Development Of Chemical Tools To Investigate Protein S-Glutathionylation In Response To Metabolic Alteration

Kusal Theekshana Gayan Samarasinghe
Wayne State University,

Follow this and additional works at: https://digitalcommons.wayne.edu/oa_dissertations
Part of the Biochemistry Commons, and the Chemistry Commons

Recommended Citation
Samarasinghe, Kusal Theekshana Gayan, "Development Of Chemical Tools To Investigate Protein S-Glutathionylation In Response To Metabolic Alteration" (2017). Wayne State University Dissertations. 1869.
https://digitalcommons.wayne.edu/oa_dissertations/1869

This Open Access Dissertation is brought to you for free and open access by DigitalCommons@WayneState. It has been accepted for inclusion in Wayne State University Dissertations by an authorized administrator of DigitalCommons@WayneState.
DEVELOPMENT OF CHEMICAL TOOLS TO INVESTIGATE PROTEIN S-GLUTATHIONYLATION IN RESPONSE TO METABOLIC ALTERATIONS

by

KUSAL T. G SAMARASINGHE

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: CHEMISTRY (Biochemistry)

Approved By:

________________________________________
Advisor Date

________________________________________
DEDICATION

I dedicate this thesis to my father (Leelananda Samarasinghe), my mother (Indrani Shanthilatha Samarasinghe), my siblings, my loving wife (Dhanusha Nalawansha), my loving son (Thiveyn Samarasinghe) and my dissertation advisor Prof. Young-Hoon Ahn.
ACKNOWLEDGEMENTS

Five years before, I had the privilege to join Department of Chemistry at Wayne State University as a graduate student. I am extremely happy that I am writing this thesis because, I have achieved the best in my education history. Of course, there are so many people helped me all the way through to this day. I am thankful to everyone and I will try my best to duly acknowledge everyone.

First and foremost, I want to thank my research advisor, Professor Young-Hoon Ahn for wonderful support given me throughout my stay in Ahn lab. Before everything, I thank him for allowing me to work on this exciting brand new project. Because of that, I could have accomplished many things including several awards and publications. Your guidance, valuable suggestions and motivation brought me a rewarding and gratifying graduate school experience during five years. He always wanted not only me to succeed in research but also to develop skills to be a professional, punctual, independent thinker and expand networking. He supported me to take part in international conferences to build professional networking among well-known scientists in the field. He was always behind me giving millions of suggestions and ideas. I apologize, I could not do everything, yet I wish you all the success on your exciting research and academic career.

I would also like to thank my thesis committee members Prof. Ashok Bhagwat, Prof. Mary Kay Pflum and Prof. Menq-Jer Lee for their valuable suggestions and support. I took graduate courses from both Prof. Ashok Bhagwat and Prof. Mary Kay Pflum. You are both excellent teachers and had brilliant and simple ways of delivering the subject matter that can grab any student in the class. Basic knowledge that I gained from those graduate classes really helped me to succeed my research efforts. I am thankful to Prof.
Menq-Jer Lee for providing me with certain resources and sharing his scientific and practical knowledge with me. Thank you for teaching me some cell culture techniques that I used while working with viruses. You all had a significant impact on me throughout my graduate studies at Wayne State.

All these accomplishments might not have gone smoothly without friendly and collaborative environment. I am grateful to have wonderful colleagues and their support all the time. I thank all the past and current members, including Chris Smith, Holly SantaLucia, Garret VanHecke, Dr. Sue Dagher, Dilini Kekulandara, Fidelis Ndombera, Dhanushaka M unkanatta Godage, Harshani Sewvandi, Adwale Adeleye, Maheeshi Abeywardana, shima Nagi, Adam Schlichther, Mohammed Alzamami, Nimeen Chouaib, Dhruvil Ashishkumar Patel, and Ahmed Ayantayo. I wish you all the success in your future endeavors.

I specially thank to our collaborators Prof. Eranthi Weerapana (Boston College), Prof. Albena Dinkova-Kostova (University of Dundee), and Dr. Yani Zhou (Boston College) for their suggestions, support and contributions to our research. Also, I would like to thank Department chair Prof. Matthew Allen and Melissa Barton for all the support and help in many things. I am thankful to Department of Chemistry and Wayne State University for providing me all the resources and funding to make sure a smooth flow in my research and graduate studies.

Finally, I want to thank my beloved parents for all the motivation and the guidance provided me throughout my life. My father and mother, both were always behind me and dreamt me succeed. My father always wanted me to become a cricketer, yet he dedicated many things for us to give good education. My mother always offers her kindness and
unconditional love which is the driving force behind everything. Father and mother, you are wonderful parents and I am truly gratified by your immeasurable love and care. Of course, I could not have asked for a better childhood than my wonderful sisters and my little brother. You guys are amazing and still a part of my life. I am thankful to you for all the encouragements and been there for me. I want to thank my wonderful wife and our son, for all the happiness and endless love given me. You make my life complete and joyful. My lovely wife and my best friend, you were always there for me and you have helped me in many ways by sharing your knowledge and skills to succeed in many things including this accomplishment. I am thankful to you for all the support, suggestions and encouragement. I am certain that this would not have been possible without you. Without my parents, siblings, my wonderful wife and my son I may never have accomplished this in my life. Thank you all, for the motivation, support, love and for been there for me.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................... ii

ACKNOWLEDGEMENTS ........................................................................................... iii

LIST OF FIGURES ........................................................................................................ x

LIST OF TABLES .......................................................................................................... xiv

LIST OF SCHEMES ..................................................................................................... xv

LIST OF ABBREVIATIONS .......................................................................................... xvi

CHAPTER 1 INTRODUCTION ....................................................................................... 1

1.1 Post translational modifications ....................................................................... 1

1.2 Redox homeostasis and oxidative and nitrosative stress ................................. 2

1.3 Sources of reactive oxygen and nitrogen species ............................................. 3

1.3.1 Enzyme-mediated ROS/RNS production ....................................................... 5

1.3.2 Cellular organelles ....................................................................................... 9

1.4 ROS detoxifying system ................................................................................... 14

1.4.1 Superoxide dismutase (SOD) ...................................................................... 14

1.4.2 Catalase ...................................................................................................... 15

1.4.3 Glutathione reductase ................................................................................ 15

1.4.4 Glutathione peroxidase ............................................................................. 16

1.4.5 Peroxiredoxin ............................................................................................ 16

1.4.6 Glutathione (GSH) .................................................................................... 17

1.4.7 Pyruvate .................................................................................................... 19

1.4.8 Nicotinamide adenine dinucleotide phosphate (NADPH) ............................. 20

1.5 ROS and RNS in protein oxidation ................................................................... 21
CHAPTER 3 CHARACTERIZATION OF GLUTATHIONYLATED PROTEINS IN RESPONSE TO METABOLIC ALTERATION ................................................................. 84

3.1 Introduction ........................................................................................................... 84

3.2 Approach ............................................................................................................... 86

3.3 Results .................................................................................................................. 87

  3.3.1 Characterization of HEK293-GS M4 stable cells for the generation of azido-GSH ......................................................................................... 87

  3.3.2 Mitochondrial ROS and protein glutathionylation ........................................ 88

  3.3.3 Glucose availability controls protein glutathionylation under mitochondrial dysfunction ............................................................................................... 90

  3.3.4 Glutathionylation under glycolysis inhibition and mitochondrial dysfunction .. 93

  3.3.5 Reversibility of glutathionylation controlled by glucose availability ............. 94

  3.3.6 Glutathionylation by different ETC blockers .................................................. 95

  3.3.7 ROS and glutathione levels in response to glucose deprivation and mitochondrial dysfunction .............................................................. 96

  3.3.8 Protein glutathionylation in response to glucose deprivation ....................... 98

  3.3.9 Glutathionylation in response to oxygen availability ...................................... 100

  3.3.10 Identification of glutathionylated proteins using LC-MS/MS ....................... 104

  3.3.11 Validation of glutathionylated proteins by biotin pull-down and Western blotting .............................................................. 109

3.4 Discussion ............................................................................................................ 110

3.5 Experimental procedure ...................................................................................... 114

CHAPTER 4 S-GLUTATHIONYLATION OF SERINE/THREONINE PROTEIN PHOSPHATASE 2Cα (PP2Cα) PROMOTES CANCER CELL MIGRATION UNDER METABOLIC STRESS ................................................................................ 121

4.1 Introduction .......................................................................................................... 121

4.2 Results .................................................................................................................. 123
4.2.1 S-Glutathionylation of serine/threonine protein phosphatase 2Cα (PP2Cα) 123
4.2.2 Functional effects of glutathionylation on PP2Cα................................. 128
4.2.3 Glutathionylation in response to glucose depletion in different cell lines ..... 131
4.2.4 Glutamate availability and the metabolic stress in MDA-MB-231 cells ........ 135
4.2.5 PP2Cα glutathionylation in cancer cell migration (wound healing assay) .... 137
4.2.6 PP2Cα glutathionylation in response to metabolic stress promotes MDA-MB-
231 cell invasion: transwell invasion assay.................................................... 140
4.2.7 PP2Cα glutathionylation in A431 cells ...................................................... 143
4.2.8 PP2Cα glutathionylation activates c-Jun N-terminal kinase (JNK) in MDA-MB-
231 cells ........................................................................................................... 146

4.3 Discussion................................................................................................... 148
4.4 Experimental procedure ........................................................................... 151
APPENDIX A - CHAPTER 2 SUPPORTING FIGURES ......................................... 161
APPENDIX B – CHAPTER 3 SUPPORTING FIGURES ........................................ 167
APPENDIX C – CHAPTER 4 SUPPORTING FIGURES ........................................ 178
APPENDIX D - COPYRIGHT PERMISSIONS ..................................................... 181
REFERENCES.................................................................................................... 189
ABSTRACT ........................................................................................................ 235
AUTOBIOGRAPHICAL STATEMENT.................................................................... 237
# LIST OF FIGURES

Figure 1.1 – Schematic representation of ROS and RNS production, and antioxidant system, i.e. redox homeostasis vs. oxidative stress................................. 2

Figure 1.2 – The major intracellular sources of ROS and RNS. ................................. 5

Figure 1.3 – NOX enzyme-mediated ROS ..................................................................... 7

Figure 1.4 – ROS production at mitochondrial complex I ................................................. 11

Figure 1.5 – Mitochondrial complex III electron movement and Q-cycle ...................... 12

Figure 1.6 – Chemical structures of the reduced gluathione (GSH) and the oxidized glutathione (GSSG) .................................................................................. 18

Figure 1.7 – An illustration of protein cysteine oxidation ............................................ 22

Figure 1.8 – Chemical mechanisms of protein S-glutathionylation and chemical reactions for reactive intermediate formation ........................................ 25

Figure 1.9 – Glutaredoxin (Grx) for de-glutathionylation ............................................. 26

Figure 1.10 – A scheme for identification of S-glutathionylation by BIOGEE ............ 32

Figure 1.11 – The example of a biotin switch method .................................................... 33

Figure 1.12 – A strategy to identify S-glutathionylated proteins using biotin-spermine and glutathionylspermidine synthetase (GspS) ............................................ 35

Figure 2.1 – A scheme for GSH and azido-GSH biosynthesis ..................................... 40

Figure 2.2 – A schematic diagram for detection of Glutathionylation by clickable GSH ........................................................................................................... 41

Figure 2.3 – Crystal structure of glutathione synthetase ............................................. 43

Figure 2.4 – Purification and kinetic analysis of GS mutants ....................................... 44

Figure 2.5 – LC-MS analysis of *in vitro* biosynthesis of clickable GSH .................... 47

Figure 2.6 – GS M4 overexpression in cells ................................................................. 48

Figure 2.7 – LC-MS analysis of biosynthesis of clickable GSH ............................... 50
Figure 2.8 – LC-MS analysis of biosynthesis of other GSH derivatives .................. 51

Figure 2.9 – Biosynthesis of clickable GSH using L-HPG and L-PG ....................... 52

Figure 2.10 – Analysis of glutathionylated proteins using metabolically synthesized azido-GSH or biotin alkyne ................................................................. 54

Figure 2.11 – Studying incorporation of amino acid derivatives to protein biosynthesis .................................................................................................................. 56

Figure 2.12 – Reversibility of glutathionylated proteins ........................................ 58

Figure 2.13 – Visualization of glutathionylated proteins by fluorescence imaging ...... 60

Figure 2.14 – Identification of glutathionylated proteins ........................................ 61

Figure 3.1 – The work flow for identifying glutathionylated proteins in response to mitochondrial ROS .................................................................................................. 86

Figure 3.2 – Evaluation of azido-glutathione (N3GSH) biosynthesis, cellular thiol concentration and redox enzyme levels in HEK293-GS M4 cells ................ 88

Figure 3.3 – Glutathionylation in response to mitochondrial ROS generation .......... 89

Figure 3.4 – Glutathionylation in response to depletion of glucose and glutamate .... 90

Figure 3.5 – Protein glutathionylation is highly dependent on glucose metabolism in the presence of the electron transport chain blocker ........................................ 91

Figure 3.6 – Glutathionylation under physiological glucose concentration .......... 92

Figure 3.7 – Induction of glutathionylation by treatment of 2-deoxyglucose (2-DG) with electron transport chain blockers ......................................................... 93

Figure 3.8 – Glutathionylation is reversed by re-addition of glucose .................... 95

Figure 3.9 – Glutathionylation in response to ETC blockers and glucose deprivation .................................................................................................................. 97

Figure 3.10 – ROS and glutathione levels in HEK293-GS M4 stable cells .............. 98

Figure 3.11 – Clickable GSH approach for detection of protein glutathionylation in response to different glucose concentrations ............................................... 99

Figure 3.12 – Glutathionylation under a limited oxygen supply ............................ 101
Figure 3.13 – Oxygen availability regulates protein glutathionylation.......................... 102
Figure 3.14 – Levels of HIF1α and redox enzymes in response to glucose-oxygen depletion.................................................................................................................. 103
Figure 3.15 – Detection of the effect of re-oxygenation on protein glutathionylation ......................................................................................................................... 104
Figure 3.16 – Biotin pull-down and elution for analysis of glutathionylated proteins ......................................................................................................................... 105
Figure 3.17 – Proteomic analysis of glutathionylated proteins ........................................ 106
Figure 3.18 – Gene ontology analysis of identified glutathionylated proteins................. 108
Figure 3.19 – Validation of glutathionylated proteins by pull-down and Western blotting .................................................................................................................. 110
Figure 3.20 – Schematic representation of glucose and mitochondrial metabolism in redox homeostasis and glutathionylation......................................................... 112
Figure 4.1 – Crystal structure of PP2Cα ........................................................................ 124
Figure 4.2 – In cellulo and in vitro glutathionylation studies for identification of modified cysteine residue in PP2Cα .......................................................................... 125
Figure 4.3 – Schematic representation of visualization of PP2Cα glutathionylation by proximity ligation assay ................................................................................. 127
Figure 4.4 – Visualization of PP2C glutathionylation by proximity ligation assay ....... 128
Figure 4.5 – Functional effects of PP2Cα glutathionylation ............................................. 130
Figure 4.6 – Co-immunoprecipitation assay for mGluR3 and PP2Cα ......................... 131
Figure 4.7 – Glutathionylation in different cell lines ..................................................... 132
Figure 4.8 – Different redox responses in MCF7 and MDA-MB-231 cells ................. 134
Figure 4.9 – Rescue of metabolic stress by pyruvate, glutamine and glutamate .......... 136
Figure 4.10 – Wound healing assay with MDA-MB-231 cells ..................................... 138
Figure 4.11 – Glutathionylation in MDA-MB-231 cells in response to a low glucose concentration .................................................................................................... 139
Figure 4.12 – Diagram of transwell set up in a 24-well plate for Investigating cancer cell invasion ................................................................. 141

Figure 4.13 – Transwell invasion assay with MDA-MB-231 cells ......................... 142

Figure 4.14 – Comparison of glutathionylation among MCF7, MDA-MB-231 and A431 cells ........................................................................................................................................... 144

Figure 4.15 – Effect of PP2Cα glutathionylation on A431 cell migration ............... 145

Figure 4.16 – Evaluation of phosphorylation levels of several PP2Cα substrates that are involved in cell migration ........................................................................................................ 147

Figure 4.17 – Cell migration induced by PP2Cα glutathionylation under metabolic Stress ......................................................................................................................... 150
LIST OF TABLES

Table 1.1 – Different forms of ROS and RNS................................................................. 4
Table 2.1 – Kinetic data of GS WT and GS mutants with different amino acids........ .45
Table 2.2 – PCR program for GS amplification .......................................................... 68
Table 2.3 – PCR program for quick change mutagenesis........................................... 70
Table 2.4 – Quick change primers for GS mutants ..................................................... 71
LIST OF SCHEMES

Scheme 1.1 – Fenton’s reaction................................................................. 4
Scheme 1.2 – NOX enzyme-catalyzed univalent reduction of molecular oxygen........ 6
Scheme 1.3 – ROS production by xanthine oxidase.............................................. 8
Scheme 1.4 – ROS production by nitric oxide synthase........................................... 8
LIST OF ABBREVIATIONS

ROS - Reactive oxygen species
RNS - reactive nitrogen species
NADPH - Nicotinamide adenine dinucleotide phosphate hydrate
NADH - Nicotinamide adenine dinucleotide hydrate
TNF - tumor necrosis factor
PDGF - platelet-derived growth factor
EGF - epidermal growth factor
NOX - NADPH Oxidases
DUOX1 - dual oxidase 1
DUOX2 - dual oxidase 2
XO - Xanthine oxidase
XDH - Xanthine dehydrogenase
FAD – Flavin adenine dinucleotide
FMN - Flavin mononucleotide
DNA - Deoxyribonucleic acid
NOS - Nitric oxide synthase
ATP - Adenosine triphosphate
TCA - Citric acid cycle
OXPHOS - oxidative phosphorylation
ETC - Electron transport chain
SOD - Superoxide dismutase
MnSOD - Mitochondrial manganese containing superoxide dismutase
MAO-A - Monoamine oxidases
RET - Reverse electron transfer
AMA- Antimycin A
ER - Endoplasmic reticulum
UPR - Unfolded protein response pathway
PDI - Protein disulfide isomerase
GR - Glutathione reductase
GSH - Glutathione
GSSG- oxidized glutathione
GPx - Glutathione peroxidases
Prx - Peroxiredoxins
GCL - γ-glutamyl-cysteine ligase
GS - Glutathione synthetase
GST - Glutathione S-transferases
Grx1 - Glutaredoxin 1
Grx2 - Glutaredoxin 2
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
PTP1B - Protein-tyrosine phosphatase 1B
Srx - Sulfdiredoxin
NF-Kb – Nuclear factor kappa-light-chain-enhancer of activated B cells
MMP - Matrix metalloprotease
ERK - Extracellular-signal regulated kinase
Nm23 - Nucleoside diphosphate kinase
BIOGEE - Biotinylated glutathione ethyl ester
Strep-HRP - Streptavidin-horseradish peroxidase
IAM - Iodoacetamide
NEM - N-ethylmaleimide
DTT - Dithiothreitol
TCEP - Tris(2-carboxyethyl)phosphine
LC-MS – Liquid chromatography- mass spectrometry
GspS - Glutathionylspermidine synthetase
GspA - Glutathionylspermidine amidase
biotin-Gsm - Biotin-glutathionylspermine
GS M4 - Glutathione synthetase mutant F152A/S151G
L-AzAla - L-azido alanine
L-HPG - L-homopropargyl glycine
L-PG - L-propargyl glycine
PK - pyruvate kinase
LDH- lactate dehyrogenase
PEI-Max - Polyethylenimine
PBS - Phosphate-buffered saline
TBS - Tris-buffered saline
AF647 - Alexa Fluor 647
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HSP90 – Heat shock protein 90
EDTA – Ethylenediaminetetraacetic acid
LB - Luria broth
PCR - Polymerase chain reaction
BSA – Bovine serum albumin
dNTP - Deoxynucleotide
IPTG - Isopropyl-1-thio-β-D-galactopyranoside
HEK293 - Human embryonic kidney 293 cells
HEK293-GS M4 - GS M4 HEK293 cells stably expressing GS M4
DMEM - Dulbecco’s Modified Eagle’s Medium
FBS - Fetal bovine serum
SDS - Sodium dodecyl sulfate
TEMED - N, N, N’, N’-tetramethylthlenediamine
APS - ammonium persulfate
FL-IA - fluorescein-iodoacetamide
TBTA - Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
THPTA - Tris(3-hydroxypropyltriazolylmethyl)amine
AMP - Adenosine monophosphate
AMPK – AMP-activated kinase
N₃-GSH – azido-glutathione
2-DG - 2-deoxyglucose
HIF1α - hypoxia inducible factor 1 α
OGD/R – Oxygen and glucose deprivation followed by re-oxygenation
DCFH-DA - 2,7-dichlorodihydrofluorescein diacetate
mGluR3 - metabotropic glutamate receptor 3
PLA - Proximity ligation assay

pNPP - para-nitrophenyl phosphate

PP2Cα –Ser/Thr protein phosphatase 2C alpha

PP2A - protein phosphatase 2A

MCF7 - Michigan cancer foundation-7 (Breast cancer cell line)

