW (http://www.genome.jp/tools/clustalw/) and Newick string generated was viewed in T-Rex (http://www.trex.uqam.ca/index.php?action=align).

Table 1.1
Summary of the most reported *GSTP1* SNPs

Allele annotation	Nucleotide change	Amino acid change	Variant ID	Variant identification	Validation
GSTP1*B	313A>G	Ile105Val	rs1695	Ali-Osman et al., 1997)	Yes
GSTP1*C	313A>G 341C>T	Ile105Val Ala114Val	rs1695 rs1138272	Ali-Osman et al., 1997)	-
GSTP1*D	341C>T	Ala114Val	rs1138272	(Ali-Osman, Akande, et al., 1997)	Yes
No annotation	506G>A	Gly169Asp	rs41462048	(Kitteringham et al., 2007)	No
No annotation	505G>A	Gly169Ser	rs746884675	-	Yes by frequency

Interestingly, GSTP1 undergoes co-operative self-preservation from physical or chemical stress, where occupancy of Cys48 in one of the units of the dimer causes enzyme inactivation and shielding of the counter-part Cys48 in the other subunit leading to an asymmetric conformation (Ricci et al., 2003). Treatment of GSTP1 with a fluorescent maleimide-derivative that forms stable adducts with Cys residues revealed that Cys48 is the only reactive Cys residue, suggesting that Cys15, Cys102, and Cys170 are not accessible to this reagent (Lo Bello et al., 1990).

1.3.2 Role of human GSTP1 in conjugating compounds of toxicological relevance

Just like other GSTs, the presence of GSTP1 is protective, preventing the damage of cell structures (including DNA) from reactive compounds. Multidrug resistance protein (MRPs) are ATP-binding cassette (ABC) transporter proteins, important for the efflux of a variety of endogenous and exogenous compounds, including organic anions conjugated with GSH, glucuronide and sulfate (Cole & Deeley, 2006). MRP1 (gene symbol *ABCC1*) and GSTP1

overexpressed in MCF7 cells work synergistically to detoxify ethacrynic acid through the formation and efflux of an ethacrynic acid GSH conjugate (Morrow et al., 1998). Dinitrosyl-dithiol-iron complexes are formed within cells from nitric oxide (NO) in a GSTP1-dependant manner and transported by MRP1 (Suryo et al., 2012).

Sulphorophane induces antioxidant response element (ARE)-containing genes, such as *GSTP1* and MRPs (ABCCs) (Sibhatu et al., 2008). Sulphorophane can be conjugated with GSH by GSTP1 and transported by MRP1 (Sibhatu et al., 2008). Arsenic trioxide is used as a chemotherapeutic drug, the presence of GSTP1, arsenite and GSH results in transport of arsenic triglutathione [$As(GS)_3$] by MRP1 (Leslie et al., 2004). This evidence suggests that GSTP1 catalyzes the formation of $As(GS)_3$ from As^{III} and GSH. 4-Nitroquinoline 1-oxide (4NQO) is genotoxic and formation of a 4NQO-GS is catalyzed by GSTP1 and dependent on MRP1 for efflux from the cell (Sibhatu et al., 2008).

Acrolein is an unsaturated aldehyde product of cigarette smoke that forms adducts with proteins causing cellular damage, it is also a GSTP1 substrate. The aorta of *Gstp1*(-/-) mice exposed to acrolein presented higher levels of acrolein-adduct and diminished acetylcholine - induced arterial relaxation than $Gstp1^{+/+}$ mice, suggesting that GSTP1 protects endothelial cells from acrolein, potentially through catalysing the formation of a GSH conjugate (Conklin et al., 2009).

Benzyl isothiocyanate (BITC), 8-anilino-1-naphthalene sulfonate (ANS) and 1-chloro-2,4-dinitrobenzene (CDNB) are GSTP1 substrates and have been used to show that GSTP1 has at least three distinguishable binding sites. Cys48 and Tyr104 are required for BITC binding, however, GSTP1-Tyr103Phe and GSTP1-Tyr103Ser bound CDNB with similar affinity As GSTP1-WT, while BITC binding to GSTP1 was reduced by mutation of Tyr to a Ser not a Phe,

suggesting that BITC binding to GSTP1 requires the aromatic ring present in the Tyr and the Phe, meanwhile either mutation decreases ANS binding. Together, these data suggest that BITC, CDNB and ANS don't share identical binding sites (Ralat & Colman, 2004).

1.3.3 Role of human GSTP1-binding compounds unrelated to detoxification

Zeaxanthin is a xanthophyll carotenoid metabolized by xanthophyll-binding proteins (XBPs) in the retina, within the macula (Billsten et al., 2003). Purification of membrane extracts from human macula revealed that GSTP1 is the XBP in the macula, it binds to zeaxanthin with high specificity and affinity [K_d of 0.33 μM (3*R*,3′*R*-zeaxanthin) and K_d of 0.52 μM (3*R*,3′*S-meso*-zeaxanthin)] (Bhosale et al., 2004).

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a major cyclooxygenase-2 (COX2) product derivative from arachidonic acid present in the plasma membrane, a PDG_2 metabolite that is highly increased in inflammatory responses (Shibata et al., 2002). 15d-PGJ_2 binds to GSTP1 and induces tetrameric oligomerization (Sanchez-Gomez et al., 2013), the process is irreversible, it involves residue Cys102 and inhibits protein activity (Sanchez-Gomez et al., 2010; Sanchez-Gomez et al., 2007).

1.3.4 GSTP1 gene regulation

Aberrant expression of GSTP1 can occur in certain tumours and how this occurs is not completely understood yet (Townsend & Tew., 2003). *GSTP1* transcription was shown to be dependent on the Activator protein 1 (AP-1)-response element in leukaemia cells (Duvoix et al., 2004). However, *GSTP1* expression is mostly regulated by the GSTP1 enhancer I (GPE1), which allows *GSTP1* expression when the AP1 is absent. GPE1 also contains two [12-O-

tetradecanoyl-phorbol-13-acetate (TPA)-response element (TRE)] within, which has no activity by themselves but act as a synergistic enhancer together (Diccianni et al., 1992; Okuda et al., 1990).

Other factors are able to promote *GSTP1* expression. *GSTP1* was also shown to be transcriptionally activated by p53 through direct interaction with a p53-binding motif within intron 4 of *GSTP1* (Lo et al., 2008). In addition, a tumour necrosis factor (TNF) alpha-inducible nuclear factor-kappa B (NF-kB) binding site is contained in the sequence located between -323/-314 within the promoter of *GSTP1* and NF-kB activation of *GSTP1* expression in K562 cells was demonstrated (Morceau et al., 2004). Interestingly, GSTP1 was demonstrated to regulate NF-kB signalling itself (Jones et al., 2016).

Downregulation of *GSTP1* expression also has implication in pathogenesis of certain diseases, such as cancer, and has been extensively studied. The most common pathogenic mechanism is epigenetic silencing of the *GSTP1* promoter via hypermethylation of CpG islands (Lin et al., 2001).

Transcription factors involved in response to cellular stress, are able to activate transcription of *GSTP1*. The nuclear factor erythroid-2 (NFE2)-related factor 2 (Nrf2) upregulates genes involved in anti-oxidant response to restore cellular redox homeostasis. *GSTP1* has an antioxidant response element (ARE) in its promoter, which serves as a binding site for Nrf2 (Hayes et al., 2010).

1.3.5 GSTP1 and cancer incidence

*GSTP1*overexpression is a hallmark of drug-resistance whereas, *GSTP1* silencing characterizes tumour development and poor prognosis. Thus, expression of *GSTP1* seems to be cell-specific

and understanding the mechanisms regulating *GSTP1* expression is important to identify the role of GSTP1 in diverse pathological conditions.

1.3.5.1 Prostate cancer

Prostate cancer is associated with the epigenetic silencing of the *GSTP1* gene, hypermethylation of the cytosine-guanine-rich sequences in the *GSTP1* promoter prevents protein transcription (Lin et al., 2001). Hypermethylation of *GSTP1* in prostate cancer has been well established, and it is currently being studied as a potential molecular biomarker (Nakayama et al., 2004). Indeed, it has been proposed that *GSTP1* silencing could be used to differentiate between prostate cancer and benign prostatic hyperplasia, which are not differentiated by prostate-specific antigen test (PSA) (Woodson et al., 2008).

1.3.5.2 Ovarian cancer

In contrast with prostate cancer, GSTP1 is widely overexpressed in solid tumours when compared with healthy neighbouring tissue (Shea et al., 1988). Increasing levels of GSTP1 is associated with the development of anti-cancer drug resistance. For example, overexpression of GSTP1 in ovarian tumour cell lines has been shown to increase cell migration and invasion. Consistent with this, knockdown of *GSTP1* in a cisplatin-resistant cell line (A2780DPP) showed reduced cell migration and proliferation and increased response to cisplatin and carboplatin (Sawers et al., 2014).

1.3.5.3 Triple-negative breast cancer (TNBC)

Estrogen, progesterone and epidermal growth factor receptors are the three main chemotherapeutic targets in breast cancer therapy. Triple negative breast cancer (TNBC) lack all of these receptors and patients have poor prognosis, which reflects the challenges in the therapeutics (Bianchini et al., 2016). GSTP1 was identified as a novel TNBC therapeutic target

as it promotes TNBC survival via regulation of the glycolytic pathway and lipid metabolism (Louie et al., 2016). *GSTP1* was expressed in estrogen receptor-positive (ER+) breast cancer, whereas, hypermethylation of CpG island prevented *GSTP1* expression in estrogen receptor-negative (ER-) human breast cancer cell lines similar to prostate cancer tissues (Jhaveri & Morrow, 1998; Millar et al., 1999)

1.3.5.4 Gliomas

GSTP1 is overexpressed in gliomas and phosphorylated by epidermal growth factor receptor (EGFR), a tyrosine kinase receptor that promotes cell growth (Okamura et al., 2009). Upon phosphorylation, GSTP1 complexes with c-Jun supressing downstream signalling, which results in cell survival through reduced apoptosis (Okamura et al., 2015).

1.3.5.5 Osteosarcoma

Increased levels of GSTP1 are also present in osterosarcoma cell lines and this is associated with doxorubicin and cisplatin resistance (Huang et al., 2007; Pasello et al., 2008). 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), an anti-tumour compound, specifically inhibits GSTP1 in cisplatin (CDDP)-sensitive (U-2OS cells) and -resistant (U-2OS/CDDP cells) human osteosarcoma cell lines. Interestingly, the same group has shown that tumour necrosis factor (TNF) receptor-associated factor 2 TRAF2-GSTP1 complex is dissociated by NBDHEX to restore TRAF2-ASK1 (Apoptosis signal-regulating kinase 1) signalling prolonging activation of JNK (Sau et al., 2012). TRAF2 is a part of the TNF superfamily signalling complex; thus these findings suggest a role for GSTP1 in signal transduction.

1.3.5.6 Hodgkin's Lymphoma

GSTP1 polymorphisms are likely relevant for cancer development and patient response to treatment. Hodgkin's lymphoma patients homozygous for GSTP1*B had a 100% survival rate

over 5 years, this was reduced to 43% for individuals homozygous for GSTP1*A, suggesting that a treatment selective for different GSTP1 variants could improve prognosis (Hohaus et al., 2005).

Many studies have suggested that GSTP1 causes anti-cancer drug resistance (Huang et al., 2007; Sawers et al., 2014), however, other studies have failed to correlate GSTP1 levels in solid tumours with resistance to anti-cancer treatment. Interestingly, a study on human GSTP1 overexpressed in MCF7 cells demonstrated that cisplatin-resistance was not associated with the catalytic activity of GSTP1, rather an unidentified mechanism (Peklak-Scott et al., 2008). This conflicting result could be because drug resistance may be associated with the ability of GSTP1 to regulate signalling pathways important for cell proliferation and cell survival rather than detoxification by inhibiting programmed cell death (Townsend & Tew, 2003). More details regarding GSTP1 and signalling pathways will be discussed in Section 1.3.8.

1.3.6 GSTP1 and other diseases

In addition to cancer, GSTP1 may have a role in the etiology of many other diseases, some of the most reported pathologies are described below.

1.3.6.1 Neurodegenerative diseases

Oxidative stress is thought to play a central role in the pathophysiology of neurodegenerative diseases through the accumulation of DNA damage and loss of protein function. It is yet to be understood whether oxidative stress can trigger these diseases or if oxidative stress is a consequence of these disease (Andersen, 2004; Kim et al., 2015).

Some studies show a role for *GSTP1* SNPs in the development of certain neurodegenerative diseases. Alzheimer's disease (AD) is a type of dementia caused by neuronal

loss. It has been proposed that the *GSTP1*C* variant acts in association with the apolipoprotein E epsilon4 allele (ApoE) in beta-amyloid-mediated neurodegeneration via oxidative stress and apoptosis, leading to AD (Bernardini et al., 2005). Another study associated AD patients harbouring *GSTP1*C* with a faster decline in cognitive function compared to *GSTP1*A* patients (Spalletta et al., 2007). Interaction of p25 (a proteolytic derivative of p35, which is a Cdk5 activator), with the serine-threonine kinase, cyclin dependent kinase-5 (Cdk5), causes oxidative stress and neurodegeneration. AD is characterized by increased levels of p25 and GSTP1*A was found to interact with Cdk5 to reduce interaction of p25 with Cdk5, thus promoting neuroprotection (Sun et al., 2011).

GSTP1 was also associated with occurrence of Parkinson's disease (PD). GSTP1 was found at higher levels of the synaptosomal fractions from the frontal cortices of patients at various stages of PD in comparison with their age-matched PD controls (Shi et al., 2009). It is understood that generation of ROS from xenobiotics and endogenous metabolites can cause cumulative damage, as a detoxification enzyme it is expected that GSTP1 protects from PD development and progression. Indeed, the variant GSTP1*B was associated with increased risk of sporadic PD (Lotharius & Brundin, 2002; Vilar et al., 2007).

1.3.6.2 Respiratory tract diseases

Air pollution, tobacco smoke and other environmental substances affect the epithelia lining of the respiratory tract, especially lungs. Generation of excessive ROS leads to excessive oxidation of GSH, thus an imbalance in the GSH:GSSG ratio (Fitzpatrick et al., 2011). *GSTP1* variants are associated with variation in the inflammatory response of the airways (Spiteri et al., 2000). Preliminary data suggests that GSTP1*C is more frequent in asthmatic patients than in controls (Fryer et al., 2000).

Idiopathic pulmonary fibrosis (IPF) is a spontaneous and progressive interstitial fibrosis of the lungs with a poor prognosis (Kim et al., 2015). IPF presents increased activity of the Fas cell surface death receptor (FAS), a tumour necrosis factor receptor superfamily (TNF receptor) member, mediator of cell apoptosis, fibrosis and altered GSH redox balance (Lopez et al., 2009; Waring & Mullbacher, 1999). GSTP1 promotes *S*-glutathionylation of FAS and directly interacts with it, therefore, GSTP1 became a target for treatment of IPF (McMillan et al., 2016). GSTP1 levels are increased in type II epithelial cells from IPF patients and administration of the GSTP1 inhibitor, TLK117, decreased caspase activation, thus decreased programed cell death and total S-glutathionylation, suggesting that it is an effective IPF treatment target (McMillan et al., 2016).

1.3.7 Subcellular localization of GSTP1

Protein translation occurs in the cytosol; the nascent protein is translocated to different cellular compartments. This process is regulated by the presence of signal sequences, which are recognized by specific proteins residing on each organelle. Subcellular translocation of proteins for compartmentalization is often assisted by chaperones. For example, chaperones can protect hydrophobic sites on the newly synthesized protein from being exposed until it is inserted to membranes. Disruption of a signal sequence affects subcellular sorting potentially leading to the development of diseases (Hung & Link, 2011; Sirover, 2012).

Some proteins can be localized in multiple compartments in a single cell. Post-translational modification and protein-protein interactions are the main switch for regulation of protein localization, as it can modulate tertiary and quaternary protein structure and accessibility to signal peptides (Sirover, 2012).

GSTK is the only GST that resides exclusively in the mitochondria and peroxisome, it contains a cleavable mitochondrial-targeting sequence at the NH_2-terminus (Raza, 2011). A putative signal sequence Ala-Arg-Leu for peroxisome localization was also identified at the COOH-terminus of the human GSTK, and localization to this organelle was confirmed in HepG2 cells (Morel et al., 2004). In addition, the MAPEGs are associated with cellular membranes, their functions vary between synthesis of leukotriene C_4 (LTC_4) and prostaglandin E_2 (PGE_2), as well as GSH transferase activity (Jakobsson et al., 1999; Martinez Molina et al., 2008).

GSTP1 is a multifunctional protein, therefore, it is reasonable to think that regulated intracellular distribution of GSTP1 would promote optimal localization for specific functions and response to external signals.

Even though GSTP1 is reported predominantly as a cytosolic protein, localization in multiple compartments has been reported. GSTP1 was found in the nuclei of glioma cells (Ali-Osman, Brunner, et al., 1997) and the amino acids 195 - 208, within the C-terminal region were shown to be essential for nuclear translocation (Kawakatsu et al., 2011). In addition, a variety of cancer cell lines have shown GSTP1 accumulated in the nucleus after doxorubicin treatment (Goto et al., 2001). Complementary to that, GSTP1 nuclear export was found to occur; lectin, an inhibitor of the nuclear transport, prevented GSTP1 translocation and accumulation in the nucleus (Kamada et al., 2004). It is possible that nuclear translocation of GSTP1 occurs to protect the DNA from damage; oxidative stress was induced in the human colon cancer cell line (HCT8) by hydrogen peroxide (H_2O_2), leading to the formation of lipid peroxidation products and GSTP1 accumulation in the nuclei (Kamada et al. 2004). It was suggested that the binding

of lectins to Leu-rich residues of GSTP1 prevented its nuclear export, thus increasing nuclear accumulation (Goto et al., 2001; Kamada et al., 2004).

In addition to the nuclear localization, GSTP1 was found to translocate to the mitochondrial compartment, and at least one Arg residue within the NH_2-terminus is required for mitochondrial localization (Raza, 2011). Oxidative stress caused by rotenone and antimycin A damage mitochondria and result in the reduction of the mitochondria membrane potential. Overexpression of GSTP1 diminished the toxic effect of ROS on the mitochondria, suggesting that GSTP1 is protecting the organelle from damage (Goto et al., 2009).

Recently, it was proposed that GSTP1 is an ER-resident protein that catalyses *S*-glutathionylation within the endoplasmic reticulum (ER) (Ye et al., 2016). It is an intriguing suggestion as GSTP1 does not have the signal peptide required for translocation from the cytosol to the ER. GSTP1 *S*-glutathionylation activity was defined based on the ability of proteins in liver cell lysate of *Gstp1/p2*$^{+/+}$ and *Gstp1/p2*$^{-/-}$ mice, and immunoprecipitation of *S*-glutathionylated proteins, which is an indirect method. Upon subcellular fractionation, GSTP1 was identified in the ER fraction, which does not necessarily mean that GSTP1 is in the ER lumen, it could be anchored to the cytosolic side of the ER membrane. In another experiment, GSTP1 was shown to reduce the cytotoxic effect of thapsigargin (ThG) and tunicamycin (TuM) (ER-stress inducers) (Ye et al., 2016). Together, these results do not provide direct evidence that GSTP1 is an ER-resident protein.

During studies of arsenic triglutathione transport by the multidrug resistance protein 1 (MRP1/ABCC1), GSTP1 was unexpectedly found to be associated with the plasma membrane of the doxorubicin resistant small cell lung carcinoma (H69AR) and the parental H69 cell lines (Leslie et al., 2004). More recently, our lab has shown endogenous and stably transfected

GSTP1 is also associated with the plasma membrane of HEK293T and MCF7 cell lines, respectively (Qazi et al., 2011). Further characterization of plasma membrane sheets isolated from MCF7-GSTP1 cells showed that GSTP1 is not removed from the plasma membrane after KI treatment, known to disrupt the electrostatic association of peripheral proteins with the plasma membrane or by alkaline carbonate, which is stronger and could also disturb stronger interactions (Qazi et al., 2011). In addition to reports for cell lines, GSTP1 co-localizes with the integral membrane protein Mrp2 in the luminal plasma membrane of rat brain capillaries (Bauer et al., 2008).

1.3.8 Signalling pathways and GSTP1

Attention to the role of GSTP1 in signalling pathways compared with detoxification is more recent. GSTP1 was found to directly interact with the C-terminus of the serine-phosphatase c-jun-kinase 1 (JNK1) (Wang et al., 2001). JNK1 (or mitogen-activated protein kinase 8) is important for cell proliferation, differentiation, and regulation of transcription (Dhanasekaran & Reddy, 2008). The interaction of GSTP1 with JNK prevents JNK from translocating to the nucleus, thus preventing the downstream phosphorylation of target transcription factors, such as c-jun, p53, STAT3, and Elk1 which are important for regulation of cell proliferation and induction of apoptosis (Adler et al., 1999). JNK is activated in response to a variety of cell-stressors. Under oxidative stress, the GSTP1-JNK interaction is broken and JNK is released and activated, therefore, through JNK1 GSTP1 can modulate cell proliferation and the induction of apoptosis (De Luca et al., 2012; Wang et al., 2001). In addition, GSTP1 regulation by EGFR increases cell proliferation and adhesion through JNK, protein kinase B (AKT), and mitogen-

activated protein kinase (MAPK) pathways. Dysfunction of EGFR is associated with development of cancer (Okamura et al., 2015; Tomas et al., 2014)

Tyr4-, Tyr8-, and Tyr199-GSTP1 have been shown to be phosphorylated by EGFR in brain tumour cells (Okamura et al., 2015). Phosphorylation of Tyr198 promotes GSTP1 association with JNK. As a consequence, JNK is inhibited and programmed cell death is not activated, allowing continued cell proliferation (Okamura et al., 2015). EGFR activation phosphorylates GSTP1 after EGF binding. Mutation of Tyr to Phe identified Tyr8 and Tyr199 as phosphorylation sites on GSTP1 in a human glioblastoma cell line highly expressing EGFR (U87MG.wtEGFR) (Okamura et al., 2009). In the same study, stimulation of EGFR caused increased GSTP1 phosphorylation and increased cisplatin resistance.

GSTP1 is also phosphorylated by other kinases, such as protein kinase C (PKC) (Singh et al., 2010). It has been proposed that PKC and cAMP-dependent protein kinase (PKA) hyper-phosphorylate GSTP1 in the presence of GSH, and increase catalytic activity as measured using the CDNB assay. Ser43 and Ser185 are the putative phosphorylation sites identified (Lo et al., 2004).

Furthermore, GSTP1 directly interacts with TNF receptor-associated factor 2 (TRAF2), and the binding affinity decreases when GSTP1 is bound to GSH or the GSTP1 inhibitor NBDHEX (bound to the substrate site), further experiments suggested that Cys48 and Cys102 are important for TRAF2 interaction (De Luca et al., 2014). In another study, GSTP1 inhibited TRAF2-induced activation of JNK and p38 but not of NF-kB and also attenuated apoptosis signal-regulating kinase 1 (ASK1) (Wu et al., 2006). Indeed, overexpression of GSTP1 caused inhibition of JNK-ASK1 pathway (Wu et al., 2006).

1.3.9 Post-translational modifications of GSTP1 in addition to phosphorylation

Several post-translational modifications of GSTP1 have been identified to date, many of them being phosphorylation, as described in Section 1.3.8. Protein post-translational modification by GSH has been discovered and is referred to as *S*-glutathionylation (Dalle-Donne et al., 2007). Due to the relevant role of GSH in cellular redox balance it is likely that this post-translational modification is important for the regulation of proteins in response to oxidative stress. Characterization of *S*-glutathionylation as a post-translational modification led to the identification of GSTP1 as a protein that not only undergoes, but also catalyses protein glutathionylation, contributing to what has been referred to as the 'glutathionylation cycle' (addition of GSH onto protein Cys residues followed by removal) (Tew & Townsend, 2011). *S*-glutathionylation of Cys48 and Cys102 has been shown to prevent the JNK-GSTP1 interaction as well as GSTP1 multimerization (Balchin et al., 2013; Townsend et al., 2009).

Nitric oxide (NO) has a well-established role as a physiological mediator in the cardiovascular system. Recently, NO has emerged as a selective modifier of Cys residue thiols referred to as *S*-nitrosylation. This post-translational modification can occur spontaneously from free NO or NO-GSH, regulating signalling pathways and cellular redox (Hess et al., 2005) (Martinez-Ruiz & Lamas, 2004). GSTP1 is *S*-nitrosylated, and interacts with dinitrosyl diglutathionyl iron complexes (DNDGIC) and *S*-nitrosoglutathione (GSNO), which serves as a NO carrier. DNDGIC binding does not affect GSTP1 catalytic activity, whereas GSNO induces a negative cooperativity. In the presence of GSH, Cys48 becomes less accessible to the solvent and less likely to bind to GSNO. Cys48 and Cys102 are modified by DNDGIC and at only Cys48 by GSNO (Bocedi et al., 2013; Cesareo et al., 2005; Tellez-Sanz et al., 2006).

1.3.10 Evidence for lipid modification of GSTP1

As described in section 2.1, GSTP1 has been found to strongly associate with the plasma membrane. For strong association of cytosolic protein onto membrane to happen, a hydrophobic component on the protein would facilitate membrane association. In this thesis we are investigating potential lipid modifications of GSTP1.

1.4 Lipidation

Co- and post-translational modification of cytosolic proteins by lipids (lipidation) is of great interest because this process can change the hydrophilic nature of the protein and increase the likelihood of interaction with cell membranes. Therefore, lipidation can be important for regulation of protein subcellular localization, function, structure, and protein-protein interactions (Ward et al., 2013).

Lipidation processes are classified according to the type of lipid bound to the protein. The most studied lipidation processes in eukaryotic cells are: glycophosphatidylinositol (GPI) anchoring, cholesteroylation, isoprenylation and fatty acylation. A GPI anchored protein has a GPI molecule attached to its C-terminus, but only occurs on proteins processed through the ER. Therefore it occurs on secreted and plasma membrane proteins (Mayor & Riezman, 2004).

Cholesteroylation is the covalent modification of a protein by cholesterol. It is important for the association of proteins with the outer leaflet of the plasma membrane, preventing the secreted protein from being released into the extracellular environment. The only example of cholesteroylated proteins to date are the Hedgehog (Hh) proteins. Full length Hh are subjected to autocatalytic cleavage in the ER and the resulting NH_2-terminal fragment undergoes palmitoylation and cholesteroylation in the ER lumen (Buglino & Resh, 2012; Jeong &

McMahon, 2002). Overall, the Hh are important for morphogenesis. Studies have confirmed their implication in neural development, thus, Hh cholesteroylation is a potential target for brain and spinal cord disease therapies (Ferent & Traiffort, 2015)

Isoprenes are unsaturated branched hydrocarbons, and modification of proteins with isoprenes (isoprenylation) is used to anchor into intrinsically disordered (not rich in cholesterol) plasma membrane regions (Jang et al., 2015). Isoprenylation requires a well-defined consensus sequence, the CAAX box. The nature of the X in the CAAX sequence determines the type of isoprene binding. If X is a Met, Ser, or Gln, the CAAX box will be recognized by a farnesyltransferase and bind to farnesyl (resulting in farnesylation), if X is a Leu, a geranylgeranyltransferase will add a geranyl onto the protein (resulting in geranylation) (Casey, 1992; Fu & Casey, 1999).

1.4.1 Fatty acylation and fatty acids

Cells in the body obtain fatty acids from diet or through *de novo* synthesis. Exogenous palmitate is preferred over *de novo* synthesis in fibroblasts (Yao et al., 2016). When the intake of fatty acids is too high, they are esterified to triglycerides and stored within the cells (Walther & Farese, 2012). On the other hand, starvation induces hydrolysis of triglycerides back to fatty acids and these are broken down by β-oxidation in eukaryotic mitochondria to generate ATP (Kawano & Cohen, 2013; Lopaschuk et al., 2010). Fatty acylation is the modification of proteins by fatty acids. Generally, acylation can be characterized according to the type of fatty acid, enzymes involved and the residue modified. Myristoylation and palmitoylation are the two major classes of acylation (Resh, 1999, 2016), and are described below.

1.4.1.1 Myristoylation

Myristoylation is the irreversible co-translational addition of myristate, a 14 carbon saturated fatty acid (14:0), onto an N-terminal glycine of a protein [Gly-X_3-X_4-X_5-(Ser/Thr/Cys)$_6$]. This process requires a class of enzymes called N-myristoyltransferases (NMT), which are highly selective for myristate. Myristoylation is mostly characterized by its ability to modify cellular localization, but it can also promote protein interactions. Even though myristoylation is irreversible, the association with cellular membranes is not. Generally, it is a dynamic process regulated by a switch mechanism, where the myristate is internalized or exposed in response to cellular signals; for example, upon binding of an interacting protein which leads to conformational modification (Farazi et al., 2001; Martin et al., 2011; Martin et al., 2008).

1.4.1.2 Palmitoylation

Palmitoylation is the most common name of the post-translational fatty acylation of a protein by a broad range of saturated fatty acids, ranging from C8 to C18 carbon length, not only palmitate. The reaction mostly occurs from the transfer of the fatty acid from fatty acid Coenzyme A onto Cys residues via the formation of a thioester bond (*S*-palmitoylation) (Berthiaume, 2014).

Palmitoylation can be autocatalytic (auto-palmitoylation), non-enzymatic or enzymatic (Lopaschuk et al., 2010). The zinc finger-DHHC-type-palmitoyl transferase (zDHHC) family of integral transmembrane enzymes catalyse protein palmitoylation and its own autopalmitoylation. The DHHC is a conserved domain required for palmitate binding (Lemonidis et al., 2015).

The subcellular localization of zDHHCs varies depending on the isoform, they can be located in the ER, Golgi, or plasma membrane (Lemonidis et al., 2015). The zDHHC active site faces the cytosol to palmitoylate proteins located in the cytosol. Many zDHHC have been identified but not many specific substrates have been characterized to date (Korycka et al., 2012).