MAPK - Mitogen-activated protein kinase

JNK - c-Jun N-terminal kinase

SMAD - Mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA
CHAPTER 1 INTRODUCTION

1.1 Post translational modifications

Human genome (~20,000-25,000 genes) encodes more than 1 million proteins (1). The higher complexity of the proteome has been found during last two decades due to the development of technology in identifying post-translational modifications (PTMs) (1,2). Proteins carry unique functions within the cellular context and their maturation, localization, function and interactions with other proteins or nucleic acids regulated by PTMs. A PTM typically occurs at the specific type of amino acid residues within the protein. Many PTMs are reversible and depend on the accessibility of the enzymes that catalyze the formation and removal of a specific PTM. Local environment around the modification also important (3). For example, the sensitivity of protein cysteine oxidation depends on the solvent exposure and the presence of positively charge amino acids in the local environment. In nature, most of PTMs are reversible and involved in regulating protein function. Due to the reversibility nature, PTMs are playing an important role in cell signaling pathways by turning on or off the function of signaling molecules in response to different physiological and pathological stimuli (4). For instance, kinases catalyze protein phosphorylation and involved in regulating cellular signaling. Phosphatases are a class of enzymes that can remove phosphate groups from serine, threonine or tyrosine, thereby reversing the effect of phosphorylation (5). Similarly, protein oxidative modifications, including sulfenylation, nitrosylation and glutathionylation are also important for regulation of redox signaling pathways associated with important cellular processes such as cell proliferation, apoptosis, differentiation and migration (6).
1.2 Redox homeostasis and oxidative and nitrosative stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered as by-products of cellular metabolism and catalytic reactions (7). In nature, cells evolved in a way that regulate the production and the removal of ROS and RNS. High levels of reactive species can damage cellular components and dysregulate numerous cellular processes by inactivating or hyperactivating signaling proteins. Under physiological conditions, ROS and RNS are mainly involved in regulation of redox signaling pathways by reversible cysteine oxidation (6). Therefore, it is crucial to have a balance between ROS production and detoxification for a maximum productivity of cellular functions.

![Diagram: Schematic representation of ROS and RNS production, and antioxidant system, i.e. redox homeostasis vs. oxidative stress.](image)

Redox homeostasis is the maintenance of the balance between the production and removal of reactive species (Figure 1.1) (8). Dysregulation of cellular metabolism and improper ROS disposal system can result in a high level of ROS leading to an oxidative or nitrosative stress (Figure 1.1).
Oxidative and nitrosative stress associated with many disease conditions, including diabetes, cardiovascular diseases, neurodegenerative diseases and cancer (9,10). The major molecular mechanism of ROS and RNS is oxidation of cysteine residues in proteins. Protein cysteine thiols are sensitive towards the change in cellular redox state (11). Under control generation of ROS, cysteine residues can undergo reversible oxidation. On the other hand, an increased level of ROS can irreversibly oxidize cysteine residues of a protein. Many oxidative modifications, if not all, affect protein function. The reactive cysteine residues can present in the active site or in distal sites. For example, protein phosphatase has a cysteine residue in the active site that is highly susceptible to oxidation (5). Oxidation of surface cysteine residues act as a redox sensor of the protein as well as a protective mechanism for spreading the effect of ROS into the other critical cysteines. Oxidation of cysteine residues away from the active site may not affect enzyme activity, but it alters protein-protein interactions and protein structure that can affect protein function (12). Among various cysteine oxidations, S-glutathionylation is reversible and referred to as formation of disulfide bond between protein cysteine and a glutathione (GSH). S-Glutathionylation regulates protein function not only under physiological concentrations of ROS/RNS, but also under oxidative or nitrosative stress (13).

1.3 Sources of reactive oxygen and nitrogen species

The existence of reactive species such as ROS and RNS are first identified nearly six decades ago in 1954 and predicted to be related with aging process (14-16). Mostly, free radicals, such as super oxide anion (O$_2^-$) and nitric oxide (NO$^+$) can produce from different intracellular processes. These free radicals are highly reactive and have a very
short half-life (nanoseconds) (17). Further reactions can generate other free radicals or non-radical ROS and RNS (Table 1.1).

**Table 1.1- Different forms of ROS and RNS.**

<table>
<thead>
<tr>
<th>Reactive Oxygen Species (ROS)</th>
<th>Reactive Nitrogen Species (RNS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radicals:</strong></td>
<td><strong>Non-Radicals:</strong></td>
</tr>
<tr>
<td>( \text{O}_2^- ) Superoxide</td>
<td>( \text{H}_2\text{O}_2 ) Hydrogen peroxide</td>
</tr>
<tr>
<td>( \text{OH} ) Hydroxyl</td>
<td>( \text{HOCl}^- ) Hypochlorous acid</td>
</tr>
<tr>
<td>( \text{ONOO}^- ) Peroxynitrite</td>
<td></td>
</tr>
<tr>
<td><strong>Radicals:</strong></td>
<td><strong>Non-Radicals:</strong></td>
</tr>
<tr>
<td>( \text{NO}^- ) Nitric Oxide</td>
<td>( \text{ONO}^- ) Peroxynitrite</td>
</tr>
<tr>
<td>( \text{ROONO} ) Alkylperoxynitrites</td>
<td></td>
</tr>
<tr>
<td>( \text{NO}^+ ) Nitroxy anion</td>
<td></td>
</tr>
</tbody>
</table>

Due to the presence of one or more unpaired electrons in a single orbit, free radicals are highly unstable and quickly react with other species or converted to less reactive and stable forms. For example, after generation of \( \text{O}_2^{-} \), these radicals are converted into less reactive and stable non-radical hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) by superoxide dismutase (18). Hydrogen peroxide is more cell permeable than \( \text{O}_2^{-} \) and diffuses to other cellular compartments to mediate redox signaling under physiological concentrations. NO can be reacted with \( \text{H}_2\text{O}_2 \) to generate more reactive peroxynitrite (\( \text{ONOO}^- \)). Similarly, \( \text{H}_2\text{O}_2 \) can further generate more reactive radicals, such as hydroxyl (\( \cdot \text{OH} \)), hydroperoxyl radicals (\( \cdot \text{OOH} \)) (Scheme 1.1).

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \cdot \text{OH}^- \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{2+} + \cdot \text{OOH} + \text{H}^+
\end{align*}
\]

**Scheme 1.1 – Fenton’s reaction.** Metal ion can react with \( \text{H}_2\text{O}_2 \) and have electron transfer ability. \( \text{H}_2\text{O}_2 \) can be either oxidized or reduced by metal ion and generate hydroxyl radicals as byproducts.

However, intracellular ROS and RNS generation can divide into two broad categories, including extracellular sources and intracellular sources. Extracellular sources include small molecule drugs, pollutants, tobacco and radiation. Radiolysis of water can also lead to formation of \( \cdot \text{OH} \), \( \cdot \text{OOH} \) and \( \text{O}_2^{-} \) (19). Intracellular sources are more diverse and readily involve in generation of ROS and RNS through their biochemical reactions.
Intracellular sources can further divide into two main classes. ROS and RNS generated as a byproduct of enzyme activity or as a result of cellular processes at different cellular organelles.

**Figure 1.2 – The major intracellular sources of ROS and RNS.** NADPH oxidase, nitric oxide synthase and mitochondrial ETC are responsible for producing intracellular ROS. This figure adopted from Kira et al. 2014, with permission from copyright clearance center (20).

### 1.3.1 Enzyme-mediated ROS/RNS production

#### 1.3.1.1 NADPH oxidases

NADPH oxidase is a multi-subunit membrane complex that closely interacts with growth factor receptors and mediate the superoxide production in response to different exogenous stimuli, including tumor necrosis factor (TNF), angiotensin II, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (21-25). There are seven members of NADPH family oxidases (also known as NOX enzymes) which include NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1(DUOX1) and DUOX2 (26,27). NOX
enzymes generate lower levels of ROS that are involved in regulating signal transduction, innate immune system and modulation of the extracellular matrix (28). NOX enzymes also play an important role in host defense against toxic microorganisms. For example, Neutrophils that have a high level of NOX enzymes can generate a large amount of ROS as a response to host pathogen interaction. During phagocytosis, neutrophils internalize microorganisms into a phagosome and deactivate them with NOX derived ROS (29). In addition to neutrophils, NOX enzymes are expressed in other immune or non-immune cells, such as endothelial cells, monocytes and smooth muscle cells (27). NOX enzymes composed with five subunits: three subunits, such as p47phox, p67phox, p40phox are localized to the cytosol while other two subunits gp91phox (catalytic subunit gp91phox or NOX2; homologous to NOX1 and NOX4) and p22phox are found in membrane.

\[ 2O_2 + \text{NAD(P)H} \rightarrow 2O_2^- + \text{NADP}^+ + H^+ \]

Scheme 1.2 – NOX enzyme catalyzed univalent reduction of molecular oxygen.

Upon activation, the cytosolic part, p47phox undergoes phosphorylation and forms complex with other two subunits, p67phox and p40phox. The three-subunit complex translocate to the membrane and produces superoxide by the single electron reduction of molecular oxygen. In this reaction, NADPH acts as the electron donor, and one electron is transferred through flavin and heme containing catalytic gp91phox subunit to molecular oxygen (30).
Figure 1.3 – NOX enzyme mediated ROS. Growth factor can activate NOX multicomplex membrane protein through activation of PI3K and small GTPase RAC1. One electron reduction of molecular oxygen by NOX enzyme will produce superoxide anion to extracellular space, which will be further dismutated to $\text{H}_2\text{O}_2$ and transferred to the cell through aquaporin. Since $\text{H}_2\text{O}_2$ has a higher half-life, $\text{H}_2\text{O}_2$ can mediate the effect to oxidizing effect to sensitive cysteines in a range of signaling proteins. This figure adopted from Kira et al. 2014, with permission from copy rights clearance center (20).

The inability to generate enough ROS due to mutations in NOX2 catalytic subunit associates with frequent or periodic bacterial and fungal infections, a condition called chronic granulomatous disease (31). Therefore, a controlled production of ROS by NOX enzymes is an essential physiological process that helps to keep cells healthy. ROS produced by NOX enzymes are also involved in redox signaling that can mediate cell growth, proliferation, survival and motility (32,33).
1.3.1.2 Xanthine Oxidases

Xanthine oxidase (XO) is involved in ROS generation under pathological conditions. Xanthine dehydrogenase (XDH) and XO are two forms of xanthine oxidoreductase. Fundamental chemical reaction of XO is the oxidation of hypoxanthine into uric acid. In this reaction, XO catalyzes the reduction of molecular oxygen to produce $H_2O_2$ and $O_2^-$(34).

$$XO + H_2O + O_2 \rightarrow \text{Uric acid} + H_2O_2$$

$$XO + O_2 \rightarrow XO^{-1} + O_2^-$$

Scheme 1.3 – ROS production by xanthine oxidase.

XO uses NADPH as an electron donor to produce NO· and ONOO-. XO catalyzes the conversion of cellular nitrate into nitrite and NO·. It is also known that XO is feedback inhibited by NO· (35).

1.3.1.3 Nitric oxide synthase (NOS)

NOS enzymes are a class of NO· producing enzymes and there are three isoforms of NOS. NOS constitutively expressed in neuronal cells and endothelial cells are known as nNOS and eNOS, respectively (36,37). These enzymes are regulated by calcium ion levels to produce NO·. Calcium ion binds to calmodulin which is a calcium binding protein, and this complex can bind to NOS (38-40). In the presence of other essential cofactors (FAD, FMN, NADPH, tetrahydrobiopterin and heme), NOS produces NO· by converting L-arginine to L-citrulline (41-43). In addition to NO· production, NOS also generate superoxide under uncoupling conditions.

$$\text{NOS} + \text{L-Arginine} + O_2^- + \text{NADPH} \rightarrow \text{NO}^+ + \text{L-Citrulline} + \text{NADP}^+$$

Scheme 1.4 – ROS production by nitric oxide synthase.
NO can react with superoxide to give rise other reactive chemicals such as ONOO\(^-\). NO\(^-\) plays an important role in vasculature including cell adhesion, inhibition of platelet aggregation, and leukocyte interaction with the endothelium (44).

The third isoform of NOS is inducible NOS (iNOS) that mediates inflammatory processes in macrophages. Macrophages form phagosome by engulfing and killing invading pathogens. iNOS plays an indispensable role in deactivating pathogens, and iNOS is constantly active in producing NO, independent of calcium levels (45). NO\(^-\) is highly diffusible across cell membrane and have a long half-life (~15 seconds). Therefore, NO\(^-\) can act on neighboring cells and react with other free radicals, proteins, and DNA.

### 1.3.2 Cellular organelles

#### 1.3.2.1 Mitochondria

Mitochondrion is the power house of the cell. It produces the energy that is required for the normal cellular functions. Mitochondria produce adenosine triphosphate (ATP) by oxidative phosphorylation. These organelles are composed with inner and outer double membrane, inter membrane space and matrix (46). A typical mitochondrion is about 2.5 \(\mu\)m in diameter and the number of mitochondrion within a cell can vary depending on the cell type (47).

Mitochondrial metabolism consists with citric acid cycle (TCA) and oxidative phosphorylation (OXPHOS). These metabolic processes are mainly powered by the availability of metabolic intermediates, including pyruvate, fatty acids and amino acids (46). TCA cycle produces the electron carriers, such as NADH and FADH, which shuttled to the electron transport chain (ETC) to synthesize ATP. During electron transfer, isolated mitochondria produce \(O_2^{•-}\) by consuming 1-2% of the available oxygen for respiration.
The complex I and complex III are the major sites for O$_2$•− generation. Mitochondrial superoxide dismutase (mSOD) convert the unstable O$_2$•− to stable and less reactive H$_2$O$_2$. In addition to ETC, several other mitochondrial enzymes can also generate ROS. For example, cytochrome b5 reductase and monoamine oxidases (MAO-A) are both found in outer mitochondrial membrane and generate O$_2$•− and H$_2$O$_2$ respectively (49,50). Cytochrome b5 reductase uses NADPH as the substrate while MAO-A catalyzes the oxidation of biogenic amines to generate ROS (49,51). There are several other mitochondrial enzymes found in inner mitochondrial membrane and known to generate ROS, including dihydroorotate dehydrogenase, dehydrogenase of α-glycerophosphate and succinate dehydrogenase (52-54). Aconitase found in mitochondrial matrix and produces hydroxyl radicals through Fenton’s reaction (55,56). α-Ketoglutarate dehydrogenase catalyzes the oxidation of α-ketoglutarate to succinyl-CoA. Inhibition of α-Ketoglutarate dehydrogenase and low levels of NAD$^+$ are known to produce ROS (57-59).

**ROS generation by complex I**

Complex I, also known as NADH-ubiquinone oxidoreductase, is a transmembrane multi-protein complex and is located in the inner membrane of mitochondria (60). Complex I is the entry site for electron carriers, such as TCA cycle derived NADH. Complex I hold FMN, seven ion-sulfur (FeS) centers, and CoQ (61,62). Two electrons from NADH transfer to CoQ through the shielded FeS centers. Therefore, it is less likely to transfer electron from FeS clusters to molecular oxygen. Therefore, ROS will be minimally produced during this process. Rotenone is a pharmacological inhibitor of complex I and is known to generate ROS (63). Rotenone binds to the CoQ binding site,
and blocks electron transfer from FeS to CoQ, thereby generating ROS (64,65). Superoxide production by complex I depends on the redox state of FMN and NADH/NAD\(^+\) ratio. In contrast, when mitochondria are respiring under normal conditions, superoxide generation at complex I is minimal (66). An additional mechanism of ROS production at complex I is the reverse electron transfer (RET) (48,66-68). When ATP production is low and CoQH\(_2\)/CoQ ratio is high, electrons are moving backward from CoQH\(_2\) towards Complex I. ROS production by RET was inhibited when complex I is treated with rotenone, indicating a back flow of electrons to complex I is through CoQ-binding site. Therefore, electrons can further push back to the FMN site and oxidize NAD\(^+\) to form the reduced NADH and superoxide (69).

![Figure 1.4 – ROS production at mitochondrial complex I](image)

Figure 1.4 – ROS production at mitochondrial complex I. Under increased NADH levels, FMN can be in fully reduced form and generate superoxide by a reaction with molecular oxygen at a FMN site. Revers electron transfer (RET) can generate ROS at CoQ-binding site of complex I. This figure adopted from Kira et al. 2014, with permission from copy rights clearance center (20). ROS generation in complex III
Mitochondrial complex III, Ubiquinone: cytochrome c reductase, shuttles electrons from CoQH₂ to cytochrome c. Complex III holds 11 polypeptides, three heme groups and an FeS center (70). During electron transfer, complex III interact with CoQ at Qi and Q₀ sites. Isolated mitochondria treated with CoQH₂ produced a large amount of superoxide (54,71-73). Antimycin A (AMA), myxothiazol and stigmatellin are known complex III inhibitors. AMA binds to Q₁ site and blocks electron transfer from Q₀ site to Q₁ site (Figure 1.5). Therefore, ROS are produced as a result of leakage of electron to molecular oxygen by ubisemiquinone at Q₀ site (Figure 1.5). In contrast, Myxothiazol and stigmatellin interact with Q₀ site by blocking electron transfer from CoQH₂ to Q₀ site and Q₀ to FeS center respectively. Myxothiazol and stigmatellin do not produce superoxide, indicating that blocking Q₁ site is the major reason for ROS production within the complex III (74,75). Under normal conditions, in the absence of AMA, complex III does not produce a significant amount of superoxide compared to RET in complex I.
Figure 1.5 – Mitochondrial complex III electron movement and Q-cycle. Antimycin A inhibits electron flow from Q₀ to Q₁ site and increases ROS production at a Q₀ site. This figure modified from Zorov et al. 2014, with permission from copyright clearance center (76).

1.3.2.2 Peroxisome

Peroxisome is a small organelle found in the cytoplasm of every eukaryotic cells (77). Peroxisome is also known as microbodies, and it has a catabolic activity towards long chain fatty acids, branched chain fatty acids, D-amino acids, and detoxification of ROS (78). Peroxisome-associated catabolic enzymes produce ROS and RNS. For example, peroxisomal oxidases catalyze the oxidative catabolism of fatty acids, purines and D-amino acids and transfer electrons directly to molecular oxygen. Acyl-CoA oxidase, xanthine oxidase, urate oxidase, D-amino acid oxidase and several other oxidases are readily available in peroxisome for catabolic processes and involved in generating ROS, such as H₂O₂ and O₂⁻⁻ (79-81). In addition to ROS, peroxisome also produces NO⁻ by iNOS (82). Although peroxisome consists with ROS detoxifying enzymes, such as catalase, H₂O₂ and NO⁻ can still penetrate the cytoplasm and participate either in redox signaling or oxidative damage.