Other non-zDHHC palmitoyl acyl transferases (PATs) exist within the secretory pathway, ER resident proteins are palmitoylated by membrane-bound *O*-acyl transferase (MBOAT) via *O*-palmitoylation. The MBOAT family members identified to date are: Protein-serine *O*-palmitoleoyltransferase porcupine (PORCN), Hedgehog acyltransferase (HHAT), ghrelin *O*-acyl transferase (GOAT) (Nile & Hannoush, 2016; M. S. Taylor et al., 2013).

Depalmitoylation occurs via cytosolic acyl protein thioesterases (APT), which cleave the thioester bond between the fatty acid and the Cys residue, rendering a reversible and dynamic character to this modification. There are four types of APTs, two of them (APT1 and APT2) are cytosolic proteins responsible for the depalmitoylation of soluble proteins, whereas the other two palmitoyl-protein thioesterases (PPT1 and PPT2) are localized in the lysosome (Kong et al., 2013).

N-Ras is one of the most well characterized palmitoylated proteins and the mechanism of palmitoylation-depalmitoylation is being used as a model to study other palmitoylated proteins (Yap et al., 2010).

Palmitoylation was first thought of as a cellular mechanism to increase hydrophobicity of a soluble protein and promote membrane anchoring. This concept has evolved, and palmitoylation is found to be important for many other aspects of protein function. Examples of this are the regulation of G protein coupled receptor, where palmitoylation regulates receptor

desensitization, thus its function (Goddard & Watts, 2012). Syntaxin 1, synaptosome associated protein 25 (SNAP25) and vesicle-associated membrane protein 2 (VAMP2) are SNARES proteins required for exocytosis of neuronal vesicles. These three SNARES are palmitoylated and palmitoylation is required for vesicle fusion (Prescott et al., 2009);

The mitochondrial 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) synthase, is an enzyme that produces energy from lipids via ketogenesis, it interacts with peroxisome proliferator-activated receptor alpha (PPARα, a nuclear receptor also required for ketogenesis. A study has shown that HMG-CoA and PPARα directly interact, and that this interaction is modulated by palmitoylation (Kostiuk et al., 2010). The ATP-binding cassette transporter A1 (ABCA1) transports phospholipids and cholesterol to form beta-high density lipoproteins. Palmitoylation of ABCA1 by DHHC8 was shown to be required for plasma membrane localization, thus transport of the lipids (Singaraja et al., 2009); other studies have shown that palmitoylation regulates the trafficking of plasma membrane proteins (Hancock et al., 1989).

1.4.1.3 Irreversible palmitoylation

As described in Section 1.4.1.2, palmitoylation is a dynamic and reversible process, however, irreversible palmitoylation can occur. *N*-palmitoylation on the NH_2-terminal Cys of the Sonic hedgehog (Shh) occurs in the lumen of the endoplasmic reticulum, right after cleavage of the signal sequence, by the palmitoyl acyl transferase HHAT (Buglino & Resh, 2012). Moreover, it has been proposed that NH_2-terminal palmitoylation is mediated by attachment of the palmitate to the SH- available on the Cys2, and the transfer occurs because the formation of an amide bond is energetically favourable (Taylor et al., 2001).

Wnt3a is *O*-palmitoylated by fatty acids of 13 to 16 carbons length onto Ser209. Wnt3 palmitoylation is catalyzed by the PORCN and it is required for Wnt3 secretion. Glycosylation is also required but not sufficient for secretion (Gao & Hannoush, 2014).

The hormone ghrelin needs to be *O*-acylated by the GOAT to bind to the ghrelin receptor. Other than an *O*-acylation, an interesting feature of this hormone is that the modification occurs with a medium sized fatty acid of 8 carbons (caprylic acid) (Al Massadi et al., 2011).

1.4.2 Physiological function of multiple lipidation

A single protein may have multiple lipidation domains to regulate protein localization and function. H-Ras undergoes isoprenylation followed by palmitoylation; isoprenylation itself is not sufficient to anchor H-Ras to cellular membranes but increases affinity. The addition of palmitate is required for membrane anchoring (Hancock et al., 1989). Palmitoylation does not occur for prenylated K-Ras. Instead it contains a polybasic domain that interacts with the polar head of acidic phospholipids on the cellular membranes, strengthening the binding (Apolloni et al., 2000). Proteins can be palmitoylated by one or more palmitate, they can also combine palmitoylation with other lipidation process. Just like palmitoylation, the myristoylation process can be regulated by a single attachment of C14 fatty acid or it can be followed by palmitoylation. Alternatively, myristoylation could be accompanied with a polybasic domain, which often serves as switch that exposes the fatty acid chain by increasing affinity with the negatively charged inner leaflet of the plasma membrane (Cadwallader et al., 1994).

Dual acylation is also a common feature of protein lipidation. The affinity to cellular membranes of a dual-lipidated protein is strong and the process is more likely governed by

enzymes than dependant on affinity (Shahinian & Silvius, 1995). Mutagenesis of the NH_2-terminal Gly of the endothelial nitric oxide synthase (e-NOS) prevents protein myristoylation and palmitoylation by blocking its targeting to cellular membranes (Robinson & Michel, 1995).

In terms of protein anchoring, it is worthwhile to highlight that different lipid anchoring can partition the proteins to different membrane microdomains and with different strength. While isoprenes can lead proteins to disordered regions of membranes due to its branched structure, palmitate and myristate tend to drive proteins to cholesterol-rich regions. However, myristoylation itself is not sufficient for the stable anchoring and it will often require a double lipidation process or a positively charged amino acid sequence to increase affinity with the polar head of the inner leaflet of membranes (Martin et al., 2011)

1.4.3 Methods for detection of palmitoylation.

Detection of protein fatty acylation can be very challenging as the body does not produce antibodies against these molecules. With that, radiolabelled lipids have been used to characterize lipidated proteins from mammalian cells. Metabolic labelling of cells with radiolabelled fatty acids allows the identification and characterization of palmitoylation of any protein from any cell line. However, these methods can be very hazardous due to the amount of radioactivity required to manipulate during metabolic labelling (e.g. ^{125}I). In addition, detection of palmitoylation could take days or even months when tritiated (^{3}H) palmitate is used (Tsai et al., 2014).

To overcome the barriers of using radiolabelled lipids for metabolic labelling and indirect methods, scientists have developed methods that are faster and less hazardous using biorthogonal chemistry (Sletten & Bertozzi, 2011). Biorthogonal chemistry modifies biological

molecules by inserting chemical groups, creating analogues which are not toxic or disruptive to the biological system. Cells and animals can be treated with these analogues and produce physiologically meaningful results. The next step is the detection of the analogue, with a tag that can be detected with traditional biochemical methods, for example using fluorophores and biotin. The tags cannot directly detect the analogue itself, but must be chemically attached.

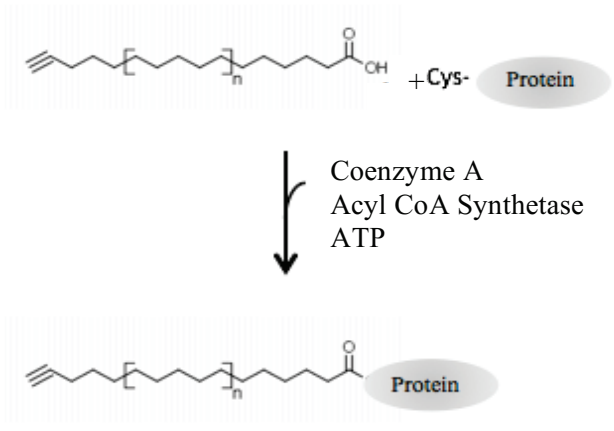
In the current thesis, a palmitic acid analogue (ω-alkynyl-palmitate) was used to metabolically label cells. The proteins acylated with ω-alkynyl-palmitate are immunoprecipitated then tagged with azido-biotin using copper(I)-catalyzed azide-alkyne [3+2] cycloaddition (click chemistry) (Figure 1.2). The protein-fatty acid-biotin complex formed that can be analysed by western blotting and detected by neutravidin.

This strategy has been optimized based on the Staudinger ligation (Sletten & Bertozzi, 2011; Yap et al., 2010). The cycloaddition is realized *in vitro* after isolation of the protein of interest. This is a copper-catalyzed process, therefore, toxic to the cells and animal (Baskin et al., 2007).

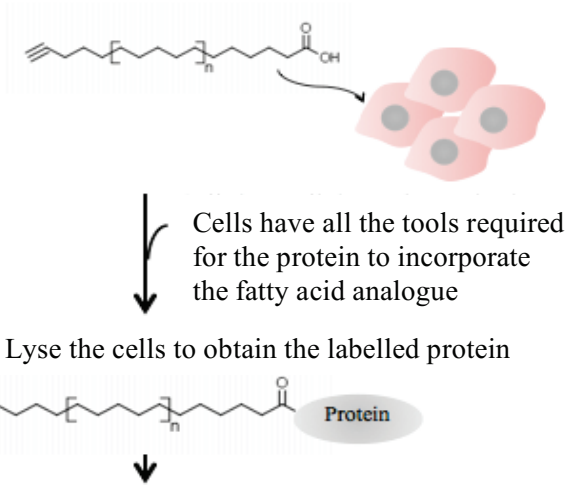
Indirect methods have also been developed to detect protein palmitoylation. Acyl–biotin exchange (ABE) identifies possible *S*-palmitoylation sites. Free Cys residues of immunoprecipitated protein are blocked by *N*-ethylmaleimide (NEM) and treated with hydroxylamine (NH_2OH) to cleave thioester bonds (removing palmitate from Cys, supposed palmitoylation sites). The free Cys is labelled with a thiol-specific reagent containing a probe that can be detected by western blot or imaging (e.g. biotin) or a fluorophore (Hannoush & Sun, 2010). Alternatively, ABE can be used for lipidomic profiling of palmitoylated proteins from a cell type or tissue. In this case, the lipidated protein tagged by ABE method is pulled down from the cells and identified by mass spectrometry (MS) analysis, this strategy has been named multi-

A) Protein labelling.

A.1 *In vitro* labelling.



A.2 Metabolic labelling



B) Click chemistry.

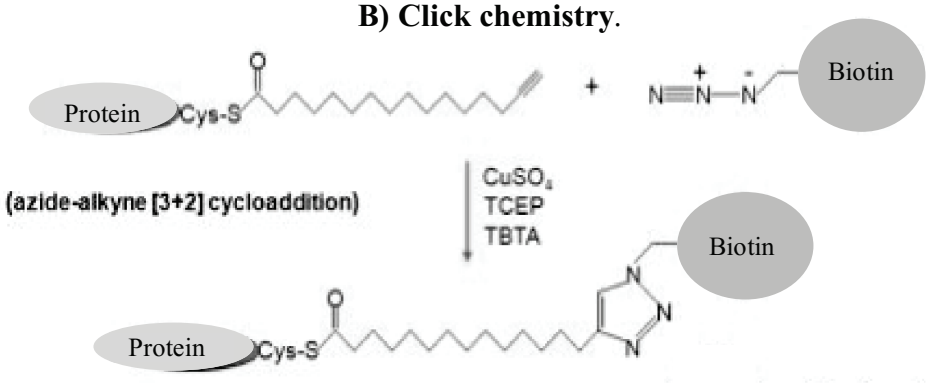


Figure 1.2 Detecting palmitoylation *in vitro* and after metabolic labelling of cells using the palmitate analogue (ω-alkynyl palmitate). (A) Protein labelling **(A.1)** ω-alkynyl palmitate is activated into its CoA ester prior to labelling proteins *in vitro*. **(A.2)** Cells are metabolically labelled with ω-alkynyl-palmitate analog, and immunoprecipitated**. (B)** Proteins acylated with ω-alkynyl palmitate are tagged with biotin using copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition (click chemistry). Figure and legend modified from (Yap et al., 2010). Modified from: Megan C. Yap et al., Rapid and selective detection of fatty acylated proteins using ω-alkynyl-fatty acids and click chemistry. 2010 51:(6) 1566-1580. doi:10. 1194/jlr.D002790

dimensional protein identification technology (MuDPIT) (Wan et al., 2007). In 2013, MS analysis has been optimized to identify palmitoylated peptides of purified proteins (Ji et al., 2013) and we have applied their strategy to identify palmitoylation sites as described in section 2.2.16.

1.5 Rationale and Objectives

Investigation of GSTP1 is required for a complete understanding of the multiple functions of this protein. GSTP1 is a hydrophilic protein that was shown to strongly associate with plasma membrane (Qazi et al., 2011). GSTP1 has a hydrophilic character; it does not associate with the cellular membranes through ionic interactions; and it does not have a hydrophobic domain that could be inserted into membranes. Therefore, I am investigating the role of GSTP1 lipidation in membrane association.

Overall objective

To determine the mechanism and relevance of the interaction between GSTP1 and the plasma membrane.

Hypothesis

Palmitoylation is responsible for the association of GSTP1 with the cellular membranes and regulates GSTP1 function.

Objective 1: Determine if GSTP1 is palmitoylated.

Optimization of metabolic labelling of MCF7 cells stably expressing GSTP1 with ω-alkynyl-palmitate and click chemistry.

Objective 2: Determine if palmitoylation of GSTP1 is via thioester bond.

In order to identify the palmitoylation sites, we utilized metabolic labelling and click chemistry on Cys mutants of GSTP1 expressed in MCF7 cells. In addition, we identified amino acids of GSTP1 modified *in vitro* by palmitoylation by LC-MS/MS.

Objective 3: Characterize the influence of palmitoylation on GSTP1 function.

Characterization of the subcellular localization of the palmitoylated GSTP1 was evaluated by immunohistochemistry. Subcellular fractionation of MCF7 cells expressing GSTP1 and metabolically labelled for click chemistry identified subcellular fractions containing palmitoylated GSTP1. In addition, the catalytic activities of the subcellular fractions were characterized.

Chapter 2 Glutathione Transferase P1 is Modified by Palmitate.

Chapter 2

2.1 Overview

Glutathione transferases (GSTs) are phase II biotransformation enzymes with well defined roles in detoxification (Hayes et al., 2005). Biotransformation by GSTs occurs through the nucleophilic addition of reduced glutathione (GSH, γ-Glu-Cys-Gly) to electrophilic compounds, generally rendering the electrophiles less reactive and more hydrophilic, consequently, preventing cell damage. The human GSTs are classified as mitochondrial, microsomal and cytosolic, where the cytosolic enzymes are sub-classified as alpha (A), pi (P), omega (O), mu (M), sigma (S), theta (T) and zeta (Z) (Habig et al., 1974; Hayes et al., 2005). GSTP1 is the most abundant GST isoform, in humans it is found in most cell types and tissues, with the exception of healthy adult hepatocytes (Raijmakers et al., 2001).

In addition to its role in detoxification, GSTP1 is a key regulator of signalling pathways, and involved in cell proliferation, cell death, and survival (Tew & Townsend, 2011). GSTP1 interacts with and regulates TNF receptor associated factor 2 (TRAF2), which mediates signal transduction from members of the TNF receptor family (De Luca et al., 2014), and c-Jun N-terminal kinases (JNKs) important for the activation of downstream regulators of cell survival and death (De Luca et al., 2012).

GSTP1 is a negatively charged soluble protein (Bhosale et al., 2004). Therefore, it is expected that the protein resides in a hydrophilic environment (Goto et al., 2001; Raza, 2011). GSTP1 is localized in multiple cellular compartments, it is found predominantly in the cytosol, and to a lesser extent mitochondria (Goto et al., 2009), and nuclei (Kamada et al., 2004). Surprisingly, endogenous GSTP1 was found in plasma membrane enriched vesicles prepared from the human small cell lung cancer cell line H69AR (Leslie et al., 2004) and HEK293T cells

(Qazi et al., 2011). Further to this, GSTP1 stably expressed in MCF7 cells was found to associate with plasma membrane sheets almost as strongly as the integral membrane protein Na^+/K^+-ATPase (Qazi et al., 2011). This led us to the hypothesis that GSTP1 was modified by a lipid and the lipidation process promotes GSTP1 association with cellular membranes.

Post-translational modification can be used as a strategy to coordinate dynamic localization, quaternary and tertiary protein structure, and/or activity. Co- and post-translational modification of proteins by lipids (lipidation) increases hydrophobicity, thus the affinity with cellular membranes. Proteins can localize and compartmentalize dynamically within the cell and these processes are especially important for multi-functional proteins. Farnesylation, cholesteroylation, glycosylphosphatidylinositol modification, irreversible fatty acylation (myristoylation) and reversible fatty acylation (palmitoylation) are the best studied type of lipid modifications (Mayor & Riezman, 2004; Nadolski & Linder, 2007; Resh, 2013).

GSTP1 is unlikely to be farnesylated or myristoylated because its amino acid sequence does not contain a CAAX motif (required for farnesylation) or an NH_2-terminal Gly (required for myristoylation) (Fu & Casey, 1999). GSTP1 cannot be cholesteroylated because this modification occurs in the ER lumen (Resh, 2013), and GSTP1 does not have a signal peptide sequence required for translocation to the ER. Thus, post-translational modification of GSTP1 by palmitate was a logical lipidation process to investigate.

Palmitoylation is a dynamic and reversible post-translational modification of proteins by long chain fatty acids, mostly palmitate (saturated 16 carbons). Palmitoylation can be important for membrane association, protein-protein interactions, and/or protein function (Dietrich & Ungermann, 2004; Linder & Deschenes, 2007). Cysteines (Cys) are by far the most common palmitoylation (*S*-palmitoylation) sites (Berthiaume, 2014; Salaun et al., 2010). A

family of 23 palmitoyl acyl transferases (PATs) that can catalyse the acylation of Cys residues of substrate proteins with palmitate are present in the mammalian genome (Lemonidis et al., 2015). These Asp-His-His-Cys (DHHCs) PATs are localized to membranes of the endoplasmic reticulum (ER), Golgi complex, and plasma membrane with the DHHC domaing facing the cytosol. The DHHC sequence is located in the PAT cytosolic domain, and is essential for *S*-palmitoylation of their substrates (Greaves & Chamberlain, 2011).

Palmitoylation of non-Cys residues has been reported, but to a much lesser extent. *O*-palmitoylation of Ser residues is mediated by enzymes localized in the ER lumen (Matevossian & Resh, 2015). The most studied *O*-palmitoylated protein is the secreted Wnt3a, which is acylated by fatty acids 14-16 carbons in length at Ser209 (Gao & Hannoush, 2014). *N*-palmitoylation of an NH_2-terminal Cys residues via amide linkage was reported for Sonic Hedgehog (Shh) and this is mediated by hedgehog acyltransferase (Hhat), within the secretory pathway (Buglino & Resh, 2008). To our knowledge there are no reports of Thr palmitoylation but this modification is considered biochemically possible due to the presence of a hydroxyl group on the side chain (Okubo et al., 1991)

N-palmitoylation onto an internal Lys is relatively rare. It was previously reported in a bacterium (Hackett et al., 1994) and recently the TNF-α cytokine was shown to be acylated on a Lys by myristate (Jiang et al., 2016).

In this thesis, human GSTP1 has been identified as a palmitoylated protein in MCF7 cells. Surprisingly, GSTP1 is palmitoylated on at least one non-Cys residue. Moreover, GSTP1 requires long metabolic labelling to incorporate the fatty acid, suggesting that modification is not as dynamic (it potentially has a slow turnover) as the classic *S*-palmitoylation. We characterized PAT-independent palmitoylation of human GSTP1 (purified from *E. coli) in vitro*

and identified Cys48, Cys102, and Lys103 as PAT-independent palmitoylation sites using LC-MS/MS. Interestingly, we have found that the cytosolic and membrane-associated GSTP1 are palmitoylated, but only the cytosolic GSTP1 is capable of catalysing the GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB). Moreover, we have shown that GSTP1 purified from the cytosolic fraction associates with plasma membrane enriched vesicles and the amount of GSTP1 associated is not increased by pre-treatment with palmitoyl CoA.

2.2 Methods

2.2.1 Materials

Rabbit pAb anti-GSTP1 (GS72) was from Oxford Biomedical Research (Rochester Hill, MI), rabbit pAb anti-Na^+/K^+-ATPase (H-300) was from Santa Cruz Biotechnology (Dallas, TX). The AffiniPure donkey anti-rabbit IgG conjugated to Cy™3 was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The Alexa Fluor® 488 conjugated goat anti-rat IgG was from Life Technologies (Grand Island, NY). The horseradish peroxidase (HRP) conjugated goat anti-rat, anti-mouse, and anti-rabbit IgG were from Thermo Scientific (Rockford, IL). Agarose affinity gel beads conjugated to mouse anti-V5 antibody (V5-10), and the mouse mAb anti-biotin (BN-34) were purchased from Sigma-Aldrich (Oakville, ON). The rabbit pAb anti-V5 was purchased from Rockland (Limerick, PA).

ω-alkynyl-palmitate was synthesized as described previously (Yap et al., 2010). Asp-N was purchased from Promega (Madison, WI) and RapiGest SF®-186001861 was purchased from Waters (Milford, MA). EDTA-free Complete™ protease inhibitor cocktail tablets and X-tremeGENE™9 transfection reagent were purchased from Roche Applied Science (Laval, QC). 3-(N-morpholino) propanesulfonic acid (MOPS), bicinchoninic acid (BCA) assay, neutravidin-

HRP, immobilized S-hexyl GSH agarose beads and Geneticin®(G418) were from Thermo Scientific (Rockford, IL). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Acyl-Coenzyme A (CoA) synthetase, Tris-(benzyltriazolylmethyl) amine (TBTA), palmitoyl CoA, Tris-carboxyethylphosphine (TCEP), lithium CoA (LiCoA), 1-chloro-2,4-dinitrobenzene (CDNB), magnesium chloride ($MgCl_2$), 1,4- dithiothreitol (DTT), Trisma-base, Tris-HCl, reduced L-glutathione (GSH), bovine serum albumin (BSA), $CuSO_4$, ATP, acetonitrile, *N*-ethylmaleimide (NEM), 4′,6-diamidino-2-phenylindole (DAPI), Duolink® *in situ* detection reagent orange, Duolink® *in situ* PLA® probe anti-mouse PLUS affinity purified donkey anti-mouse IgG (H+L), Duolink® *in situ* PLA ® probe anti-rabbit MINUS affinity purified donkey anti-rabbit IgG (H+L), Duolink® *in situ* wash buffers, were purchased from Sigma-Aldrich (Oakville, ON, Canada). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). The human GSTP1 expressed in and purified from *E.coli* (GS70) was purchased from Oxford Biomedical Research (Michigan, USA). DAKO mounting medium was from Agilent Technologies (Santa Clara, CA).

2.2.2 Cell lines

The human breast adenocarcinoma cell line (MCF7) and the SV40-transformed HEK293 (HEK293T) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). MCF7 and HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% or 7.5% fetal bovine serum (FBS), respectively and 4 mM L-glutamine. Routine testing for *Mycoplasma sp.* contamination of cell lines was performed using the ATCC Universal Mycoplasma Testing Kit (Manassas, VA). MCF7 cells were used in the majority of experiments because they are

known to have very little endogenous GSTP1 due to *GSTP1* silencing by hypermethylation of the CpG dinucleotides at the 5' transcriptional regulatory region (Lin et al., 2001).

2.2.3 Generation of V5 tagged GSTP1 expression vectors

The pBluescriptSK(−) GSTP1 construct was a kind gift from Dr. Philip G. Board (Australian National University, Canberra). pcDNA3.1(+) containing the full-length open reading frame of human GSTP1 [pcDNA3.1(+) GSTP1] was generated as previously described (Qazi et al., 2011). A construct of pcDNA3.1(+) GSTP1 containing a V5 tag at the NH_2-terminus [pcDNA3.1(+) V5_GSTP1] was generated by PCR amplification of pcDNA3.1(+) GSTP1 using the forward primer 5'...AAAAA*GGATCC*ACCATGGGTAAACCTATTCCTAATCCT CTTCTTGGTCTTGATTCTACA**CCGCCGTATACCGTGG**...3' with a *BamHI* restriction site indicated in italics, the V5 tag sequence is underlined and the GSTP1 sequence is in bold. The reverse primer used was 5'...CGGGCCCTCTAGA*CTCGAG***TCACTGTTTCCCGTTG CCATTGATGG**...3' with a *XhoI* restriction site indicated in italics and GSTP1 sequence indicated in bold.

A construct of pcDNA3.1(+) GSTP1 containing a V5 tag at the COOH-terminus [pcDNA3.1(+)-GSTP1_V5] was generated by PCR amplification of pcDNA3.1(+)-GSTP1 using the forward primer 5'...TACCGAGCTC*GGATCC***GCCATGCCGCCGTATACCGTG** ...3'with a *BamHI* restriction site indicated in italics and the GSTP1 sequence in bold. The reverse primer used was'...TTT*CTCGAG*CTATGTAGAATCAAGACCAAGAAGAGGATTA GGAATAGGTTTACC**CTGTTTCCCGTTGCCATTGATG**...3' with an *XhoI* site indicated in italics, the V5 tag sequence is underlined and the GSTP1 sequence is in bold. The PCR product and empty pcDNA3.1(+) vector were digested with *BamHI* and *XhoI* and then ligated.

The correctness of the pcDNA3.1(+)-V5_GSTP1 and pcDNA3.1(+) GSTP1_V5 constructs were confirmed by sequencing (The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON).

Table 2.1
Forward primers used for generating the GSTP1 mutants by site-directed mutagenesis.

Mutant	**Primer Sequence (5' to 3')**
Cys15Ser	TTCGAGGCCGCTCC**A**GC GCCCTGCG
Cys48Ser	CTCACTCAAAGCCTCC**A**GCCTATACGGGC
Cys102Ser	GGAGGACCTCCGC**A**GCAAATACATCTCCC
Cys170Ser	CCTAGCCCCTGGC**A**GCCTGGATGCGTTC
Cys15Ala	CAGTTCGAGGCCGC**GCG**GCGGCCCATGC
Cys48Ala	CTCACTCAAAGCCTCC**GCG**CTATACGGGC
Cys102Ala	GGAGGACCTCCGC**GCG**AAATACATCTCCC
Cys170Ala	CCTAGCCCCTGGC**GCG**CTGGATGCGTTC
Cys102Ala/Lys103Arg	GTGGAGGACCTCCGC**GCCGC**ATACATCTCCCTCATCTAC

Nucleotides mutated are highlighted in bold.

2.2.4 Site-directed mutagenesis

GSTP1 mutants were generated using the Agilent QuikChange Lightning site-directed mutagenesis kit (Mississauga, ON). Mutagenesis was performed using the pcDNA3.1(+)-V5_GSTP1 construct as the PCR template, according to the manufacturer's instructions. Mutagenic primers [Sigma-Aldrich (Oakville, ON) or Integrated DNA Technology (Coralville, IA)] were designed to generate four individual Cys to Ser mutants (V5_GSTP1-C15S, V5_GSTP1-C48S, V5_GSTP1-C102S, V5_GSTP1-C170S) (sequences in Table 2.1). A Cys-less V5_GSTP1 mutant was also generated; where all four Cys residues within GSTP1 were mutated to Ser [V5_GSTP1-Cys15/Cys48/Cys102/ Cys170Ser, named GSTP1_4X(Cys to Ser). Lastly, a Lys103Arg mutation was added to a GSTP1_4X(Cys to Ala) [V5_GSTP1-Cys15/Cys48/Cys102/Cys170Ala, named GSTP1_4X(Cys to Ala)] and it was named GSTP1-4X(Cys to Ala)/Lys103Arg [primer sequences in (Table 2.1)]. The presence of the mutants and

the lack of unintentional mutations were confirmed by sequencing the entire GSTP1 coding region (The Center for Applied Genomics, The Hospital for Sick Children, Toronto, ON).

2.2.5 Generation of stable MCF7 cell lines expressing wild-type GSTP1 tagged with V5 at the NH_2 and COOH termini (V5_GSTP1 and GSTP1_V5, respectively) and mutant V5_GSTP1

Generation of the MCF7-GSTP1 (untagged) and MCF7-vector stable cell lines was described previously (Qazi et al., 2011). MCF7 cells stably expressing pcDNA3.1(+)-V5_GSTP1, -GSTP1_V5, V5_GSTP1-4X(Cys to Ser) or -V5_GSTP1-4X(Cys to Ala)K103R (named MCF7-V5_GSTP1, MCF7-GSTP1_V5, MCF7-V5_GSTP1-4X(Cys to Ser), or MCF7-V5_GSTP1-4X(Cys to Ala)/Lys103Arg, respectively) were generated as described previously (Qazi et al., 2011), with minor modifications. Briefly, MCF7 cells were seeded at $3x10^5$ cells per well in a 6-well plate and transfected with 1 μg DNA using 3 μl X-tremeGENE9, according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were selected in 1 mg/ml of G418, and approximately two weeks later the selected cells were cloned by limiting dilution. GSTP1 protein levels in G418-resistant cell clones were determined by immunoblotting with the rabbit anti-GSTP1 pAb (1:5000). Cells were then maintained with media as described in section 2.2.2, supplemented with 600 μg/ml of Geneticin® G418.

2.2.6 Transient transfection of MCF7 and HEK293T cells.

HEK293T and MCF7 cells were seeded at a density of $6.5 x10^5$ and $1x10^6$ cells per T-25 flask, respectively. Twenty-four h later, cells were transfected at a 3:1 ratio of X-tremeGENE9:DNA, according to the manufacturer's instructions (Roche Applied Sciences, Penzberg, Germany).

2.2.7 Preparation of dextran-coated charcoal (DCC) stripped-FBS

To generate fatty acid free (FAF) FBS, dextran-coated charcoal (DCC) was prepared by incubating 200 ml of Dextran-70 (0.1%), Tris-HCl (10 mM, pH 7.4) with activated charcoal (2.5%), and left to stir overnight at 4°C. The suspension was then spun at 2800 g for 10 min to pellet the charcoal. The supernatant was discarded and 500 ml of FBS was added to DCC, shaking at 56°C for 30 min followed by 2800 g centrifugation for 10 min at room temperature. The charcoal was discarded and the FAF-FBS supernatant was filtered through a 0.45 μm Millipore pre-filter, then filtered through a 0.22 μm filter. Aliquots of FAF-FBS were stored at -20°C.