1.3.2.3 Endoplasmic reticulum (ER)

Endoplasmic reticulum (ER) is an organelle found in most cell types, while absent in red blood cells (83). ER associates with protein synthesis, and lipid metabolism. The accumulation of unfolded or misfolded proteins in the ER can lead to ER stress. As a response to the ER stress, unfolded protein response pathway will be activated (UPR) (84,85). During UPR, protein disulfide isomerase (PDI) is catalyzing the formation of disulfide bond between cysteine residues of target proteins. Endoplasmic reticulum oxidoreductin 1 (ERO1) can reduce the oxidized PDI through electron transfer from PDI.
to molecular oxygen in FAD-dependent manner (86,87). During this process, there is a higher chance to generate $O_2^{•−}$ and $H_2O_2$. In ER associated oxidative protein folding, many disulfide exchange reactions occur. Therefore, as a side reaction of disulfide formation, electrons can leak and react with molecular oxygen to produce ROS. Nearly 25% of ROS produced in the ER are estimated to be generated as a byproduct of oxidative disulfide bond formation (88). In addition, the constitutively active NOX4 is known to generate ROS under ER stress. Interestingly, during phagocytosis, PDI directly interacts with NOX4 and produces superoxide (89).

1.4 ROS detoxifying system

Control generation of free radical species and other reactive species are involved in regulation of cellular processes. However, over the time, continues production of these species can accumulate inside the cell, which would be deleterious to the cell. Therefore, cells have evolved with ROS detoxifying system, also called an antioxidant system. Antioxidant system broadly divided into two major categories, including redox enzymes and small-molecule antioxidants. Superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and peroxiredoxins are examples of redox enzyme that play a vital role in maintaining redox homeostasis by neutralizing the harmful effect of ROS. Small molecule antioxidants include glutathione, vitamin A, C, and E, β-carotene, zinc, selenium, NADPH and pyruvate that are found in green vegetables (90).

1.4.1 Superoxide dismutase (SOD)

During ETC, highly reactive and unstable $O_2^{•−}$ produced as a by-product of respiration. Manganese-containing Mitochondrial superoxide dismutase (MnSOD) or SOD2 are solely found in mitochondrial matrix, and neutralizes $O_2^{•−}$ to produce less
reactive H$_2$O$_2$ (91). The dismutase activity depends on the level of MnSOD expression, and does not require other cofactors. However, overexpression of MnSOD closely associates with deficiencies in development and fertility in mice (92), suggesting that a controlled level of MnSOD or ROS is necessary for cellular function. MnSOD can be found in range of tissues including heart, liver, muscle, brain and spleen, showing the importance of MnSOD (93).

1.4.2 Catalase

Catalase found in most organisms, including plants, fungi, aerobic and anaerobic microorganisms. Catalase is a homo-tetramer and each subunit holds one porphyrin heme group that reacts with H$_2$O$_2$ (94). Catalase catalyzes the conversion of H$_2$O$_2$ to molecular oxygen and water with a high turnover number (millions of H$_2$O$_2$ molecules per second). Since, mitochondria and peroxisomes are generating a large amount of O$_2$$^-$, catalase is mostly found in mitochondrial matrix and peroxisomes (95). The lack of catalase activity increases the risk of developing obesity, fatty liver and type 2 diabetes (96).

1.4.3 Glutathione reductase

Glutathione reductase (GR) is an essential antioxidant enzyme for maintaining the balance between oxidized glutathione (GSSG) and reduced glutathione (GSH). Typically, the ratio of a reduced form over an oxidized form of glutathione represents the redox state of the cell. In normal conditions, the ratio of GSH/GSSG is high (97). GSH is an essential antioxidant that involves in detoxifying intracellular ROS (98). Therefore, reduction of GSSG back to GSH is important, and this process catalyzed by GR. GR uses NADPH as the reducing source, and a FAD prosthetic group used as an electron carrier during the
catalysis (99). Two catalytic cysteine residues (Cys58 and Cys63) are in proximity with NADH and FAD. In general, catalytic mechanism of GR divided into two steps. In the first step, NADPH will reduce oxidized GR. In the second step, GSSG is reduced by two catalytic cysteine residues in GR, releasing two GSH molecules. (100). Since many of antioxidant activities of GSH take place in mitochondria, GR mostly found in mitochondrial matrix (101,102). In addition to the detoxification of ROS, GR activity is also important in glutathione homeostasis because GSSG cannot cross the membrane (103).

1.4.4 Glutathione peroxidase

Glutathione peroxidases (GPx) are a family of selenium-containing enzymes that scavenge H\textsubscript{2}O\textsubscript{2} and use two GSH molecules to restore its catalytic function (104). There are at least eight different isoforms of GPx (GPx1-8), which are widely expressed in different tissues and found in different subcellular locations, including mitochondria (105). Mitochondrial Gpx1 provides the protection against oxidative stress by detoxifying H\textsubscript{2}O\textsubscript{2} produced by SOD. Active site selenocysteine in Gpx1 detoxify H\textsubscript{2}O\textsubscript{2} to water. During the catalysis selenocysteine oxidizes to selenol. Oxidized Selenocysteine will be then reduced back with the reaction of two consecutive oxidation and reduction steps by GSH (104). GPx1-knockout mice did not show any apparent complications, compromised growth and fertility, and a severe damage under oxidative stress. However, lower levels of GPx were associated with type 2 diabetes, multiple sclerosis and coronary heart diseases, suggesting a role of GPx in maintaining redox homeostasis (106-109).

1.4.5 Peroxiredoxin

Peroxiredoxins (Prx) can efficiently detoxify H\textsubscript{2}O\textsubscript{2} and lipid peroxides with the activity of other redox enzymes, such as thioredoxin and thioredoxin reductase (110). In
addition to scavenging mitochondrial hydroperoxides, Prx are also involved in redox signaling (111). Peroxiredoxins categorized into six isoforms, including Prx1-Prx6. Prx3 and Prx5 are ubiquitously expressed in different tissues including mitochondria (112). Therefore, Prx3 and Prx5 considered as mitochondrial peroxides, and their overexpression protect against \( \text{H}_2\text{O}_2 \)-induced oxidative damage in different tissues. All peroxiredoxins are sharing the same catalytic mechanism, and broadly divided into two categories depending on the number of cysteine residues in the active site. After one catalytic round, Prx active site cysteines undergo catalytically inactive oxidation state. In order to restore enzymatic activity, the oxidized form of peroxiredoxins are reduced by thioredoxin, ascorbate or GSH. The oxidized thioredoxin formed during the reaction, then reduced by thioredoxin reductase using NADPH as the reducing source.

1.4.6 Glutathione (GSH)

Glutathione is a tripeptide composed of glutamate, cysteine and glycine. GSH is the most abundant thiol-containing small molecule that acts as an antioxidant in mammalian and plant cells. GSH is involved in many cellular events, including ROS scavenging (113). GSH concentration largely varies among subcellular locations where GSH concentration is in the range of 2-10 mM (114). Since mitochondria handle more than 90% of total ROS production in the cell, mitochondria have a higher concentration of GSH than other organelles. GSH is biosynthesized intracellularly while it is degraded outside of the cell (114). Two consecutive enzyme catalyzed reactions biosynthesize GSH in cells. Firstly, g-glutamyl-cysteine ligase (GCL) catalyzes the formation of \( \gamma \)-glutamyl-cysteine peptide bond between cysteine and glutamate. Typically, cysteine is a limiting amino acid, and GSH inhabits GCL. Therefore, GCL catalyzed first step is
considered as the rate limiting step of GSH biosynthesis. Secondly, glutathione synthetase (GS) catalyzes the coupling of γ-glutamyl-cysteine with glycine to form glutathione (γ-glutamyl-cysteinyl-glycine or GSH). (this topic will discuss in chapter 2, section 2.1). GSH is stable and present in high concentrations, because, it is evolved in a way that can resist to intracellular peptidases and γ-glutamyl cyclotransferase due to the γ-glutamyl-cysteine linkage and the presence of glycine residue respectively (115).

![Figure 1.6 – Chemical structures of the reduce gluathione (GSH) and the oxidized glutathione (GSSG).](image)

GSH and GSSG are involved in maintaining the redox state of the cell with the activity of other antioxidant agents and reducing sources, such as NADPH and FADH₂ (116). An Intracellular redox potential of GSH/GSSG couple is estimated to be in the range of -250 mV to -150 mV (116). GSH can either directly react with free radicals or take part as a cofactor in other redox enzymes. GSH can directly react and detoxify hydroxyl radicals or peroxynitrite (116,117). Direct reaction of GSH with free radicals can generate thyl radicals and finally generating GSSG (118). Gpx uses glutathione to detoxify ROS, such as H₂O₂, organic peroxides and lipid peroxides. In this reaction, GSH is oxidized to GSSG. Increased GSSG level is an indicative of oxidative stress. GSSG in the ER serves as the main oxidizing source that induce formation of disulfide bond in
nascent polypeptides, which is a critical step in protein maturation (119). In addition, GSH can directly react with RNS, such as NO\(^{-}\). In this reaction, NO\(^{-}\) first reacts to give nitrosonium ion (NO\(^{+}\)) by a copper-catalyzed reaction, which subsequently react with GSH to form GSNO, S-nitrosoglutathione (120). Formation of GSNO is involved in transportation of less stable NO\(^{-}\), and GSNO reacts with the redox-sensitive cysteine residues of proteins, which is known as protein nitrosylation.

**1.4.7 Pyruvate**

Pyruvate is a 3-C metabolic intermediate and the final product of glycolysis. D-Glucose is the major precursor for pyruvate synthesis. Glucose is breaking down to pyruvate by 9 sequential enzyme catalyzed reactions. Hexokinase catalyzes the first reaction of glycolysis by converting glucose to glucose-6-phosphate. As a result of several other enzymatic reactions, fructose1,6-bisphosphate will be produced as an intermediary metabolite. Fructose1,6-bisphosphate then break down into two 3-C units by fructose bisphosphate aldolase. These two 3-C units are further metabolized in to phosphoenolpyruvate. Finally, pyruvate kinase catalyzes the synthesis of pyruvate from phosphoenolpyruvate. Overall, in glycolysis, one glucose molecule generates two pyruvate molecules, two NADH, two ATP and two water molecules (121). In addition to the metabolic role, pyruvate non-enzymatically detoxifies O\(_2\)^{•−} and H\(_2\)O\(_2\) to acetate and CO\(_2\) (122). Physiological antioxidant properties of pyruvate are dependent on the metabolic state of the cell. For example, when non-respiring diplomonads were supplemented with physiological concentrations of pyruvate, the production of H\(_2\)O\(_2\) was diminished compared to the respiring saccharomyces cerevisiae (122). Therefore, the availability of glucose is an important determinant of antioxidant capacity of the cell.
1.4.8 Nicotinamide adenine dinucleotide phosphate (NADPH)

Well known antioxidant function of NADPH is to provide the reducing power to other redox enzymes to maintain redox homeostasis. For example, the ratio of GSH/GSSG is decreasing under increased production of ROS, due to the accumulation of GSSG inside the cell. GR can reduce GSSG to regenerate GSH by using NADPH. Also, when catalase binds to NADPH, it dramatically increases the life time of catalase, thereby indirectly involves in detoxification of H$_2$O$_2$ (123). In addition to its indirect antioxidant function, NADPH also known to act as a direct ROS scavenger and found in high concentrations (~5 mM) in mitochondria (124). Mitochondrial NADPH can be generated via isocitrate dehydrogenase and malic enzyme in a NADP$^+$ dependent manner (125). Also, NADPH is produced by hydride transfer from NADH, during proton transfer across the membrane, is also common (126). A significant portion of NADPH pool is synthesized by pentose phosphate pathway (PPP) (127). Since, glucose-6-phosphate is the preferred substrate for PPP, glucose availability is an important factor in determining the NADPH biosynthesis (127).

Usually, peroxynitrite derived reactive species such as tricarbonate anion (CO$_3^{--}$) and nitrogen dioxide (NO$_2^-$) are involved in damaging macromolecules such as proteins and nucleic acids. Therefore, alteration in protein structure and function will lead to pathological conditions (128,129). Since, a large portion of ROS are produced within the mitochondria, higher concentration of NAD(P)H is needed to detoxify them. Because, several reports showed that NADPH can acts as a non-enzymatic ROS scavenger by directly involving in detoxification of peroxynitrite derived CO$_3^{--}$ and NO$_2^-$. During this reaction, O$_2^{--}$ and H$_2$O$_2$ are produced and detoxified by SOD and catalase (130).
1.5 ROS and RNS in protein oxidation

Mitochondrial respiration and other enzymatic reactions can generate ROS and RNS. ROS and RNS are involved in different cellular processes by modifying redox sensitive proteins associated with signaling. Reactive species can damage normal protein function by irreversible protein oxidation (24,131). ROS can react with protein thiols to form both reversible and irreversible modifications such as sulfenic acid, sulfinic and sulfonic acids. Protein function can be finely regulated or permanently inactivated by cysteine oxidation (132). For example, glutathionylation, a reversible protein cysteine oxidation, is not only involved in protecting reactive cysteine from irreversible oxidation, but also it plays an important role in redox signaling. Glutathionylation is a well-studied reversible oxidative modification, where it refers to the formation of disulfide bond between a protein thiol and GSH (Figure 1.7 A) (133). On the other hand, the irreversible cysteine oxidation, such as sulfonic acids, can inhibit enzyme activity by directly modifying active site cysteine residues or other cysteines that are important for maintaining the structure (134).

Glutathione is the major low molecular weight thiol in cells and acts as an antioxidant to protect the cell from oxidative stress. Glutathionylation can form through several transient intermediates, including sulfenic acid or nitrosothiol. While glutathionylation can occur non-enzymatically, a few redox enzymes, including glutathione transferase pi and glutaredoxin, are reported to enzymatically catalyze the formation and reversal of glutathionylation, respectively (135).

Multiple proteins are regulated by glutathionylation in important cellular events, such as proliferation, migration, cell signaling, and apoptosis (136). However, their
functional importance is much less understood due to lack of chemical tools. Development of effective biochemical tools for identifying and characterizing protein glutathionylation will help profiling target proteins and furthering our understanding of glutathionylation in redox regulation.

Figure 1.7 - An illustration of protein cysteine oxidation. Reactive cysteines can react with $\text{H}_2\text{O}_2 / \text{O}_2^-$ and reactive nitrogen species to generate sulfenic or nitrosothiol, which can act as intermediates for forming protein glutathionylation. Glutathionylation can take place chemically or enzymatically by GSH and GSTP respectively. B) A Prolonged exposure to reactive species can form irreversible cysteine modifications, such as sulfinic and sulfonic acids.

1.5.1 Protein S-glutathionylation

Under both physiological and oxidative stress conditions, a subset of protein cysteines is oxidized (13). Among many thiol oxidations, protein glutathionylation is one of the major oxidations. S-glutathionylation is mostly found in redox-sensitive cysteine residues that depends on the local environment around the cysteine residue (137). The addition of a GSH molecule to a protein cysteine can increase its overall negative charge
and an increase in molecular weight by 305 Da. The downstream effect of such modifications depends on the site of modification in the protein structure (137). For example, S-glutathionylation in the active site cysteine residue can enhance or inhibit its catalytic activity. S-Glutathionylation in cysteine residues distal to active site can alter protein function by interfering with binding partners or affecting protein structure (138).

The susceptibility of cysteine thiol to undergo S-glutathionylation depends on several factors. The first important determinant is accessibility of cysteine residues to proximal donors of S-glutathionylation, such as superoxide, nitric oxide GSSG and nitrosoglutathione (139). Secondly, the local environment that can increase the nucleophilicity of cysteine thiol is also one of the major factors in determining the modification site of a protein. Proximity of cysteine residue to basic amino acids such as lysine, arginine and histidine can increase the formation of thiolate anion by increasing its nucleophilicity (3). Selenocysteine has a lower pKₐ value at physiological pH (~7.4), due to hydrogen bonding with neighboring charged residues, including lysine. Cysteines that are coordinated with metal ions, such as Mg²⁺ or Zn²⁺ can also lower the pKₐ of cysteine thiol making them more nucleophilic (140). Because of low availability of reactive cysteines and thiol reactive radicals, only a limited number of cysteine residues is glutathionylated under physiological conditions. Under oxidative stress, there is a higher chance for most of cysteine thiols to get oxidized through a disulfide bond with GSH. Reversible S-glutathionylation regulates many of cellular processes under both normal and pathological conditions. Therefore, it is important to understand chemical and enzymatic routes of formation of protein S-glutathionylation and deglutathionylation.

**S-glutathionylation from chemical reactions:**
In general, S-glutathionylation occurs by a thiol exchange reaction. A thiolate anion can nucleophilically attack GSSG or another protein-GSH adduct. In this reaction, GSH forms disulfide with a protein cysteine residue. Alternatively, different oxidative intermediates of protein cysteine, including sulfenic acid, can react with glutathione to form disulfide (Figure 1.8 A). But, there are several other chemical mechanisms that facilitate formation of S-glutathionylation (Figure 1.8 B). Typically, ROS-mediated reactions and RNS-mediated reactions are more common but it is very difficult to dissect an exact mechanism for S-glutathionylation. In the presence of ROS, such as O$_2$$^•$-, thiols in GSH or protein cysteine residues can oxidize to a highly reactive thiolate anion (GS-) or a thyl radical (GS). These reactive intermediates can react with H$_2$O$_2$ to form sulfenic acid (GSOH and PSOH) (equation 1). Further, GSOH can react again with GS- to generate GSSG (equation 2). Protein thiolate can easily react with these glutathione intermediates (GS-, GS- and GSSG) to form S-glutathionylated cysteines (equation 3).

RNS are also involved as intermediates in S-glutathionylation. Several proteins, including GAPDH and p21Ras, are known to undergo both S-nitrosation and S-glutathionylation. RNS, such as NO$^•$ and N$_2$O$_3$, are involved in generation of nitrosothiols, such as GSNO and PS-NO. As in Figure 1.8, the resulting RNS intermediates can react to give S-glutathionylation.
Figure 1.8 – Chemical mechanisms of protein S-glutathionylation. A) The mechanisms for S-glutathionylation via intermediates of thiolate and sulfenic acid. B) The chemical equations for generating ROS and RNS intermediates that are mediate S-glutathionylation.

**Enzyme catalyzed mechanism of S-glutathionylation:**

In addition to chemical formation of S-glutathionylation, enzymatic formation and removal of glutathionylation is also known. Glutathione S-transferases (GST’s) are a class of enzymes that decrease the pKₐ of thiol group in GSH, thus increasing the nucleophilicity and the reactivity of GSH for S-glutathionylation. GSTP is one of seven in GST family and is reported to increase S-glutathionylation in specific substrates. For example, oxidation of catalytic cysteine in peroxiredoxin to sulfenic acid can inhibit its peroxidase activity. GSTP interacts with peroxiredoxin and facilitates the enzymatic formation of S-glutathionylation of the active site cysteine, hence restoring the catalytic activity. Other enzymes, such as glutaredoxin 1 (Grx1), glutaredoxin 2 (Grx2) and gamma-glutamyl transpeptidase also play a role in S-glutathionylation. The enzyme-catalyzed S-glutathionylation may occur on the less reactive cysteine in a specific protein. For example, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) does not possess cysteine residue with a low pKₐ. However, glutathionylation occur by reacting with GS- as
the proximal donor that is catalyzed by glutaredoxin. In addition, Grx catalyzes S-glutathionylation in several other proteins, including actin and protein-tyrosine phosphatase 1B (PTP1B).

![Diagram](image)

**Figure 1.9 – Glutaredoxin (Grx) for deglutathionylation.** Grx catalyzes deglutathionylation through A) monothiol and B) dithiol mechanism. GSSG is produced as result of both reactions. GR reduces GSSG to generate reduced glutathione (GSH) with NADPH as a reducing source. This figure adopted from Mashamaite et al. 2015, with permission from Portland press (141).

Another extensively studied role of Grx is its deglutathionylating function (6). Grx catalyzes a removal of GSH from protein cysteine residues by the disulfide-exchange mechanism through the reaction of catalytic cysteine of Grx or by GS⁻ (142). First, catalytic cysteine of Grx attacks and reduces disulfide on a target protein. During the first step, the catalytic cysteine of Grx forms a disulfide with GSH. Secondly, a thiolate anion of
glutathione can restore the catalytic cysteine by forming GSSG (monothiol mechanism). In dithiol mechanism, catalytic cysteine forms a disulfide with an adjacent cysteine, which releases reduced glutathione. An Oxidized form of Grx enzyme can be reduced to a catalytically active form by two molecules of glutathione. In both mechanisms, GSSG is released and will be reduced back to GSH by GR. In addition to Grx, sulfiredoxin (Srx) is also engaged in deglutathionylation of several proteins, such as PRX1, actin and PTP1B (143). Since glutathionylation of catalytic cysteine in PTP1B inactivates its phosphatase activity, deglutathionylation plays a crucial role in regulating important signaling pathways.

1.5.2 Other protein cysteine modifications

In the presence of H$_2$O$_2$, reactive cysteine residues can be oxidized to form reversible sulfenic acid (Figure 1.7 A). Several proteins are known to form sulfenic acid, including Prx, NADH peroxidase, NF-kB, PTP’s and several mitogen-activated protein kinases (MAPK’s) (132,144). Identification of sulfenic acid formation in proteins has been approached indirectly by using a biotin-switch method and by direct methods, such as azido- and alkyne-containing dimedone derivatives (145,146). Alternatively, the sulfenic acid adducts formed by reacting with dimedone has been probed by using an antibody (147). Sulfenic acid formation is labile to form glutathionylation. Alternatively, a constant exposure to ROS can oxidize sulfenic acid irreversible cysteine oxidations such as sulfinic acid and sulfonic acid. However, it is recently found that sulfinic acid can be reduced back to sulfenic acid by Srx enzyme (148). A Prolonged exposure to ROS, Prx can be oxidized to sulfinic acid. Srx selectively reduce the sulfinic acid to sulfenic acid in plant and mammals (149,150). Sulfinic acid formation has been reported in several studies, including matrix metalloproteases (MMP’s) and the Parkinson’s disease protein, Dj-1.
In addition to ROS, NO- can react with protein thiols to form S-nitrosylated proteins. Like S-glutathionylation, S-nitrosylation is involved in regulating different cellular processes, such as trafficking, apoptosis and neural transmission (152-154).

1.6 Protein S-glutathionylation and its relevance to human diseases

1.6.1 Regulation of protein function by S-glutathionylation

Protein thiols can be oxidized irreversibly to sulfinic and sulfonic acids under oxidative stress. Not only the irreversible oxidation can lead to the inactivation of protein function, but also result in the subsequent protein degradation (155). Protein S-glutathionylation serves two important functions under increased production of ROS or RNS. First, S-glutathionylation protects protein thiols from permanent oxidation. Second, the reversible S-glutathionylation involves in regulating protein function by inactivating or altering its structure and protein-protein or protein-DNA interactions. The reversibility of S-glutathionylation allows for protein thiols to be reduced back to sulfhydryl form while restoring the protein function. Some protein cysteine residues are glutathionylated even under basal conditions indicating a regulatory function in different cellular processes (156). S-glutathionylation can inhibit or activate protein activity, or stabilizes protein structure (157). For instance, some of metabolic enzymes, including tyrosine hydroxylate, aldose reductase, creatine kinase and GAPDH, are inhibited upon glutathionylation (158-161). S-glutathionylation of Cys186 and Cys181 in carbonic anhydrase III activate and inhibit its phosphatase activity, respectively (162). In HIV-1 protease, there are two conserved cysteine residues (163). While, glutathionylation of Cys67 is involved in stabilization and activation of protease activity, glutathionylation of Cys95 is associated with inhibition of protease activity (163).
S-glutathionylation plays important roles in cellular signaling pathways in many physiological processes, such as proliferation, apoptosis, differentiation and immunity (164). Many kinases and phosphatases are found to be regulated by S-glutathionylation. Protein kinase A, MAPK/extracellular -signal regulated kinase or ERK, PTP1B, and MAPK/ERK kinase kinase (MEKK1) are glutathionylated under physiological and stress conditions (4,165). Most of kinases are inhibited upon S-glutathionylation. For example, an active site cysteine residue (Cys199) of protein kinase A (PKA) is glutathionylated, which inactivates the kinase activity in a reversible manner (166). S-glutathionylation of small GTPase, Ras, activates downstream signaling of ERK and AKT. Angiotensin II induces glutathionylation of Ras, which is involved in hypertrophy in both smooth muscles and cardiac myocyte (167,168). Also, Ras glutathionylation is involved in inhibition of insulin signaling in endothelial cells (169). In addition to signaling proteins, S-glutathionylation regulates several transcription factors such as NF-Kb and Pax-8 (170,171). NF-kB is S-glutathionylated in both p65 and p50 subunits, which interferes with its binding to DNA. Therefore, NF-kB glutathionylation can inhibits the transcription of targeted genes (169,172).

1.6.2 Protein S-glutathionylation in human diseases

Age-related human pathologies are associated with increased production of ROS and RNS (173). Diabetes is a complication where body does not respond to insulin or has insufficient production of insulin due to destruction of β-cells (174). In both conditions, glucose in blood can be found in a high level. This can lead to an excessive supplementation of metabolites to mitochondria and generate increased amount of ROS, which leads to mitochondrial dysfunction (175). In addition to an increased production of
ROS, type 1 and type 2 diabetes show low levels of GSH and Gpx (176,177). Due to the oxidized environment in diabetics, a high level of GSSG can induce protein S-glutathionylation. S-glutathionylation of certain proteins are considered as biomarkers for identification of several disease conditions in human. Due to the difficulty of access in human tissue samples, identification of S-glutathionylation in body fluids such as blood are more common. For example, an increased level of S-glutathionylated hemoglobin is a characteristic of type 2 diabetes (178). Hemoglobin S-glutathionylation may be a potential biomarker for different pathologies, including hyperlipidemia, iron deficiency anemia, chronic renal failure and hemodialysis (179-181).

Cancer cells rapidly proliferate partially due to the loss of control of different cellular events involved in apoptosis, metabolism and redox homeostasis (182). Cancer cells rely on aerobic glycolysis, namely Warburg effect (183). The increased glucose metabolism is found to support the essential antioxidant activity by generating pyruvate and NADPH.

In a solid tumor, tumor cells largely experience a short of nutrients, which may augment metastasis of tumor cells. Interestingly, several metastasis-related proteins are found to be regulated by S-glutathionylation. Nucleoside diphosphate kinase (Nm23) is considered as a tumor suppressor, and the decreased mRNA levels and mutations of Nm23 are identified in several metastatic cancers, including breast and colon (184). S-glutathionylation of Nm23 at Cys109 can suppress its activity and increases the metastatic properties (185). Similarly, actin S-glutathionylation inhibits its polymerization and deglutathionylation by Grx1 can facilitate the actin polymerization (186,187). Since the actin polymerization and depolymerization are essential to cell migration, S-glutathionylation of actin may play an important role in controlling cancer cell migration.
under altered redox state (188). Moreover, MAPK, such as p38, JNK and ERK, play key roles in regulating cell migration (189-191). ERK is involved in regulating cell movement by phosphorylating myosin light chain kinase or calpain while p38 governs the directionality of the cell migration through phosphorylating MAPK-activated protein kinase 2/3. A recent study revealed that ROS produced by NOX4 induce MAPK phosphatase 1 (MKP-1) glutathionylation and subsequent inactivation of phosphatase activity, thereby facilitating the monocyte migration (192). These data provide evidences for a possible role of S-glutathionylation of MKP1 or other MKPs in cancer cell migration.

1.7 Analytical and biochemical methods for detection of S-glutathionylation

S-glutathionylation is the most extensively studied protein oxidation in an array of human diseases. Over the years, several analytical and biochemical approaches have been developed to visualize and identify glutathionylated proteins and their modification sites. Most common detection method is an antibody approach. A range of antibodies have been developed for different epitopes including glutathione, glutathione-BSA, glutathione-glutaraldehyde-BSA, glutathione conjugated to Kyhole Limpet Hemocyanin, and glutathione-conjugated protein complexes. Antibodies against glutathione derivatives have been widely used for Western blot analysis and for immunoprecipitation of individual glutathionylated protein (193-195).

Other detection methods include an indirect biotin-switch method, biotinylated glutathione ethyl ester (BIOGEE), radiolabeled GSH, isotope-labeled GSH and tagged GSH using glutathionyl-spermidine synthetase (196-198).
1.7.1 Biotinylated glutathione ethyl ester (BIOGEE)

Biotinylated glutathione ethyl ester generated by derivatizing GSH molecule at amine group of glutamate residue. This biotin tagged-GSH is not cell permeable. Therefore, a carboxyl group of GSH molecule was modified to be an ethyl ester group. It is expected that intracellular esterase can hydrolyze ethyl group once getting into the cell. The released biotin-GSH could modify any protein cysteine residue in response to ROS (Figure 1.10).

![Figure 1.10](image)

**Figure 1.10 – A scheme for identification of S-glutathionylation by BIOGEE.** This figure adopted from Samarasinghe *et al.* (199).

Biotin-derivatized oxidized glutathione was also used for studying S-glutathionylation (200). BIOGEE has been used to identify various glutathionylated proteins in response to ROS and growth factors (201,202). The biotin group allows to enrich biotinylated glutathionylated proteins by streptavidin agarose beads. Using this method, glutathionylated proteins can be visualized in a Western blot using streptavidin-horse radish peroxidase (strep-HRP). This method was also used with mass spectrometry method in identifying glutathionylated proteins. After performing biotin pull-down, glutathionylated proteins was digested by trypsin and the subsequent elution allows to
identify modified peptides by mass spectrometry analysis (202). Further, this method was modified to identify and quantify the degree of glutathionylation among different ROS. Such improvements include addition of isotope-labeled biotin-GSH and the cleavable tags to identify modification sites (197,202).

### 1.7.2 Biotin-switch method

Another method is a biotin switch method. In this method, S-glutathionylated proteins are identified indirectly (203). First, cells are induced for glutathionylation by ROS. After cell lysis, all free thiols are blocked by reacting with thiol-reacting agents, such as iodoacetamide (IAM) or N-ethylmaleimide (NEM). After irreversible blocking of all unmodified thiols, S-glutathionylated cysteine residues are reduced by dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP) or a mutant form of Grx3 (202,204,205). The reduced cysteine residues then react with biotin-NEM or biotin-IAM. Biotinylated proteins that represent S-glutathionylated proteins can be visualized by Western blotting (Figure 1.11). Similar to BIOGEE, biotin-switch method was used with mass spectrometry (LC-MS/MS). Therefore, identification of glutathionylated proteins as well as their cysteine modification sites can be deduced using biotin-switch method. A modified version of biotin-switch method has been reported and successfully used to visualize glutathionylated proteins within different compartments of fixed tissues or cells (205).
**Figure 1.11 – The example of a biotin switch method.** After inducing glutathionylation, the remaining cysteine residues were blocked with N-ethylmaleimide (NEM) and subsequent reduction by Grx3 will expose glutathionylated cysteine residues. Then cysteine residues are reacted with biotin-containing cysteine reactive reagent for the enrichment and identification by LC-MS/MS. This figure adopted from Samarasinghe *et al.* 2015 (199).

### 1.7.3 Biotin-glutathionylspermin

GSH is present in a high concentration in eukaryotic cells as well as in prokaryotes. In several types of bacteria, while a carboxylic group of a glycine residue in GSH molecule is further modified with spermidine by glutathionylspermidine synthetase (GspS). The synthesized gluathionylspermidine can be hydrolyzed by glutathionylspermidine amidase (GspA) (206-208). In a recent study, GspS was used to generate biotin-glutathionylspermine (biotin-Gsm) in mammalian cells to identify S-glutathionylated proteins by LC-MS/MS (209). In this method, first, GspS is transiently expressed into cells. Biotin-spermine was then incubated to generate biotin-Gsm inside the cell. Because biotin-Gsm was synthesized inside the cell, this method can avoid an incubation of an exogenous modified GSH, such as BIOGEE, in a high concentration, which may minimize the perturbation of an intracellular redox status. In the presence of ROS stimulus, biotin-Gsm can undergo S-glutathionylation (Figure 1.12). Biotin-Gsm modified proteins can be visualized by Western blotting and identified by LC-MS/MS. This approach was used to identify 1400 modified cysteine residues in LC-MS/MS analysis.
Figure 1.12 – A strategy to identify S-glutathionylated proteins using biotin-spermine and glutathionylspermidine synthetase (GspS). Transfection of GspS followed by the incubation of biotin-spermine generates biotin-glutathionylspermine (biotin-Gsm). In the presence of ROS, sensitive cysteine residues can be modified by biotin-Gsm and the subsequent LC-MS/MS analysis can identify glutathionylated proteins. This figure adopted from Samarasinghe et al. 2015 (199).

1.8 Current dissertation work in examining S-glutathionylation

Protein S-glutathionylation is implicated in a range of human diseases including age-related diseases such as diabetes, neurodegenerative diseases, cardiovascular diseases and cancer. Many of these diseases are associated with altered metabolism and cellular redox homeostasis. For example, cancer cells rely on a high rate of glucose uptake in order to provide enough energy for rapidly proliferating cells. Many cancer cells are shown to produce high levels of ROS and RNS. S-glutathionylation is an oxidative cysteine modification that regulates protein function due to its reversible nature.
Identification and evaluation of functional consequences of S-glutathionylation in such proteins allow basic understanding for development of therapeutics in the future. However, despite several available biochemical approaches, identification and characterization of S-glutathionylated proteins are challenging.

In this dissertation work, I will describe development of a novel chemical method to detect S-glutathionylated proteins, and the application to identify S-glutathionylated proteins in response to metabolic alteration. Moreover, functional roles of S-glutathionylation in cancer metastasis will also be elucidated.

In chapter 2, development of a clickable glutathione approach for detection of glutathionylation will be discussed. In this study, we developed a selective and versatile method for identifying and characterizing glutathionylation in cells by using a mutant of glutathione synthetase (GS). We have engineered the active site of GS to catalyze an incorporation of azido-Ala in place of glycine. This generates the azide-containing glutathione in cells, which undergoes glutathionylation upon ROS stimulus. The subsequent bioorthogonal copper-catalyzed 1,3-dipolar cycloaddition reaction (click reaction) with biotin-alkyne or fluorophore-alkyne allows a direct enrichment or detection of glutathionylated proteome by biotin pull-down and fluorescence imaging, respectively. The method presents a unique metabolic labeling of intracellular glutathione without disturbing thiol-redox states and a chemical method for characterization of glutathionylated proteins in response to ROS.

Chapter 3 will describe identification of glutathionylated proteins induced in response to glucose metabolism and/or mitochondrial dysfunction Mitochondrial ROS (mROS) are the key components of cellular damage and redox signaling. Cancer,
ischemia and other pathological conditions are accompanied by elevated levels of mROS. Although glucose metabolism is known to be associated with redox regulation, glutathionylation in response to glucose metabolism remains unknown. To address this, we employed a clickable glutathione approach in a HEK293-GS M4 stable cell line that stably overexpresses the mutant of glutathione synthetase (GS M4). glutathionylation was induced in response to antimycin A (AMA), glucose starvation, a combination of AMA and glucose starvation. Interestingly, glutathionylation was reversed by the addition of glucose or pyruvate, suggesting that reversibility of glutathionylation is regulated by glucose availability under mitochondrial dysfunction. We identified over 1200 glutathionylated proteins by LC-MS/MS analysis including several new glutathionylated proteins, including PP2Cα. PP2Cα is a metal-dependent protein phosphatase involved in metabolic stress and AMPK signaling. We found that PP2Cα is glutathionylated at C314 residue in response to metabolic stress, and glutathionylation of PP2Cα disrupted its interaction with mGluR3, an important metabotropic glutamate receptor associated with neurological disorders. Together, this study provides the importance of glucose availability in regulating protein glutathionylation under mitochondrial dysfunction.

Chapter 4 will describe the functional role of PP2Cα S-glutathionylation in cancer cell migration and invasion in response to metabolic alterations. PP2Cα is a serine/threonine protein phosphatase which acts as a negative regulator of stress responsive pathway. First, glutathionylation was systematically analyzed under a limited concentration of glucose and oxygen availability. We found different glutathionylation pattern in different cell lines in response to low glucose. Glutathionylation was selectively induced in an invasive breast cancer cell line MDA-MB 231 compared to non-invasive
MCF7 cells, indicating a distinct redox response by metastatic cancer cells. Further, an incubation of L-glutamic acid reversed the effect of low glucose. We also showed that oxygen availability is also important for glucose deprivation-induced protein glutathionylation. Moreover, we found that PP2Cα glutathionylation enhances cancer cell migration potentially by dephosphorylating and inactivating JNK under metabolic stress in MDA-MB231 cells. Altogether, our data reveal an important regulatory role of PP2Cα glutathionylation in cancer cell migration in response to metabolic stress.
CHAPTER 2 METABOLIC SYNTHESIS OF CLICKABLE GLUTATHIONE FOR STUDYING PROTEIN GLUTATHIONYLATION

2.1 Introduction

Protein glutathionylation is implicated in redox signaling under controlled generation of reactive oxygen species by cytokines and growth factors (210). Most of available chemical methods suffer from poor specificity and/or sensitivity. To overcome these limitations and to develop a reliable method for identification of glutathionylated proteins, we developed a chemical method which uses enzyme engineering, metabolic synthesis and biorthogonal chemistry (199,211). First, we generated clickable glutathione inside the cell which excludes the incubation of high concentrations of exogenous glutathione. Glutathione biosynthesis is catalyzed by two enzymatically catalyzed reactions (Figure 2.1). In the first step, γ-glutamylcysteine ligase (GCL), which is the rate limiting enzyme, catalyzes the formation of an amide bond between a side chain carboxylic group of glutamate (Glu) and an amine group of cysteine (Cys) to generate γGlu-Cys dipeptide. The second step of glutathione biosynthesis is catalyzed by glutathione synthetase (GS). GS catalyzes the formation of tripeptide (γGlu-Cys-Gly) by ligating glycine (Gly) with a dipeptide γGlu-Cys (212,213).

We planned to incorporate a small clickable functional group onto a glycine residue in glutathione. We envisioned that we can add clickable moiety on Gly of glutathione by engineering GS. Since GCL involve in the rate-limiting step of glutathione biosynthesis, metabolic synthesis of clickable glutathione may not affect the total glutathione or thiol concentration in the cell (214). In addition, a glutathione-degrading enzyme, γ-glutamyl transpeptidase, retains the specificity towards Glu in glutathione. Therefore, we further envisioned that clickable glutathione may be degraded by glutathione-catabolic pathways
(215). Also, interestingly, there are other naturally existing glutathione derivatives containing β-Ala, Ser, or Glu in place of Gly in glutathione in plants (216), which may justify the modification in clickable glutathione.

### Biosynthesis of glutathione

$$\text{Glu} + \text{Cys} \xrightarrow{\gamma\text{GluCys ligase}} \gamma\text{Glu-Cys} \xrightarrow{\text{GSH synthetase}} \gamma\text{Glu-Cys-Gly (GSH)}$$

### Biosynthesis of clickable glutathione

$$\gamma\text{Glu-Cys} + \text{L-AzAla, L-HPG or L-PG} \xrightarrow{\text{Glutathione Synthetase Mutant}} \text{Clickable GSH}$$

Figure 2.1- A scheme for GSH and azido-GSH biosynthesis. The first reaction is catalyzed by γGluCys ligase to generate dipeptide γGlu-Cys. Glutathione synthetase catalyzes the second step of GSH biosynthesis. GS mutant catalyzes azide- or alkyne-containing Gly derivatives to form clickable GSH.

### 2.2 Design of clickable glutathione

Our design is based on a synthesis of azido-glutathione or glutathione containing alkyne group in situ in cells. We thought that the GS active site could be engineered to accommodate azide-or alkyne-containing Gly derivatives. Overexpression of GS mutant allows metabolic synthesis of clickable GSH inside the cell. First, transient overexpression of a GS mutant in HEK 293 cells will metabolically synthesize clickable glutathione in the
presence of azide- or alkyne- containing Gly derivatives, such as L-azidoAlanine (L-AzAla), L-homopropargylglycine (L-HPG) or L-propargylglycine (L-PG). After glutathionylation is induced by incubation of exogenous H$_2$O$_2$, cells are lysed in the presence of a thiol-blocking reagent. The subsequent bio-orthogonal click reaction with appropriate alkyne or azide containing biotin or fluorophore is expected to selectively detect glutathionylation by Western or in-gel fluorescence respectively (Figure 2.2). This novel approach provides not only detection of glutathionylation by gel analysis, but also identification of glutathionylated proteins by mass spectrometry analysis.