2.2.8 Metabolic labelling of cells with ω-alkynyl palmitate.

Metabolic labelling of cells with ω-alkynyl-palmitate (Figure 1.2), was adapted from a method described previously (Yap et al., 2010). Briefly, HEK293T and MCF7 cells transiently or stably expressing GSTP1 were grown until 70-80% confluency, then the culture medium was exchanged for DMEM with 10% FAF-FBS and ω-alkynyl-palmitate (100 μM). The ω-alkynyl-palmitate was dissolved in DMSO and diluted in the FAF medium right before labelling started. The final DMSO concentration in the culture media was 0.1%. The unlabelled cells were maintained in DMEM contaning the regular FBS and the same amount of DMSO (vehicle) as the labelled cells. The FAF medium containing ω-alkynyl-palmitate was replaced daily for 24-48 h, unless indicated otherwise.

2.2.9 Immunoprecipitation of wild-type and mutant forms of GSTP1

After metabolic labelling with ω-alkynyl-palmitate, cells were washed with cold PBS,

harvested, and lysed with cold EDTA-free RIPA buffer [0.1% SDS, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium-deoxycholate, 2 mM $MgCl_2$, EDTA-free Complete^{TM} protease inhibitor cocktail] for 15 min at 4°C. Cell lysates were centrifuged at 16,000 *g* for 10 min at 4°C and the post-nuclear supernatant collected. GSTP1 was then immunoprecipitated from this fraction by incubating with agarose beads conjugated to mouse anti-V5 antibody (V5-10) overnight at 4°C. Beads were then spun down at 16 000 *g* for 10 min and washed with 1 ml of cold PBS with 0.1% Triton-X, 3-5 times. GSTP1 was eluted from the beads by incubating for >10 min at room temperature with Laemlli buffer or subjected to click chemistry (Section 2.2.10),

2.2.10 Detection of GSTP1 metabolically labelled with ω-alkynyl-palmitate using click chemistry.

Immunoprecipitated GSTP1 was eluted from the agarose beads conjugated to mouse anti-V5 antibody (V5-10), by incubating at 80°C for 15 min in 50 mM HEPES, pH 7.4, with 1% SDS. The beads were then pelleted by centrifuging at 2800 g and the supernatant containing GSTP1 collected. The supernatant was then incubated with click reaction components [Tris-(benzyltriazolylmethyl) amine (100 μM), $CuSO_4$ (1 mM), Tris-carboxyethylphosphine (1 mM), and azido-biotin (100 μM)] at 37°C for 30 min, protected from light. The click reaction was stopped by the addition of a 10X volume of ice-cold acetone and incubated overnight at 4°C to precipitate proteins. To determine the alkali sensitivity of palmitoylation, samples were treated with NaOH (0.1 M) at room temperature for 1 h after being eluted from the beads. The samples were then neutralized by adding an equal volume of HCl (0.1 M). Proteins were then processed and subjected to click chemistry as described above. Precipitated proteins were then pelleted at

16 000 *g* for 15 min, the supernatant discarded and the pellet resuspended in Laemmli buffer containing DTT (30 mM). Samples were heated at 95°C for 5 min, resolved on 11% SDS-PAGE then electrotransfered to a PVDF membrane. Biotinylated ω-alkynyl-palmitate-labelled GSTP1 was detected with neutravidin-HRP (1:20000) and total GSTP1 loading was detected with rabbit pAb anti-GSTP1 (GS72) (1:5000).

2.2.11 Identification of GSTP1 proximity with ω-alkynyl-palmitate using *in situ* proximity ligation assay (PLA)

MCF7-V5_GSTP1and MCF7-vector cells were seeded at $2x10^5$ cells per well of a 6-well plate on glass cover slips and grown overnight. Cells were then metabolically labelled with ω-alkynyl-palmitate for 24-48 h. Coverslips were washed twice with PBS, fixed with ice-cold methanol at -20ºC, and permeabilized with Triton-X 100 (0.1%) in PBS for 15 min at room temperature. Cells were washed twice with 1X PBS and then the click reaction components were added onto the coverslips and incubated at 37ºC for 1 h in the dark. After the click reaction, coverslips were washed three times with 1X PBS and incubated with blocking buffer [BSA (3%) + Triton-X 100 (0.1%) in 1X PBS] for 1 h at room temperature. Rabbit pAb anti-V5 (1:500) and mouse mAb anti-biotin (BN-34) (1:500) were diluted in blocking buffer and added to the coverslips for 1-2 h at room temperature. Cells were washed with washing buffer [BSA (0.3%) + Triton (0.1%) in 1X PBS] and from this step on, reagents from the DUOLINK® PLA kit were used according to the manufacturer's instructions (OLINK Bioscience, Uppsala, Sweden). Briefly, coverslips were washed with washing buffer A, probed with anti-mouse PLUS and anti-rabbit MINUS 1:500, where PLUS and MINUS stands for 5' to 3' reverse and forward oligonucleotides, respectively, and incubated for one hour at 37ºC. Coverslips were

washed with washing buffer A and incubated with 1 U of ligase in ligation buffer for 30 min at 37ºC, then washed twice with washing buffer A. The ligation product was amplified with 5 U of polymerase in amplification buffer containing the orange detection reagent [excitation 554 nm and emission at 579 nm ($Cy^{TM}3$)] and DAPI, for 100 min at 37ºC. All of the reactions with the PLA kit were done in a humidified chamber. Coverslips were mounted onto slides with DAKO mounting medium and stored at 4ºC protected from light until visualization. In addition to the MCF7-vector negative control, MCF7-GSTP1 cells not labelled with ω-alkynyl-palmitate, not subjected to click chemistry or not having PLA performed were used as negative controls.

2.2.12 Immunofluorescence imaging

Cells were viewed with a 60X oil immersion objective with a fully motorized inverted fluorescence microscope (Leica DMI6000 B, Wetzlar, Germany) coupled to a X-Cite®exacte fluorescence light source, equipped with Quorum Mac 6000 system and the Angstrom illumination system associated with Optigrid (Guelph, ON, Canada). The MetaMorph software (Molecular devices, Sunnyvale, CA) was used for multi-dimensional acquisition that allows the measurement of multiple Z sections. Images were captured with a Flash 4.0 camera (Hamamatsu) and analysed by Volocity® (Improvision, Perkin Elmer, Waltham, MA).

2.2.13 Synthesis of ω-alkynyl-palmitate-CoA

ω-alkynyl-palmitate-CoA was freshly synthesized prior to use in experiments, as described previously (Kostiuk et al., 2008). Briefly, ω-alkynyl-palmitate (1 mM) was incubated with Li CoA (1 mM), ATP (5 mM), Triton X-100 (2.5%), and acyl CoA synthetase (1 U/ml) in acylation

buffer (Tris-HCl 20 mM, pH 7.4 , $MgCl_2$ 10 mM, EGTA 200 μM, DTT 2 mM) at 37ºC for 30 min protected from light.

2.2.14 Characterization of PAT-independent modification of GSTP1 with ω-alkynyl palmitate-CoA and detection using click chemistry.

Commercially available recombinant human GSTP1 (0.5 μg) expressed in and purified from *E. coli* was incubated with ω-alkynyl-palmitate-CoA (100 μM) for 20 min at room temperature for *in vitro* palmitoylation. After the incubation, click reaction components [Tris-(benzyltriazolylmethyl) amine (100 μM), $CuSO_4$ (1 mM), Tris-carboxyethylphosphine (1 mM), and azido-biotin (100 μM)] in HEPES (50 mM) containing SDS (1%) were added and incubated at 37°C for 30 min, protected from light. The click reaction was stopped and GSTP1 precipitated by the addition of a 10X volume of ice-cold acetone overnight at -20°C. Precipitated protein was pelleted at 16 000 *g* for 15 min at 4°C and resuspended in Laemmli buffer containing DTT (30 mM). Samples were heated at 95°C for 5 min then resolved on 11% SDS-PAGE and electrotransfered to a PVDF membrane.

To detect non-covalent interactions of ω-alkynyl-palmitate-CoA with GSTP1, GSTP1 was incubated with either ω-alkynyl-palmitate or LiCoA alone, as described above for ω-alkynyl-palmitate-CoA.

2.2.15 Biochemical characterization of PAT-independent GSTP1 palmitoylation

To determine if the PAT-independent covalent modification of GSTP1 by ω-alkynyl-palmitate is through an oxyester, thioester, or amide bond, biochemical aproaches previously applied to characterize palmitoylation of other proteins were utilized (Kostiuk et al., 2009). To begin with,

the functional group on potentially palmitoylated amino acids Cys and Lys were blocked with the covalent modifier NEM at either pH 7.4 (to block Cys residues) or pH 10 (to block Lys and Cys residues). Thus, GSTP1 from *E. coli* (1 µg) was incubated in the dark with or without NEM (10 mM) in MOPS (50 mM at either pH 7.4 or 10), at room temperature, for 30 min. NEM-treated GSTP1 was then reacted with ω-alkynyl-palmitoyl-CoA, ω-alkynyl-palmitate, or Li-CoA, and subjected to the click reaction. To determine if GSTP1 was covalently modified by palmitate through an ester bond, additional treatments were done. NaOH breaks oxyester and thioester bonds, but not amide bonds. Palmitoylated GSTP1 (2 µg) was incubated with NaOH (0.1 M) for one h at room temperature. NaOH treatment was stopped by the addition of HCl (at a 1:1 molar ratio with NaOH). GSTP1 was precipitated with ice-cold acetone overnight and subjected to click chemistry followed by 11% SDS-PAGE and immunoblotting, as described above.

2.2.16 Mapping non-PAT mediated GSTP1 palmitoylation sites by LC-MS/MS

GSTP1 (2 µg) was incubated with palmitoyl-CoA or CoA alone, (as described in 2.2.14) and then digested for 6 h with 0.6 µg Asp-N (which cuts on the NH2-terminal side of Asp and Cys residues) in 0.05% surfactant RapiGest SF® at 37ºC. The digestion was stopped with trifluoroacetic acid (TFA, 1%) and dried using a speed vacuum. The samples were enriched for palmitoylated peptides using column chromatography with POROS R1 50 resin. Palmitoylated peptides were eluted stepwise in 60% and 80% acetonitrile and analysed by liquid chromatography, using a C18 analytic column and analysed by electrospray tandem mass spectroscopy (LC/MS/MS) on a LTQ-Orbitrap XL mass spectrometer as described previously (Ji et al., 2013).

2.2.17 Subcellular fractionation of MCF7-GSTP1 cells

MCF7-GSTP1 cells (2 x 10^7) were re-suspended in tris-sucrose-buffer (50 mM Tris, pH 7.4, 250 mM sucrose) containing $CaCl_2$ (250 μM) and EDTA free Complete™ protease inhibitors, and disrupted by N_2 cavitation as described previously (Loe et al., 1996). Cell homogenates were centrifuged at 2000 *g* at 4ºC for 10 min to remove nuclei and unbroken cells. 500 μl of supernatant was transferred to 1.7 ml ultracentrifuge tubes, centrifuged at 100 000 *g* for 30 min at 4°C (Beckman TLA 110 rotor). Supernatent was collected (490 μl of cytosolic fraction) while ellular membranes (P100) were washed once with 1X PBS and re-suspended in 50 μl Tris (10 mM, pH 7.4). For immunoprecipitation of GSTP1, the cellular membranes were re-suspended in 500 μl Tris (10 mM, pH 7.4) + SDS (0.1%). BCA or Bradford protein assays were performed to determine protein concentrations, and the fractions were either used fresh or stored in aliquots at -80ºC (cellular membrane fractions) or -20°C (cytosolic fractions).

2.2.18 Catalytic activity of total, cytosolic, and crude membrane fractions isolated from MCF7-vector and MCF7-GSTP1

1-chloro-2,4-dinitrobenzene (CDNB) is a well established and commonly used GST substrate (Habig et al., 1974) that is conjugated with GSH to form 2,4-dinitrophenyl-S-glutathione (DNP-SG). The enzymatic activity of subcellular fractions (total, cytosol, or cellular membranes) isolated from MCF7-GSTP1 and MCF7-vector cells was evaluated using CDNB. Assays were performed in 96 well plates and activity measured by monitoring the formation of DNP-GS at 340 nm ($\Delta\varepsilon$ = 9600 M^{-1} cm^{-1}) every 30 seconds for 10 min, in 100 mM potassium phosphate buffer (K_2PO4) pH 6.8 at 25°C (Habig et al., 1974). CDNB (1 mM) was added to 3 μg of protein (input, cytosol, and membrane), followed by GSH (2.5 mM). Spontaneous reaction of GSH with

CDNB (blank) was subtracted from the absorbance of the GSTP1-catalysed reactions. The rate was calculated over 300 s and expressed as mean ± SD of at least three independent experiments. In parallel with the CDNB, 5 μg of each fraction was analysed by western blotting to determine the relative level of GSTP1 in each fraction (Figure 2.8A) by densitometry and that value was used to normalize the final absorbance (mean of abosorbance triplicates – mean of blank absorbance triplicate) of CDNB catalytic activity. Relative levels of GSTP1 were quantified using ImageJ.

2.2.19 GSTP1 purification from MCF7-GSTP1 cells

The cytosolic fraction of MCF7-GSTP1 was incubated with 300 μl of immobilized *S*-hexyl-GSH-agarose beads for 1 h at 4ºC, centrifuged at 10000 *g* for 10 min at 4ºC and then beads were washed with Na_2HPO_4 , pH 6.8, and NaCl 150 mM. Protein was eluted with 20 mM GSH in Na_2HPO_4 , pH 8.0 and NaCl 150 mM. Eluted protein was concentrated using a speed vacuum, then the protein concentrations were determined at 280 nm using a NanoDrop 2000 (Wilmington, DE, USA). Eluted and concentrated protein (3 μg) was resolved on 11% SDS-PAGE. The gel was fixed for 30 min in fixation buffer (isopropanol 25%, acetic acid 10%), stained for 2 h with Coomassie 250 (60 mg/L) for 30 min followed by overnight destaining in acetic acid 10%, then the gel was dried.

2.2.20 Characterization of GSTP1 association with MCF7 plasma membrane-enriched vesicles

Plasma membrane-enriched vesicles were prepared from MCF7 cells using N_2 cavitation, as previously described (Loe et al., 1996). Total protein concentrations were determined using the

BCA assay according to the manufacturer's instructions. Purified human GSTP1 from *E.coli* or MCF7-GSTP1 cells was incubated with or without palmitoyl-CoA (100 μM) in sodium phosphate buffer K_2HPO_4 (100 mM, pH 7.4) at 25ºC for 20 min. Vesicles (20 μg of protein) were then added and incubated for 30 min at 37ºC, samples were then centrifuged at 100000 *g* at 4ºC for 1 h. Supernatants were collected and diluted in Laemmli buffer whereas the pellet was washed once with 100 μl of Tris buffer (10 mM, pH 7.4), and then resuspended in Laemmli buffer. Samples were resolved on 11% SDS-PAGE, transferred onto a PVDF membrane and immunostained with rabbit pAb anti-GSTP1 (GS72) (1:5000), equalness of vesicle loading was detected using the rabbit pAb anti-Na^+/K^+-ATPase (H-300) (1:10000).

2.3 Results

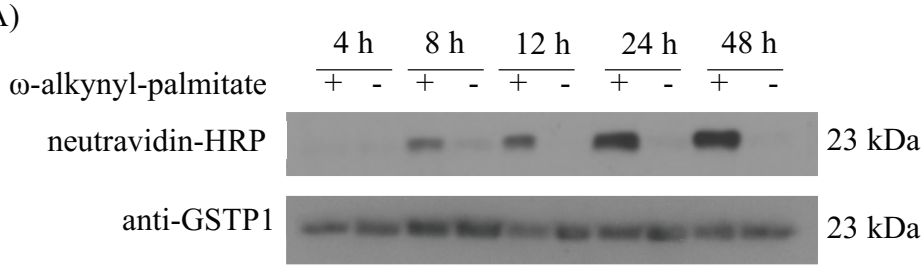
2.3.1 V5_GSTP1 is post-translationally modified by palmitate in MCF7 cell lines

To determine if GSTP1 is post-translationally modified by palmitate, MCF7 cells stably expressing GSTP1 tagged at the NH_2-terminus (MCF7_V5_GSTP1) were metabolically labelled with ω-alkynyl-palmitate and harvested at multiple time points over four to forty-eight h. Cells were lysed, GSTP1 was immunoprecipitated with V5 antibody conjugated to agarose beads, eluted from the beads, and subjected to click chemistry to label ω-alkynyl-palmitate with biotin (methods summarized in Figure 1.2). Samples were analysed by western blotting, palmitoylated GSTP1 was detected with neutravidin-HRP and total GSTP1 was detected with rabbit pAb anti-GSTP1 (GS72) (1:5000). We report the novel observation that GSTP1 is post -translationally modified by palmitate (Figure 2.1A, top panel) as evidenced by the detection of a neutravidin-HRP signal (revealing the presence of a GSTP1-palmitate-biotin complex). This signal was only detected for GSTP1 immunoprecipitated from cells metabolically labelled with

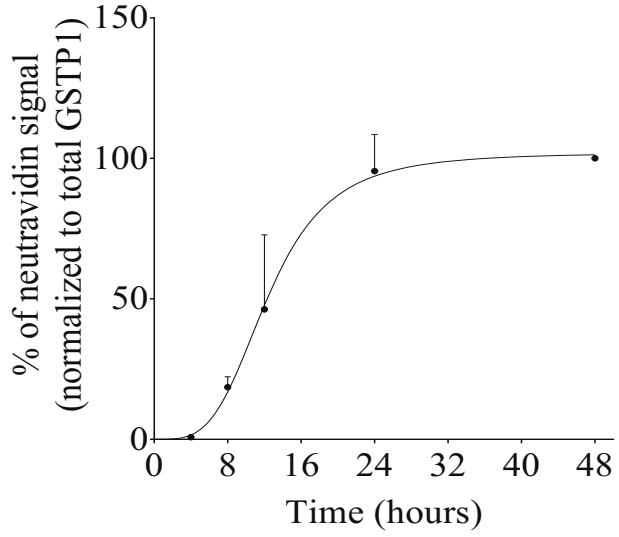
ω-alkynyl-palmitate and not cells treated with a vehicle control. Interestingly, GSTP1 palmitoylation was not detected until eight h after initiating metabolic labelling with ω-alkynyl-palmitate and plateaued at 24 h (Figure 2.1A & B). These data could suggest that palmitoylation turnover is not as fast and dynamic as it is for prototypical palmitoylated proteins such as H- and N-Ras, which require less than four hours of metabolic labelling for detection of palmitoylation (Yap et al., 2010). Metabollic labelling with ω-alkynyl-palmitate was done for a minimum of 24 h for all future experiments.

Previous studies have shown that a green fluorescence protein tag on the COOH terminus of GSTP1 prevents its targeting to the nucleus, possibly through a conformational change (Kawakatsu et al., 2011). Thus it was important to test if the V5 tag position might influence palmitoylation. MCF7 cells transfected with GSTP1 tagged with the V5 epitope at either the NH_2-terminus (MCF7-V5_GSTP1) or COOH-terminus (MCF7-GSTP1_V5) were metabolically labelled with ω-alkynyl-palmitate, subjected to click chemistry, and analysed by western blotting. Consistent with the time course data (Figure 1A and B), a strong neutravidin-HRP signal was detected for GSTP1 from MCF7-V5_GSTP1 cells metabolically labelled with ω-alkynyl-palmitate (Figure 2.1C, left most lane). In contrast, no neutravidin signal was detected for GSTP1 from MCF7-GSTP1_V5 cells metabolically labelled with ω-alkynyl-palmitate (Figure 2.1C third lane) or for either cell line treated with vehicle control (Figure

A)


4 h
8 h
12 h
24 h
48 h
+ - + - + - + - + -
ω-alkynyl-palmitate
neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa

B)


% of neutravidin signal
(normalized to total GSTP1)
150
100
50
0
0
8
16
24
32
40
48
Time (hours)

C)

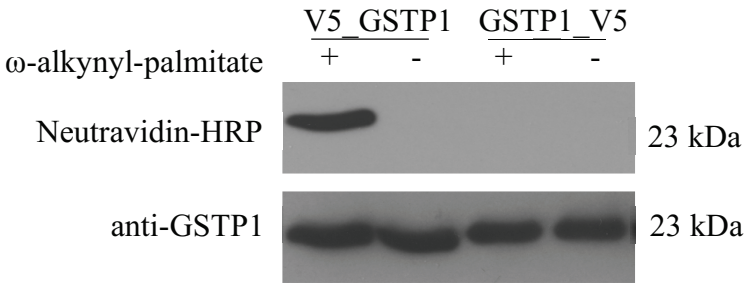

V5_GSTP1
GSTP1_V5
+ - + -
ω-alkynyl-palmitate
Neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa

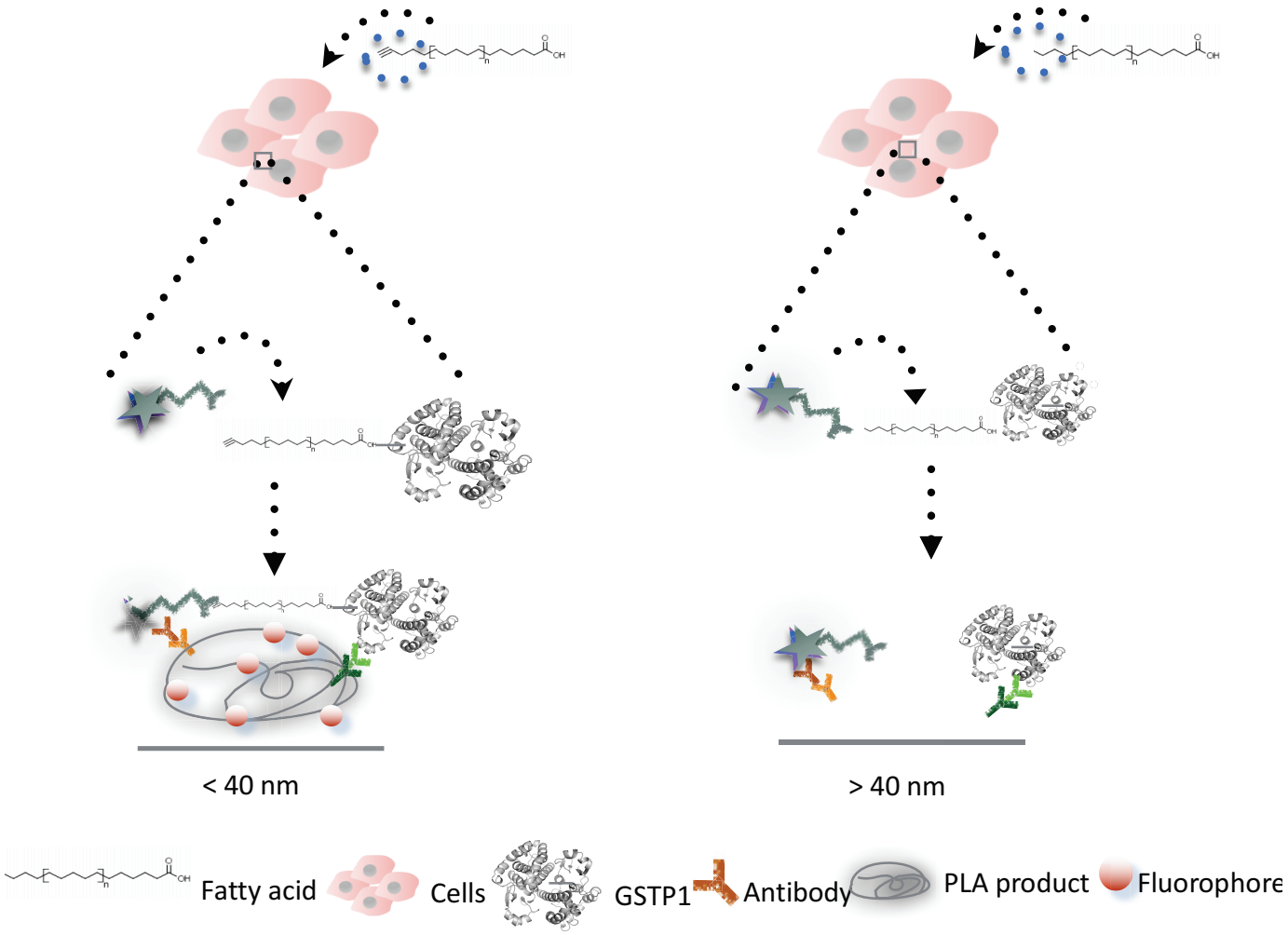
Figure 2.1 *Palmitoylation of GSTP1 in MCF7 cells*

MCF7-V5_GSTP1 or MCF7-GSTP1_V5 cells were metabolically labelled with ω-alkynyl-palmitate (+) or vehicle control (-). Cells were lysed and GSTP1 was immunoprecipiated with agarose beads conjugated to mouse anti-V5 antibody (V5-10), subjected to click chemistry, resolved on 11% SDS-PAGE and transferred to a PVDF membrane. Palmitoylated GSTP1 was detected with neutravidin-HRP (1:20000) and total GSTP1 was detected with rabbit pAb anti-GSTP1 (GS70) (1:5000). **(A)** Time course of GSTP1 metabolically labelled for 4, 8, 12, 24 and 48 h. *(Top panel)* Signal from neutravidin-HRP corresponding to palmitoylated GSTP1. *(Bottom panel)* Signal from anti-GSTP1 antibody corresponding to total GSTP1. **(B)** Densitometry on the bands detected by neutravidin-HRP and anti-GSTP1 in (A) was performed using ImageJ software. The relative level of palmitoylated GSTP1 (neutravidin-HRP) was expressed as % of total GSTP1 plotted over time. Symbols represent means +SD from at least 3 independent experiments. **(C)** MCF7- V5_GSTP1 and -GSTP1_V5 cells were metabolically labelled with ω-alkynyl-palmitate for 24 h (*Top panel*). Signal from neutravidin-HRP corresponding to palmitoylated GSTP1. (*Bottom panel*) Signal from anti-GSTP1 antibody corresponding to total GSTP1. Shown is a representative blot and similar results were obtained in three additional independent experiments.

2.1C). Thus, the V5 epitope tag at the COOH-terminus prevents GSTP1 palmitoylation and the NH_2-terminal V5_GSTP1 was used for all future experiments.

2.3.2 GSTP1 is found in close proximity with ω-alkynyl-palmitate by proximity ligation assay.

GSTP1 palmitoylation was further characterized by a specialized adaptation of an *in situ* proximity ligation assay (PLA) for the detection of palmitoylated proteins (Figure 2.2A). This technique has been described previously for Wnt proteins (Gao & Hannoush, 2014). MCF7-V5_GSTP1 and MCF7-vector cell lines were metabolically labelled with ω-alkynyl-palmitate, fixed, subjected to click chemistry, and the PLA performed utilizing antibodies directed towards the V5 tag of GSTP1 and biotin (to detect palmitoylated protein). As expected, GSTP1 (green) was distributed throughout the cell cytosol and nuclei (Figure 2.2.B.i). The PLA product, indicating a close proximity between GSTP1 and ω-alkynyl-palmitate (orange) was also distributed throughout the cytosol and nuclei (Figure 2.2.B.ii). Localization of GSTP1 (green) and the PLA product (orange) was overlapping in many regions of the cell including stippled structures around and within nuclei (Figure 2.2.B.iii). Several regions of only GSTP1 (green) were also present, showing the presence of GSTP1 not in close proximity with ω-alkynyl-palmitate. These data provide evidence that specific populations of GSTP1 are palmitoylated and that palmitoylation potentially influences cellular localization.


< 40 nm
> 40 nm
Fatty acid
Cells
GSTP1
Antibody
PLA product
Fluorophore

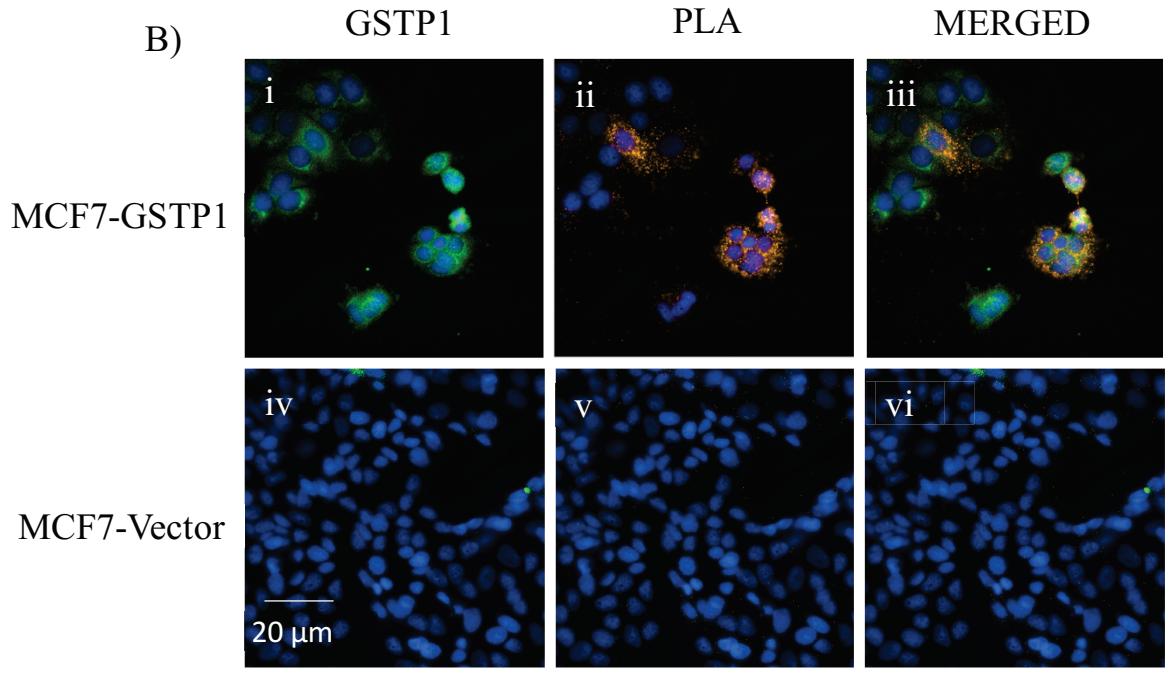

B)
GSTP1
PLA
MERGED
MCF7-GSTP1
MCF7-Vector
i
ii
iii
iv
v
vi
20 µm

Figure 2.2 *Detection of cellular GSTP1 palmitoylation by a modified proximity ligation assay*

(A) Schematic of the modified PLA. Cells metabolically labelled with ω-alkynyl-palmitate labelled with azido-biotin followed by detection of the fatty acid and the protein of interest with primary antibodies. The close proximity generates PLA producet that is detected by an oligonucleotide with a fluorophore, images can be seen by microscopy (left panel). When cells are metabolically labelled with palmitate in place of ω-alkynyl-palmitate, the click chemistry (cycloaddition reaction) does not occur and the PLA product is not formed and the close proximity cannot be detected. This can be used as a negative control **(B)** MCF7-V5_GSTP1 and MCF7-vector cells were metabolically labelled with ω-alkynyl-palmitate for 24 h, fixed, permeabilized and subjected to click chemistry. Cells were incubated with mouse mAb anti-biotin (BN-34) (1:500) and pAb rabbit anti-GSTP1(GS72) (1:250) for reaction with the biotinylated fatty acid analogue and GSTP1, respectively. Cells were then incubated with secondary antibodies PLUS and MINUS probe and ligated. The ligation product was amplified and detected with Duolink® *in situ* detection reagent (orange), nuclei (blue) were detected with DAPI and GSTP1 (green) was detected with anti-rabbit Alexa488 (1:500). Cells were viewed with a Leica fluorescence microscope at a 60X magnification. **(i-iii)** MCF7-V5_GSTP1 cells **(iv-vi)** MCF7-vector control displayed. Similar results were obtained for multiple cells from two additional independent experiments. MCF7-vector was used as negative control. In addition MCF7-V5_GSTP1 not treated with ω-alkynyl-palmitate, not subjected to click chemistry or not subjected to PLA were also used with results similar to MCF7-vector cells. Images were acquired in Volocity.