Figure 2.2 - Schematic diagram for the detection of glutathionylation by clickable GSH.

2.3 Results

2.3.1 Generation of glutathione synthetase mutants and kinetic analysis

Glutathione synthetase is a specific enzyme towards its substrates including glycine. In the active site, there are several amino acids which are important for specificity towards glycine. We noticed in the crystal structure of GS (PDB:2HGS) that two amino acids (F152 and S151) are in a close proximity to a glycine residue of GSH (Figure 2.3.
A). We thought that a bulky structure of F152 in the GS active site does not allow formation of glutathione derivatives containing other amino acids than glycine.

Active Site AAs in GSH synthetase
Figure 2.3 – Crystal structure of GS WT and rearranged active site structures of GS M4. A) Crystal structure and active site amino acid residues of glutathione synthetase enzyme. B) Model structures of the active site in glutathione synthetase (GS) WT and mutants. Space filling (upper) and stick (bottom) models of GS mutants are drawn from GS WT (PDB:2HGS) by using PyMol program. The structure of azido-Ala is drawn in an arbitrary conformation that appears to have a low steric hindrance.

Therefore, we thought we can mutate F152 and/or S151 in the active site of GS to provide an extra space that may accommodate the azide- or alkyne-containing glycine derivatives (Figure 2.3 B). We have generated several GS mutants with mutation of F152 and S151 residues. GS WT and GS mutants were overexpressed in bacteria and purified by affinity chromatography (Figure 2.4 A and Figure A.2.1) and characterized by an ADP-coupled enzyme assay with pyruvate kinase (PK) and lactate dehydrogenase (LDH)
Initial rates were fitted to the Michaelis-Menten equation. \( K_m \) and \( k_{cat} \) values were calculated for all GS mutants and GS WT (Figure 2.4 C and Figure A.2.2).

![Figure 2.4](image)

**Figure 2.4 – Purification and kinetic analysis of GS mutants.** A) GS WT and GS mutants were purified with high purity. B) Kinetic assays by enzyme coupled reactions using ATP consumption. C) Michaelis-Menten plot for L-AzAla and GS M4 (F152A/S151G).

Kinetic data for all GS enzymes are summarized in Table 2.1. Since GS WT is specific towards Gly, it did not catalyze other amino acids. GS WT catalyzes Gly with a high catalytic efficiency (\( k_{cat}/K_m = 1095 \)) and has a high binding affinity to Gly. Interestingly, GS M1 (F152G) did not catalyze Gly and displayed a poor binding affinity towards Gly compared to L-AzAla. The mutation of bulky F152 to a small glycine may have created an enough space to accommodate glycine derivatives. GS M1 catalyzed L-AzAla with high catalytic efficiency compared to other amino acids. Although GS M1 prefers L-AzAla over other substrates, GS M1 was not ideal. The binding affinity is still poor and the \( K_m \) value was high (2.46 mM). Also, the \( k_{cat}/K_m \) value is 25 times lower than \( k_{cat}/K_m \) value for Gly by GS WT. We further hypothesized that a bigger space in the active site pocket will increase the binding affinity of GS mutant towards L-AzAla.
Therefore, we generated a double mutant, GS M2 (F152G/S151G), that may give a more space to accommodate L-AzAla in the active site of GS. Although, GS M2 has a decreased $K_{cat}$ value for L-AzAla, GS M2 improved the binding affinity by 10-fold when compared to GS M1. Overall, GS M2 showed a 5-fold higher catalytic efficiency with azido-Ala than GS M1, and only 6 times lower catalytic efficiency than GS WT for glycine. However, it was found that GS M2 can catalyze both L-Met and L-AzAla with the similar catalytic efficiency, suggesting that GS M2 lost its selectivity towards L-AzAla.
In addition, we noticed that GS M3 (S151G) with a small size of mutation showed significantly improved the selectivity towards L-AzAla over L-Met. Thus, we sought to mutate F152 to alanine which may reduce the active site space in GS M2. Indeed, we observed that the mutation of F152A together with S151G (GS M4) improved the selectivity towards L-AzAla ($K_m = 0.09$ mM) over Met ($K_m = 0.17$ mM). GS M4 has the highest catalytic efficiency ($k_{cat}/K_m = 630.6$) towards L-AzAla. In addition, GS M4 had a three-fold higher catalytic efficiency for L-AzAla over L-Met. GS M4 also could catalyze both L-HPG and L-PG with a similar or slightly lower $k_{cat}/K_m$ value to L-AzAla. Overall, in vitro kinetic data provide evidences that GS M4 can efficiently catalyzes L-AzAla to synthesize clickable glutathione.

**2.3.2 Biosynthesis of clickable glutathione in HEK293 cells**

In vitro kinetic data showed that GS M4 is the most efficient GS mutant with azido-Ala. However, GS M4 can also catalyze L-Met albeit 3-fold lower catalytic efficiency than azido-Ala. Therefore, we analyzed in vitro synthesis of glutathione derivatives by GS WT and GS M4. Gly, L-AzAla, L-Met, γGlu-Cys and ATP were incubated with GS WT and/or GS M4, as shown in Figure 2.5. The synthesis of glutathione derivatives was analyzed by LC-MS with or without modification by fluorescein iodoacetamide (FL-IA). When the reaction mixture reacted with FL-IA, the masses corresponding to endogenous GSH (m/z 308) and azido-GSH (m/z 363) shifted to m/z 695 and m/z 750, respectively. Masses corresponding to endogenous GSH, azido-GSH and GSH containing L-Met (Met-GSH) were extracted and overlaid in LC-MS analysis. In the presence of GS WT, the only mass corresponding to endogenous GSH was detected without observing other masses for azido-GSH and met-GSH (Figure 2.5 A and Figure A.2.3). In contrast, GS M4 synthesized
the glutathione derivatives containing azido-Ala, Met, or Gly, but with the highest intensity for azido-glutathione (Figure 2.5 B). In the presence of both GS WT and GS M4, there is still significant amount of azido-glutathione in comparison to endogenous glutathione (Figure 2.5 C).

Figure 2.5 - LC-MS analysis of in vitro biosynthesis of clickable glutathione. Individual GS enzymes were incubated with γGlu-Cys, amino acids and ATP. The reaction mixture was then treated with FL-IA and injected to LC-MS. The masses of endogenous GSH, azido-GSH and met-GSH were extracted and overlaid. A) GS WT B) GS M4 C) GS WT and GS M4 were incubated with other reactants and injected to LC-MS.

Next, we analyzed metabolic synthesis of clickable glutathione in cells. For mammalian cell transfection, we produced a mammalian vector that can express GS M4
(pCDNA3.1-GS M4) with n-terminal FLAG tag. A transient transfection of FLAG-tagged GS M4 into HEK293 cells induced approximately 15-20-fold overexpression of the GS mutant compared to endogenous GS WT (Figure 2.6 A). First, we analyzed total thiol concentrations in cell lysate to check whether metabolic synthesis of clickable glutathione interfere with intracellular redox state. Cells were lysed by three freeze-thaw cycles and protein free lysates were analyzed for free thiols using bromobimane assay.

Figure 2.6 – GS M4 overexpression in cells. A) FLAG tagged GS M4 was transiently transfected to HEK 293 cells and overexpression of GS M4 probed with anti-GS and anti-flag. B) Intracellular thiol levels were determined by bromobimane assay. After transfection of GS M4, cells were incubated with different concentrations of L-AzAla for 20 h. Cells were lysed by freeze-thaw cycles and protein free lysates were subjected to bromobimane assay.

An incubation of increasing concentrations of L-AzAla did not change the total thiol concentration when compared to cells without transfection of GS M4 and incubation of L-
AzAla. The data indicated that metabolic synthesis of clickable glutathione by GS M4 may not alter intracellular redox state.

We further demonstrated the biosynthesis of azido-GSH in cells. L-AzAla, L-HPG or L-PG were incubated for 20 h with GS M4-transfected cells. Cells were lysed by several freeze-thaw cycles in 1X PBS with or without fluorescein-iodoacetamide. Protein free cell lysates injected to LC-MS. The masses corresponding to endogenous GSH (m/z 308) and azido GSH (m/z 363) were extracted and overlaid. only endogenous GSH was found in cells without expression of GS M4 (Figure 2.7 A and 2.8 A). Masses corresponding to other glutathione derivatives including azido-GSH were not detected in LC-MS analysis. In contrast, when HEK 293 cells were transfected with GS M4 and incubated with L-AzAla, we observed a mass ion peak for azido-GSH together with endogenous GSH (Figure 2.7 B). The amount of azido GSH was gradually increased with an increasing incubation time of L-AzAla. Incubation of L-AzAla for 6 h, 20 h and 40 h increased the amount of azido-GSH up to 10%, 40% and 70% of endogenous GSH, respectively (Figure 2.7 C). In addition, the amount of azido-GSH was increased dose dependently when cells were incubated with increasing concentrations of L-AzAla (Figure A.2.4). When HEK293 cells without transfection of GS M4 were incubated with L-AzAla, we observed only one mass ion peak which is corresponding to endogenous GSH (Figure 2.8 B). We also analyzed GS M4 selectivity towards L-AzAla over other amino acids for synthesizing glutathione derivatives in cells.
Figure 2.7 - LC-MS analysis of biosynthesis of clickable GSH. A) Protein free lysates from control cells without azido-Ala and GS M4 transfection were injected to LC-MS. Masses corresponding to endogenous GSH and azido-GSH were extracted and overlaid. B) Cell lysates were prepared after transfection of GS M4 and incubation of azido-Ala to HEK29C3 cells. Protein free lysate were injected to LC-MS. C) Cells were lysed and derivatized with FL-IA after incubating azido-Ala for different times.
Figure 2.8 - LC-MS analysis of biosynthesis of other GSH derivatives. A) Masses corresponding to different glutathione derivatives were extracted and overlaid with HEK293 cells in the absence of azido-Ala. B) Masses for GSH and azido-GSH were analyzed from HEK293 cells in the absence of GS M4 overexpression after incubation of azido-Ala. C) Masses corresponding to GSH, azido-GSH, L-Met containing GSH (Met-GSH), alanine containing GSH (Ala-GSH) and reduced form of azido-GSH (aminoalanine-GSH) were analyzed from cells after transfection of GS M4 and an incubation of azido-Ala.
Importantly, overexpression of GS M4 and incubation of L-AzAla led to a selective synthesis of azido-GSH. We did not detect any glutathione derivatives containing either methionine (Met-GSH) or alanine (Ala-GSH) (Figure 2.8 C). Despite the fact that an azide group can be reduced to an amine group in an intracellular environment, we did not observe a mass (m/z 724) corresponding to a reduced form of azido-GSH (glutathione with amino-alanine) indicating the relatively high stability of an azido group inside the cell (Figure 2.8 C).

**Figure 2.9 - Biosynthesis of clickable GSH using L-HPG and L-PG.** Cell lysates prepared from cells with A) L-HPG and B) L-PG were subjected to LC-MS analysis after modifying with FL-IA.

Moreover, we checked the biosynthesis of GSH derivatives containing an alkyne group. HEK293 cells were transfected with GS M4 and incubated with L-PG or L-HPG. An incubation of L-PG or L-HPG produced their corresponding glutathione derivatives (Figure 2.9 A and B). We detected a lower intensity of GSH containing L-HPG when compared to GSH containing L-PG. L-HPG is a methionine surrogate which is known to be incorporated into proteins. These LC-MS study further provides the evidence that clickable GSH can be synthesized upon expression of the GS M4 mutant in cells.
2.3.3 Analysis of glutathionylated proteins by in-gel fluorescence and Western blotting

Metabolic tagging of clickable glutathione is a selective and versatile chemical approach to detect glutathionylated proteome in cells. To induce glutathionylation in HEK293 cells, GS M4 transfected cells were incubated with L-AzAla for 20 h followed by the incubation of hydrogen peroxide (H₂O₂). Then, cells were lysed and subjected to click reaction with fluorophore-alkyne or biotin-alkyne in the presence of CuBr. We used CuBr instead of common CuSO₄/TCEP method because of disulfide in glutathionylation. After one hour of click reaction, glutathionylation was visualized by in-gel fluorescence or chemiluminescence with streptavidin-HRP. All negative controls without GS M4, L-azido-Ala, or H₂O₂ (Figure 10 A lanes 1-7) did not show fluorescence signals for glutathionylation. In contrast, in the presence of GS M4, L-AzAla and H₂O₂ (Figure 10 A lane 8), click reaction produced numerous fluorescence bands corresponding to glutathionylated proteins. To confirm that the fluorescence signal results from glutathionylated proteins, we treated cell lysate (used in lane 8 Figure 10 A) with DTT prior to click reaction with rhodamine-alkyne. A treatment of DTT showed disappearance of fluorescence signal suggesting that the fluorescence signal results from a disulfide bonds formation between azido-GSH and protein cysteine residues in response to ROS stimulus (Figure 10 A lane 12). We observed the similar result after click reaction with biotin-alkyne and the subsequent Western blotting analysis with streptavidin-HRP (Figure A.2.5 A).
Figure 2.10 - Analysis of glutathionylated proteins using metabolically synthesized azido-GSH. A) HEK 293 cells were incubated with or without L-Azido-Ala and induced with 1 mM H₂O₂ or B) varying concentration of H₂O₂ for 15 minutes, followed by cell lysis and click reaction. Glutathionylation probed as shown above.

To assess the sensitivity of clickable glutathione approach, GS M4 overexpressing cells were treated with an increasing concentration of H₂O₂ (0.1-1 mM) (Figure 2.10 B). There was a significant intensity of glutathionylated proteins even at a low concentration of H₂O₂ (100 μM) (Figure 2.10 B lane 6). We did similar experiment by varying the L-Azido-Ala concentration, which showed similar levels of detection for glutathionylation even with a low concentration of L-Azido-Ala (0.3 mM) (Figure A.2.5 B). We also analyzed glutathionylation in different cell lines using the clickable glutathione approach. GS M4 was transiently transfected to three different cell lines including HEK293, HBEC3-KT and A549 cells. After incubation of L-AzAla, cells were induced for glutathionylation. The
intensity of glutathionylation was similar between HEK 293 and HBEC3-KT cells while A549 cells displayed a relatively lower signal of glutathionylation in response to H$_2$O$_2$ (Figure A.2.6). These data demonstrated that clickable glutathione can be used to study glutathionylation in different cell lines in response to ROS stimuli.

Several clickable glycine derivatives can be incorporated into proteins during *de novo* protein biosynthesis. For example, L-HPG is a well-known methionine surrogate. It has been shown that L-HPG can be incorporated into proteins (218). We further compared detection of glutathionylation using L-azido-Ala versus L-HPG and L-PG. HEK293 cells expressing GS M4 were incubated with clickable Gly derivatives (L-HPG or L-PG). After ROS stimulation and click reaction with rhodamine alkyne or rhodamine azide, glutathionylation was detected by in-gel fluorescence (Figure 2.11). In the absence of GS M4, L-AzAla or H$_2$O$_2$, cells did not produce significant amount of signal compared to the cells with all three components (Figure 11 lane 1-3 compare with lane 4). The diminished fluorescence signal in L-azido-Ala incubated cells without GS M4 overexpression and H$_2$O$_2$ incubation (Figure 11 lane 2), indicates that L-azido-Ala is not incorporated into proteins during protein biosynthesis. In contrast, an incubation of L-HPG alone (Figure 11 lane 6) produced numerous bands even without GS M4 and ROS stimuli, suggesting that L-HPG is incorporated into proteins.
Table 2.11 – Studying possible incorporation of amino acid derivatives to protein biosynthesis. After incubating L-AzAla, L-HPG and L-PG separately with or without GS M4 overexpression and ROS stimuli, prepared cell lysates were subjected to click reaction with rhodamine alkyne and glutathionylation detected by in-gel fluorescence.

An incubation of L-PG did not produce any labeling of proteins (Figure 11 lane 9-11). After treatment of H₂O₂, there was a significant signal for glutathionylation albeit a slightly lower intensity of signals when compared to glutathionylation detected by L-azido-Ala or L-HPG (Figure 11 lane 12). From this study, we concluded that L-AzAla is the most efficient clickable amino acid for identifying protein S-glutathionylation.
2.3.4 Reversibility of azido-glutathionylated proteins

Glutathionylation is a highly dynamic post translational modification due to the reversibility of the disulfide bond. Glutaredoxin (GRX1) is a well-known redox enzyme that catalyzes the reduction of disulfide bond in glutathionylated proteins and GRX1 is involved in maintaining the redox balance in the cell (219). We carried out in vitro GRX1 assay to examine whether azide group in clickable glutathione interferes with GRX1-mediated deglutathionylation. GS M4-transfected cells were incubated with L-AzAla and treated with H$_2$O$_2$ for 15 minutes prior to lysis. The lysates were then incubated with recombinant GRX1. glutathionylation was significantly decreased after incubation with GRx1 when compared to without GRX1 (Figure 2.12 A). This suggests that a small azide functional group in glutathione may be tolerated for deglutathionylation by GRX1 (compare lane1 and 2 in Figure 2.12 A).

Next, we evaluated protein deglutathionylation in HEK293 cells. After transfecting and incubating L-AzAla, cells were induced with H$_2$O$_2$ for glutathionylation. After 15-minute induction, cell culture medium was replaced with a fresh medium without H$_2$O$_2$ for different time points. cells were then lysed at 0, 0.5 and 1 h time points and were analyzed for glutathionylation after click reaction with biotin-alkyne.
Figure 2.12 – Reversibility and clickable glutathionylated proteins. After incubating L-AzAla with GS M4 overexpressing HEK293 cells and ROS stimuli cell lysates were prepared and subjected to click reaction with rhodamine alkyne and glutathionylation detected by in-gel fluorescence. A) Lysates were incubated with purified GRX1 protein and subjected to click reaction, probed by fluorescence. B) HEK293 Cells were washed with PBS for different time points after induction with H₂O₂ for 15 minutes. Then click reaction was performed with rhodamine-alkyne to assess reversibility of glutathionylation.

Deglutathionylation was readily induced after removing H₂O₂ from cells (lane 4 and lane 3, Figure 2.12 B). These data confirm that deglutathionylation can be detected by azido-GSH, showing that clickable glutathione can be used to detect a reversible change of glutathionylation in cells.

2.3.5 Fluorescence imaging

The clickable glutathione approach can be used to study various aspects of protein glutathionylation. For example, we envision that clickable glutathione can be used to
visualize glutathionylated proteins and the localization by fluorescence imaging. This technique excludes the incubation of primary and secondary antibodies for visualization. In addition, a clickable glutathione approach can be coupled with a proximity ligation, a sensitive technique that is used to study protein-protein interactions and to visualize PTMs in individual proteins by fluorescence imaging (220). Therefore, we sought to demonstrate fluorescence imaging of glutathionylated proteins in cellular context. HEK 293 cells were grown on a glass cover slip and L-AzAla was incubated in cells with or without GS M4 transfection. Cells were then induced for glutathionylation by 15 min incubation of 1 mM H₂O₂. Cells were fixed by cold methanol and washed with PBS to remove an excess amount of cellular glutathione. Then, click reaction was performed with Alexa Fluor 647 alkyne (AF647). Cells were then extensively washed and mounted on glass cover slips using a mounting solution containing DAPI.

Cells incubated with L-azido-Ala in the presence of GS M4, produced a strong fluorescence signal (Figure 2.13 E). Glutathionylated proteins were localized in the cytoplasm and in the nucleus. On the other hand, cells without GS M4 did not produce any fluorescence signal for glutathionylation (Figure 2.13 B), supporting that clickable glutathione can be used to visualize glutathionylated proteins using fluorescence imaging.
Figure 2.13 – Visualization of glutathionylated proteins by fluorescence imaging. L-AzAla was incubated with HEK 293 cells with (D-F) or without (A-C) GS M4 for 20 hours. Cells were fixed, blocked and subjected to click reaction with AF647-alkyne. Fluorescence images were captured for DAPI (A, D) and Cy5, for glutathionylation (B, E). Overlaid images shown in (C and F).

### 2.3.6 Identification of individual glutathionylated proteins

We further sought to couple a clickable glutathione approach with mass spectrometry techniques to identify new glutathionylated proteins. As a proof of concept, we carried out a pull-down experiment of glutathionylated proteins. To enrich glutathionylated proteins, cell lysates were subjected to click reaction with biotin-alkyne. Then, glutathionylated proteins were bound to streptavidin agarose beads (221). Proteins were then eluted by heating in a SDS loading buffer, and the eluted proteins were separated by running SDS-PAGE. Glutathionylated proteins were visualized by silver staining. There were significant amount of glutathionylated proteins when compared to the control (Figure 2.14 A lane 4).
Also, this method can be used to probe individual glutathionylated proteins by immunoprecipitation and Western blotting. After metabolic labeling, individual glutathionylated proteins were immunoprecipitated using relevant primary antibodies and subjected to on-bead click reaction with biotin alkyne. PTP1B and Hsp90 are known to undergo glutathionylation. Therefore, PTP1B and Hsp90 were selected for the analysis of glutathionylation by immunoprecipitation and Western blotting (5,222). After immunoprecipitation and on bead-click reaction (223), the eluted protein were probed for glutathionylation with strep-HRP.

Figure 2.14 – Identification of glutathionylated proteins. A) Lysates subjected to click reaction with biotin alkyne and biotinylated protein were enriched with streptavidin-agarose beads, and eluted. Glutathionylated proteins were visualized by silver staining. B) After metabolic labeling, HSP90 and PTP1B were immunoprecipitated, followed by on bead-click reaction with biotin alkyne. Eluted proteins were probed as shown above.
There was a strong signal of glutathionylation for both PTP1B and HSP90 after incubation of H$_2$O$_2$ (Figure 2.14 B). These data showcase that clickable glutathione can be used to enrich glutathionylated proteins and the potential mass spectrometric studies.

2.4 Discussion

Protein glutathionylation is a reversible cysteine oxidative modification that controls protein function (6). Glutathionylation is the disulfide bond formation between a GSH and cysteines in proteins that take place in the presence of ROS or RNS. Many proteins are likely to be regulated by glutathionylation (156). Therefore, identification of protein glutathionylation would make a significant contribution to understanding protein glutathionylation. Although there are several methods to study protein glutathionylation, most of tools still suffer from limitations (224). To overcome these limitations, we developed a versatile chemical method, which metabolically synthesizes clickable glutathione by a mutant of glutathione synthetase.

Glutathione biosynthesis is an enzyme catalyzed two-step process where the first enzyme GCL catalyzes the rate limiting step of the formation of dipeptide γGlu-Cys (213,225). The second enzyme GS catalyzes a ligation of Gly with γ-Glu-Cys to form γGlu-Cys-Gly tripeptide (GSH). we sought to engineer a GS active site in a way that catalyzes azide- or alkyne-containing Gly derivatives to make clickable glutathione. After performing a series of site-directed mutagenesis within vitro GS kinetic assay, we found GS M4 efficiently catalyzes L-AzAla, L-PG and L-HPG to generate clickable glutathione.

Because L-AzAla, L-HPG and L-PG are amino acid derivatives, there is a possibility of these amino acids being incorporated into proteins during translation. Consistent with previous studies, our data indicated that L-HPG was significantly
incorporated into protein biosynthesis while L-AzAla and L-PG were not incorporated into proteins (226). Although L-PG was not incorporated into proteins, L-PG did not detect glutathionylation sensitively. Therefore, we concluded that L-AzAla is the best Gly derivative to generate clickable glutathione inside the cell and to study protein glutathionylation.

Next, LC-MS analysis confirmed efficient metabolic synthesis of azido-GSH. Importantly, metabolic synthesis of azido-GSH did not affect the total thiol content of the cell. Clickable glutathione approach sensitively detected glutathionylated proteins in response to low concentrations of H$_2$O$_2$. Compared to other methods, azido functional group is small and tolerated by cellular redox enzymes such as GRX1. Pulse-chase experiment, where cells were induced for de-glutathionylation by removing ROS containing medium, showed that azido-GSH containing proteins readily undergo deglutathionylation by intracellular redox enzymes. In vitro GRX1 assay data also supported towards similar conclusion that azido-GSH can be tolerated by redox enzymes unlike other bulky glutathione derivatives such as BIOGEE.

One advantage of clickable glutathione approach is it can couple with mass spectrometry for the identification of glutathionylated proteins and their modified cysteine residues. Our data indicated that azido-GSH can use to enrich glutathionylated proteins by biotin pull-down. Bioorthogonal click reaction allows selective detection and enrichment of glutathionylated proteins. This method can be improved to identify those proteins after trypsin digestion and LC-MS/MS analysis. Further, this method can be used to identify modified cysteine residues by using a cleavable biotin-alkyne with click reaction. Selective cleavage of glutathionylated peptides can identify by mass
spectrometry. In addition to identification by mass spectrometry, azido-GSH also can be used to identify or used to validate glutathionylated proteins by Western blotting. Immunoprecipitation of individual proteins and on bead click reaction with biotin-alkyne identified several known glutathionylated proteins by probing with streptavidin-HRP. This, can also be approached by doing biotin pull-down with clicked lysate and subsequent analysis of eluted proteins by primary antibodies against protein of interest. Another important application of clickable glutathione approach is that visualization of glutathionylated proteins by fluorescence imaging in live or fixed cells. Our data indicated that glutathionylated proteins can be readily visualized by cellular fluorescence without using antibodies. This approach can further improve to visualize individual glutathionylated proteins by coupling with other advanced technologies such as proximity ligation assay. In future, these applications will allow to study the effect of site specific glutathionylation on protein interaction and their cellular localization. Overall, we have generated GS M4 mutant that catalyzes the synthesis of azido-GSH with high catalytic efficiency, in cells. Thus, our chemical approach with clickable glutathione provides a chemoselective and versatile handle for studying glutathionylation without altering the thiol content of the cell.

2.5 Experimental procedure

2.5.1 Reagents and materials

N-Ethylmaleimide (NEM), 5-(Iodoacetamido)fluorescein (FL-IA), phosphoenol pyruvate (PEP), adenosine 5′-triphosphate disodium salt hydrate (ATP), type II rabbit muscle lactate dehydrogenase (LDH), type II rabbit muscle pyruvate kinase (PK), γ-glutamylcysteine, β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate
(NADH), L-glycine (Gly), L-alanine (Ala), L-methionine (Met), L-valine (Val), copper(I) bromide, anti-FLAG antibody and anti-GS antibody were purchased from Sigma Aldrich. Streptavidin-horseradish peroxidase (HRP) was purchased from Thermo Scientific. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from GE healthcare (U.K). Polyethyleneimine “MAX” was purchased from Polysciences, Inc (USA). 3-Azido-L-alanine HCl (L-AzidoAla) was purchased from Jena Bioscience (Germany). L-homopropargyl-glycine (HPG) and L-propargyl-glycine (PG) were purchased from Life Technologies and Pep-Tech Corporation, respectively. Ni-NTA agarose was purchased from QIAGEN. EDTA-free protease inhibitor cocktail was purchased from Roche. All cell culture reagents and medium were purchased from Life Technologies.

2.5.2 Competent cell preparation

DH5α cells were used to extract plasmid DNA and BL21 cells were used for protein overexpression. To generate chemically competent DH5α and BL21 cells, first, a tiny amount from both cell stocks were streaked on two separate agar plates without any antibiotic. Agar plates were incubated in a bacterial incubator at 37 °C for overnight (approximately 16 h). On the following day, a single colony was inoculated in a 5 mL of Luria broth (LB) medium without any antibiotic and incubated in a bacterial shaker for 16 h. LB media (5 mL) containing bacterial cells were diluted into an autoclaved 1 L LB medium and incubated in a bacterial shaker until OD600 reach to 0.4. Then cells were transferred into autoclaved, ice-cold centrifuge bottles and cells were harvested by spinning at 5000 rpm for 15 minutes. After this step, all other steps were done on ice or at 4 °C. Cells were washed once with sterile filtered ice-cold 100 mL of 100 mM MgCl2
solution and centrifuged to remove supernatant. Then cells were re-suspended in 100 mL of 100 mM CaCl$_2$ and incubated for 20 minutes. After centrifugation, supernatant was removed and the resultant pellet was re-suspended in 100 mL of 85 mM CaCl$_2$ and 15% glycerol (v/v) and incubated for another 30 minutes. After the 30 minute incubation, cells were collected by centrifugation and re-suspended in 10 mL of 85 mM CaCl$_2$ and 15% glycerol (v/v) and aliquoted (100 µL) into autoclaved and chilled eppendorf tubes. Competent cells were flash frozen in liquid nitrogen and stored in -80°C freezer.

2.5.3 Long term storage of bacterial cells

A single colony was inoculated in 5 mL of LB media and incubated for 16 h in a bacterial shaker at 37°C. In the following day, a portion (750 µL) of overnight culture was mixed with 250 µL of autoclaved 65% glycerol in an eppendorf tube, flash froze in liquid nitrogen and stored in -80°C freezer.

2.5.4 Bacterial transformation

An aliquot of competent DH5$\alpha$ or BL21 cells were thawed in ice. Once competent cells are thawed, 50 µL of cells were mixed with ice-cold plasmid DNA (50 ng) and incubated on ice for 20 minutes. Then tubes were incubated in a heat block at 42°C for 45 seconds and immediately placed on ice for another 2 minutes. Then cells were mixed with 400 µL of super optimal broth with catabolite repression (SOC) medium and incubated for 30 minutes in a bacterial shaker at 37°C. Then cells were briefly collected by centrifugation at 4000 rpm for 5 minutes and a half of media was removed and cells were re-suspended in the rest of medium and evenly spread on agar plates containing appropriate antibiotic. Plates were then incubated in a bacterial incubator at 37°C for 16
h. Always included a negative control (cells without DNA) for the confirmation and to check for possible contamination.

2.5.5 Plasmid DNA isolation (mini and maxiprep)

A clump from cell stock containing plasmid DNA was inoculated in LB (5 mL for miniprep and 250 mL for maxiprep) containing appropriate antibiotic and incubated in a bacterial shaker (200 rpm) at 37 °C for 16 h. Overnight 5 mL culture was centrifuged (4000 rpm for 5 min) and cell pellet was resuspended in 250 µL of P1 solubilizing buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNAse A). Then cells were incubated with 250 µL of P2 lysis buffer (200 mM sodium hydroxide, 1% SDS) for 3-5 minutes (no more than 5 minutes). Lysis buffer was neutralized with the addition of 350 µL N3 buffer (3.0 M potassium acetate, pH 5.5) and incubated for 5 minutes at room temperature. Cell debris were precipitated by centrifugation at 13 000 rpm for 10 minutes. Clear cell lysate was carefully transferred to DNA binding spin columns without any solid debris. Extracted plasmid DNA was bound to the membrane after high speed centrifugation at 13 000 rpm for 1 minute and flow through was discarded. Then spin columns were washed twice with 500 µL of washing buffer (Qiagen) and liquid was discarded by high speed centrifugation after each wash. Bound DNA was eluted with 50 µL water by centrifugation at high speed for 1 minute. DNA concentrations were measured by nanodrop before using in cloning procedures. For DNA purification by maxiprep, manufacturer protocol was followed as described in protocols under product number K0491 from ThermoFisher Scientific.

2.5.5 Subcloning

2.5.5.1 Polymerase chain reaction (PCR)
GS WT gene was amplified from pCMV6-GS vector by performing Polymerase chain reaction (PCR). PCR was carried out using a thermocycler (Eppendorf Mastercycler Gradient). Approximately 50 ng of plasmid DNA (2 μL from 50 ng/μL stock) containing GS WT gene incubated with 5 μL of 10X ultra pfu buffer, (200 mM Tris, 100 mM (NH4)2SO4, 100 mM KCl, 1% TritonX-100, 20 mM MgSO4, 1 mg/mL BSA, pH 8.8), forward and reverse primers (1 μL from 10 μM stocks), and dNTPs (2 μL of 10 mM; 2.5 mM of each base). The final PCR reaction volume adjusted to 49 μL by adding deionized water. After mixing well, 1 μL of Pfu ultra-enzyme was added and incubated in a thermocycler programmed as indicated in the Table 2.2. Amplified DNA was purified by gel extraction method after running a DNA agarose gel as described in QIAGen gel extraction kit (cat # 28704).

**Table 2.2 - PCR program for GS amplification.**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temp (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>45 sec</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1.5 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>15 min</td>
<td>1</td>
</tr>
</tbody>
</table>

**2.5.5.2 Restriction digestion**

GS WT gene was inserted into pET28A (Novagen Catalogue number – 698643) bacterial expression vector after double restriction digestion with NdeI and HindIII. Amplified GS WT (1 μg) were incubated with 5 μL of 10X cutsmart NEB buffer and 1 μL from each NdeI (20,000 units/mL) and HindIII-HF (20,000 units/mL) restriction enzyme, 1
μL from 10 mg/mL BSA stock and finally reaction volume was set to 50 μL with deionized water. After mixing well, reaction mixture was incubated in an incubator at 37 °C. After 1 h of incubation, 10 μL from 6X DNA loading dye (10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA) was added and digested GS gene was separated in an agarose gel (120 mV for 30 minutes) and purified by gel extraction. Similarly, restriction digestion by same restriction enzymes and gel extraction was performed for empty pET28A bacterial expression vector and used for ligation.

2.5.5.3 Ligation

Double digested GS WT and pET28A were mixed in 1:3 ratio (0.020 pmol and 0.060 pmol) with 2 μL of 10X T4 DNA ligase buffer, 1 μL from 100 mM ATP stock, 1 μL from T4 DNA ligase (400,000 units/ml) and final reaction volume set to 20 μL with deionized water. Ligation mixture was mixed well and incubated in an incubator at 16 °C for 20 h. Ligase enzyme was heat inactivated by incubating ligase reaction mixture at 65 °C for 15 min. Then mixture was chilled on ice for another 15 minutes, transformed into DH5α and plated on agar plates containing 50 μg/mL Kanamycin according to the protocol described in 2.5.4. A single colony was inoculated in LB containing 50 μg/mL Kanamycin and incubated for 16 h and plasmid DNA (pET28A-GSWT) was purified using the protocol described in section 2.5.5. After purification, DNA concentration was measured by nanodrop and ligation was confirmed by DNA sequencing.

2.5.6 Site-directed QuickChange mutagenesis

GS mutants were generated by QuickChange mutagenesis. pET28A-GSWT was used as the template to generate GS M1 (F152G). pET28A-GSWT 50 ng was mixed with
5 μL of pfu buffer. Quick change forward and reverse primers (all quick-change primers are listed in Table 2.4) were incubated in two separate PCR tubes with template and pfu buffer. After adjusting the volume to 49 μL with deionized water, 1 μL of pfu ultra enzyme was added to each tube. Two tubes containing above components were incubated in a thermocycler and subjected to 2 thermocycles as indicated in the Table 2.3. After that, reaction mixtures in two tubes were mixed together and incubated in the thermocycler for another 18 cycles using the program shown in table 2.3. Reaction mixture was then incubated with 1 μL of Dpn1 (20,000 units/mL) for 1 h at 37 °C. Finally, 2 μL from Dpn1 digested PCR reaction was transformed into DH5α using the protocol described in section 2.5.4.

Table 2.3 - PCR program for QuickChange mutagenesis.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temp (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>45 sec</td>
<td>2 or 18</td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>20 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 - QuickChange primers for GS mutants.

<table>
<thead>
<tr>
<th>Mutant enzyme</th>
<th>Primer sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-primer</td>
<td>GAA ATC AAC ACC ATC TCT GCC AGC GGT GGG GGC CTG GCC TCC</td>
</tr>
</tbody>
</table>
### 2.5.7 Generation of expression vectors

Wild type GS (GS WT) gene was amplified by PCR using a forward primer with NdeI restriction site (5'-GGT GGT CATATG GCC ACC AAC TGG TGG GGG AGC-3') and a reverse primer with Hind III restriction site (5'-GGT GGT AAGCTT CTA CAC AGG GTA TGG GTT GTC CAG GAC TGC TGC-3'). Amplified GS WT PCR products were subcloned into the pET28a bacterial expression vector. All GS mutant (M1, M2, M3 and M4) constructs were made using pET28a-GS WT as the template by site-directed quick-change mutagenesis (Primers used in QuickChange mutagenesis listed in the Table S1). GS M2

<table>
<thead>
<tr>
<th>GSM1</th>
<th>R-primer GGA GGC CAG GCC CCC ACC GCT GGC AGA GAT GGT GTT GAT TTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSM2</td>
<td>F-primer GAA ATC AAC ACC ATC TCT GCC GGC GGT GGG GGC CTG GCC TCC</td>
</tr>
<tr>
<td></td>
<td>R-primer GGA GGC CAG GCC CCC ACC GCC GGC AGA GAT GGT GTT GAT TTC</td>
</tr>
<tr>
<td>GSM3</td>
<td>F-primer GAA ATC AAC ACC ATC TCT GCC GGC TTT GGG GGC CTG GCC TCC</td>
</tr>
<tr>
<td></td>
<td>R-primer GGA GGC CAG GCC CCC AAA GCC GGC AGA GAT GGT GTT GAT TTC</td>
</tr>
<tr>
<td>GS M4</td>
<td>F-primer GAA ATC AAC ACC ATC TCT GCC GGC GCT GGG GGC CTG GCC TCC</td>
</tr>
<tr>
<td></td>
<td>R-primer GGA GGC CAG GCC CCC AGC GCC GGC AGA GAT GGT GTT GAT TTC</td>
</tr>
</tbody>
</table>
clone was PCR amplified using a forward primer with HindIII restriction site and N-terminal FLAG-tag sequence (5'-GGT GGT AAG CTT ATG GAT TAC AAG GAT GAC GAT GAC AAG ATG GCC ACC AAC TGG GGG AGC-3') and a reverse primer with XhoI restriction site (5'-GGT GGT CTCGAG CTA CAC AGG GTA TGG GTT GTC CAG GAC TGC-3'). Amplified GS M2 PCR product was sub-cloned into the pCDNA3.1(+) mammalian expression vector. QuickChange mutagenesis was performed using pCDNA3.1-GS M2 as the template to introduce Kozak sequence with a forward primer (5'-GCT AGC GTT TAA ACT TAA GCT TGC CAC CAT GGA TTA CAA GGA TGA CG-3') and a reverse primer (5'-CGT CAT CCT TGT AAT CCA TGG TGG CAA GCT TAA GTT TAA ACG CTA GC-3'). pCDNA3.1-GS M2 plasmid was used to generate GS M4 for mammalian expression. For glutaredoxin (GRX1), the gene was amplified by PCR using a forward primer (5'-GAC GAC CAT ATG GCT CA GAG TTT GTG AAC TGC-3') and a reverse primer (5'-GGT GGT AAG CTT CTA GTG GTA TGG GTT GTC CAG GAC TCC AAT CTG CTT TAG-3'). GRX1 PCR product was subcloned into pET28a. All cloned plasmids were confirmed by DNA sequencing of entire open reading frames.

2.5.8 Bacterial protein expression and purification

All the bacterial expression constructs were transformed into *E. coli* BL21 (DE3) cells and transformed cells were inoculated in Luria broth (LB) media containing Kanamycin (50 μg/mL), and incubated overnight at 37 °C in a bacterial shaker. Growing bacterial cells were diluted into 1L of LB media containing kanamycin. Cells were grown at 37 °C until OD$_{600nm}$ reached at 0.8 and then induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.4 mM) at 16 °C. After 20 h of induction, cells were collected by centrifugation at 4,000 rpm for 20 min at 4 °C. Collected cells were re-suspended in
lysis buffer (50 mM Tris-HCl pH 8.3, 300 mM NaCl and 10 mM imidazole) and lysed by double passing through a chilled French press at 1,000 psi. After removing the cell debris by high speed centrifugation at 14,000 rpm for 30 min at 4 °C, supernatant was incubated with Ni-NTA beads (prewashed with lysis buffer) for 1 h at 4 °C. Lysates were then drained and beads were washed three times with wash buffer (50 mM Tris-HCl pH 8.3, 300 mM NaCl) containing 25 mM imidazole and His-tagged GS protein was eluted with elution buffer (50 mM Tris-HCl pH 8.3, 300 mM NaCl) containing 300 mM imidazole. Positive fractions were collected and dialyzed against 50 mM Tris buffer pH 7.4, 10% glycerol and 1 mM DTT overnight, followed by concentrated using a centrifugal filter device (Millipore). Protein concentration was measured by Bradford assay (Bio-Rad) using bovine serum albumin as the standard. Grx1 was expressed as described above, but with induction at 37 °C for 5 h.