2.3.3 Individual GSTP1 Cys to Ser mutants retain palmitoylation.

S-palmitoylation of Cys residues is the most common palmitoylation type. GSTP1 has four Cys residues (Cys15, Cys48, Cys102, and Cys170) the location of each is indicated in the cytosolic GSTP1-1 dimer X-ray crystal structure (bound to the GSH conjugate of ethacrynic acid) in (Figure 2.3A). To determine if any of the Cys residues are modified by palmitate they were individually mutated to Ser (V5_GSTP1-Cys15Ser, V5_GSTP1-Cys48Ser, V5_GSTP1-Cys102Ser, and V5_GSTP1-Cys170Ser) and expressed transiently in HEK293T cells. Transfected cells were metabolically labelled with ω-alkynyl-palmitate, GSTP1 was immunoprecipitated using agarose beads conjugated to mouse anti-V5 antibody (V5-10), then subjected to click chemistry and analysed by western blotting with neutravidin-HRP and rabbit pAb anti-GSTP1 (GS72) (Figure 2.3B). If GSTP1 contains a single Cys residue that is palmitoylated it was expected that a complete loss in neutravidin signal would be observed for one of the Cys to Ser mutants. Somewhat surprisingly, all four individual Cys to Ser mutants retained a neutravidin/ palmitoylation signal. Each mutant had its specific negative control (cells treated with vehicle in place of ω-alkynyl-palmitate), and as expected, no signal was detected. These results could suggest that Cys residues are either not utilized for palmitoylation or that more than one Cys, or a combination Cys residues and another amino acid are palmitoylated.

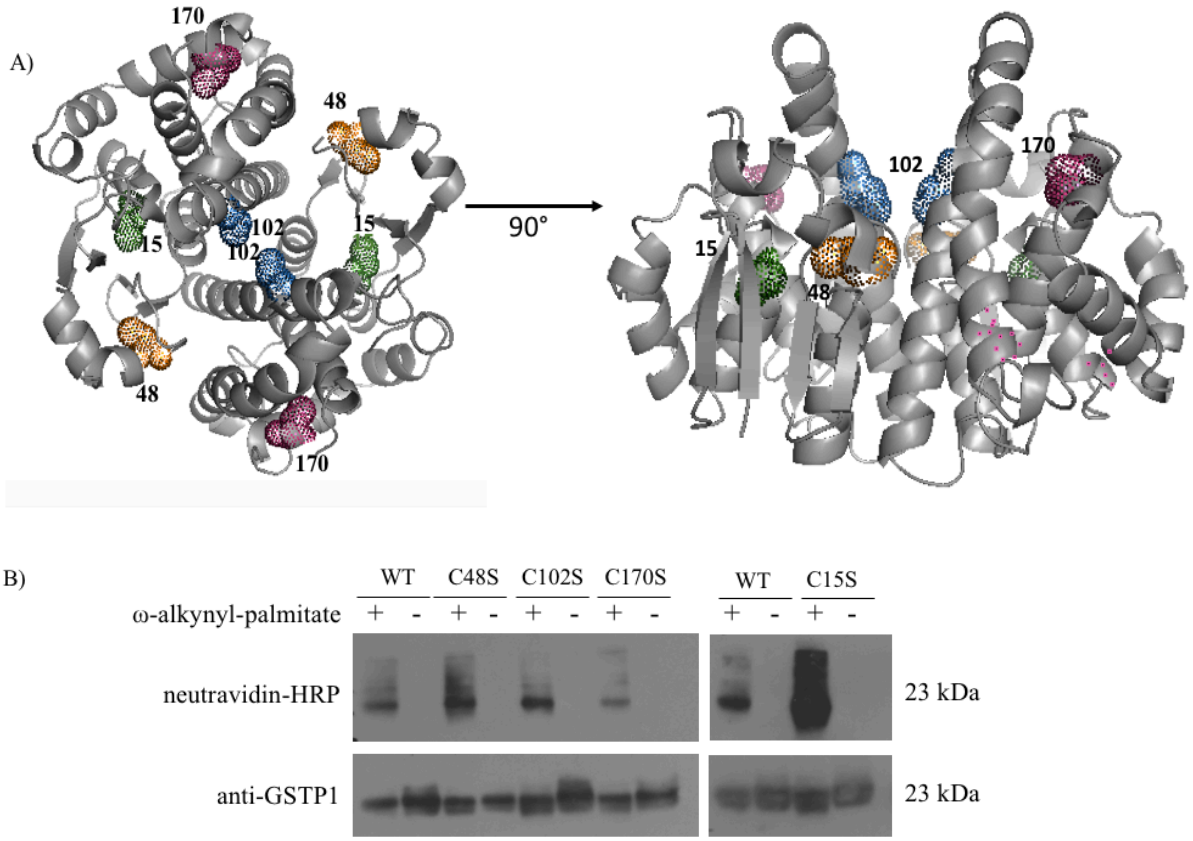


Figure 2.3 *Palmitoylation status of human GSTP1 after mutation of individual Cys residues to Ser.*

(A) Visualization of Cys residues present on GSTP1 dimer were identified in the PBD:3GSS crystal structure and visualized in Pymol; Cys15 (green), Cys48 (orange), Cys102 (blue) and Cys170 (pink). **(B)** pcDNA3.1-V5_GSTP1-WT, -Cys15Ser, -Cys48Ser, -Cys102Ser or-Cys170Ser were transiently transfected in HEK293T cells, metabolically labelled with ω-alkynyl-palmitate, immunoprecipiated and subjected to click chemistry. Samples were resolved on 11% SDS-PAGE and transferred to a PVDF membrane. (*Top panel*) Palmitoylated GSTP1 was detected by neutravidin-HRP (1:20000) and (*bottom panel*) total GSTP1 by rabbit pAb anti-GSTP1 (1:5000).

2.3.4 Cys-less GSTP1 retains palmitoylation and GSTP1 palmitoylation is resistant to NaOH

To investigate the possiblity that multiple Cys residues in GSTP1 are palmitoylated, a Cys-less GSTP1 mutant was generated by mutating all Cys residues to Ser (V5_GSTP1_4XCys to Ser). MCF7 cells stably expressing this construct [MCF7-V5_GSTP1_(4XCys to Ser)] were metabolically labelled with ω-alkynyl-palmitate, immunoprecipitated with agarose beads conjugated to mouse anti-V5 antibody (V5-10), subjected to click chemistry, and analysed by western blotting with neutravidin-HRP and rabbit pAb anti-GSTP1 (GS72). The Cys-less mutant retained a strong palmitoylation signal (Figure 2.4). Ester bonds are susceptible to alkaline hydrolysis and treatment with NaOH is a useful tool for characterizing the nature of the chemical bond of protein acylation (Kostiuk et al., 2009). To determine if V5_GSTP1-WT and-4X(Cys to Ser) are modified by palmitate through an ester bond (either oxy- or thioester), samples were treated with NaOH. Palmitoylation of V5_GSTP1-WT and GSTP1_4X(Cys to Ser) mutant were not sensitive to NaOH treatment (Figure 2.4). Together, these data suggest that GSTP1 is modified by palmitate through a non-ester bond, possibly through an amide bond, which is resistant to NaOH

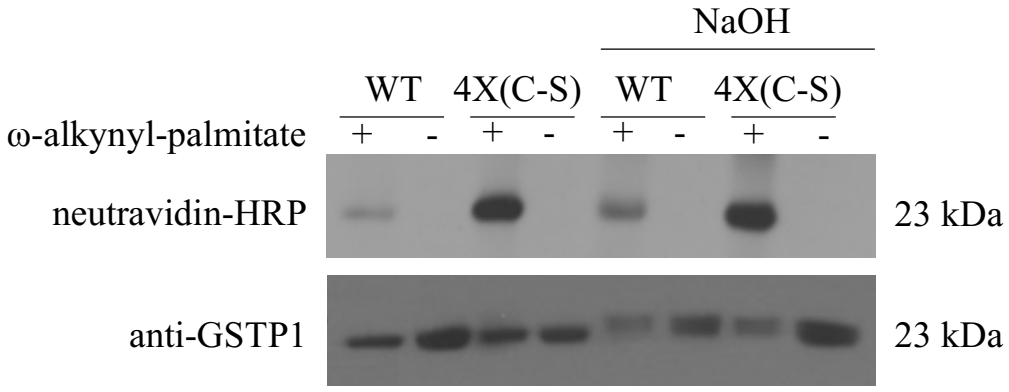


Figure 2.4 *Characterization of palmitoylation of V5_GSTP1_4X(Cys to Ser) stably expressed in MCF7 cells*

MCF7 cells expressing V5_GSTP1-WT and V5_GSTP1-4X_(Cys-Ser) were metabolically labelled with ω-alkynyl-palmitate for 24-48 h, immunoprecipitated with agarose beads conjugated to mouse anti-V5 antibody (V5-10), and subjected to click chemistry. One half of each sample was treated with NaOH (0.1 M) for one h, the other half was kept at neutral pH. Samples were resolved on 11% SDS-PAGE and transferred to a PVDF membrane. Neutravidin-HRP (1:20000) and pAb rabbit anti-GSTP1-GS72 (1:5000) were used to detect palmitoylated and total GSTP1, respectively. Similar results were obtained for an additional independent experiment.

2.3.5 GSTP1 can be palmitoylated in the absence of a PAT in vitro and this depends on Coenzyme A (CoA).

Due to the atypical nature of GSTP1 palmitoylation through at least one non-ester bond it was impractical to take a traditional site-directed mutagenesis approach for determining which residues are palmitoylated. Thus, a strategy to map sites on GSTP1 by analysing peptide fragments using LC/MS/MS was developed. Initially, we took the simplest approach and labelled recombinant human GSTP1 purified from *E. coli* with palmitoyl-CoA *in vitro*. Prior to mass spectrometry analysis, GSTP1 palmitoylated *in vitro* was biochemically characterized.

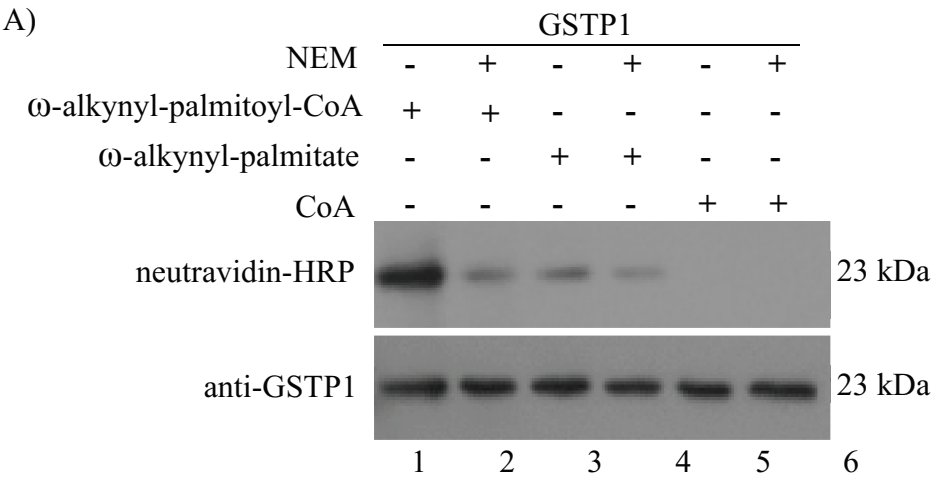
CoA is a co-factor in the synthesis and oxidation of fatty acids. It forms a thioester bond with the carboxylic acid of fatty acids; thus acting as an acyl carrier. Esterification of a fatty acid allows the transfer of the fatty acid to another available sulphydryl group linked by a covalent bond. To investigate if GSTP1 can be palmitoylated *in vitro* in the absence of a PAT, and if the association of GSTP1 with palmitate is due to a covalent modification (rather than a hydrophobic interaction), human GSTP1 purified from *E.coli* was labelled *in vitro* with ω-alkynyl-palmitate, CoA alone, or ω-alkynyl-palmitoyl-CoA, at physiological pH and room temperature. To detect the modification, the fatty acid was "clicked" with azido-biotin and analysed by western blotting with neutravidin-HRP and rabbit pAb anti-GSTP1 (GS72). A signal for neutravidin was detected for ω-alkynyl-palmitoyl-CoA labelled GSTP1 (Figure 2.5A, lane 1). To determine if one or more Cys residues are involved in the ω-alkynyl-palmitoyl-CoA labelling, GSTP1 was pre-treated with NEM to block Cys residues prior to treatment with ω-alkynyl-palmitoyl-CoA. This resulted in a large drop in neutravidin signal suggesting that a Cys residue is modified by palmitate, at least *in vitro* (Figure 2.5A, lane 2). The palmitoylation signal for GSTP1 treated with ω-alkynyl-palmitate without CoA was much weaker than for ω-

alkynyl-palmitoyl-CoA (Figure 2.5A lane 3 versus lane 1) and was reduced by pre-treatment with NEM (Figure 2.5A, lane 3 versus lane 4). As expected, samples prepared in the presence of CoA alone (with and without NEM) did not have a neutravidin signal (Figure 2.5A, lanes 5 and 6). Thus, GSTP1 is an enzyme that can be covalently modified by palmitate *in vitro,* likely on a Cys residue, in the absence of a PAT.

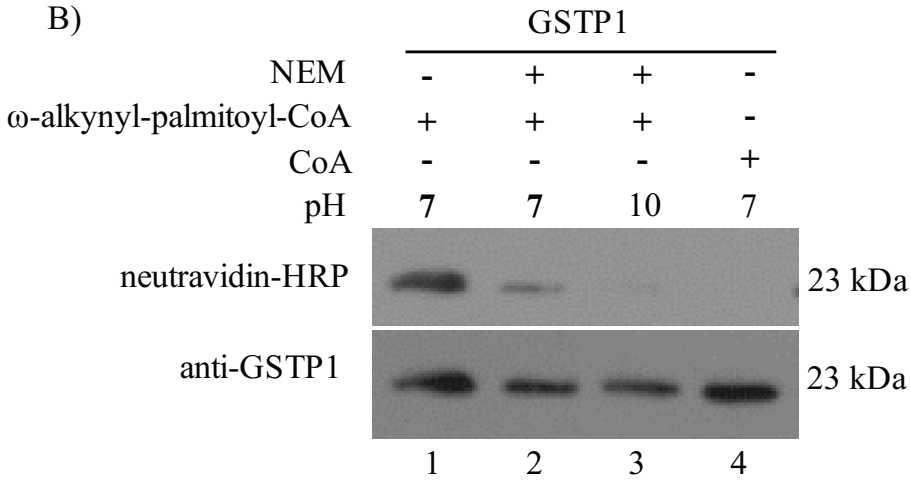
To further characterize the type of bond formed between palmitate and GSTP1, NEM was used to block free Cys residues at neutral pH, and Cys plus Lys residues at alkali pH; *in vitro* palmitoylation of GSTP1was then compared with non-NEM treated controls. Purified GSTP1 was treated with ω-alkynyl-palmitoyl CoA at physiological pH and consistent with Figure 2.5A, a strong neutravidin signal was detected which was reduced when GSTP1 was pretreated with NEM at pH 7 (Figure 2.5B, lane 1 versus lane 2). Pretreatment of GSTP1 with NEM at pH 10 resulted in a further reduction of neutravidin signal (Figure 2.5B, lane 3 versus lane 2), suggesting that Cys and Lys residues could be palmitoylation sites. CoA was used as a negative control and did not show any signal for palmitoylation (Figure 2.5B, lane 4),

Human GSTP1 purified from *E. coli* was labelled with ω-alkynyl-palmitoyl CoA and incubated with NaOH (0.1 M) for one h, then subjected to click chemistry. Consistent with data from MCF7-V5_GSTP1 cells metabolically labelled with ω-alkynyl-palmitate (Figure 2.4), GSTP1 *in vitro* palmitoylation was resistant to NaOH treatment, providing further support for the involvement of an amide bond (Figure 2.5C).

A)


GSTP1
NEM - + - + - +
ω-alkynyl-palmitoyl-CoA + + - - - -
ω-alkynyl-palmitate - - + + - -
CoA - - - - + +
neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa
1 2 3 4 5 6

B)


GSTP1
NEM - + + -
ω-alkynyl-palmitoyl-CoA + + + -
CoA - - - +
pH 7 7 10 7
neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa
1 2 3 4

C)

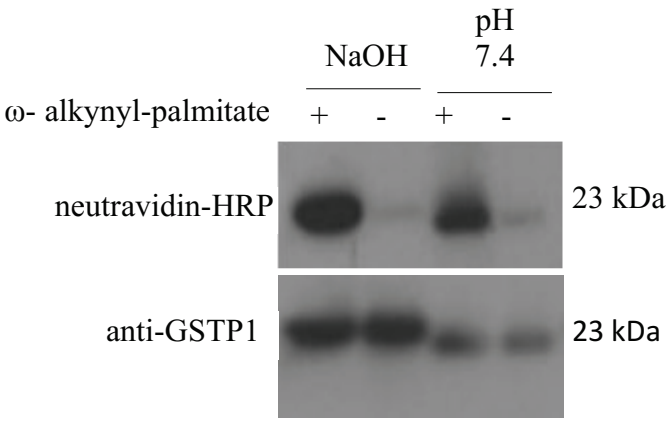

NaOH
pH 7.4
ω- alkynyl-palmitate + - + -
neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa

Figure 2.5 *Biochemical characterization of PAT-independent palmitoylation of human GSTP1 purified from E.coli* .

GSTP1 was subjected to a series of chemical treatments pre- or post-palmitoylation *in vitro* and resolved on a 11% SDS-PAGE and transferred to a PVDF membrane. Palmitoylated GSTP1 was visualized with neutravidin-HRP (1:20000) and total GSTP1 detected with rabbit pAb anti-GSTP1 (1:1000). **(A)** GSTP1 was incubated in the presence or absence of NEM with ω-alkynyl-palmitoyl-CoA (lanes 1 and 2, respectively), ω-alkynyl-palmitate (lanes 3 and 4) or CoA (lanes 5 and 6). **(B)** Cys and Lys residues were blocked by incubating purified GSTP1 with NEM (10 mM) at pH 7 and 10 (lanes 2 and 3, respectively), then *in vitro* palmitoylation using ω-alkynyl-palmitoyl-CoA was done. Samples were analysed by western blotting. **(C)** *In vitro* palmitoylated GSTP1 was treated with NaOH (0.1 M) or MOPS at pH 7 for 1 h and then analysed by western blotting.

2.3.6 Identification of in vitro palmitoylated GSTP1 residues by LC-MS/MS.

Identification of a hydrophobic component, such as palmitate, bound to a hydrophilic peptide is analytically challenging because fatty acids are likely to be lost in the mobile phase or trapped on the reverse-phase column. In addition, when the bond between the protein and the fatty acid is an ester, the chances of detecting the modification are even lower when compared with a more stable amide bond. A strategy to analyze fatty acylated peptides by MS was previously developed (Ji et al., 2013), and was used to analyze recombinant human GSTP1 purified from *E.coli*. GSTP1 was labelled *in vitro* with palmitoyl-CoA or CoA alone then digested into peptides with trypsin or endoproteinase Asp-N and analysed by LC-MS/MS. The following palmitoylated peptides were identified: ^{25}DQGQSWKEEVVTVETWQEGSKAS$^{48}C_{palm}$LYGQ LPKFQ^{57} and a single peptide with both Cys102 and Lys103 palmitoylated $^{102}C_{palm}{}^{103}K_{palm}$YISLIY TNYEAGKDDYVK^{121}] (Figure 2.6). This is the first identification of a protein with an internal Lys modified by palmitate *in vitro*.

GSTP1 co-crystallized with ethacrynic acid-GSH [PBD:3GSS, (Oakley et al., 1997)] was analysed using Pymol to determine the spatial distribution of the *in vitro* palmitoylated residues (Figure 2.6C). Cys48 (orange), Cys102 (blue) and Lys103 (red) are distributed along the dimer interface (Hegazy et al., 2004; B. Wu & Dong, 2012). The conservation of these three amino acids, for different GSTP1 orthologs is shown in Table 2-2 and for different GST isoforms in Table 2-3. Lys103 was completely conserved in GSTP1 for all species examined. Similarly, Cys48 was highly conserved with the exception of conversion to an Ala in *Xenopus laevis* GSTP1. Cys102 was conserved in all species except for mouse, rabbit, and *Xenopus laevis*. The conservation of all three amino acids was low for other human GST isoforms (Table 2-3).

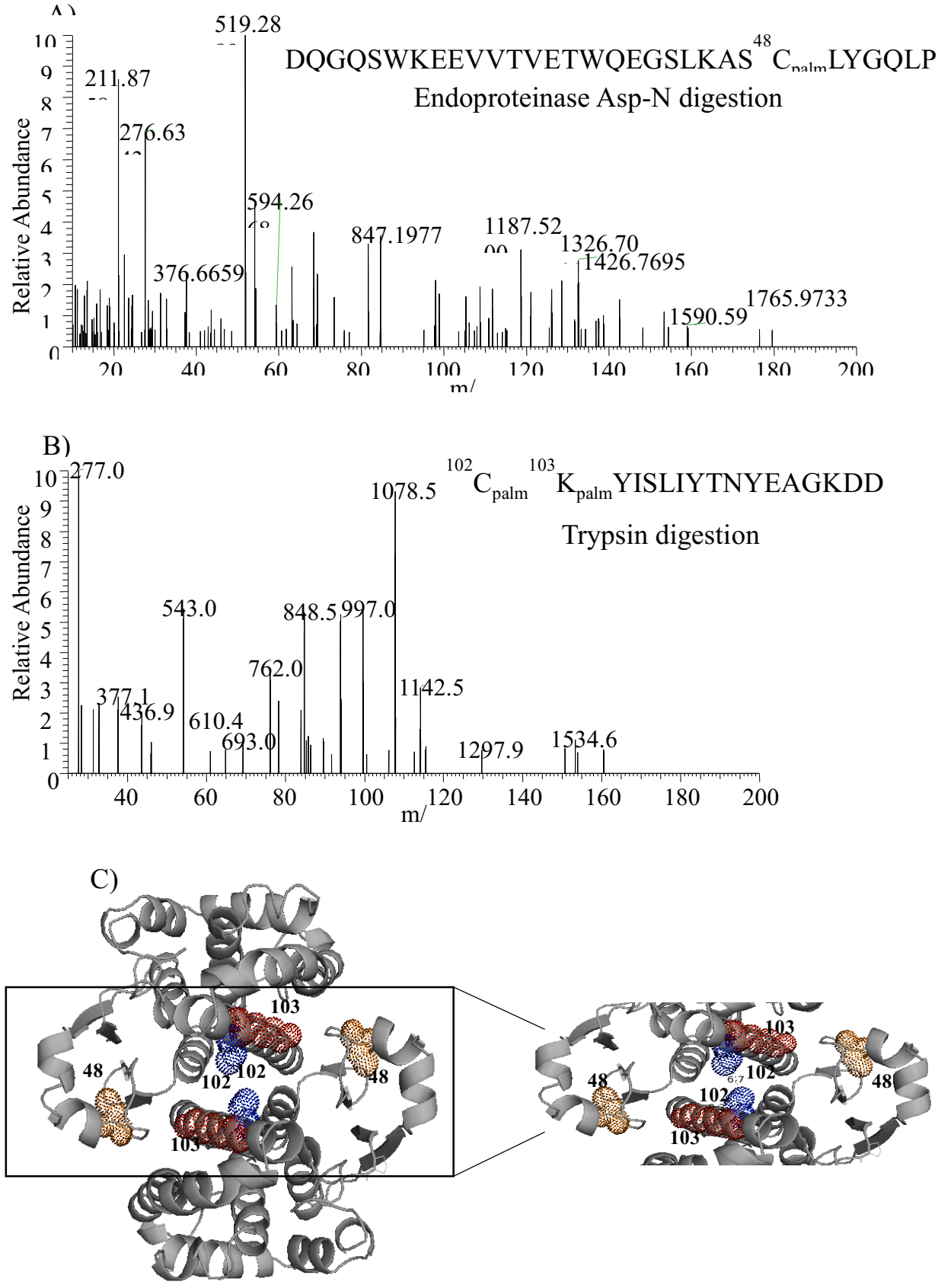

A)
519.28
DQGQSWKEEVVTVETWQEGSLKAS48 Cpalm LYGQLP
Endoproteinase Asp-N digestion
211.87
276.63
594.26
847.1977
1187.52
1326.70
1426.7695
376.6659
1590.59
1765.9733
Relative Abundance
m/
B)
277.0
1078.5
102 Cpalm 103 Kpalm YISLIYTNYEAGKDD
Trypsin digestion
543.0
848.5
997.0
762.0
1142.5
377.1
436.9
610.4
693.0
1297.9
1534.6
Relative Abundance
m/
C)
103
48
102
102
48
103
103
102
48
102
48
103

Figure 2.6 *Mapping of palmitoylated amino acids on in vitro palmitoylated GSTP1 LC-MS/MS.* Human GSTP1 purified from *E.coli* was *in vitro* palmitoylated and digested with proteases into peptides. After enriching for palmitoylated peptides, the samples were analysed by LC-MS/MS. **(A)** *(top spectrum)* GSTP1 peptides obtained by Asp-N digestion identified Cys48 and *(lower spectrum)* tryptic digestion identified Cys102 and Lys103 as palmitoylated amino acids. **(B)** Spatial distribution of *in vitro* palmitoylated Cys48, Cys102, and Lys103 of GSTP1 identified by LC-MS/MS was visualized in Pymol using the crystal structure (PBD 3GSS). Distances between Cys102 from one monomer to the Cys102 of another monomer is 6.0 Angstroms, whereas the distance between the side chain of Lys103 and Cys 102 whithin the same monomer is 9.8 Angstroms.

Table 2.2
Conservation of the *in vitro* palmitoylated amino acids among different GSTP1 orthologues. Residue number of the human GSTP1 is being used as reference.

Species	Cys48	Cys102	Lys103
Human	Cys	Cys	Lys
Mouse 1	Cys	Gly	Lys
Mouse 2	Cys	Gly	Lys
Rat	Cys	Cys	Lys
Chimpanzee	Cys	Cys	Lys
Gorilla	Cys	Cys	Lys
Rabbit	Cys	Ile	Lys
Pig	Cys	Cys	Lys
Frog	Ala	Gln	Lys

Table 2.3
Conservation of the *in vitro* palmitoylated amino acids among different human GST isoforms. Residue number of the human GSTP1 is being used as reference.

Species	Cys48	Cys102	Lys103
GSTP1	Cys	Cys	Lys
GSTM1	Leu	Met	Gln
GSTM2	Leu	Met	Gln
GSTM3	Leu	Thr	Gln
GSTM4	Leu	Asn	Gln
GSTM5	Leu	Met	Glu
GSTA1	Leu	Glu	Met
GSTA2	Leu	Glu	Met
GSTA3	Leu	Glu	Met
GSTA4	Leu	Glu	Leu
GSTA5	Leu	Glu	Met
GSTO1	Asn	Ser	-
GSTO2	His	His	-
GSTS	Leu	Ser	Cys
GSTT1	Asn	Arg	Ser
GSTT2	Asn	Arg	Thr

2.3.7 Metabolic labelling of MCF7 cells expressing GSTP1_4X (Cys to Ala)/Lys103Arg mutant retains palmitoylation

Our LC-MS/MS results showed that GSTP1 palmitoylation *in vitro* can occur on at least amino acids Cys48, Cys102, and Lys103, and these sites are candidates for palmitoylation in cells. To determine if cellular palmitoylation of GSTP1 was lost if these residues were no longer available for palmitoylation, a V5_GSTP1_4X_Cys-less (Cys to Ala)/Lys103Arg mutant construct was generated. This was done in the Cys-less construct because even though mass spectrometry analysis (Figure 2.6) did not identify the Cys15 or Cys170 as palmitoylated sites the potential of these sites to be modified still exists. It is possible that peptides containing palmitoylated Cys15 and Cys170 could have been missed in our analysis for a few reasons. It is possible that the hydrophobic peptides were retained in the column or lost during sample preparation (due to instability). Furthermore, Cys15 and Cys170 could potentially require a PAT for palmitoylation and for this reason could be utilized sites within cells but not *in vitro*.

The V5_GSTP1_4X_Cys-less (Cys to Ala)/Lys103Arg mutant was stably expressed in MCF7 cells, subjected to metabolic labelling followed by click chemistry and then analysed by western blotting with neutravidin-HRP and rabbit pAb anti-GSTP1 (GS72). Expression of V5_GSTP1_4X_ (Cys to Ala)/Lys103Arg mutant in MCF7 was ~80% lower than V5_GSTP1-WT (Figure 2.7A, bottom panel). Palmitoylation of V5_GSTP1_4X_Cys-less (Cys to Ala)/Lys103Arg was retained (Figure 7A), and when the palmitoylation (neutravidin) signal was normalized for GSTP1 level, palmitoylation was reduced by only ~20% (Figure 2.7B). This result suggests that palmitoylation of GSTP1 occurs at additional or different sites other than those utilized during *in vitro* palmitoylation . This is not entirely surprising due to the presence

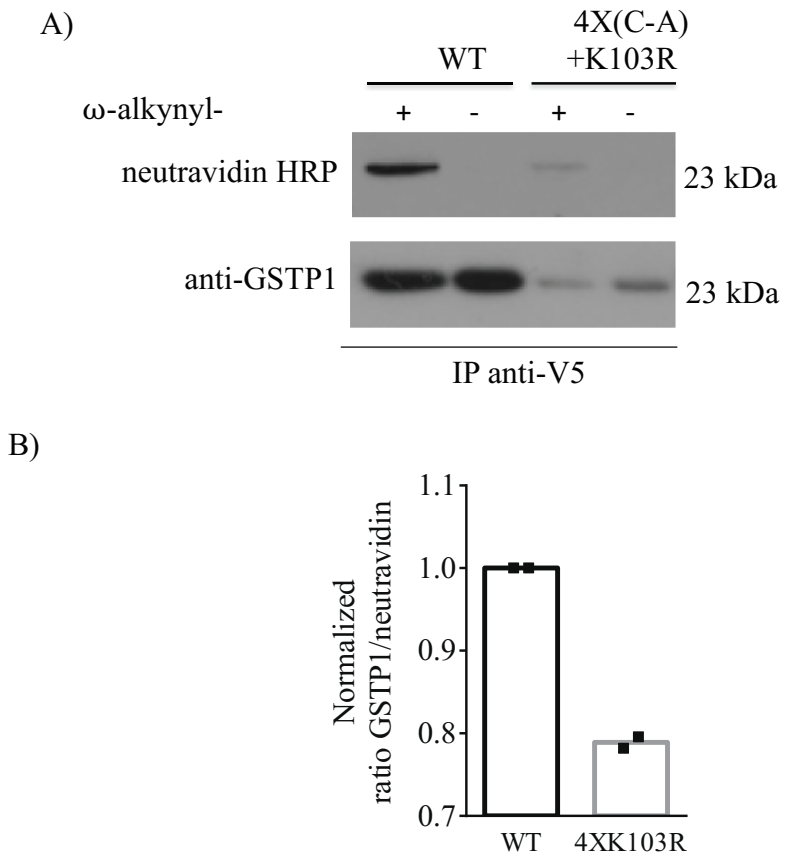


Figure 2.7 *Characterization of V5_GSTP1-4X(Cys to Ala)K103R palmitoylation.*

(A) The V5_GSTP1-4X(Cys to Ala)K103R mutant was generated and stably expressed in MCF7 cells. This cell line and MCF7-V5_GSTP1-WT were then metabolically labelled with ω-alkynyl-palmitate for >24 h. Cells were lysed and GSTP1 immunoprecipitated with agarose beads conjugated to mouse anti-V5 antibody (V5-10), subjected to click chemistry, resolved on 11% SDS-PAGE and transferred to a PVDF membrane. Palmitoylated GSTP1 was visualized with neutravidin-HRP (1:20000) and total GSTP1 with rabbit pAb anti-GSTP1 (1:5000). **(B)** Densitometry on neutravidin and anti-GSTP1 was performed using ImageJ software. The relative level of palmitoylated GSTP1 (neutravidin-HRP) normalized to GSTP1 are plotted. Bars represent means of two independent experiments and symbols represent relative levels of individual experiments.

of cellular PATs that might catalyse the addition of palmitate to residues that cannot be modified *in vitro*.

2.3.8 Less GSTP1 is associated with the cellular membrane than the cytosolic fraction and palmitoylated GSTP1 is present in both fractions.

To quantify the relative level of GSTP1 in the cytosol compared with cellular membranes, subcellular fractions of MCF7-GSTP1 cells were isolated and analysed by western blotting. GSTP1 was detected in the total cell homogenate (input), cytosolic, and membrane fractions using the rabbit pAb anti-GSTP1 (GS70) (Figure 2.8A). To compare GSTP1 levels in the membrane and cytosolic fractions, bands were quantified using imageJ and plotted relative to the level in the cytosolic fraction, equal total protein loading was confirmed by staining the blot with amido black. The total cellular membrane pellet had 67% less GSTP1 than the cytosolic fraction ($p < 0.05$) (Figure 2.8B).

To identify the subcellular fraction in which palmitoylated GSTP1 resides, MCF7-V5_GSTP1 cells were metabolically labelled with ω-alkynyl-palmitate and cytosolic and total cellular membrane fractions isolated. GSTP1 was then immunoprecipitated using agarose beads conjugated to mouse anti-V5 antibody (V5-10) directly from the cytosolic fraction, while the membrane fraction was first solubilized using SDS (0.1%). Samples were then subjected to click chemistry. Samples were resolved on SDS-PAGE and transferred to a PVDF membrane. GSTP1 palmitoylation and total GSTP1 were detected with neutravidin-HRP or rabbit pAb anti-GSTP1 (GS72), respectively. Palmitoylated GSTP1 was present in the membrane and the cytosolic fraction. The differences in conditions for immunoprecipitation from the cytosolic versus the membrane fractions prevents comparison of relative levels of total and palmitoylated

GSTP1 between the cytosol and membranes. Comparison of the ratio of palmitoylated GSTP1/total GSTP1 from cytosol (0.5) with the ratio of the membrane-associated GSTP1 (0.7) shows that palmitoylated GSTP1 is greater in the membrane fraction than the cytosolic fraction. The existence of palmitoylated GSTP1 forms in the cytosol suggest that palmitoylation could be important for multiple functions, such as dimer structure, oligomerization or signalling, not only membrane association (Figure 2.8C).

2.3.9 GSTP1 associated with the cell pellet does not catalyze the GSH conjugation of CDNB.

CDNB is well characterized as a substrate for GSTs and GSTP1 catalyzes its conversion to DNP-GS (Habig et al., 1974). Although GSTP1 strongly associates with the plasma membrane (Qazi et al., 2011), the influence of this localization on GSTP1 function was not previously characterized. The rate of DNP-GS formation by GSTP1 in the total cell lysate (input), cytosolic fraction, and total cellular membranes (pellet) fraction was determined. The rate of DNP-GS formation for the cytosolic fraction from MCF7-GSTP1 was 0.27 nmol of DNP-GS/mg protein/min and not significantly different from the total input of MCF7-GSTP1 at 0.23 nmol of DNP-GS/mg protein/min. In contrast, GSTP1 activity in the membrane fraction of MCF7-GSTP1 was only 0.02 nmol of DNP-GS/mg protein/min which was not significantly different from the formation of DNP-GS by total input and subcellular fractions isolated from MCF7-vector cells (negative control) (Figure 2.8D). GSTP1 in the membrane fraction did not demonstrate any activity even after normalization for GSTP1 levels, suggesting that the GSTP1 associated with the membranes is not catalytically active (at least for CDNB) (Figure 2.8D). In summary, palmitoylated GSTP1 is present in the membranes and cytosol, however, only cytosolic GSTP1 has the ability to catalyze the formation of DNP-GS from CDNB.

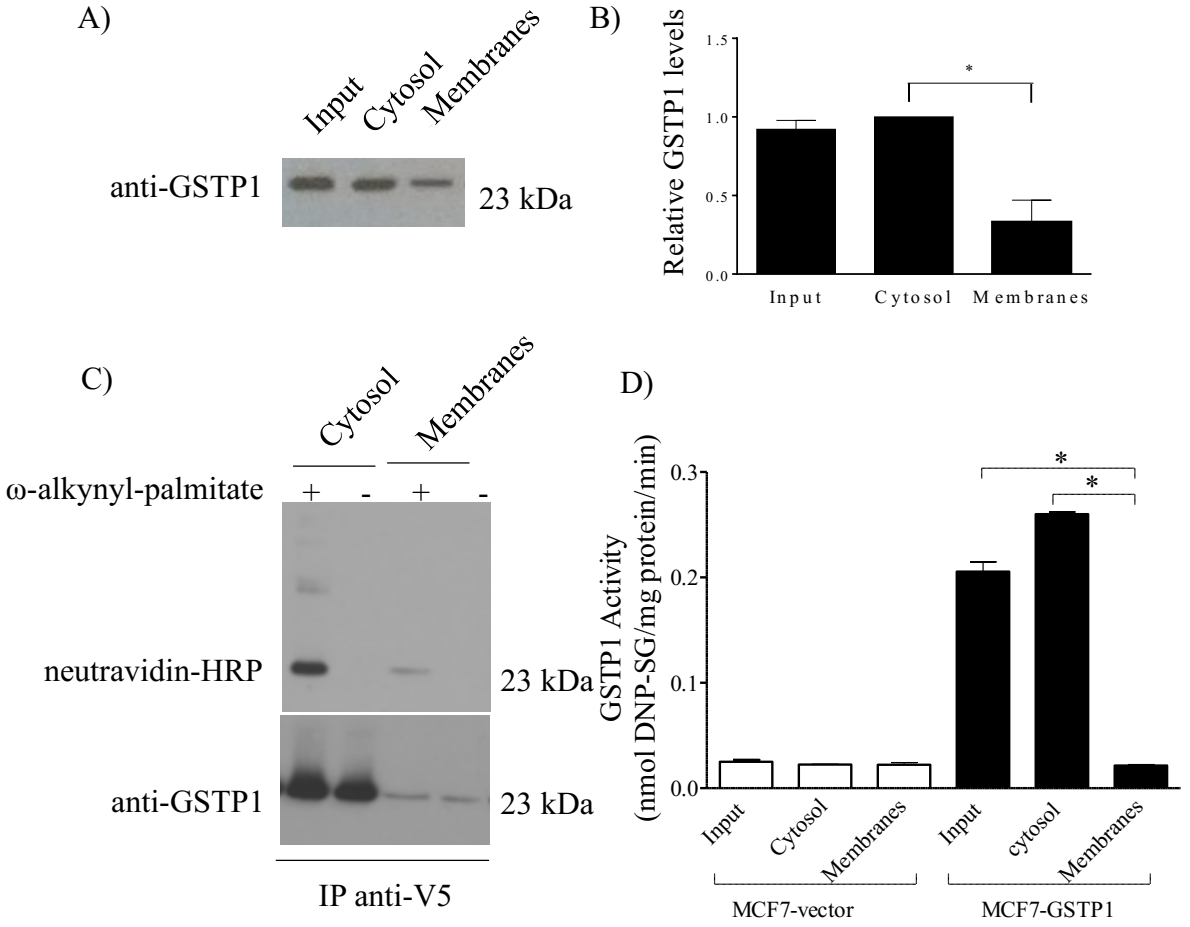


Figure 2.8 *Characterization of GSTP1 levels, palmitoylation and activity from total, cytosolic and cellular membrane fractions isolated from MCF7 cells.*

MCF7-V5_GSTP1 or MCF7-GSTP1 and MCF7-vector mantained with FBS condition was lysed by N_2 cavitation, nuclei and unbrokn cells were removed by centrifuging at 2000 *g*. Supernatant (input) was collected and cytosolic fraction was separated from cellular membranes by analytical centrifugation at 100000 *g* for 1 h. **(A)** Total level of GSTP1 in the input (5 μg of protein), cytosol (5 μg of protein) and cellular membrane (5 μg of protein) fractions

concentrated by 90% in comparison to the cytosol and input were determined by resolving samples on 11% SDS-PAGE and tranferred onto a PVDF membrane. Palmitoylated GSTP1 were detected with neutravidin-HRP (1:20000) and total GSTP1 with rabbit pAb anti-GSTP1 (1:5000). **(B)** Densitometry of anti-GSTP1 in Figure 2.8.A was performed using ImageJ software. The relative level of GSTP1 (anti-GSTP1) from each fraction was plotted with bars representing means (±SD). Statistical significance was calculated by one-way ANOVA with post-hoc Tukey honest significant difference (HSD) from at least three independent experiments, where (*) $p < 0.05$. **(C)** GSTP1 was immunoprecipitated from cytosolic and membrane fractions of MCF7-V5_GSTP1 cells metabolically labelled with ω-alkynyl palmitate (+) or FBS-containing medium (-) using agarose beads conjugated to mouse anti-V5 antibody (V5-10) (as described in section 2.2.9), and subjected to click chemistry, resolved on a 11% SDS-PAGE and tranferred onto a PVDF membrane. (*top panel*) Palmitoylated GSTP1 was detected with neutravidin-HRP (1:20000) and (*bottom panel*) total GSTP1 with rabbit pAb anti-GSTP1 (1:5000). Palmitoylated GSTP1 from cytosolic and membrane fractions (neutravidin-HRP) and total GSTP1(anti-GSTP1) from MCF7-V5_GSTP1 are shown. **(D)** GSTP1 activity of input, cytosol and membrane fraction from MCF7-vector and MCF7-GSTP1. Three μg of cytosol, input and membranes were incubated with 1 mM CDNB and 2.5 mM GSH. Formation of the DNP-GS conjugate was measured at 340 nm. Abosorbance values were normalized for blanks and spontaneous formation of DNP-GS in the absence of cellular fraction. Rates were normalized for relative GSTP1 levels estimated by densitometry in Figure 2.8.A, with bars representing means (±SD) of at least three independent experiments. ** $p \leq 0.001$ (one-way ANOVA with post-hoc Tukey HSD).

2.3.10 Purified GSTP1 associates with MCF7 plasma-membrane enriched vesicles.

To determine if palmitoylation influences the ability of GSTP1 to associate with plasma membrane enriched vesicles prepared from untransfected MCF7 cells. Thus, human GSTP1 purified from *E. coli* or MCF7-GSTP1 cells grown under conditions with regular FBS (GSTP1-FBS) or charcoal stripped FAF-FBS (GSTP1-FAF), were subjected to *in vitro* palmitoylation and incubated with plasma membrane-enriched vesicles. Samples were pelleted by ultracentrifugation and analysed by immunoblotting. GSTP1 was detected with rabbit pAb anti-GSTP1 (GS72) and the rabbit pAb anti-Na^+/K^+-ATPase (H-300) was used as a loading control for plasma membrane-enriched vesicles. GSTP1 is not expressed endogenously in MCF7 cells, therefore vesicles alone did not have any detectable signal for GSTP1 (Figure 2.9, left lane in all blots). GSTP1 from *E. coli* (Figure 2.9A & B), as well as MCF7 cells GSTP1-FBS (Figure 2.9C & D) and GSTP1-FAF (Figure2. 9E & F) associated with the plasma membrane-enriched vesicles in the absence of *in vitro* palmitoylation. After *in vitro* palmitoylation, no significant increase in the amount of GSTP1 interacting with the MCF-7 plasma membrane enriched membrane vesicles was detected for human GSTP1 purified from *E. coli*, or MCF7-GSTP1 grown under regular or FAF conditions ($p > 0.05$) (Figure 2.9A to E, right hand lane and column). Thus, before and after *in vitro* palmitoylation GSTP1 is capable of associating with cellular membranes.

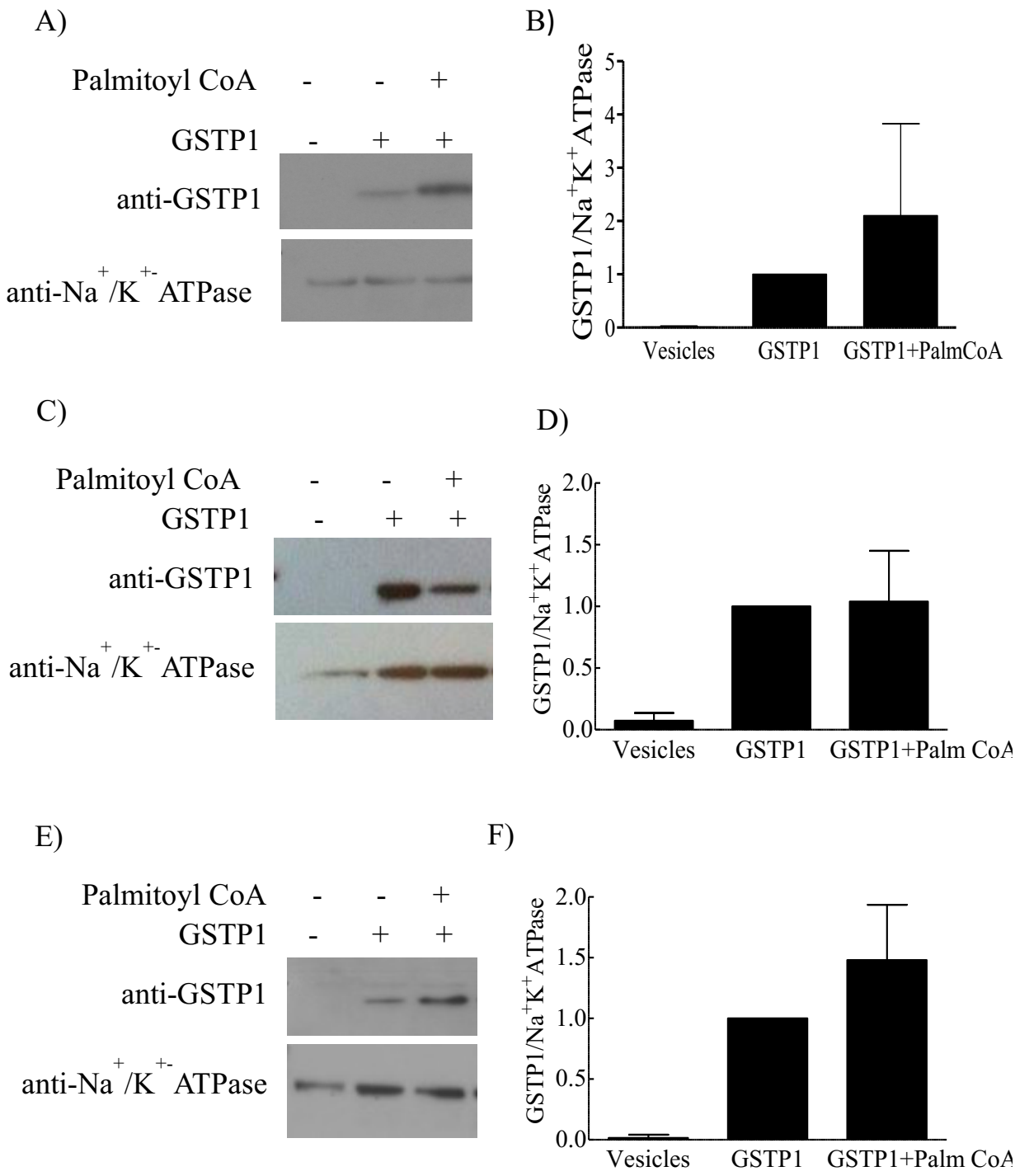

A)
Palmitoyl CoA - - +
GSTP1 - + +
anti-GSTP1
anti-Na+/K+-ATPase
B)
GSTP1/Na+K+ATPase
0
1
2
3
4
5
Vesicles
GSTP1
GSTP1+PalmCoA
C)
Palmitoyl CoA - - +
GSTP1 - + +
anti-GSTP1
anti-Na+/K+-ATPase
D)
GSTP1/Na+K+ATPase
0.0
0.5
1.0
1.5
2.0
Vesicles
GSTP1
GSTP1+Palm CoA
E)
Palmitoyl CoA - - +
GSTP1 - + +
anti-GSTP1
anti-Na+/K+-ATPase
F)
GSTP1/Na+K+ATPase
0.0
0.5
1.0
1.5
2.0
Vesicles
GSTP1
GSTP1+Palm CoA

Figure 2.9 *Association of purifed GSTP1 with MCF7 plasma membrane-enriched vesicles*

Human GSTP1 purified from (A, B) *E. coli* (*E. coli* GSTP1) or (C-F) MCF7-GSTP1 cells grown in the presence of (C,D) regular FBS (GSTP1-FBS) or charcoal stripped fatty acid free FBS (GSTP1-FAF) were tested for their ability to interact with MCF7 plasma membrane enriched vesicles. *E. coli* GSTP1, GSTP1-FBS, and GSTP1-FAF were left untreated or *in vitro* labelled with ω-alkynyl-palmitoyl-CoA. Untreated and *in vitro* palmitoylated GSTP1 were incubated with MCF7 plasma membrane-enriched vesicles for 30 min at 37°C. MCF7 vesicles without GSTP1 were incubated under identical conditions. Membranes were pelleted by ultracentrifugation at 100000 *g* for 1 h, washed with PBS 1X + Triton-X-100 0.1%, resolved by 11% SDS-PAGE, and transferred onto a PVDF membrane. Membranes were immunoblotted for GSTP1 using the pAb rabbit anti-GSTP1(GS72) (1:5000). Equality of vesicle loading was determined by probing for the integral plasma membrane protein Na^+/K^+-ATPase using the rabbit pAb anti- Na^+/K^+-ATPase (1:10000). (A, C, E) A representative blot is shown for each source of GSTP1 and similar results were observed in at least three additional independent experiments. (B, D, F) Densitometry analysis of GSTP1 and Na^+/K^+-ATPase bands was performed with ImageJ. GSTP1 associated with the membrane was normalized for protein loading as needed the Na^+/K^+-ATPase signal. GSTP1 signal was normalize to protein loading (Na^+/K^+-ATPase) and calculate the expression relative to GSTP1 only (set at 1) to be able to compare palmitoylated and non-palmitoylated GSTP1 between experiments. Bars represent the mean ± SD of at least 3 independent experiments * $p< 0.01$ (one-way ANOVA followed by a Tukey HSD post-hoc test)

2.4 Discussion

In the current chapter, the hypothesis that GSTP1 is modified by lipids to allow its strong association with cellular membranes was addressed. Using western blot and a specialized PLA, we found that GSTP1 is post-translationally modified by palmitate, and the modification can take longer to incorporate when compared to other palmitoylated proteins (Yap et al., 2010). Furthermore, *in vitro* palmitoylation suggests GSTP1 palmitoylation can occur in the absence of PATs. Typically, proteins undergo palmitoylation via *S*-palmitoylation of a Cys residue. We found that GSTP1 Cys residues are not the only requirement for GSTP1 palmitoylation, as a Cys-less GSTP1 mutant retained palmitoylation after metabolic labelling with ω-alkynyl palmitate. MS analysis of *in vitro* palmitoyl CoA labelled GSTP1 identified Cys48, Cys102 and Lys103 as (at least *in vitro*) modification sites. Modification of a Lys residue by palmitate was not our only surprising observation, we also found that GSTP1 associates with cellular membranes and there was no significant difference between GSTP1 subjected to *in vitro* palmitoylation or not. Although, after *in vitro* palmitoylation GSTP1-FAF and GSTP1 purified from *E. coli* seemed to have more affinity for the membranes than the GSTP1-FBS, the trend was not significant. Even though lipidation would be an alternative for a cytosolic protein with hydrophilic character to associate with membrane, fatty acylation was the only lipidation process possible for GSTP1, according to what is understood about lipidation to date. Surprisingly, not only the membrane but also the cytosolic GSTP1 is palmitoylated and GSTP1 from the membrane cellular fractions does not catalyse the formation of DNP-GS from CDNB.

Standard protocols for the detection of palmitoylated proteins require four hours of metabolic labelling with ω-alkynyl-palmitate (Yap et al., 2010). Surprisingly, we found that GSTP1 required longer metabolic labelling times with ω-alkynyl-palmitate (>8 h reasonable

levels of palmitoylation start to be detected) for palmitoylation to occur. Interestingly, GSTP1 has been reported to have a half-life of 8 to 9 h that varies according to the cell line (Ali-Osman, Akande, et al., 1997; Shen et al., 1995). The longer metabolic labelling required for the detection of palmitoylation might suggest that GSTP1 must be turned over prior to incorporation of ω-alkynyl-palmitate or the process could be co-translational. Together, these data suggest that palmitoylation could be more stable and less dynamic for GSTP1 than other proteins, and consistent with the modification of GSTP1 by palmitate through an amide bond.

Further support for the modification of GSTP1 by palmitate through an atypical bond came from studying individual GSTP1 Cys to Ser mutants as well as a Cys-less mutant (V5_GSTP1_4XCys to Ser). Indeed, the individual Cys mutants and Cys-less GSTP1 retained strong levels of palmitoylation, demonstrating that palmitoylation can occur at non-Cys sites. Treatment of palmitoylated GSTP1-WT and GSTP1_4XCys to Ser with a strong base (NaOH) did not cleave palmitate. Thio and oxy-ester bonds are cleaved by strong base, suggesting that GSTP1 is palmitoylated through an amide bond. Due to the dynamic nature of *S*-palmitoylation, these data do not rule out the possibility that GSTP1 Cys residues are utilized. Thus, we conclude that GSTP1 is palmitoylated on at least one non-Cys residue, likely through an amide bond, potentially at a Lys residue.

Palmitoylation at non-Cys sites could potentially occur at one or more of the twelve Lys, ten Ser, or nine Thr residues contained in GSTP1. Due to the number of potential sites and the possibility of palmitoylation occurring at multiple sites, a traditional site-directed mutagenesis approach to map the location of palmitoylation was not feasible. *In vitro* palmitoylation of GSTP1 was used as a simple strategy to set up conditions for sequencing of GSTP1 peptides for palmitoylated amino acids by LC-MS/MS. To begin with, *in vitro* palmitoylation of GSTP1

was characterized. We found that GSTP1 is palmitoylated in the absence of a PAT, after treatment with ω-alkynyl-palmitoyl CoA, but not ω-alkynyl-palmitate suggesting a covalent modification occurs (not just hydrophobic binding). *In vitro* palmitoylation of GSTP1 was markedly reduced when the protein as treated with NEM at pH 7 (blocks Cys) before subjecting GSTP1 to *in vitro* palmitoylation and further reduced when treated with NEM at pH 10 (blocks Cys and Lys), suggesting that *in vitro* palmitoylation occurs through a thioester and amide bond.

Although application of MS to lipidomic research is quite new (Shevchenko and Simons, 2010), we were able to apply a protocol recently optimized to analyze peptides modified by fatty acids and identify Cys48, Cys102, and Lys103 as modified by palmitate through a PAT-independent process (Ji et al, 2013). Cys48 is located in the NH_2-terminal half of GSTP1 whereas Cys102 is at the dimer interface (Hegazy et al., 2004). Both sites are known to be important for NO storage and *S*-glutathionylation (Bocedi et al., 2013; Tew & Townsend, 2011). Therefore, *S*-palmitoylation of Cys48 and Cys102 may be the result of synchronized cross-talk with other post-translational modifications or GSH interaction, thus regulating GSTP1 function; such cross-talk could be important for regulating cellular redox balance. To our knowledge, there is no literature regarding the influence of Lys103 on GSTP1 structure or function. Lys residues are frequently the target of ubiquitin, SUMO and acetyl groups.

Cys48 and Lys103 are highly conserved for GSTP1 between species, suggesting that palmitoylation could occur at these sites in those species if it is depending only on the presence of these residues. Cys102 was not conserved in mouse, rabbit, or *Xenopus laevis* and in this case, the presence of a palmitoylated Cys could be an evolutionary advance of human GSTP1, which permits reactions (such as palmitoylation) to occur.

Although, GSTP1 is palmitoylated *in vitro*, the identification of Cys48, Cys102, and Lys103 does not mean that other sites are not modified *in vitro* or by a PAT within cells. A Cys-less GSTP1 with a Lys103Arg mutation (GSTP1-4X(Cys to Ala)/Lys103Arg) was stably expressed in MCF7 cells, metabolically labelled with ω-alkynyl-palmitate and the palmitoylation status determined. The Cys-less mutant was used to introduce the Lys103Arg mutation because for reasons described in Section 2.3.7, Cys15 and Cys170 (although not detected by MS) could still be palmitoylated. Indeed, prediction by CSS-Palm (http://csspalm.biocuckoo.org) suggests that Cys15 and Cys170 are substrates for DHHC-PATs. When corrected for protein levels, GSTP1-4X(Cys to Ala)/Lys103Arg retained a similar palmitoylation signal as GSTP1-WT. These data suggest that there are palmitoylation sites within the cells in addition to or different from Cys48, Cys102, and Lys103.

We are aware of the limitations of the analysis of *in vitro* labelled GSTP1. It identifies PAT-independent palmitoylated residues only. Nevertheless, the fact that GSTP1 is a palmitoylated protein was used as an advantage to setup optimal conditions to analyze GSTP1 purified from cells (thus, palmitoylated by PATs, if that is the case). GSTP1 was purified from MCF7s maintained in medium containing FBS, FAF or FAF + ω-alkynyl-palmitate. No palmitoylation was detected for FAF + ω-alkynyl-palmitate or FBS using Fourier transform mass spectrometry (FTMS) (Appendix 1). The potential reasons for this and future experiments are described in Appendix 1.

Fatty acid modification that results in amide bond formation has been reported previously. For example, the NH_2-terminal position of the Cys residue of the Sonic Hedgehog is palmitoylated and the modification was mediated by an acyl transferase named Hhat (Chamoun et al., 2001). Aquaporin 0, a transmembrane protein, can also be acylated on an

internal Lys by oleic acid (Schey et al., 2010). It was surprising that a stable amide bond was formed *in vitro*, without the requirement of an enzyme, and at neutral pH. It has been suggested that palmitoylation of Lys residues could be an artifact of sample preparation prior to MS analysis (Ji et al., 2016), however, samples were prepared at neutral pH. Thus, the Lys modification even if it occurs during sample preparation might be possible physiologically.

In cells, the mechanism by which an internal Lys residue is modified by palmitate is also unknown. While *N*-myristoyltransferases (NMTs) add myristate (C14:0) to proteins through an amide bond, they have a ligand pocket specific in length to myristate, and require an N-terminal Gly (Martin, 2011). Despite the lack of understanding, fatty acylation of Lys residues has been reported (Hornemann, 2015). Enzymes known to remove palmitate from Lys have also been identified (Jiang et al., 2013). Sirtuin 6 (SIRT6) was recently found to de-palmitoylate *N*-palmitoylation of an internal Lys residue of the tumour necrosis factor α (TNFα), and this is important for the regulation of its secretion (Jiang et al. 2014).