2.5.9 Enzyme assay

Kinetics parameters for GS WT and other GS mutants were determined by measuring consumption of NADH with ATP-regenerating system, pyruvate kinase (PK) and lactate dehydrogenase (LDH). The NADH consumption was monitored by UV absorbance at 340 nm. The reaction mixture (0.2 mL) contained 100 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 25 mM MgCl₂, 0.2 mM NADH, 2 mM phosphoenolpyruvate (PEP), 5 mM ATP, 15 units of LDH, 10 units of PK, 2 mM γ-glutamylcysteine and 0.5 μg of GS enzyme. Different amino acids, including Gly and L-azido-Ala, were used as substrates ranging from 0.01 mM to 5 mM while γ-glutamylcysteine was kept at saturated levels. Reactions were started by adding 0.5 or 1 μg of GS WT or mutants, and the decrease of absorbance was measured at 340 nm using DU730-Beckman coulter UV/Vis
spectrophotometer. To calculate initial rates for different substrate concentrations, the linear region of the curve was considered. Initial rates were then fitted to the Michaelis-Menten equation \( V = \frac{V_{\text{max}} [S]}{K_m + [S]} \) using Graphpad prism 5.01 to calculate \( K_m \) and \( K_{\text{cat}} \) values for individual substrates.

2.5.10 Cell culture

Human embryonic kidney (HEK) 293 cells were grown in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum (FBS) and 5 U/mL of penicillin and 5 μg/mL of streptomycin. Cells were plated at least two days before being subjected to any experimental procedure in a humidified incubator at 37 °C and 5% CO₂.

2.5.11 Starting a new cell line

A cell stock stored in liquid nitrogen vapor phase was taken out and thawed in 37 °C water bath. Then cells were centrifuged at 1000 rpm for 5 minutes and supernatant was discarded. Cell pellet was resuspended in 1mL of complete medium and transferred to a 10 cm dish containing 9 mL of complete DMEM medium. Cells were incubated in a humidified incubator at 37 °C and 5% CO₂.

2.5.12 Mammalian cell splitting

When HEK293 cells are nearly 80-90% confluent, cells were washed with 10 mL of warm PBS. Then, 1 mL of 0.25% trypsin-EDTA solution was added to a 10 cm dish (0.5 mL for 6 cm dish) and incubated for 3-5 minutes at 37 °C. After trypsinization, 10 mL of complete DMEM medium was added to neutralize trypsin activity and cells were resuspended to avoid clumping. Then 1 mL of trypsinized cells were transferred to fresh
sterile 10 cm dishes containing 9 mL of complete DMEM medium and incubated in the humidified incubator at 37 °C and 5% CO₂.

2.5.13 PEI-max transfection

When cells were about 70-80% confluent, GS M4 was transfected using polyethylenimine (PEI-max) as the transfecting agent. A stock solution of PEI-max was prepared by dissolving 10 mg of PEI-max in 1 mL of autoclaved deionized water. Then solution was basified with 6 M NaOH until pH reached 7-7.5. After adjusting pH, stock solution was sterile filtered using a syringe filter (0.2 µm, 13 mm). Working solution of 1 mg/mL PEI-max solution was prepared by 10 times dilution of 10X stock solution in sterile water. First, cell culture media was removed from 10 cm dishes and replaced with 9 mL of DMEM without penstrep and FBS. To two 1.5 eppendorf tubes, 500 µL of DMEM without penstrep and FBS was added. To a one tube, 12 µL of 1X PEI-max was added and to the other tube, 5 µg of DNA was added and incubated for 10 minutes in room temperature. After ten minutes, both tubes were mixed together and a single tube containing PEI-max and DNA was incubated for another 15 minutes at room temperature. Then 1 mL of DNA-PEI-max complex was added dropwise to a single 10 cm dish, mixed gently and incubated in a mammalian cell culture incubator. After 6 h, medium containing DNA-PEI-max complex was removed and cells were incubated for another 18 or 42 h in fresh complete DMEM medium.

2.5.14 Cell lysis

To detect glutathionylation sensitively, cells must lyse immediately after removing ROS containing medium. Lysis buffer was prepared by adding 1 mL of 10% SDS, 1 mL of IGEPAL, 100 µL of TWEEN-20 to 98 mL of Tris buffer saline (TBS, pH-7.4, 150 mM
NaCl). TBS lysis buffer can store in cold room for months. To make 1 mL of complete lysis buffer, 100 µL of protease cocktail mixture (prepared by dissolving one crushed protease tablet in 2 mL of sterile water) and 50 µL from 1 M N-ethylmaleimide (NEM) were mixed with 850 µL of TBS lysis buffer. Lysis buffer was kept on ice until use. Cells were washed once with ice-cold PBS and all remaining liquids were aspirated in to the waste container. Then 500 µL of lysis buffer was immediately added on to the cells and cells were scraped out with a cell lifter. Cells were collected by lifting cell culture dish to a 45-degree angle and with a 1mL pipet. Tubes containing cells and lysis buffer were placed in a rotary shaker for 30 minutes at 4 °C. After incubation, cell debris were removed and clear cell lysate was collected to a fresh eppendorf tube after centrifugation at 14,000 rpm for 20 minutes.

2.5.15 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gel was prepared using separating and stacking buffers. Prior to that, glass plates (1.5 mm, BioRad) were washed and dried before mounting on the gel preparing rack. Separating buffer mixture for 12% gel was prepared by mixing 2 mL of 4X Tris/SDS buffer (1.5 M Tris, 0.24 M HCl; pH 8.8, 0.4% SDS), 2.4 mL of 40% acrylamide (BioRad; 37:1), 3.62 mL of distilled water, 80 µL of 10% (w/v) of freshly made ammonium persulfate (APS) and 8 µL of N, N, N', N'-tetramethylthlenediamine (TEMED; Sigma) and carefully added in between the glass plate up to ¾ of the short glass plate. After pouring separating layer, methanol or isopropanol was immedietly added over the separating layer. After 30 minutes, stacking buffer mixture was prepared (by mixing 1 mL of 4X Tris/SDS buffer (1.5 M Tris, 0.24 M HCl; pH 6.8, 0.4% SDS), 0.5 mL of 40% acrylamide, 2.48 mL of distilled water, 20 µL of fresh 10% (w/v) APS, and 4 µL TEMED)
and poured over the separating layer after removing methanol or isopropanol and drying. Immediately after pouring the stacking layer, 10-well or 15-well comb was put through the glass plates and kept for another 30 minutes before using. Once samples are ready for electrophoretic separation, comb was removed and transferred to the SDS-PAGE running container together with electrodes. Then, SDS-running buffer (1X; 0.025 M Tris; 0.05 M glycine 0.5% SDS) was poured up to the level indicated in the container and closed the lid before running for desired time at 150 V or 200 V. After running the gel, glass plates were separated, gel was exposed and washed with water before staining or Western blotting.

2.5.16 Coomassie staining

After running SDS-PAGE, protein containing gel was washed twice in distilled water and fixed in fixing solution (50% methanol and 10 glacial acetic acid) for 30 minutes. Then fixing solution was discarded and the gel was stained in staining solution (0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% glacial acetic acid) for 1 h after microwaving for 1 minute at full power or incubated overnight without microwaving. Before destaining, gel was washed three times with distilled water to remove excess staining solution. Then the gel was destained in 40% methanol and 10% glacial acetic acid overnight. The destained gel stored in 5% glacial aceic acid until imaging.

2.5.17 Western blotting

To transfer protein bands in SDS-PAGE gel to a PVDF membrane, Westen blotting was carried out. First, stacking layer of the gel was removed and separating layer was washed several times using distilled water and transfer buffer (10% methanol, 24 mM Tris and 194 mM glycine). To make the gel sandwich, black side of the cassette was placed
on the surface of the bench and pre-wetted fiber pad was placed on the top of it. Then pre-wetted filter paper and the gel was placed on the fiber pad one after the other. Then pre-wetted membrane was placed on the top of the gel followed by the second filter paper and fiber pad to complete the sandwich. At this stage, it is critical to remove any air bubbles from the membrane before closing and locking the cassette. Place the cassette in the module facing black side of the cassette to black side of the module. Take the whole set up to the transfer container with an ice block and fill the tank with transfer buffer. Transfer is carried out at 90 V for 2 h on ice. After the transfer, cassette was unlocked by unclamping the white latch and fiber and filter paper was removed. The membrane was washed once with 1X TBST and proceeded to the antibody probing.

2.5.18 Metabolic labeling and click reaction

At 70-80% confluence, 2.5 μg of GS M4 plasmid was transfected using PEI-Max as the transfecting agent in 6 cm dishes. After 48 hours, different concentrations of L-azido-Ala, HPG and PG were incubated (separately in individual experiments) with cells for another 20 h or varying time periods. Cells were then treated with hydrogen peroxide for 15 min. For glutathionylation turn-over studies, the medium containing hydrogen peroxide was removed and replaced by fresh medium. Cells were then lysed in TBS-Tween lysis (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% IGEPAL, 0.1% TWEEN-20) containing 50 mM N-ethylmaleimide and EDTA-free protease inhibitor. Protein concentration was determined by Bradford assay. For click reactions, 100 μg of cell lysates were precipitated by adding cold acetone (4 times volume to cell lysates) and incubation at -20 °C for 1 hour. The precipitate was re-dissolved in 100 mM Tris buffer pH 7.4. Lysates were then mixed with click reagents, [0.1 mM biotin-alkyne, rhodamine-
alkyne or rhodamine-azide, 0.2 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) and 0.4 mM CuBr] for 1 h at RT. To monitor in vitro deglutathionylation by Grx1 enzyme, lysate was incubated with 10 µg of purified GRX1 in the presence of 1.5 mM GSH for 30 min at 37 °C. Lysates were then subjected to click reactions. Lysates labeled by click reaction were resolved on 12% SDS-PAGE, followed by Western blotting or direct detection of in-gel fluorescence. After transferring to PVDF membrane, the membrane was blocked with 5% BSA in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) and incubated with streptavidin-HRP (1:1000) in 5% BSA in TBST for 2 h at RT, and analyzed for chemiluminescence to visualize biotinylated proteome. Anti-GS (1:1000) and anti-FLAG antibody (1:1000) and appropriate HRP-conjugated secondary antibodies were used to visualize expression levels of GS WT or M4 mutant.

2.5.19 LC-MS analysis of glutathione

To monitor in vitro biosynthesis of glutathione and glutathione derivatives, in vitro assay was analyzed by LC-MS (SHIMADZU, Nexera X2). In a reaction mixture, 5 mM γ-glutamylcysteine, 10 mM ATP, 5 mM Gly, 5 mM Met and 5 mM Azido-Ala were incubated with GS WT, GS M4, or a mixture of GS WT and GS M4 in a ratio of 1:5 for 2 h at 37 °C. The reaction was quenched by spin-filtering enzymes with centrifugal device (MW cutoff 3,000 Da, Millipore). The filtrate free of proteins was treated with fluorescein-iodoacetamide (FL-IA) (1.5 mM) for 1 h at RT and injected into LC-MS. To monitor biosynthesis of azido-glutathione in cells, cells were lysed in 1xPBS by repeating freeze-thaw three times in the presence or absence FL-IA. Lysates were spin-filtered through centrifugal device (MW cutoff 3,000 Da) to remove proteins. Proteins-free filtrate was then injected into LC-MS. In mass ion chromatogram, the masses corresponding to
glutathione or glutathione derivatives were extracted. Individual mass extraction ion chromatograms were overlaid.

2.5.20 Bromobimane assay

To calculate the overall thiol content, protein-free lysates were incubated with 0.5 mM bromobimaneS5 in 0.1 M Tris buffer for 30 min at 25 °C in the dark. Fluorescence (λex 390 nm and λem 478 nm) was measured using Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek).

2.5.21 Fluorescence imaging of glutathionylation

HEK 293 cells were grown on glass cover slips in a six well plate. At about 40% confluence, HEK 293 cells were transfected with GS M4 mutant, using PEI-Max as the transfecting agent. Cells with or without GS M4 mutant were incubated with L-azido-Ala (1 mM) for 20 h, and treated with H2O2 (1 mM) for 15 min. Cells were then washed with PBS once, fixed with cold methanol for 15 min at -20 °C, and washed with PBS three times. All free thiols were blocked by N-ethylmaleimide (50 mM) for 15 min at RT. After washing with PBS, cells were blocked with 1xTBS containing 0.1% TWEEN-20 and 3% BSA for 30 min at RT. Cells were rinsed twice with PBS, and subjected to click reaction in 1xTBS in the presence of TBTA (0.1mM) and CuBr (0.2 mM) and Alexa Fluor 647-alkyne (Invitrogen) (20 μM) for 30 min at RT. After extensive washing with PBS, the glass cover slips were mounted on microscope slides with a mounting solution containing DAPI. Slides were stored at 4 °C in dark until imaging. Fluorescence images were monitored and captured with Olympus fluorescence microscopy (BX 41) for nucleus staining with a DAPI channel (λex 350 nm, λem 470 nm) or for glutathionylation with Cy5 channel (λex 650 nm, λem 670 nm).
2.5.22 Enrichment of glutathionylated proteins by streptavidin-agarose

HEK 293 cell lysates (2 mg) were prepared after GS M4 transfection and incubation of L-AzidoAla (1 mM). Lysates were subjected to click reaction with biotin-alkyne. Reaction was performed at RT for 1 h in 1xTBS (0.5 mL) containing 0.2 mM biotin-alkyne, 0.4 mM TBTA and 1 mM CuBr with gentle vortex at least twice during the reaction. Each reaction tube was incubated with 1 mL of ice-cold acetone for 30 minutes at -20 °C. Precipitated proteins were centrifuged at 6500xg for 4 min. Protein pellets were washed with cold methanol (0.5 mL) twice, followed by centrifugation. Proteins were re-dissolved with a brief sonication in 1xTBST (0.2 mL) containing 1.2% SDS. The final SDS concentration was diluted to 0.2% by adding 1xTBST (1 mL). Proteins were then mixed with pre-washed streptavidin-agarose beads (100 μL) for 1 h at RT. Streptavidin-agarose beads were washed three times with 1xTBST and eluted with 1X SDS loading buffer (100 μL). Proteins were separated by SDS-PAGE. Enriched glutathionylated proteins were visualized by silver staining.

2.5.23 Identification of individual glutathionylated proteins (Immunoprecipitation and on bead click)

HEK 293 cell lysates (1 mg) were prepared after GS M4 transfection and incubation of L-AzidoAla (1 mM). Lysates were incubated with primary antibodies for PTP1B (SantaCruz) and HSP90 (BD Bioscience) at 4 °C. After 1 hour, cell lysates were incubated with pre-washed protein G agarose (Invitrogen) (50 μL) overnight at 4 °C. After washing three times with 1xTBST (0.5 mL), proteins on beads were subjected to on bead-click reaction with biotin-alkyne. Click reaction was performed in 1xTBST containing biotin-alkyne (0.2 mM), TBTA (0.4 mM) and CuBr (1 mM) for 20 h at 4 °C. Proteins on
beads were then washed with TBST (1 mL) three times and eluted with a SDS-loading buffer. Eluted samples were separated on SDS-PAGE, and probed with streptavidin-HRP (1:1000, 3% BSA in TBST) or primary antibodies for PTP1B (1:1000 dilution) and HSP90 (1:1000 dilution), followed by secondary antibodies.

2.5.24 Determination of protein concentration using Bradford protein assay

Cell lysates were diluted by 20X and 40X using distilled water. First, 19 µL and 39 µL of distilled water were transferred to two 500 µL eppendorf tubes. Then 1 µL from lysate was mixed in each tube to give 20X and 40X diluted lysates. Out of 20 µL and 40 µL of total diluted mixture, 10 µL was pipetted out and transferred to two wells of clear 96-well plate. Then 200 µL from quick start 1X Bradford reagent was mixed and air bubbles were removed using a needle. Absorbance was then measured at 595 nm using a plate reader. Lysate concentrations were calculated using the standard curve equation. For standard curve, 10 mg/mL BSA was serially diluted to make 0.5, 0.4, 0.25, 0.15 and 0.05 mg/mL. In a similar manner, 10 µL from each standard BSA solution was mixed with 200 µL of quick start 1X Bradford reagent and absorbance was measured at 595 nm.

2.5.25 Silver staining

After electrophoresis, gel was washed for 5 minutes with distilled water. Washing continued for another two times before fixation. Then gel was incubated in fixing solution (30% ethanol, 10% acetic acid) for 15 minutes. Fixing solution was discarded and fix again with fresh fixing solution for 15 minutes. After fixation, gel was washed twice with 10% ethanol solution for 10 minutes at room temperature followed by two time washing with distilled water for another 10 minutes. Sensitizer solution (Pierce Silver Stain Kit, catalog number 24612) was prepared by mixing 25 µL of sensitizer solution in 10 mL of
distilled water and the gel was sensitized for 1 minute and two washings with distilled water for 1 minute each. Next, gel was stained in stain working solution (0.5 mL of Silver Stain Enhancer in 10 mL of Stain solution) for 30 minutes. Gel was washed with distilled water for 20 seconds twice quickly before developing the stain with developer working solution (0.25 µL of Enhancer in 10 mL of Developer solution) for 3-5 minutes until protein bands appear. Staining was quenched by the addition of 5% acetic acid solution. Gel was washed well with distilled water before taking pictures.
CHAPTER 3 CHARACTERIZATION OF GLUTATHIONYLATED PROTEINS IN RESPONSE TO METABOLIC ALTERATION

3.1 Introduction

Glucose metabolism provides necessary metabolites for generation of ATP via the electron transport chain (ETC) in mitochondria (227). Reactive oxygen species (ROS) are produced in mitochondria as a byproduct of respiration (228). Many cancer cells have an elevated level of ROS when compared to normal cells, partially due to their increased cellular metabolism (229). Importantly, glucose or its metabolite is fluxed into pentose phosphate pathway (PPP) to generate NADPH, a reducing source for many redox enzymes that are important for maintaining redox homeostasis. Therefore, cancer cells appear to rely on excess glucose availability and its metabolism for both energy generation and redox homeostasis (230). An elevated level of mitochondrial ROS in cancer cells can trigger redox-mediated signaling associated with cancer cell phenotypes, such as increased proliferation rate, decreased apoptosis, cell survival and migration (231-234). Similarly, high levels of mROS were observed in pancreatic β cells, in response to high glucose or hyperglycemia (235-237).

On the other hand, a low concentration of glucose in cells also induces generation of ROS (238,239). One study showed that increased levels of H$_2$O$_2$ and superoxide were produced in isolated rat β cells when they were exposed to a low concentration of glucose (0-5 mM) (240). Similar response was observed also in several insulin-secreting cells (241,242). Cells with a low level of glucose can produce superoxide specifically by mitochondrial complex 1. ROS production is further intensified, partially due to a low expression level or activity of anti-oxidant enzymes (243-246). Under these conditions, it was shown that AMP activated kinase (AMPK) initiates apoptosis in pancreatic β cells.
Since AMPK activation is interconnected with mTOR/autophagic pathway, a low level of cellular glucose also plays a role in autophagy (248,249).

In addition, it is reported that glucose depletion together with hypoxia increases the production of ROS via activating unfolded protein response (UPR) (250). The UPR is known to induce ROS by several enzyme-catalyzed reactions, such as protein disulfide isomerase and ER oxidoreductin 1 (251,252). UPR is also associated with cell death by inducing apoptosis through activating ATF4, C/EBP-homologous protein (CHOP) and caspases-12, highlighting significant cellular phenotypes in response to glucose depletion or metabolism (250,253,254). It is further shown that glucose deprivation induces cell death in MCF7/ADR cells via activation of mitogen-activated protein kinase (MAPK) pathway (255).