S-palmitoylation typically has a cycle of palmitoylation and de-palmitoylation regulated by PATs and thioesterases, respectively (Conibear & Davis, 2010). This process permits dynamic localization as the protein is likely to be palmitoylated while in the membrane and depalmitoylated in the cytosol. However, it is possible that the fatty acid modification is important for functions unrelated to membrane association. Palmitoylation could stabilize the quaternary or tertiary structure of GSTP1, for dimerization, oligomerization or association with other proteins. It could create a hub of protein complex that is tightly regulated and important for signalling. Curiously, we found that Cys48, Cys102, and Lys103, present in the hydrophobic interface of the dimer are palmitoylated (at least *in vitro*). Within the dimer interface a 'lock and key' motif is formed by the Tyr50 in the NH_2-terminus of one monomer, which wedges

into a hydrophobic pocket in the COOH-terminus of the other monomer (amino acid 80 to 110) (Hegazy et al., 2004). Palmitoylation of amino acids in this vicinity could help stabilize the dimer.

It turns out that GSTP1 is palmitoylated not only in the membrane fraction, but the cytosolic fraction. This is consistent with the complexity of the data presented in this chapter, where palmitoylation likely occurs at different sites. It could also influence multiple GSTP1 functions because different palmitoylation sites may lead to different roles for this protein. Interestingly, in contrast with the cytosolic GSTP1, the membrane-associated GSTP1 has an undetectable ability to conjugate CDNB with GSH, this could suggest that GSTP1 association with cellular membranes is mostly related to the signalling function of GSTP1, rather than the catalytic function, which is important for detoxification. However, GSTP1 substrates in addition to CDNB should be tested. The reversible nature of *S*-palmitoylation could be important for regulating oligomerization for GSTP1 or for interaction with other proteins. This could bring new insight into the mechanism that GSTP1 functions in such diverse ways.

Understanding GSTP1 function goes beyond its detoxification capacity and knowing the significance of its expression in a variety of diseases makes understanding the cell biology of GSTP1 a necessity. Strategic cellular localization and determination of protein partners are key factors for optimal activity of a multifunctional protein.

It is the first time that a protein from the GST family is reported to be palmitoylated. Our objective was to characterize GSTP1 association with the plasma membrane upon palmitoylation. We have identified GSTP1 as a palmitoylated protein, however, we have not found all the residues modified by palmitate. Identification of all palmitoylated residues is required to compare the cellular membrane association of a GSTP1-WT with a non-

palmitoylated GSTP1 mutant. Yet, we obtained preliminary data that support the presence of palmitoylated GSTP1 in the cytosol and the plasma membranes. In addition, GSTP1 purified from cells was able to interact with cellular membranes. Furthermore, the membrane-associated GSTP1 was found not to have catalytic activity towards CDNB, suggesting that cytosolic and membrane-associated GSTP1 do not behave the same way. Together, these findings will be important to understand the function of GSTP1 association with the plasma membrane once all the palmitoylation sites are characterized.

In this work, we have contributed to new insights into the cell biology of GSTP1, through the identification of a post-translational modification and relating this to the importance of understanding the switches that regulate GSTP1 activity. Identifying the occurrence of a regulated lipidation mechanism on GSTP1 is a first step towards the complete understanding of the fundamental biology of GSTP1. This could be translated into advances for major diseases such as cancer and neurodegenerative diseases as well as its complications such as drug resistance and involvement in metastasis.

Chapter 3 General discussion

3.1 Overview

Identifying GSTP1 as a palmitoylated protein was an important step in understanding the multifunctional character of GSTP1. We discovered that a Cys residue is not the only requirement for GSTP1 palmitoylation and palmitoylation is resistant to alkali treatment with NaOH. In addition, this modification is not only a feature of the membrane-associated GSTP1 but also the cytosolic. *In vitro* studies of purified GSTP1 suggest that palmitoylation might be possible in absence of PATs and mass spectrometry analysis identified Cys48, Cys102 and Lys103 as palmitoylation sites *in vitro*.

3.2 GSTP1 requires a long metabolic labelling time

Standard protocol requires four hours of metabolic labelling to detect palmitoylation of proteins (Yap et al., 2010). Surprisingly, we found that MCF7-V5_GSTP1 cells require longer ω-alkynyl-palmitate metabolic labelling (8 h) for detection of GSTP1 palmitoylation. These data suggest that palmitoylation could be more stable and less dynamic for GSTP1 than other palmitoylated proteins. Interestingly, GSTP1 has a half-life of 8-9 h and this might correspond with a requirement of GSTP1 to turn over to incorporate ω-alkynyl-palmitate (Shen et al., 1995)

Future work: Future work should include pulse-chase experiments with GSTP1 metabolically labelled with ω-alkynyl-palmitate. Translation of GSTP1 will then be inhibited with cycloheximide and the half-life of palmitoylated GSTP1 determined by click chemistry.

3.3 GSTP1 is not (or not only) palmitoylated via *S*-palmitoylation

Most proteins undergo *S*-palmitoylation with a few exceptions that exist for *N*- and *O*-palmitoylation. Although we discovered that GSTP1 is a palmitoylated protein, the palmitoylation site(s) have proven difficult to identify.

Future work: The next step is to identify GSTP1 residues modified by palmitate in mammalian cells. We have analysed GSTP1 from MCF7-GSTP1 grown in the presence of regular FBS, FAF-FBS, and FAF-FBS supplemented with ω-alkynyl-palmitate cells by electrospray Fourier transform mass spectrometry (ESI-FTMS) after purification using S-hexyl GSH beads (Appendix 1). Preliminary data has shown that GSTP1 is modified by stearate, but not palmitate (Appendix 1). These results suggest that something occurs during GSTP1 purification or sample preparation that excludes or cleaves palmitoylated forms of GSTP1. Preliminary data suggests that GSTP1 purified with S-hexyl GSH agarose is not palmitoylated (Appendix 1). It is possible that the GSH binding is blocked when the palmitoylation site is utilized and this prevents the pull down of the palmitoylated protein or there is a conformational change that is blocking the GSH-binding site. Thus, to overcome this challenge GSTP1 should be purified using another method, such as adding a His-tag for purification, and analysed by MS in the same way. Furthermore, peptide analysis should be completed to identify the residue(s) modified by stearate.

Once the amino acids that are modified by palmitate and stearate are identified, a non-lipidated mutant should be generated, transfected into MCF7s, metabolically labelled and a lack of palmitoylation assessed by click chemistry, as described in Chapter 2. This mutant can then be compared to GSTP1-WT for cellular localization, membrane association, catalytic function and protein-protein interaction. Assessment of the catalytic activity is further validating the

activity and the stability of the protein and these experiments will be only possible if the mutation does not severely influence stability and levels.

3.4 Characterization of membrane-associated GSTP1

We have shown that GSTP1 in the cellular membrane fraction of MCF7-GSTP1 cells does not catalyse the conjugation of GSH to CDNB. Catalytic activity could be evaluated in the presence of multiple GSTP1 substrates, such as: 1-chloro-2,4-dinitrobenzene (CDNB), benzyl isothiocyanate (BITC), ethacrynic acid (EA) as these substrates were previously characterized to have distinct binding sites on GSTP1 (Ralat & Colman, 2004).

Our data suggests that human GSTP1 purified from mammalian cells (grown with and without free fatty acids) and *E. coli*, associated with plasma membrane enriched vesicles. It was expected that GSTP1 would associate with the membranes in a palmitate-dependent manner, however, there was no significant difference in the level of GSTP1 associated with the membrane with or without *in vitro* palmitoylation. Considerable variability was encountered during these experiments and this could be an explanation for the lack of statistical significance. There was a non-significant trend that *in vitro* palmitoylation of FAF-GSTP1 and GSTP1 from *E. coli* increased the membrane association. Furthermore, it is not clear if the purified GSTP1 from all sources is completely free of lipidation prior to *in vitro* labelling. It is expected based on data in Appendix 1 that GSTP1-FBS purified under the same conditions is modified by stearate (Figure A1.1). Therefore, it is still difficult to exclude lipidation of GSTP1 as a mechanism for its association with cellular membranes.

Future work: Purified GSTP1 from all sources (*E. coli,* MCF7-GSTP1 grown in the presence of regular and FAF-FBS) could be subjected to treatment with organic solvents to strip

the protein of fatty acids. The lipids extracted could be analysed by gas chromatography and the stripped protein used for membrane association studies with and without *in vitro* palmitoylation. The catalytic activity of GSTP1-stripped and non-stripped will be compared.

To characterize the membrane association further, biophysical approaches could be taken. To characterize the components of the membrane that GSTP1 is associating with, liposomes could be used in place of plasma-membrane enriched vesicles. The liposomes could be prepared with various types of lipids to mimic the different regions of the cellular membranes. For example, outer leaflet of the plasma membrane is richer in phosphatidylcholine whereas the inner leaflet is richer in phosphatidylserine. Using liposomes, it would also be possible to determine the constant of binding of GSTP1 with Trp fluorescence quenching. GSTP1 has two Trp residues, which would allow the titration of GSTP1 into liposome-containing medium and the fluorescence of the pellet can be measured in a concentration-dependent manner.

3.5 Subcellular localization of palmitoylated GSTP1

In this study, we have shown that GSTP1 in both the total membranes and cytosol are palmitoylated. Palmitoylation of cytosolic GSTP1 was surprising because we expected that palmitoylation would promote the association with plasma membrane. Instead, the cytosolic GSTP1 is able to retain this hydrophobic moiety even when it is not associated with the membrane. It is possible that the cytosolic fraction that is palmitoylated is just utilizing amino acids that are in the dimer interface and not exposed to aqueous solution, whereas other residues may be responsible for the membrane association.

Cellular membranes are amphipathic and the inner leaflet of the plasma membrane is negatively charged. Some proteins have loops and helices that stay in close proximity or interact with cellular membranes facilitating palmitoylation. Phosphorylation of Tyr, Ser, or Thr can increase the localized negative charge and, if a palmitoylation site is in close proximity with the phophorylation site, it might prevent the fatty acid from associating with the membrane. To further investigate the GSTP1-membrane affinity, experiments might be repeated using phosphatase inhibitors, and/or utilizing purified GSTP1 mutated at its phosphorylation sites.

In addition, immunofluorescence analysis might be done once the palmitoylation mutant is identified. Preliminary data has shown that fatty acids may influence GSTP1 association with ER membranes (Appendix II).

3.6 Cross-talk of palmitoylation with other GSTP1 post-translational modifications and/or protein-protein interactions

The two Cys residues shown to be palmitoylated *in vitro* Cys48 in the NH_2-terminus and Cys102 at the dimer interface (Hegazy et al., 2004), are modified by *S*-glutathionylation and *S*-nitrosation (Bocedi et al., 2013; Tew & Townsend, 2011). Therefore, *S*-palmitoylation of Cys48 and Cys102 may be co-ordinated and regulated with other post-translational modifications and play a role in the regulation of GSTP1 function.

3.7 Significance of GSTP1 palmitoylation in cell signalling pathways

GSTP1 association with JNK has been well characterized and it is required for JNK-activated phosphorylation of transcription factors (Wang et al., 2001). To determine if palmitoylation of GSTP1 influences JNK signalling co-immunoprecipitation of GSTP1 and JNK from MCF7-

GSTP1 treated with FAF or FBS medium and interaction followed by immunoblotting would provide evidence of the role of lipidation of GSTP1-JNK interaction. Assessment of activity, phenotype and expression of proteins downstream of JNK would further characterize the importance of GSTP1 in JNK binding. GSTP1 also interacts with TRAF2 (Wu et al., 2006), GSTP1 palmitoylation upon induction of TRAF2 would be investigated. Because TRAF2 directly interacts with GSTP1, co-immunoprecipitation of either proteins collected from cells mantained under different fatty acid condition is an alternative to explore the influence of palmitoylation on the GSTP1-TRAF2 association.

3.8 Overall Significance

This research lays the foundation for understanding the fundamental biology of GSTP1 and how palmitoylation influences its function. Understanding the cellular processes that regulate this multi-functional protein will potentially contribute to knowledge about its activity in drug metabolism, redox balance, cell signalling, and multiple diseases including its role in drug resistance and tumour aggressiveness.

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Appendix 1: Identifying palmitoylation sites of GSTP1 purified from MCF7 cells using tandem mass spectrometry.

A1.1 Overview

S-palmitoylation is the reversible and covalent modification of Cys residues by palmitate. Although less common, *O*-palmitoylation of Ser and Thr and *N*-palmitoylation of Lys residues can also occur (Okubo et al., 1991; Resh, 2016). *S*-palmitoylation of Cys residues can occur enzymatically in the cytosol by DHHC –containing palmitoyl transferases (PATs) and *O*-palmitoylation of Ser residues by membrane bound *O*-acyl transferase (MBOAT), porcupine and ghrelin O-acyltransferase (GOAT) (Al Massadi et al., 2011; Berthiaume, 2014; Buglino & Resh, 2012). In addition, palmitoyl transferase (PAT) - independent palmitoylation (or non-enzymatic palmitoylation) can occur, however, the requirements for when PAT-dependent or independent palmitoylation occurs have not yet been well defined (Dietrich & Ungermann, 2004; Wolff et al., 2000).

In vitro palmitoylation of purified proteins in combination with MS analysis can be used as a strategy to identify sites of *in vitro* palmitoylation and allow the establishment of sample preparation and analysis conditions (Ji et al., 2013). Identification of palmitoylation sites from *in vitro* modified proteins need to be interpreted carefully because it is limited to identification of PAT-independent palmitoylation sites. Furthermore, *in vitro* palmitoylation is often done using concentrations of palmitoyl CoA that exceed cellular levels. In Chapter 2 of this thesis three PAT-independently palmitoylated amino acids on GSTP1 (Cys48, Cys102, and Lys103) were identified (Figure 2.6). The influence of these three residues as well as Cys15 and Cys170 on GSTP1 palmitoylation within MCF7 cells was assessed by stably expressing the GSTP1_4X (Cys to Ala)/Lys103Arg mutant followed by metabolic labelling with ω-alkynyl-palmitate, and click chemistry (Figure 2.7). The GSTP1_4X (Cys to Ala)/Lys103Arg mutant retained palmitoylation at levels similar to GSTP1-WT. This suggests that, either different, or additional

palmitoylation sites can be utilized inside the cell. Within the cell, palmitoylation of GSTP1 could occur at sites requiring PAT addition of palmitoyl CoA. Therefore, we further investigated the palmitoylation status of GSTP1 purified from MCF7s by MS using the protocol optimized for *in vitro* palmitoylation (Ji et al., 2013) and Chapter 2.

A1.2 Methods

A1.2.1 Materials

Rabbit pAb anti-GSTP1 (GS72) was from Oxford Biomedical Research (Rochester Hill, MI), rabbit pAb anti-Na^+/K^+-ATPase (H-300) was from Santa Cruz Biotechnology (Dallas, TX). The horseradish peroxidase (HRP) conjugated anti-rabbit IgG was from Thermo Scientific (Rockford, IL).

ω-alkynyl-palmitate was synthesized as described previously (Yap et al., 2010). Asp-N was purchased from Promega (Madison, WI) and RapiGest SF®-186001861 was purchased from Waters (Milford, MA). EDTA-free Complete™ protease inhibitor cocktail tablets and X-tremeGENE™9 transfection reagent were purchased from Roche Applied Science (Laval, QC). 3-(N-morpholino) propanesulfonic acid (MOPS), Bicinchoninic acid (BCA) assay, neutravidin-HRP, glutathione-agarose, formic acid, Zeba™ spin desalting columns and Geneticin®(G418) were from Thermo Scientific (Rockford, IL). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Acyl-CoA synthetase, Tris-(benzyltriazolylmethyl) amine (TBTA), palmitoyl CoA, Tris-carboxyethylphosphine (TCEP), magnesium chloride ($MgCl_2$), acetonitrile, 1,4-dithiothreitol (DTT), Trisma-base, Tris-HCl, reduced L-glutathione, bovine serum albumin (BSA) and $CuSO_4$, were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA).

A1.2.2 Cell lines

The human breast adenocarcinoma cell line (MCF7) (Rockville, MD) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Oakville, ON, Canada) and supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS). Routine testing for *Mycoplasma sp.* contamination of cell lines was performed using the ATCC Universal Mycoplasma Testing Kit (Manassas, VA). The MCF7-GSTP1 stable cell line was previously generated (Qazi et al., 2011). The MCF7-V5_GSTP1-WT was created as described in Chapter 2, section 2.2.3.

A1.2.3 Metabolic labelling of cells with ω-alkynyl palmitate.

Metabolic labelling of cells with ω-alkynyl-palmitate was adapted from a method described previously (Yap et al., 2010). Briefly, MCF7 cells stably expressing GSTP1 were grown until 70-80% confluency, then the culture medium was exchanged for ω-alkynyl-palmitate (100 μM) in DMEM with 10% FAF-FBS or FBS alone. Medium alone was replaced daily for 24-48 h.

A1.2.4 Immunoprecipitation of wild-type and mutant forms of GSTP1

After metabolic labelling, cells were washed with cold PBS, harvested, and lysed with cold EDTA-free RIPA buffer [0.1% SDS, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium-deoxycholate, 2 mM $MgCl_2$, EDTA-free Complete^{TM} protease inhibitor cocktail] for 15 min at 4°C. Cell lysates were centrifuged at 16,000 *g* for 10 min at 4°C and the

post-nuclear supernatant collected. GSTP1 was then immunoprecipitated from this fraction using agarose beads conjugated to mouse anti-V5 antibody (V5-10) overnight at 4°C. Beads were then spun down at 16 000 xg for 10 min and washed with 1 ml of cold PBS with 0.1% Triton-X, 3-5 times. GSTP1 was eluted from the beads by incubating for >10 min at room temperature with Laemmli buffer or, subjected to click chemistry, acetone precipitated and then re-suspended in Laemmli buffer. Eluted proteins were then resolved on 11% SDS-PAGE.

A1.2.5 GSTP1 purification from MCF7-GSTP1 cells

MCF7-GSTP1 cells (2 x 10^7) were re-suspended in Tris-sucrose-buffer (50 mM Tris, pH 7.4, 250 mM sucrose) containing EDTA free Complete^{TM} protease inhibitors, and disrupted by nitrogen cavitation as described previously (Leslie et al., 2004). Cell homogenates were centrifuged at 2000 *g* at 4ºC for 10 min to remove nuclei and unbroken cells. The supernatants were transferred to 1.7 ml ultracentrifuge tubes, centrifuged at 100 000 *g* for 30 min at 4°C (Beckman TLA 110 rotor). The supernatant containing the cytosolic fraction of MCF7-GSTP1 was collected and 1 mg of total cytosolic protein was incubated with 300 µl of immobilized GSH-agarose beads for 1 h at 4ºC, centrifuged at 10000 *g* for 10 min at 4ºC and then beads were washed with Na_2HPO_4 , pH 6.8, and NaCl 150 mM. Protein was eluted with 20 mM GSH in Na_2HPO_4 , pH 8.0 and NaCl 150 mM or 2% formic acid. Eluted protein was concentrated using a speed vacuum, then the protein concentrations were determined at 280 nm using a NanoDrop 2000 (Wilmington, DE, USA). Eluted and concentrated protein was either analysed by mass spectrometry (Section A1.2.6) or subjected to click chemistry (Section A1.2.7). To check the purity of GSTP1, eluted GSTP1 (3 µg) was resolved on 11% SDS-PAGE, the gel was fixed for 30 min in fixation buffer (isopropanol 25%, acetic acid 10%), stained for 2 h with Coomassie

blue 250 (60 mg/L) for 30 min followed by overnight destaining in acetic acid 10%, then the gel was dried.

A1.2.6 Electrospray fourier transform mass spectrometry (ESI-FTMS) analysis of GSTP1 purified form MCF7 under FBS, FAF or FAF + ω-alkynyl palmitate

MCF7-GSTP1 was mantained under FBS, FAF or FAF + ω-alkynyl palmitate conditions and purifed as described in Section A1.2.6. using 2% formic acid as elution buffer. GSTP1 (2 µg) was resuspended in trifluoroacetic acid (TFA, 1%) and dried using a speed vacuum. The samples were enriched for palmitoylated peptides using column chromatography with POROS R1 50 resin. Palmitoylated peptides were eluted stepwise in 60% and 80% acetonitrile and analysed by electrospray ionisation-Fourier transform mass spectrometry (ESI-FTMS) using a 12 tesla mass spectrometer as described previously (Ji et al., 2013).

A1.2.7 *Detection of GSTP1 metabolically labelled with ω-alkynyl-palmitate using click chemistry.*

Purified GSTP1 (2 µg) or immunoprecipitated V5_GSTP1 was incubated with click reaction components [Tris (benzyltriazolylmethyl) amine (100 µM), $CuSO_4$ (1 mM), Tris-carboxyethylphosphine (1 mM), and azido-biotin (100 µM)] at 37°C for 30 min, protected from light. The click reaction was stopped by the addition of a 10X volume of ice-cold acetone and incubated overnight at 4°C to precipitate proteins. Precipitated proteins were then pelleted at 16 000 *g* for 15 min, the supernatant discarded and the pellet resuspended in Laemmli buffer containing DTT (30 mM). Samples were heated at 95°C for 5 min, resolved on 11% SDS-PAGE then electrotransfered to a PVDF membrane. Biotinylated ω-alkynyl-palmitate-labelled GSTP1

was detected with neutravidin-HRP (1:20000) and total GSTP1 loading was detected with rabbit pAb anti-GSTP1 (GS72) (1:5000).

A.1.3 Results and Discussion

A.1.3.1 *Determination of palmitoylation of GSTP1 purified from MCF7*

As shown in Chapter 2, MS analysis of *in vitro* palmitoylated GSTP1 identified three palmitoylation sites, however, mutation of these amino acids did not prevent GSTP1 from being palmitoylated in MCF7 cells. Eukaryotic cells contain PATs that are likely required for GSTP1 palmitoylation at certain sites, preventing their formation during *in vitro* labelling. To identify GSTP1 palmitoylation sites that are utilized within cells, GSTP1 was purified from MCF7-GSTP1 cells using immobilized GSH-agarose beads, eluted with 2% formic acid, and analysed by ESI-FTMS. The m/z ration of GSTP1 is 1161.50 (based on the mass calculated by the spectrum in Figure A1.1). We expected to find a peak for palmitate in the mass spectrum for GSTP1 purified from MCF7 grown in regular FBS containing media, however, it was not present, suggesting that the palmitoylated GSTP1 was not detected. Instead, a peak at a m/z ratio of 1174.8 was present, potentially indicating modification of GSTP1 by stearate (18:0) (Figure A1.1, A.). Mass spectra of GSTP1 purified from cells grown in FAF-FBS containing medium (Figure A1.1, B.) and GSTP1 grown in FAF-FBS medium containing ω-alkynyl-palmitate, (Figure A1.1, C.) did not contain peaks for either palmitate or stearate modified GSTP1.

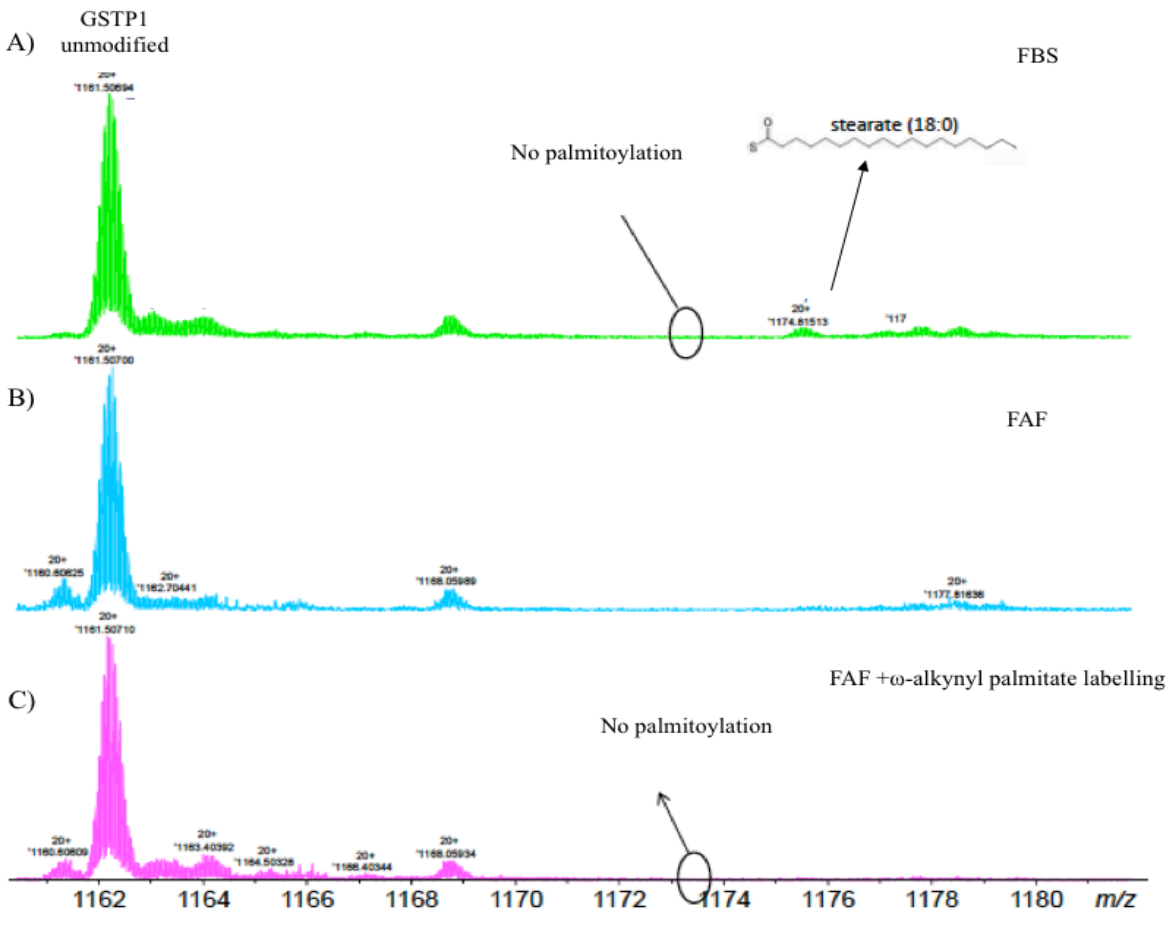


Figure A1.1 *ESI-FTMS spectra of GSTP1 purified from MCF7 maintained in FBS, FAF and FAF plus* ω-alkynyl-palmitate.

MCF7-GSTP1 grown in the presence of FBS, FAF-FBS and FAF-FBS plus ω-alkynyl-palmitate was purified from MCF7 with immobilized GSH-agarose and analysed by ESI-FTMS. **(A)** Spectrum of GSTP1 purified form MCF7 mantained in regular FBS **(B)** Spectrum of GSTP1 purified from MCF7 mantained in FAF-FBS. **(C)** Spectrum of GSTP1 purified form MCF7 mantained in FAF-FBS with the addition of ω-alkynyl-palmitate.

The detection of stearate (rather than palmitate) associated with GSTP1 in the FBS condition was surprising, however, GSTP1 might be modified by a range of fatty acids, not only palmitate. The lack of palmitoylation for GSTP1 purified from MCF7-GSTP1 cells grown in the presence of regular FBS or FAF-FBS plus ω-alkynyl-palmitate was puzzling and not consistent with our results in Chapter 2 that GSTP1 is a palmitoylated protein.

A.1.3.2 *Determination of the palmitoylation status of GSTP1 purified from MCF7-GSTP1 cells using GSH-agarose.*

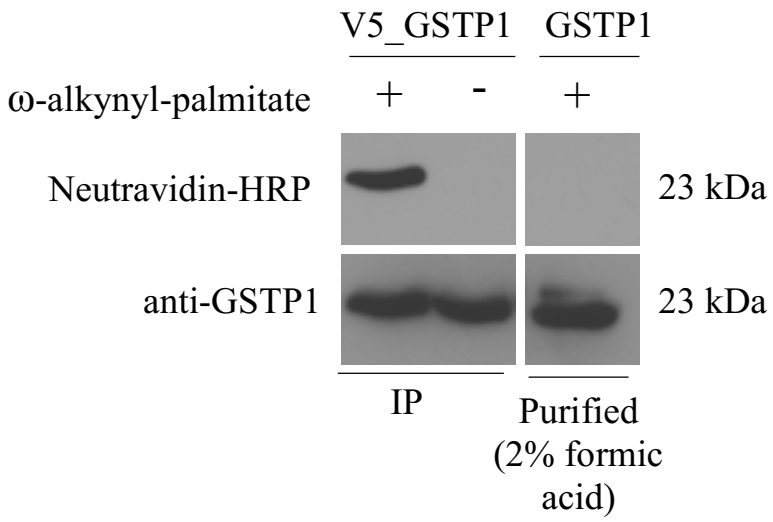
Our clear data in Chapter 2 that GSTP1 is a palmitoylated protein and the contrasting lack of palmitoylated GSTP1 detected by MS suggested that something was occurring during the GSTP1 purification and/or MS preparation to prevent its detection. We explored the possibility that palmitoylated GSTP1 is not purified efficiently compared to non-palmitoylated forms using the GSH-agarose beads. MCF7-GSTP1 cells were metabolically labelled with ω-alkynyl-palmitate and GSTP1 was purified over GSH-agarose beads and eluted with either 2% formic acid or 20 mM GSH. Eluted fractions were dialysed, subjected to click chemistry, acetone-precipitaed, resolved on 11%-SDS-PAGE and transferred to a PVDF membrane. Palmitoylated GSTP1 was detected with neutravidin-HRP (1:20000) and total GSTP1 was detected with rabbit pAb anti-GSTP1 (GS70) (1:5000). MCF7-V5_GSTP1 cells were treated under similar conditions as the MCF7-GSTP1 cells except instead of purification with GSH-agarose GSTP1 was immunoprecipitated under conditions used in Chapter 2 and served as a positive control for click chemistry. Consistent with the ESI-FTMS analysis, palmitoylated GSTP1 was not detected for GSTP1 purified over GSH-agarose beads (Figure A1.2A). To rule out that 2% FA is affecting the palmitoylation or the detection of the palmitoylation, we pulled down GSTP1

with GSH-agarose beads and eluted GSTP1 with 20 mM GSH (rather than 2% FA). No palmitoylated GSTP1 was detected in the pull down of GSTP1 eluted with GSH 20 mM (Figure A1.2B). Therefore, it is required an alternative pull down method, such as addition of a tag onto GSTP1 for purification form MCF7s to determine if GSH binding site is required for GSTP1 palmitoylation in MCF7s.

Future directions

To overcome the potential limitations of the immobilized GSH-agarose pull down, we will rely on a tag for protein pull down. A GSTP1 tagged at the NH_2-terminus stably transfected into MCF7 cells will be grown under FBS, FAF and FAF plus ω-alkynyl-palmitate will be purified from MCF7-GSTP1 cell lines. Purified protein will be detected by top down analysis (identification of intact GSTP1 palmitoylation) followed by digestion with Asp-N or trypsin for bottom up analyses (identification of the palmitoylated amino acid) by ESI-FTMS. Once the amino acid sites are identified, a mutant of GSTP1 in a plasmid construct will be made and transfected into MCF7 for validation of palmitoylation loss by metabolic labelling and click chemistry. In addition, this can be used to evaluate the consequences of the mutation in membrane association and subcellular localization using the methods described in Chapter 2 and Appendix 2.

A)


V5_GSTP1
GSTP1
ω-alkynyl-palmitate
+
-
+
Neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa
IP
Purified
(2% formic
acid)

B)

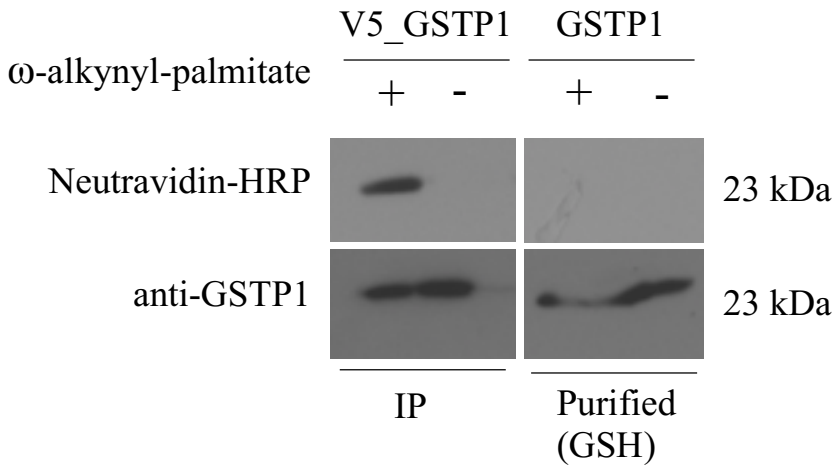

V5_GSTP1
GSTP1
ω-alkynyl-palmitate
+
-
+
-
Neutravidin-HRP
23 kDa
anti-GSTP1
23 kDa
IP
Purified
(GSH)

Figure A1.2 *Detection of GSTP1 palmitoylation in MCF7 cells after immunoprecipitation of V5_GSTP1 compared with GSTP1 purified using GSH-agarose beads.*

MCF7-GSTP1 and MCF7-V5_GSTP1 cells were metabolically labelled with ω-alkynyl-palmitate. MCF7-GSTP1 cells were disrupted with N_2 cavitation and the cytosolic fraction was isolated by ultracentrifugation. Cytosolic GSTP1 was then purified over GSH-agarose beads and eluted with **(A)** 2% formic acid or **(B)** GSH elution buffer. The eluted fractions (formic acid or GSH) were then dialysed and subjected to click chemistry. Immunoprecipitation (Ipp) of V5_GSTP1 from MCF7-V5_GSTP1 cells were run as positive controls for the click chemistry (left hand panels A and B). Cells were lysed with 0.1% SDS-RIPA buffer, immunoprecipitaed, and subjected to click chemsitry. After click chemistry, proteins (purified or IP) were acetone-precipitated, resolved on 11%-SDS-PAGE and transferred to a PVDF membrane. Palmitoylated GSTP1 was detected with neutravidin-HRP (1:20000) and total GSTP1 was detected with rabbit pAb anti-GSTP1 (GS70) (1:5000). All panels shown in **(A)** are cropped from the same blot as are all panels in **(B)**.

Appendix 2 Influence of fatty acids on GSTP1 cellular localization

A2.1 Overview

Glutathione transferase P1 (GSTP1) is an enzyme critical for detoxifying electrophiles through conjugation with reduced glutathione (GSH). For a long time, GSTP1 research focussed on this role, more recently, GSTP1 has been identified as an important regulator of signalling (Wang et al., 2001). Furthermore, GSTP1 can regulate the function of other proteins by post-translationally modifying them with GSH (*S*-glutathionylation) (Townsend et al., 2009). Although classically reported as a cytosolic enzyme its cellular localization also includes the mitochondria (Goto et al., 2009), nuclei (Kamada et al., 2004; Kawakatsu et al., 2011), ER (Ye et al., 2016) and plasma membrane (Qazi et al., 2011).

Lipids define the boundaries of organelles creating a spatially organized and compartmentalized system. The lipid bilayers of organelles and the plasma membrane are also the location of biological processes required for continuous survival of the cell (Eyster, 2007; van Meer et al., 2008). Cellular membranes differ in types of proteins and lipids among the compartments of the cells; for example, the amount of cholesterol in the plasma membrane is greater than that found in the endoplasmic reticulum (ER) (van Meer & de Kroon, 2011).

Proteins can be present in more than one compartment of the cell at the same time. For correct localization, specific signals determine the location, implicating a requirement for carefully controlled regulation. Subcellular localization of GSTP1 may dictate whether it will act as a detoxification enzyme (Peklak-Scott et al., 2008), a signalling protein (Wang et al., 2001), or a regulatory enzyme, as occurs during *S*-glutathionylation (Townsend et al., 2009).

Proteins are sorted to subcellular compartments based on signal sequences (Emanuelsson et al., 2007). The classic consensus sequence for nuclear import is three Lys or Arg out of five amino acids in a sequence (Kosugi et al., 2009; Robbins et al., 1991). GSTP1

has a non-classical signal sequence that allows its localization to the nucleus, it is located in the COOH-terminus within amino acids 195-208 (Kawakatsu et al., 2011). In addition, GSTP1 contains a set of polybasic amino acids (Arg 14, 19, 71, and 75) that are required for mitochondrial translocation (Goto et al., 2009).

In addition to specific signal sequence peptides, post-translational modification can regulate the association of proteins with different organelles and influence subcellular localization. Phosphorylation is known to work as a switch for proteins. The addition of phosphate groups onto Tyr, Ser, and Thr, can decrease the affinity of the peptide for the negative inner leaflet of the plasma membrane. Moreover, phosphorylation can determine cellular localization through influencing protein-protein interactions. For example, phosphorylation of c-Jun Kinase (JNK) prevents its interaction with GSTP1, which leads to JNK nuclear translocation, where it phosphorylates and activates multiple transcription factors (Adler et al., 1999; De Luca et al., 2012). Mass spectrometry–based proteomic studies have identified 19 phosphorylation sites on GSTP1 to date (Hornbeck et al., 2015). Cell based studies have confirmed the utilization of six phosphorylation sites (Tyr4, Tyr8, Ser43, Tyr64, Ser185, and Tyr199) (Okamura et al., 2015; Okamura et al., 2009; Singh et al., 2010).

We have also shown that GSTP1 is post-translationally modified by the saturated fatty acid palmitate (16:0) (Chapter 2). Palmitoylation can modify proteins to increase their affinity with cellular membranes, regulate protein function, and modify protein-protein interactions (Linder & Deschenes, 2007; Resh, 1999). Furthermore, we have shown that membrane and cytosolic forms of GSTP1 are palmitoylated, and that this modification might not necessarily be required for membrane association (Chapter 2).

Previous studies have shown that GSTP1 is present at the plasma membrane and the strength of association is comparable to the integral membrane Na^+/K^+-ATPase (Qazi et al., 2011). We have demonstrated that membrane associated GSTP1 does not conjugate 1-chloro-2,4-dinitrobenzene (CDNB), a well-established glutathione transferase substrate, with GSH.

We couldn't make a palmitoylation free GSTP1 mutant due to the complexity of the palmitoylation. Therefore, we attempted to developed tools to characterize the effect of fatty acid free (FAF) conditions (mimicking a lack of GSTP1 lipidation) on GSTP1 localization.

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A2.2 Methods

A2.2.1 Materials

Rabbit pAb anti-GSTP1 (GS72) was from Oxford Biomedical Research (Rochester Hill, MI) and rabbit pAb anti-Na^+/K^+-ATPase (H-300) was from Santa Cruz Biotechnology (Dallas, TX). The AffiniPure donkey anti-rabbit IgG conjugated to Cy™3 was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The Alexa Fluor® 488 conjugated goat anti-rat IgG was from Life Technologies (Grand Island, NY). The mouse mAb anti-GSTP1 (H-11) was from Sigma-Aldrich (Oakville, ON, Canada). The rabbit anti-giantin pAb (poly19243) was from BioLegends (San Diego,A CA) and the rabbit mAb anti-KDEL antibody (EPR12668) Alexa Fluor® 555 was from Abcam (Cambridge, UK).

X-tremeGENE 9 transfection reagent, and protease inhibitor cocktail tablets (Complete, Mini, EDTA-free) were from Roche Applied Science (Laval, QC, Canada). Geneticin®(G418) was from Thermo Scientific (Rockford, IL); paraformaldehyde (PFA), methanol, triton-X 100, palmitoyl CoA, 4′,6-Diamidino-2-phenylindole (DAPI), bovine serum albumin (BSA), were purchased from Sigma-Aldrich (Oakville, ON). DAKO mounting medium was purchased from

Agilent Technologies (Santa Clara, CA).

A2.2.2 Cell lines

The human breast adenocarcinoma cell line (MCF7) was obtained from the American Type Culture Collection - ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma-Aldrich (Oakville, ON) and supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS). Routine testing for *Mycoplasma* contamination of cell lines was performed using the ATCC Universal Mycoplasma Testing Kit (Manassas, VA).

A2.2.3 Generation of V5 tagged GSTP1 expression vectors

pBluescriptSK(−) GSTP1 was a gift from Dr. Philip G. Board (Australian National University, Canberra). pcDNA3.1(+) containing the full-length open reading frame of human GSTP1 [pcDNA3.1(+) GSTP1] was generated as previously described (Qazi et al., 2011). A construct of pcDNA3.1(+) GSTP1 containing a V5 tag at the NH_2-terminus [pcDNA3.1(+) V5_GSTP1] was generated by PCR amplification of pcDNA3.1(+) GSTP1 using the forward primer 5'...AAAAA*GGATCC*ACCATGGGTAAACCTATTCCTAATCCTCTTCTTGGTCTTGATT CTACA**CCGCCGTAT ACCGTGG**...3' with a *BamHI* restriction site indicated in italics, the V5 tag sequence underlined and the GSTP1 sequence in bold. The reverse primer used was 5'...CGGGCCCTCTAGA*CTCGAG***TCACTGTTTCCCGTTGCCATTGATGG**...3' with a *XhoI* restriction site indicated in italics and GSTP1 sequence in bold.

The PCR product and empty pcDNA3.1(+) vector were digested with *BamHI* and *XhoI* and then ligated. The pcDNA3.1(+)-V5_GSTP1 construct was confirmed by sequencing (The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON).

A2.2.4 Site-directed mutagenesis

GSTP1 mutants were generated using the Agilent QuikChange Lightning site-directed mutagenesis kit (Mississauga, ON). Mutagenesis was performed using the pcDNA3.1(+)-V5_GSTP1 construct as the PCR template according to the manufacturer's instructions. Mutagenic primers [Sigma-Aldrich (Oakville, ON) or Integrated DNA Technology (Coralville, IA)] were designed to generate a Cys-less V5_GSTP1 mutants [V5_GSTP1-Cys15/Cys48/Cys102/ Cys170Ala, named GSTP1_4X(Cys to Ala)] (primer sequences in (Table 2.1, Chapter 2). The mutant was confirmed by sequencing. To ensure that no unintentional mutations were introduced the entire GSTP1 coding region was also sequenced (The Center for Applied Genomics, The Hospital for Sick Children, Toronto, ON).

A2.2.5 Generation of stable MCF7 cell lines expressing V5_GSTP1 and GSTP1_V5 and V5_GSTP1 with mutations

The MCF7-GSTP1 and stable cell lines were generated previously (Qazi et al., 2011). MCF7 cells stably expressing pcDNA3.1(+)-V5_GSTP1, or -V5_GSTP1-4X(Cys to Ala) [named MCF7-V5_GSTP1and GSTP1-4X(Cys to Ala)] were generated as described previously (Qazi et al., 2011), with minor modifications. MCF7 cells were seeded and transfected using X-tremeGENE9. Forty-eight hours post-transfection, cells were selected in 1 mg/ml of G418, and the selected cells were cloned by limiting dilution. GSTP1 expression in G418-resistant cell populations was determined by immunoblotting with the rabbit anti-GSTP1 pAb (1:5000).

A2.2.6 Immunostaining

MCF7-GSTP1, MCF7-V5_GSTP1 or MCF7-V5_GSTP1-4X(Cys to Ala) cells were seeded at a density of $3x10^5$ cells per well of 6 well plate. After 24 h, medium was substituted for DMEM with charcoal-stripped fatty acid free (FAF) FBS, or kept in DMEM with regular FBS. Cells were washed once with PBS and then fixed with either PFA (4%) for 10 min at room temperature or with ice-cold methanol for 20 min at -20°C. PFA fixation was quenched with glycine (150 mM) and then washed with PBS, whereas, cells fixed with methanol were directly washed with PBS. Cells were permeabilized and blocked with blocking buffer (3% bovine serum albumin in PBS + 0.1% Triton-X 100) for one h at room temperature and incubated with primary antibody (1:250 – 1:500) for one h. Cells were washed with PBS at least three times for one min and incubated with secondary antibody (1:500) and DAPI. Cover slips were further washed, mounted onto slides with DAKO mounting medium, kept at room temperature overnight and stored at 4°C until visualization.

A2.2.7 Immunofluorescence imaging

Cells were viewed with a 60X objective with a fully motorized inverted fluorescence microscope (Leica DMI6000 B, Wetzlar, Germany) coupled to X-Cite®exacte fluorescence light source, equipped witth Quorum Mac 6000 system and the Angstrom illumination system associated with Optigrid (Guelph, ON). The MetaMorph software (Molecular devices, Sunnyvale, CA) was used for multi-dimensional acquisition that allows the measurement of multiple Z sections. Images were captured with a Flash 4.0 camera (Hamamatsu) and analysed by Volocity® (Improvision, Perkin Elmer, Waltham, MA).

A2.2.8 Statistical Analysis.

Data was checked for normal distribution, if normally distributed statistical analyses was performed using *t*-test with Welch's correction, when significance was considered $p < 0.05$. When data was not normally distributed, images were analysed by *t*-test and Mann-whitney U test, where $p < 0.05$.

A2.2.9 Preparation of 2-bromo-palmitate (2-BP) stock solution

300 mM of 2-BP was dissolved in 1 ml of ethanol (100%), added to 1 ml of BSA (10%) in water and incubated for 1 h at 37°C. 2-BP was prepared right before the experiment and not stored.

A2.2.10 Inhibition of palmitoylation by 2-bromo-palmitate followed by immunofluorescence.

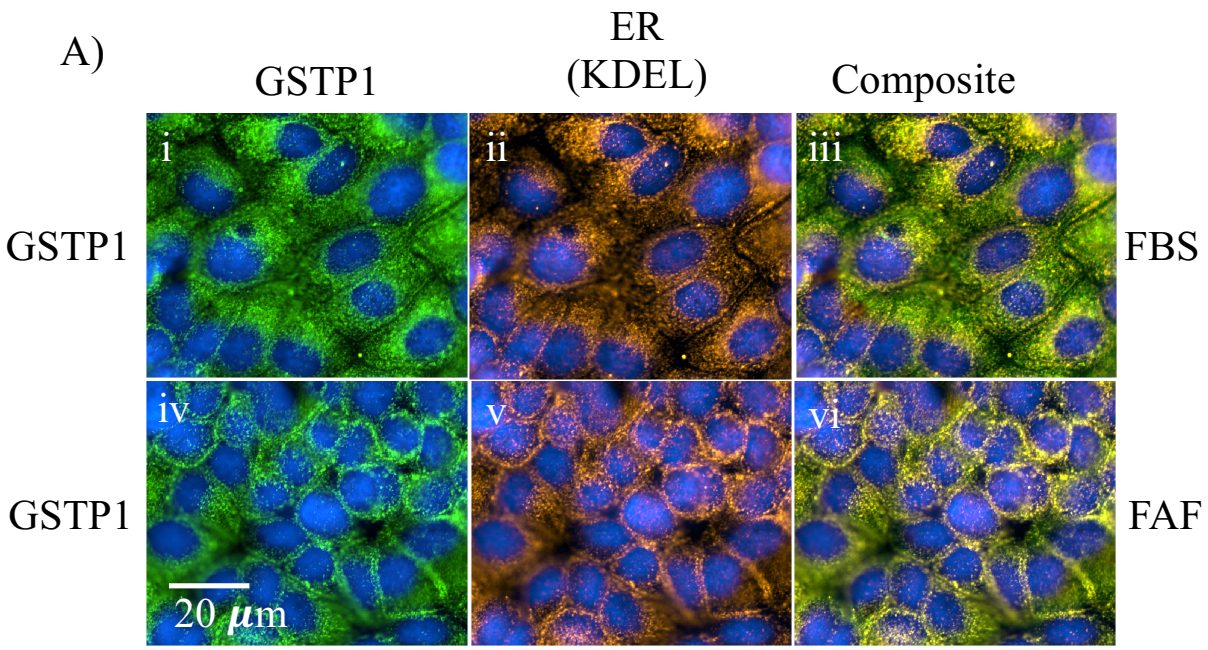
$1x10^6$ MCF7-GSTP1 cells were seeded onto coverslips in a 6 well plate and incubated for 24 h. Cells were then incubated for 4 h with DMEM medium without FBS (FAF) to deplete free fatty acids. After incubation, the medium was changed, replaced with medium containing 2-BP (100 μM) and gently added onto coverslips. Cells were then incubated at 37°C in a humidified chamber with 5% CO_2 for one h. Cells were washed with PBS 1X , incubated on ice for 20 to 30 min and then incubated with cholera toxin subunit B (CT-B) conjugated to AlexaFluor® 555 (1:1000) for 10 min to stain for plasma membrane. Cells were washed with cold PBS 1X and fixed with PFA (4%). PFA was quenched with glycine (100 mM) in PBS and washed with PBS 1X before being permeabilized with 0.1% Triton-X-100 for 1 min at room temperature. After permeabilization, cells were blocked with gelatin 0.2%, then incubated with rabbit pAb anti-GSTP1 (GS72) (1:250) for one h followed by anti-rabbit Alexa Fluor® 488 and DAPI.

Coverslips were mounted with DAKO mounting medium and visualised by fluorescence microscopy as described in section A2.2.7.

A2.3 Results and Discussion

A2.3.1 GSTP1 has increased association with the endoplasmic reticulum under FAF conditions.

To study the influence of GSTP1 palmitoylation on its cellular localization, we grew MCF7-GSTP1 cells in regular FBS or FAF medium for 24 h. Growing cells in the presence of charcoal stripped FAF medium should deplete free lipids from cells, thus preventing palmitoylation and other potential fatty acid modifications. MCF7-GSTP1 cells were triple stained with antibodies directed towards ER-resident proteins containing a KDEL sequence (ER-marker) (orange), and GSTP1 (green), as well as nuclei with DAPI (blue) (Figure A2.1). Cells were analysed by immunofluorescence. GSTP1 (Figure A2.1.i & iv), ER (Figure A2.1.ii & v) and the composite images (Figure A2.1.iii & vi) are shown for FBS (Figure A2.1. top panels) and FAF (Figure A2.1. bottom panels). GSTP1 was significantly associated with ER when grown in the presence of regular FBS (Pearson's coefficient: 0.829 ± 0.023) and FAF (Pearson's coefficient: 0.885 ± 0.066) conditions. Moreover, MCF7-GSTP1 cells grown under FAF condition were significantly more associated with the ER than MCF7-GSTP1 cells grown with regular FBS. These results indicate that GSTP1 is associated with cellular membranes of the ER. Surprisingly, when cells are depleted of fatty acids the amount of GSTP1 associated with the ER is increased by 5.6% ($p < 0.005$(*)). In summary, GSTP1 is a protein localized in close association with ER. Localization of GSTP1 with ER is not well documented, the only study reporting GSTP1 in association with ER suggests that GSTP1 is in the lumen of the


A)
GSTP1
ER
(KDEL)
Composite
i
ii
iii
GSTP1
FBS
iv
v
vi
GSTP1
FAF
20 μm

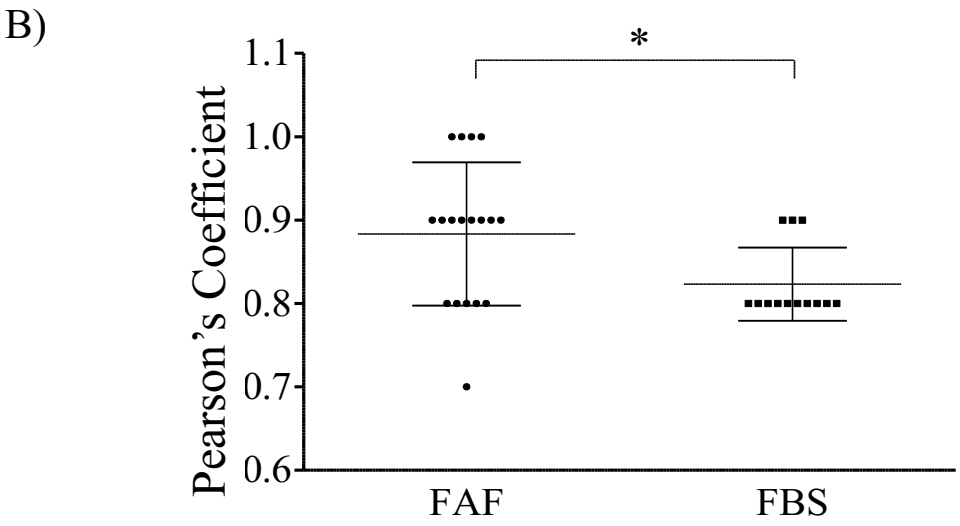

B)
*
1.1
1.0
0.9
0.8
0.7
0.6
Pearson's Coefficient
FAF
FBS

Figure A2.1 *Cellular localization of GSTP1 stably expressed in MCF7 cells grown under regular FBS and FAF-FBS conditions and co-stained for ER and nuclei.*

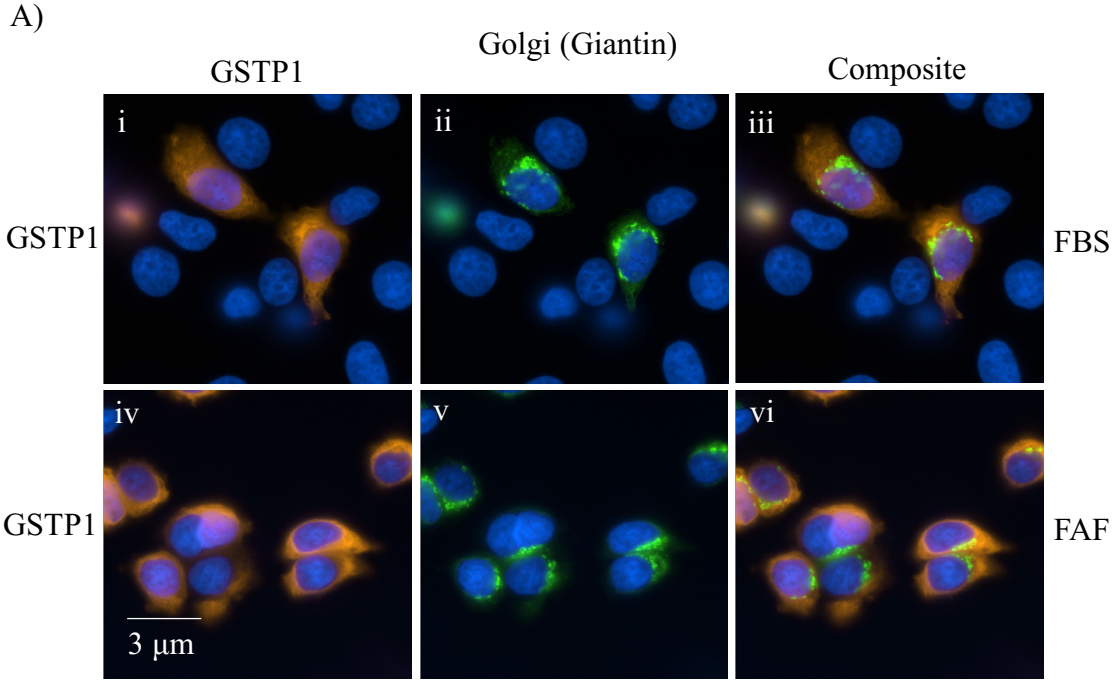
Triple staining of MCF7-GSTP1 grown in FBS **(1-iii)** or FAF **(iv-vi)** conditions for >24h. **(i, iv)** GSTP1 was detected with rabbit pAb anti-GSTP1 (GS72) and **(ii, v)** ER was detected with a mouse mAb anti-KDEL antibody coupled to a Cy3. **(iii, vi)** Composite images are shown. Co-localization analysis. Co-localization of GSTP1 grown under FBS conditions (FBS) and KDEL was compared with GSTP1 grown under FAF condition (FAF) by determining the Pearson's coefficient of multiple images in Volocity®. More than 20 cells were analysed from a single experiment. Pearson's coefficient was plotted as a vertical scatter plot. The horizontal lines represent the means ± SD and the (●) represents each image. Variance was significant for $p < 0.05$ (*) Scale bar 20 μm.

organelle (Ye et al., 2016). However, GSTP1 has no signal peptide sequence that would allow the translocation from cytosol to ER. Instead, it is possible that GSTP1 is in association with the cytosolic side of the ER membrane.

A2.3.2 GSTP1 co-staining with Golgi by immunofluorescence imaging.

GSTP1 was reported as a plasma membrane protein and also localizes to the ER. Thus, the influence of fatty acids on association of GSTP1 with the Golgi was logical to investigate, as the Golgi mediates the trafficking between ER and the plasma membrane. MCF7-GSTP1 cells grown in the presence of regular FBS or under FAF conditions were stained for GSTP1(orange) (Figure A2.2.A.i & iv) and Golgi marker giantin (green) (Figure A2.2.A.ii & v). The composite image (Figure A2.2.A.iii & iv) shows the overlapping of GSTP1 and Golgi (Figure A2.2.B). Co-localization analysis MCF7-GSTP1-FBS shows that co-staining of GSTP1 with Golgi from multiple cells is variable and the average Pearson's coefficient (mean ± SD) for FBS condition is 0.602±0.224 (Figure A2.2 i, ii & iii), whereas FAF was 0.493±0.267 (mean ± SD) (Figure A2.2, iv, v & vi). No significant difference was found between FBS and FAF treatment ($p > 0.05$) (Figure A2.2.B). To be more conclusive, further analysis with quantitation of more cells is required. To support this data, additional biochemical analysis with subcellular fractionation would be advantageous.

A)


GSTP1
Golgi (Giantin)
Composite
i
ii
iii
GSTP1
FBS
iv
v
vi
GSTP1
FAF
3 µm

B)

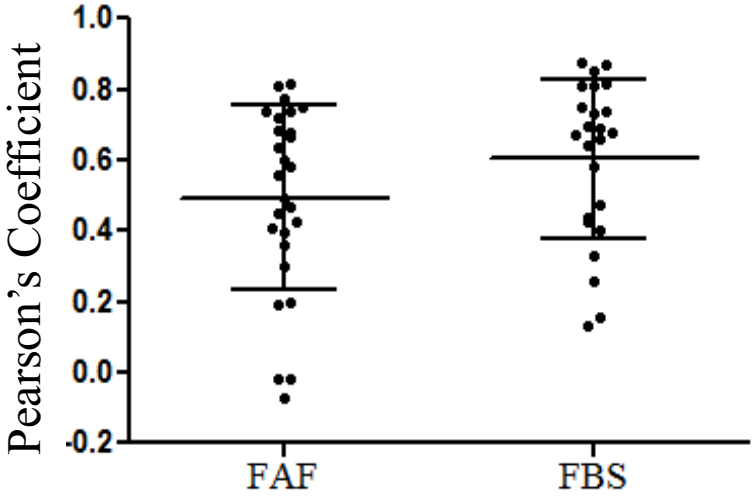

Pearson's Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
FAF
FBS

Figure A2.2 *Cellular localization of GSTP1 stably expressed in MCF7 cells grown under regular FBS and FAF-FBS conditions and co-stained for Golgi and nuclei.*

(A) Triple staining of MCF7-GSTP1 grown under FBS **(i-iii)** or FAF **(iv-vi)** conditions. Cells were grown under FBS or FAF conditions and fixed with methanol. GSTP1 was detected with mouse mAb anti-GSTP1 (H-11) and visualized with Cy3™ (orange) **(i,iv).** Golgi was detected with rabbit pAb anti-giantin and visualized with AlexaFluor488® (green) **(i,iv)** composite image visualize co-localization of GSTP1 with Golgi **(iii,vi)**. (**B)** Co-localization of GSTP1 grown under FBS conditions and giantin was compared with GSTP1 grown under FAF condition by determining the Pearson's coefficient of multiple images (>20 cells) from a single experiment using Volocity®. Pearson's coefficient was plotted as vertical scatter plot where the horizontal lines represent the means ± SD. Variance was significant for $p<0.05$ (*) using two tailed t-test with Welch correction. Scale bar 3 μm.

A2.3.3 Comparison of nuclear localization of GSTP1 under FAF or FBS condition

To investigate the influence of fatty acids on GSTP1 nuclear localization, MCF7-GSTP1 maintained under FAF and FBS condition were stained for GSTP1 and nuclei. Co-localization analysis of GSTP1 (green) and nucleus (blue) was performed with Volocity® and the Pearson's coefficient for correlation between GSTP1 and nuclei staining (Figure A2.3A). The mean (±SD) of the Persons coefficient under FBS condition was 0.37 ± 0.19 and 0.40 ± 0.21 for FAF (Figure A2.3.B). No significant difference was found between FAF and FBS treatment ($p > 0.05$).

A2.3.4 Mutation of all GSTP1 Cys residues (Cys to Ala) on GSTP1 alters subcellular localization

While investigating the subcellular localization of GSTP1 in response to exogenous fatty acids, we compared the subcellular localization of the GSTP1 WT with a Cys-less mutant, because Cys residues are target sites for palmitoylation. Immunostaining of MCF7 stably transfected with V5_GSTP1-4X(Cys to Ala)) or V5_GSTP1-WT with rabbit pAb anti-GSTP1 (GS72) (green) and nuclei (blue). The mutant V5_GSTP1-4X(Cys to Ala) was highly localized in the nuclear fraction of cells whereas the V5_GSTP1-WT was mostly present in the cytosol. To quantify this, we calculated the percentage of the ratio nuclear GSTP1/cytosolic GSTP1 for V5_GSTP1-4X(Cys to Ala)) and V5_GSTP1-WT and confirmed that V5_GSTP1-4X(Cys to Ala) is mostly localized in the nucleus and the V5-GSTP-WT in the cytosol, * = ($p < 0.001$). These data suggest that the Cys residues may have a role in the prevention of nuclear translocation, it is possible that the one or more of the Cys are required for structural conformation or interaction with other proteins. Alternatively, one or more Cys residue may be a target of post-translational modification that prevents nuclear import, because Cys48

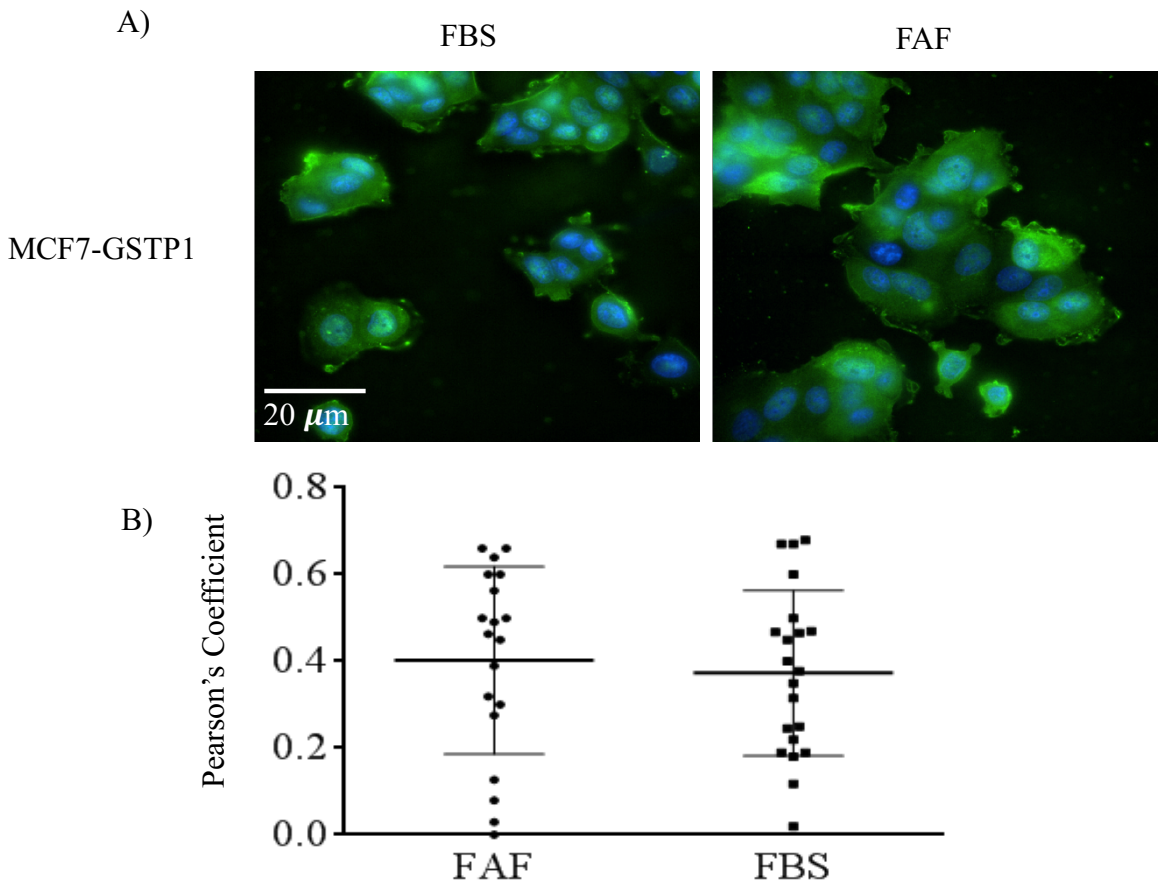


Figure A2.3 *Quantification of nuclear GSTP1 under FAF and FBS condition by immunostaining.*

(A) MCF7-GSTP1 grown in FBS **(i)** or FAF **(ii)** conditions for >24h. Cells were fixed with methanol and GSTP1 was detected with rabbit pAb anti-GSTP1 (GS72) (Green) and the nuclei was stained with DAPI (blue). **(B)** Nuclear localization of GSTP1 grown under regular FBS or FAF conditions was determined by the Pearson's coefficient of multiple images from two independent experiments in Volocity®. Pearson's coefficient was plotted as a vertical scatter plot. The mean is represented as a horizontal lines ± S.D. (•) represents the Pearson's coefficient of each image (~20 cells) from different regions of the cover slip. Variance was not significant $p>0.05$ (*) using t-test unpaired, with Welch correction. Scale bar 20 μm.

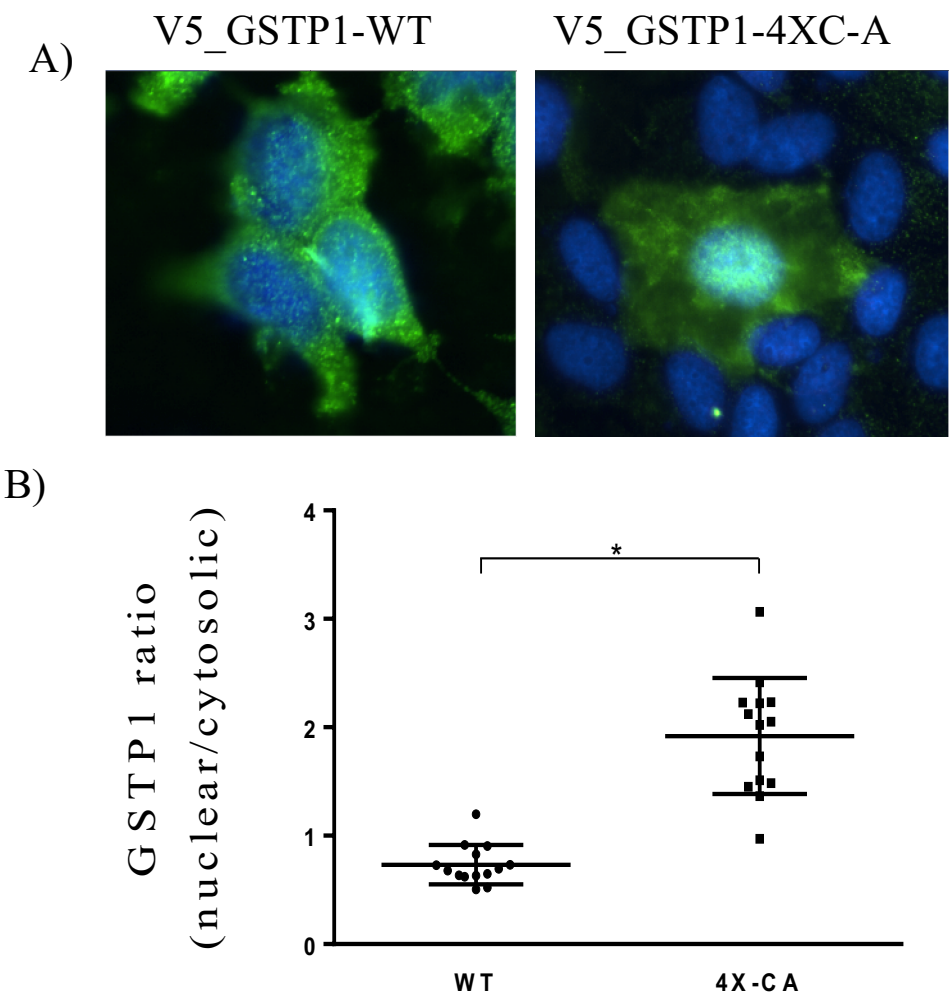


Figure A2.4 *V5_GSTP1-4X(Cys to Ala) is localized to the nucleus to a greater extent than V5_GSTP1_WT.*

MCF7 cells stably expressing V5_GSTP1 and V5_GSTP1-4X(Cys to Ala) were seeded onto coverslips and incubated for 24-48h. Cells were fixed, stained with pAb rabbit anti-GSTP1-GS72 (1:250) primary antibody and Alexa Fluor® 488 (green) and visualized by fluorescence microscopy. The ratio of nuclear to cytosolic GSTP1 was calculated using D'Agostino's K^2 test and statistical analysis with t-test unpaired, two-tailed Mann-Whitney, * = $p \leq 0.001$). The mean is represented as a horizontal lines ± S.D. Scale bar 20 μm.

and Cys102 (Chapter2) were shown to be *in vitro* palmitoylation sites, it may be worth to investigate the role of palmitoylation for nuclear translocation.

A2.3.5 Triple staining of MCF7-GSTP1 treated with the palmitoylation inhibitor 2-bromopalmitate.

2-BP is used as an inhibitor of palmitoylation (Davda et al., 2013). To investigate the effect of 2-BP on GSTP1 cellular localization, fixed MCF7-GSTP1 cells were immunostained and analysed by immunofluorescence. GSTP1 presents different cellular localization in presence of 2-BP (Figure A2.5, iv, v & iv) when compared with the untreated (Figure A2.5, i, ii & iii). However, the precise localization cannot be determined in absence of a cellular marker. This data suggests that GSTP1 or a protein that regulates GSTP1 localization is palmitoylated and palmitoylation is responsible for the changes in subcellular localization. To obtain more information, this experiment should be repeated in the presence of cellular markers as multiple independent experiments and subjected to statistical analysis. Moreover, measuring the activity of GSTP1 subcellular fractions of MCF7-GSTP1 treated or untreated with 2-BP would support data acquired with immunostaining.

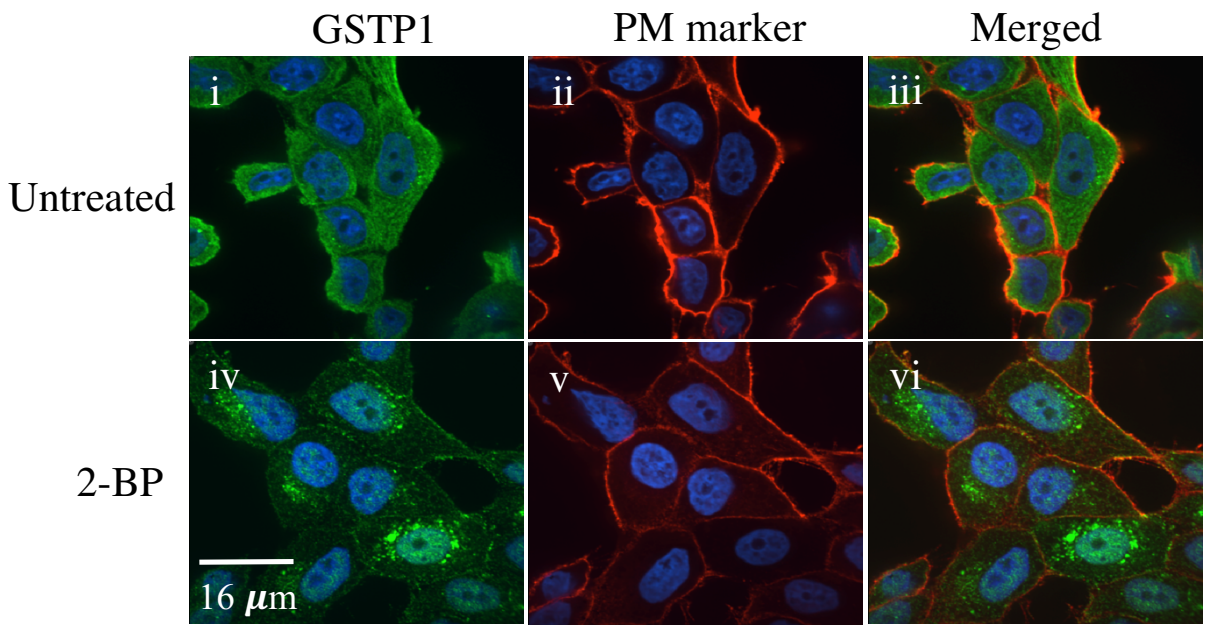


Figure A2.5 *Triple staining of MCF7-GSTP1 treated with the palmitoylation inhibitor 2-bromopalmitate.*

MCF7-GSTP1 was incubated with or without 2-bromopalmitate (2-BP) for 1 h then stained for plasma membrane (PM) with CT-B AlexaFluor®555 (1:10000)(red) and fixed with PFA (4%). Cells were immunostained for GSTP1 with rabbit anti-GSTP1 (GS70) (1:500) and detected with AlexaFluor®488 (green). Nuclei were stained with DAPI (blue). Cover slips were visualized using a fluorescence microscope at 60X magnification. Scale bar 16 μm. (i-iii) GSTP1 untreated (B) GSTP1 treated with 2-bromopalmitate (iv-vi).

Appendix 3 Investigation of GSTP1 SUMOylation

A3.1 Overview

GSTP1 is classically described as a metabolic enzyme important for protecting cells from toxic electrophiles (Hayes et al., 2005). More recently, GSTP1 was found to regulate signalling pathways that control cell proliferation and apoptosis. GSTP1 is generally described as cytosolic protein, but is also found in the nucleus, mitochondria, ER and plasma membrane (Goto et al., 2009; Kawakatsu et al., 2011; Qazi et al., 2011; Ye et al., 2016). The mechanisms regulating GSTP1 subcellular localization are not yet completely understood.

Post-translational modification can regulate protein localization, function, and interactions with other proteins. It is expected that different post-translational modifications of GSTP1 control its multiple localizations and functions. SUMOylation is the dynamic modification of proteins by small ubiquitin-related modifier (SUMO). In mammals, there are four SUMO proteins, SUMO1, SUMO2, SUMO3, and SUMO4 (Guo et al., 2004). Only SUMO2 and SUMO3 can elongate and form a chain (Tatham et al., 2001). SUMOylation can occur through an amide linkage within a canonical consensus sequence **ψ-K-X-E/D** [(where ψ stands for a hydrophobic and aliphatic amino acid, X refers to any amino acid and a negatively charged amino acid (glutamic acid or aspartic acid)] on the modified protein. In addition, SUMOylation can occur non-covalently through interacting motifs SUMO interacting motifs (SIM) on target proteins. The SIM is characterized by a hydrophobic core **([V/I]-x-[V/I]- [V/I])** flanked by a cluster of negatively charged amino acids that interact with the surface of a SUMO protein (Yavuz & Sezerman, 2014). A SIM selective for SUMO2 and SUMO3 was also identified **[I/V/L]- [D/E]-[I/V/L]-[D/E]-[I/V/L]** and others SIMs are expected to be identified (Geiss-Friedlander & Melchior, 2007; Y. Wang & Dasso, 2009). In addition, a non-consensus binding motif for SUMO2 was reported (Ouyang et al., 2009).

There are multiple roles assigned for SUMOylation, including regulation of gene transcription, DNA replication and repair, and nuclear translocation (Seeler & Dejean, 2003). Providing that SUMOylation can regulate the translocation of cytosolic proteins to the nucleus and GSTP1 is localized in the nucleus, it was of interest to investigate the possibility of GSTP1 being modified through SUMOylation.

A3.2 Methods

A3.2.1 *Materials*

Rabbit pAb anti-GSTP1 (GS72) was from Oxford Biomedical Research, Rochester Hill, MI), rabbit pAb anti-Na^+/K^+-ATPase (H-300) was from Santa Cruz Biotechnology (Dallas, TX). The AffiniPure donkey anti-rabbit IgG conjugated to Cy™3 was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The Alexa Fluor® 488 conjugated goat anti-rat IgG was from Life Technologies (Grand Island, NY). The horseradish peroxidase (HRP) conjugated goat anti-rat, anti-mouse, and anti-rabbit IgG were from Thermo Scientific (Rockford, IL). The mouse mAb anti-GSTP1 (H-11), rabbit pAb anti-SUMO1 (FL-101) and mouse mAb anti-SUMO-1 (D-11) were purchased from Sigma-Aldrich (Oakville, ON).

EDTA-free Complete™ protease inhibitor cocktail tablets and X-tremeGENE™9 transfection reagent were purchased from Roche Applied Science (Laval, QC). Bicinchoninic acid (BCA) assay, neutravidin-HRP, and Geneticin®(G418), NaCl, EDTA and EGTA, were from Thermo Scientific (Rockford, IL). Tris-HCl, BSA, *N*-ethylmaleimide (NEM), 4′,6-Diamidino-2-phenylindole (DAPI), sodium phosphate and sodium docecyl sulfate (SDS) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). DAKO mounting medium was

from Agilent Technologies (Santa Clara, CA) and Pierce™ protein G-agarose beads were from Thermo Scientific (Rockford, IL).

A3.2.2 *Cell lines*

The human breast adenocarcinoma cell line (MCF7) (Rockville, MD) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Oakville, ON, Canada) and supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS). Routine testing for *Mycoplasma sp.* contamination of cell lines was performed using the ATCC Universal Mycoplasma Testing Kit (Manassas, VA). The generation of the MCF7-GSTP1 stable cell line was previously reported (Qazi et al., 2011).

A3.2.3 *Immunoprecipitation of GSTP1 and SUMO-1*

MCF7-GSTP1 were harvested, washed with cold PBS, and lysed with cold SUMO buffer (Tris-HCl pH 7.5 20 mM, NaCl 150 mM, EDTA 100 mM, 1 mM EGTA, Triton-X-100 0.1%, sodium phosphate 25 mM, SDS 0.1%) containing freshly prepared NEM (25 mM) and EDTA-free Complete™ protease inhibitor cocktail for less than 5 min at 4°C. Cell lysates were centrifuged at 10000 *g* for 10 min at 4°C and the post-nuclear supernatant collected. To immunoprecipitate GSTP1 and SUMO, 800 µl of supernatant was incubated with 5 µl of either anti-GSTP1 (H-11) or mouse mAb anti-SUMO (D-11), respectively, overnight at 4°C. Day after, 40 ml of Protein G-agarose beads was added and incubated for 1 h at 4°C to bind with the primary anti-GSTP1 and anti-SUMO antibody. Beads were then spun down at 16000 *g* for 10 min and washed with 1 ml of cold PBS with 0.1% Triton-X-100, 3-5 times. GSTP1 was eluted from the beads by incubating >10 min at room temperature with Laemmli buffer and resolved on 11% SDS-PAGE

and electrotransferred to a PVDF membrane. The blots were probed for GSTP1 and SUMO using rabbit pAb anti-GSTP1 (GS72) (1:5000) and rabbit pAb anti-SUMO1 (FL-101) at (1:2000), respectively.

A3.2.4 *Immunofluorescence imaging*

MCF7-GSTP1 cells were fixed with 4% PFA blocked with BSA 3% + 0.1% Triton-X-100 and immunostained with rabbit pAb anti-GSTP1 (GS72) (1:250) and mouse mAb anti-SUMO (D-11) (1:250). Cells were viewed with a 60X oil immersion objective with a fully motorized inverted fluorescence microscope (Leica DMI6000 B, Wetzlar, Germany) coupled to a X-Cite®exacte fluorescence light source, equipped with Quorum Mac 6000 system and the Angstrom illumination system associated with Optigrid (Guelph, CA). The MetaMorph software (Molecular devices, Sunnyvale, CA) was used for multi-dimensional acquisition that allows the measurement of multiple Z sections. Images were captured with a Flash 4.0 camera (Hamamatsu) and analysed by Volocity® (Improvision, Perkin Elmer, Waltham, MA).

A3.3 Results, discussion and future work

A3.3.1 *Identification of GSTP1 SUMOylation.*

To start our investigation of GSTP1 SUMOylation, prediction of SUMOylated sites was done with GPS-SUMO prediction software (http://sumosp.biocuckoo.org) (Zhao et al., 2014). Three putative SUMOylation sites were identified, one based on the consensus sequence, one SIM and one not based on a consensus sequence (Table A3.1). To determine if GSTP1 is associated with SUMO, its interaction with SUMO-1 was investigated as a starting point, by co-immunoprecipitation. GSTP1 was immunoprecipitated with mouse mAb anti-GSTP1 (H-11)

and immunoblots were probed with rabbit pAb anti-SUMO1 (FL-101) (Figure A3.1). A signal for SUMOylation by SUMO-1 was identified at ~36 and 100 kDa. The molecular weight of SUMO1 is 12 kDa, but in western blots, it is usually associated with other SUMO molecules, therefore commonly detected at higher molecular weight. In addition, SUMO-1 was immunoprecipitated by mAb anti-SUMO-1 (D-11) and GSTP1 was identified at ~25 kDa with rabbit pAb anti-GSTP1 (GS72) (Figure A3.1). Overall, these co-immunoprecipitations results suggest that GSTP1 and SUMO-1 are interacting However, co-immunoprecipitation experiments should be repeated with a MCF7-vector negative control.

Future directions for co-immunoprecipitation experiments:

GSTP1 has 12 Lys residues that are potential SUMOylation sites. I have generated multiple Lys to Arg construct for screening, including triple mutants (V5_GSTP1-K30/45/55R; V5_GSTP1-K82/103/116R; V5_GSTP1-K121/128/141R and V5_GSTP1-K189/191/209R) (Table A3.2). To identify the SUMOylated Lys residues, co-immunoprecipitation of these triple mutants expressed in MCF7 cells will be performed as described in methods (section A3.2.3) and compared to GSTP1-WT. Priority will be to screen V5_GSTP1-K30/55/55R first because K30 and K45 are identified as important consensus and non-consensus SUMOylation sites (Table A3.1)

Table A3.1 Predicted SUMOylated human GSTP1 peptides

Position	Peptide	Type
30	ADQGQS**WKEE**VVTVE	Sumoylation Consensus ψ-K-X-E/D
45	TWQEGSLKASCLYGQ	Sumoylation Non-consensus
105 – 109	EDLRCKY**ISLIY**TNYEAGK	SUMO Interaction (non-covalent)

Table A3.2 Forward primers used for generating the GSTP1 mutants by site-directed mutagenesis

Mutants	**Primer sequence (5' to 3')**
V5-GSTP1-K30R	GGCCAGAGCTGGA**G**GGAGGAGGTGG
V5-GSTP1-K45R	GAGGGCTCACTCA**G**AGCCTCCTGCC
V5-GSTP1-K55R	GGCAGCTCCCCA**G**GTTCCAGGACGGAG
V5-GSTP1- K82R	GGGCTCTATGGGA**G**GGACCAGCAGGAGGC
V5-GSTP1-K103R	GACCTCCGCTGCA**G**ATACATCTCCCTC
V5-GSTP1- K116R	GAGGCGGGCA**G**GGATGACTATGTGAAGGCACTGCCC
V5-GSTP1-K121R	GATGACTATGTGA**G**GGCACTGCCCGGG
V5-GSTP1-K128R	CCCGGGCAACTGA**G**GCCTTTTGAGACCCTG
V5-GSTP1- K141R,	CCAGGGAGGCA**G**GACCTTCATTGTGG
V5-GSTP1-K189R/K191R	GCCCGGCCCA**G**GCTCA**G**GGCCTTCCTGGCC
V5-GSTP1- K209R	GGCAACGGGA**G**ACAGTGACTCGAGTC

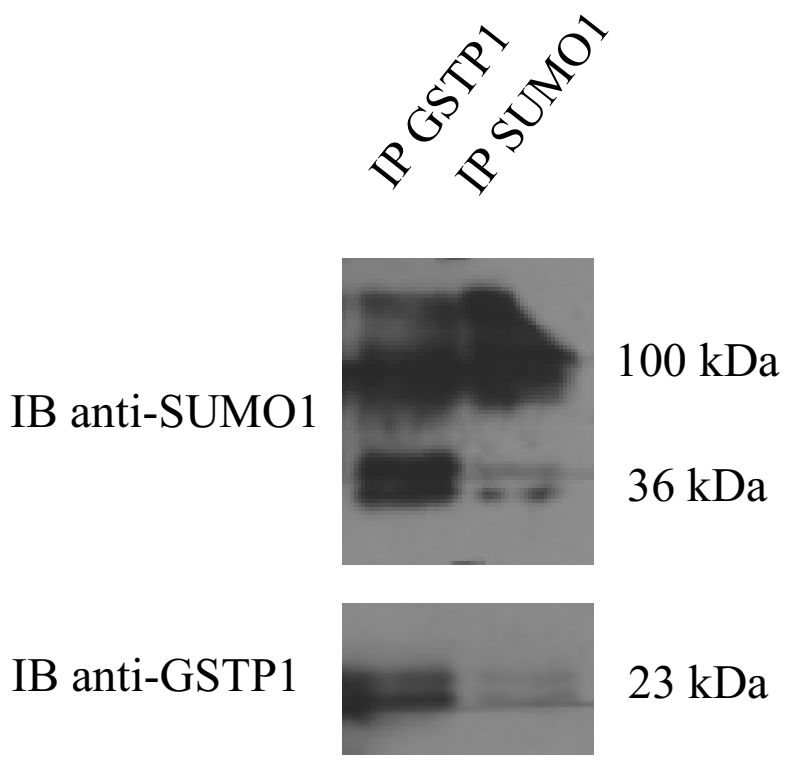


Figure A3.1 *Co-immunoprecipitation of GSTP1 and SUMO-1.*

MCF7-GSTP1 cells were lysed with SUMO buffer [Tris-HCl pH 7.5 20 mM, NaCl 150 mM, EDTA 100 mM, 1 mM EGTA, Triton-X-100 0.1%, sodium phosphate 25 mM, SDS 0.1%] with freshly added NEM (25 mM). GSTP1 was immunoprecipitated with mouse mAb anti-GSTP1(H-11) or mouse mAb anti-SUMO-1 (D-11) overnight and incubated with protein G-agarose beads for one h. Beads where washed and eluted with Laemmli buffer. Proteins were resolved on a 11% SDS-PAGE, and then electrotransferred to a PVDF membrane. GSTP1 was identified with rabbit pAb anti-GSTP1 (GS72) (1:5000) and SUMO-1 was detected with rabbit pAb anti-SUMO-1 (FL-101) (1:2000). IB, immunoblot, IP, immunoprecipitation.

A3.3.2 *Immunofluorescence of GSTP1 and SUMO-1 localization.*

Immunofluorescence is a technique complementary to the co-immunoprecipitation in the investigation of protein co-localization. GSTP1 was immunostained with rabbit pAb anti-GSTP1 (GS72) and AlexaFluor® 488 (Green) and mouse mAb anti-SUMO-1 (D-11) with anti-mouse Cy3™ (Orange) were localized throughout the cell. The signal for GSTP1 was intense around the nucleus whereas SUMO was mostly within the nucleus. There is no visual co-localization of GSTP1 and SUMO-1 Figure A3.2

Future directions for immunofluorescence studies

To further investigate the results obtained from immunofluorescence, it is required to repeat this experiment and collect a number of images from cells that allow statistical analysis of co-localization.

Overall, this preliminary data, especially from co-immunoprecipitation studies, suggest that GSTP1 is a SUMOylated protein by interacting with SUMO-1. To determine if GSTP1 interacts with SUMO2, SUMO3 and SUMO4, similar immunoprecipitation experiments should be performed for these proteins. Future work will focus on the identification of the amino acid residues of GSTP1 that interact with SUMO. Individual mutants will them be generated and compared with GSTP1-WT to determine the influence of SUMOylation on GSTP1 function.

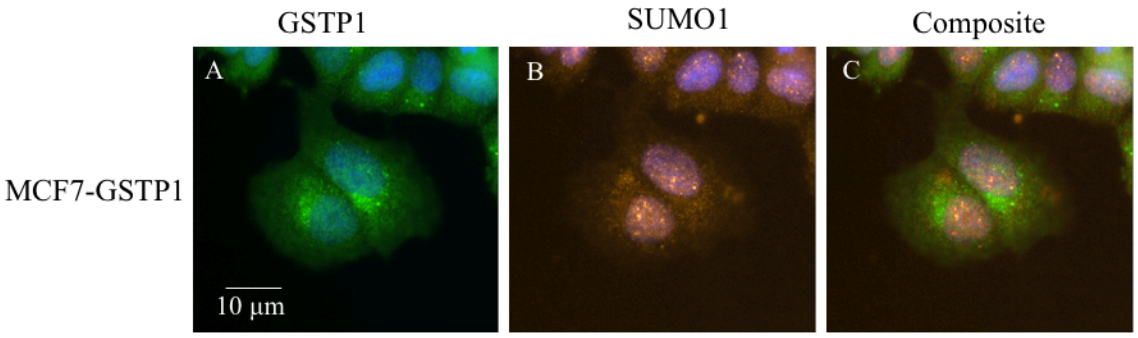


Figure A3.2 *Immunostaining of GSTP1 and SUMO-1 in MCF7-GSTP1 cells*

MCF7-GSTP1 cells were grown on coverslips, fixed with PFA (4%) and then blocked with 3% BSA + 0.1% Triton-X-100 for one h. GSTP1 was detected with rabbit pAb anti-GSTP1 (GS72) and SUMO-1 was detected with mouse mAb anti-SUMO (D-11) (1:250). GSTP1 and SUMO-1 where visualized with AlexaFluor®488 and anti-mouse Cy3™, respectively. Cells were visualized under a 60X oil immersion objective fluorescent microscope (Leica DMI6000 B, Wetzlar, Germany) associated with Optigrid (Guelph, CA).

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