NADPH is an essential reducing agent, which is important for maintaining cellular redox homeostasis (256). PPP is one of NADPH synthesizing processes in cell. In cancer cells, glucose is fluxed into PPP to produce NADPH, which is necessary for anabolic reactions and detoxification of reactive species. Thus, glucose depletion slows down NADPH production and disturbs redox homeostasis and ROS detoxification (225). Glucose depletion also increase ROS production in the mitochondrial electron transport chains, contributing to a metabolic oxidative stress in the cell (255).

Age-related diseases, including cancer and diabetes, are associated with an increased production of mROS. High levels of ROS leads to mitochondrial DNA mutations. Mitochondrial dysfunction is a common feature of many age-related diseases. Although mitochondrial dysfunction and glucose depletion are involved in production of
mROS, glutathionylation is not systematically evaluated under glucose depletion and/or mitochondrial dysfunction.

### 3.2 Approach

To induce mitochondrial ROS, we used the electron transport chain blockers. Antimycin A (AMA) and rotenone are well-known for blocking the ETC, which generates ROS at complex III and complex I, respectively (257). To investigate protein glutathionylation in the presence of AMA or rotenone, we employed our clickable glutathione approach with GS M4 mutant (211). First, we generated HEK293-GS M4 stable cells which stably overexpress a GS M4 mutant. L-AzAla was then incubated with HEK293-GS M4 cells for 20 h. After serum starvation for another 4 h, cells were treated with AMA (2 μg/mL) with or without glucose for different time points. Glutathionylated proteins were visualized by a bioorthogonal click reaction using fluorophore-alkyne or biotin-alkyne. Glutathionylated proteins were further identified by LC/MS/MS analysis (Figure 15).

![Figure 3.1 - The work flow for identifying glutathionylated proteins in response to mitochondrial ROS.](image)
3.3 Results

3.3.1 Characterization of HEK293-GS M4 stable cells for the generation of azido-GSH

First, HEK293-GS M4 cell line was evaluated for synthesis of clickable glutathione. After generation of HEK293-GS M4 stable cell line, L-AzAla was incubated for the synthesis of azido-GSH. It was found that cells generated the similar amount of azido-GSH (N$_3$-GSH) to endogenous GSH within 20 h (Figure 3.2 A). In addition, a free thiol content and several redox enzyme levels were examined in HEK293-GS M4 stable cells. HEK293-GS M4 and HEK293 cells showed the similar thiol concentrations and expression levels of redox enzymes (Figure 3.2 B and C). Therefore, these data support that the stable expression of GS M4 in HEK293/GS M4 did not alter an intracellular redox state significantly.
Figure 3.2 - Evaluation of azido-glutathione (N$_3$GSH) biosynthesis, cellular thiol concentration and redox enzyme levels in HEK293-GS M4 cells. (A) HEK293 cells, stably expressing GS M4, was incubated with (bottom) or without (top) azido-Ala for 20 h. Cell lysates were injected for LC-MS and extracted ion mass of endogenous glutathione (black) and azido-glutathione (pink) were overlaid. B) Protein free cell lysates were analyzed for free thiol content by bromobimane assay. C) Cells were lysed using TBST (0.1% TWEEN20) and checked for redox enzyme levels including TRX1 and GRX1.

3.3.2 Mitochondrial ROS and protein glutathionylation

After confirming efficient synthesis of azido glutathione in stable cells, we next evaluated glutathionylation in response to mROS. To study protein glutathionylation, HEK293-GS M4 stable cells were incubated with azido-Ala (0.6 mM) for 20 h. Cells were
then washed once with PBS. After serum starvation for 4 h, different concentrations of AMA were added to cells for different time points with or without glucose. After click reaction with fluorophore-alkyne, glutathionylated proteins were visualized by in-gel fluorescence. An incubation of AMA to KEK293/GS M4 cells for 2 h did not induce glutathionylation (Figure 3.3, lane 3). However, AMA induced glutathionylation in a time-dependent manner. Incubation of AMA for 22 h induced a significant intensity of glutathionylation (Figure 3.3, lane 4).

**Figure 3.3 - Glutathionylation in response to mitochondrial ROS generation.** HEK293-GS M4 cells were treated with L-AzAla for 20 h. Then AMA was incubated for different time points. After lysing, cell lysates were subjected to click reaction with fluorophore alkyne and analyzed by in-gel fluorescence.
3.3.3 Glucose availability controls protein glutathionylation under mitochondrial dysfunction

To investigate the role of cellular metabolism in glutathionylation, we evaluated HEK293-GS M4 cells in the presence and absence of glucose or glutamate. Cells were serum starved for 4 h and treated with glucose or glutamine free medium for 2 h. After cell lysis, lysates were analyzed for glutathionylation by in-gel fluorescence. Both glucose (Figure 3.4 lane 2) and glutamine (Figure 3.4 lane 4) removal did not induce glutathionylation within 2 h period. This suggests shorter incubation time was not sufficient to induce enough stress in the cells. Next, we evaluated how glutathionylation will be changed upon incubation of AMA in the absence of glucose.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Glucose (mM)</th>
<th>Glutamine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3.4 – Glutathionylation in response to depletion of glucose and glutamate. After incubation of azido-Ala to HEK293-GS M4 cells for 20 h, cells were subjected to the following stimulus. Cells were incubated in glucose-free medium and glutamine free medium for 2h. Cells
were lysed and performed click reaction with cy-5 alkyne and analyzed by in-gel fluorescence after running SDS-PAGE.

Cells were treated with AMA in the presence and absence of glucose. Glutathionylation was significantly induced within 1 h in response to AMA in the absence of glucose (Figure 3.5 A, lane 4, 5 and 6). On the other hand, an incubation of AMA at a high concentration of glucose (25 mM) did not induce glutathionylation (Figure 3.5 A, lane 3). These data clearly indicate that glucose availability is important to induce glutathionylation. Also, under glucose starvation, glutathionylation was induced by AMA in a dose dependent manner. Importantly, the level of glutathionylation was inversely correlated with glucose availability. When cells were incubated with decreasing concentrations of glucose in the presence of AMA, the level of glutathionylation was increased, indicating dependence of glutathionylation on glucose availability (Figure 3.5 C).
Figure 3.5 - Protein glutathionylation is highly dependent on glucose metabolism in the presence of the electron transport chain blocker. After incubation of azido-Ala to HEK293-GS M4 cells, cells were subjected to the following stimulus. (A) glucose starvation with antimycin A in a time-dependent manner, (B) glucose starvation with an increasing concentration of antimycin A, (C) decreasing glucose concentrations with antimycin A. After stimulus, cell lysates were subjected to click reaction with rhodamine-alkyne, and analyzed for in-gel fluorescence.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Antimycin A (μg/mL)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Time (h)</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Figure 3.6 - Glutathionylation under physiological glucose concentration. L-AzAla treated cells were incubated in 5 mM glucose containing medium for different time intervals with or without AMA treatment. After lysing and click reaction with cy-5 alkyne glutathionylation was analyzed by in-gel fluorescence. Increasing incubation time of antimycin A in a physiological glucose concentration (5 mM).

Similarly, glutathionylation was induced under a physiological glucose concentration (5 mM) when cells were treated with AMA for different times (Figure 3.6). HEK293-GS M4 stable cells displayed a time dependent increment of glutathionylation...
when treated with increasing concentrations of AMA under a physiological glucose concentration (5 mM). This suggests that ETC blockers alter the cellular redox state by generating high levels of ROS under limiting glucose concentrations, thereby inducing protein glutathionylation. More importantly, this study found that glucose metabolism is an important factor for controlling the level of protein glutathionylation.

3.3.4 Glutathionylation under glycolysis inhibition and mitochondrial dysfunction

To further confirm that glucose metabolism is important for protein glutathionylation, we examined glutathionylation after inhibiting glycolysis by a known pharmacological inhibitor. 2-deoxyglucose (2-DG) is an analogue of glucose which is lack of 2-hydroxyl group. 2-deoxyglucose binds and inhibits hexokinase, which decreases glycolysis (258). 2-deoxyglucose has been used to study the importance of glucose metabolism (259). In this project, we evaluated glutathionylation in response to glycolysis inhibition, which is likely to mimic glucose depletion.
Figure 3.7 - Induction of glutathionylation by treatment of 2-deoxyglucose (2-DG) with electron transport chain blockers. After incubation of azido-Ala to HEK293-GS M4 cells for 20 h, cells were serum-starved for 4 h. Cells were then incubated in glucose (5 mM) and antimycin A (2 µg/mL) with an increasing concentration of 2-DG for 2 h. After stimulus, cell lysates were subjected to click reaction with rhodamine-alkyne, and analyzed for in-gel fluorescence.

HEK293/GS M4 cells were incubated with L-AzAla (0.6 mM) for 20 h, followed by serum starvation and incubation of 2-deoxyglucose different concentrations in the presence of 2 µg/mL of AMA for 2 h. All conditions were carried out in the presence of 5 mM glucose in the medium. After cell lysis and click reaction with cy-5 alkyne, glutathionylation was analyzed by in-gel fluorescence. In the absence of AMA and 2-DG, glutathionylation did not induce in response to a physiological glucose concentration. However, glutathionylation was significantly induced when cells were treated with AMA and 2-DG under similar condition (Figure 3.7). Interestingly, glutathionylation was further increased with an incubation of increasing concentrations of 2-DG, indicating AMA induces glutathionylation in response to glycolysis inhibition (Figure 3.7).

3.3.5 Reversibility of glutathionylation controlled by glucose availability

Next, we evaluated whether glutathionylation is reversible depending on glucose availability. After inducing glutathionylation under glucose starvation with AMA, we re-introduced glucose to glucose starved cells. Interestingly, glutathionylation signal was decreased when glucose was re-introduced to cells, showing the importance of glucose availability (Figure 3.8 A lane 6-8).

Interestingly, the similar deglutathionylation was observed when pyruvate was added to cells instead of glucose. pyruvate is an intermediate metabolite of glycolysis (Figure 3.8 A lane 3-5). glutathionylation level was decreased upon addition of pyruvate.
However, addition of lactate, a reduced form of pyruvate, did not change the level of glutathionylation (Figure 3.8 B). Overall, these data prove that glucose acts as a key regulator for maintaining redox state of the cells and regulating the level of global protein glutathionylation.

<table>
<thead>
<tr>
<th>A</th>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Antimycin A (μg/mL)</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Addition after 2 h</td>
<td>-</td>
<td>-</td>
<td>Py</td>
<td>Py</td>
<td>Py</td>
<td>GL</td>
<td>GL</td>
<td>GL</td>
<td>-</td>
</tr>
<tr>
<td>Time after addition (min)</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>60</td>
<td>120</td>
<td>20</td>
<td>60</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 3.8 - Glutathionylation is reversed by re-addition of glucose.** After incubation of azido-Ala to HEK293-GS M4 cells for 20 h, glutathionylation was induced by glucose starvation with the treatment of antimycin A for 2 h. Subsequently, (A) glucose (GL, 5 mM), pyruvate (Py, 5 mM) or (B) lactate (La, 10 mM) was individually added to the cells for the indicated time periods. Cell lysates were subjected to click reaction with rhodamine-alkyne, and analyzed for fluorescence.

### 3.3.6 Glutathionylation by different ETC blockers

ETC blockers increase ROS generation at different sites in oxidative phosphorylation. In order to investigate the differential effects of ETC blockers, HEK293/GS M4 cells were treated with inhibitors of complex I and complex III. In a similar
manner to AMA, an incubation of rotenone, a complex I inhibitor, induced glutathionylation under glucose starvation. (Figure 3.9 A and B). Next, we analyzed and compared glutathionylation upon incubation of two complex III inhibitors, myxothiazol and AMA. Both complex III inhibitors induced the similar level of glutathionylation under glucose deprivation, indicating similar effects on glutathionylation by different ETC blockers.

Figure 3.9 - Glutathionylation in response to ETC blockers and glucose deprivation. A) After incubating stable cells with L-AzAla, cells were induced with rotenone different concentrations with or without glucose. B) Direct comparison of the effect of AMA and complex I inhibitor rotenone. C) Direct comparison of AMA and another complex III inhibitor myxothiazol.

3.3.7 ROS and glutathione levels in response to glucose deprivation and mitochondrial dysfunction

The ETC blockers, including AMA, are known to induce ROS and alter the redox state of the cell (260). To investigate how AMA affects cellular redox state, we analyzed ROS levels, GSH and GSSG upon incubation of AMA in the presence or absence of
glucose. HEK293/GS M4 cells were incubated with L-AzAla (0.6 mM) for 20 h. Cells were then induced for glutathionylation. The level of ROS was measured by 2′,7′-Dichlorofluorescin diacetate (DCF) for 1 h at 37 °C in phenol red free DMEM. An incubation of AMA or glucose depletion increased the level of ROS. Obviously, the level of ROS was further increased when AMA was treated to cells under glucose depletion (Figure 3.10 A).

Figure 3.10 - ROS and glutathione levels in HEK293-GS M4 stable cells. A) ROS levels were determined by DCF assay in AMA treated and non-treated cells in the presence and absence of glucose. Thiol levels were determined by bromobimane fluorescence assay after inducing with
AMA with or without glucose supply. After lysis by freeze and thawing, protein free lysates were reacted with bromobimane and fluorescence data for B) reduce thiol levels C) total thiol levels were measured using a plate reader. D) By subtracting reduce GSH from total GSH level, oxidized GSH was calculated and shown as a GSSG/GSH ratio.

The cellular level of GSH was also measured by bromobimane assay. Cells were lysed by several freeze-thaw cycles in PBS and protein-free lysates were analyzed for thiols. In the presence of AMA, the level of reduced GSH was decreased in a time-dependent manner (Figure 3.10 B). However, total thiol concentration did not change upon incubation of AMA (Figure 3.10 C). Overall, the ratio of GSSG/GSH was significantly increased when cells were treated with AMA for 2 h under glucose starvation, suggesting a significant oxidative stress in cells. These data suggest an important role for glucose metabolism in maintaining ROS levels.

3.3.8 Protein glutathionylation in response to glucose deprivation

We further evaluated glutathionylation under glucose starvation condition without using the ETC blockers. HEK293/GSM4 cells were subjected to glucose starvation with different incubation times. Depletion of glucose in HEK293/GSM4 cells did not induce glutathionylation significantly within 5 h, but induced a strong level of glutathionylation at 20 h (Figure 3.11 A and B).
Figure 3.11 - Clickable GSH approach for detection of protein glutathionylation in response to different glucose concentrations. A) Stable cells were treated with different concentrations of glucose for 5 h. Lysates were analyzed by in-gel fluorescence after the click reaction with Cy5-Alkyne. B) Stable cells were treated with glucose free media for 20 h. Then analyzed by in-gel fluorescence. C) Detection of protein glutathionylation in glucose dose dependent manner for 20 h.

Importantly, the level of glutathionylation was increased with decreasing concentrations of glucose. Interestingly, glutathionylation were readily observed even at physiological (5 mM) and hypoglycemic (3 mM) concentrations. (Figure 3.11 C). It was also found that glutathionylation was induced under glucose deprivation for 13 h (Figure B.3.1). All together, these data further support that glucose availability is an important factor for regulation of glutathionylation.
3.3.9 Glutathionylation in response to oxygen availability

It was known that oxygen availability is an important factor for generation of ROS. We next evaluated whether glutathionylation is induced depending on oxygen availability. Cells were subjected to different conditions of oxygen and glucose concentrations. L-AzAla was incubated with HEK293-GS M4 cells for 20 h. Two cell culture dishes, containing high glucose and no glucose medium were incubated under normal oxygen concentration (21%). Another two cell culture dishes were incubated with or without glucose, inside a hypoxic modular chamber saturated with low oxygen concentration (1%). Cells were lysed immediately after incubation and glutathionylation was detected by in-gel fluorescence after click reaction. Glucose starvation under normoxic condition (21% oxygen) induced a significant level of glutathionylation (Figure 3.12 lane 2 vs 1). However, glucose starvation under hypoxic condition (1% oxygen) did not show a significant level of glutathionylation induction (Figure 3.12 lane 3 and 4). These data indicated that oxygen is necessary for inducing glutathionylation under glucose deprivation.

The induction of hypoxia was confirmed by monitoring the elevated level of hypoxia inducible factor 1 α (HIF1α) (Figure 3.14 A). In addition, redox enzymes, such as GRX1 and catalase, did not significantly change under hypoxic or normoxic conditions, suggesting the detected glutathionylation difference is not due to a change in redox enzyme levels (Figure 3.14 B). Next, we evaluated glutathionylation under different oxygen concentrations. Consistently, we did not observe detectable level of glutathionylation when oxygen concentration maintained at 0 or 1% (Figure 3.13 lane 1-6). In contrast, glutathionylation was readily induced with an increasing oxygen concentration.
concentrations (5 or 21%) under glucose starvation condition. These data showed a dose dependent increment of glutathionylation in response to different oxygen supply (Figure 3.13 lane 8 and 10). Overall, these data support the fact that oxygen availability is an important factor for inducing protein glutathionylation.

<table>
<thead>
<tr>
<th>HEK293-GSM4</th>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-AzAla</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(1%) Hypoxia 13h</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.12 – Glutathionylation under a limited oxygen supply.** Stable cells were incubated with L-AzAla and incubated with or without glucose in a hypoxia chamber containing 1 and 21% oxygen. After 13 h cells were immediately lysed and performed click reaction and analyzed by in-gel fluorescence.
Figure 3.13 - Oxygen availability regulates protein glutathionylation. HEK293-GSM4 stable cells were incubated with 0.6 mM L-AzAla for 20 h and media was replaced with serum free medium containing different amounts of glucose prior to the incubation in hypoxic chamber containing 0%, 1%, 5% and 21% oxygen. Cells were lysed and analyzed by in-gel fluorescence after click reaction.
Figure 3.14 – Levels of HIF1α and redox enzymes in response to glucose-oxygen depletion. A) Cell lysates, prepared from normoxic and hypoxic conditions were analyzed by Western blotting for the induction of HIF1α and B) the detection of redox enzymes including GRX1 and catalase.

Next, we evaluated glutathionylation in a hypoxic condition with subsequent re-oxygenation. HEK293-GS M4 stable cells were first subjected to oxygen-glucose-depletion (OGD) (1% O₂ and no glucose) for 8 h. Subsequently, cells were exposed to a normal oxygen atmosphere for 0, 20 and 120 minutes under normal or depleted glucose conditions. cells were then lysed and analyzed for glutathionylation after performing click reaction with Cy5-Alkyne (Figure 3.15). Glucose deprivation or OGD did not induce a significant level of glutathionylation during 8 h. (Figure 3.15 lane 1-6). However, we observed time dependent glutathionylation in cells after OGD and re-oxygenation for 20 and 120 minutes (Figure 3.15 lane 9 and 12). Similar results were observed in HEK293-GS M4 cells after 13 h OGD and 1h re-oxygenation experiment (Figure B.3.3). Altogether, these data indicated that oxygen availability is important for inducing glutathionylation under prolonged glucose starvation.
Figure 3.15 - Detection of the effect of re-oxygenation on protein glutathionylation. After L-AzAla incubation for 20 h, HEK293-GS M4 cells were maintained under 1% oxygen with or without glucose for 8 h (OGD). Then cells were exposed to 21% oxygen for different time points (OGD/R). Cells were lysed at indicated time points and analyzed by in-gel fluorescence after click reaction with Cy-5 Alkyne.

3.3.10 Identification of glutathionylated proteins using LC-MS/MS

Glucose starvation together with AMA treatment induces glutathionylation in numerous proteins. To identify these proteins, we used a biotin pull-down experiment followed by LC-MS/MS analysis (Figure 3.16 A). Global glutathionylation was induced in
HEK293-GS M4 cells after treating AMA together with glucose deprivation. After induction, cells were lysed and subjected to click reaction with biotin alkyne.

Figure 3.16 – Biotin pull-down and elution for analysis of glutathionylated proteins. A) Approach for pull-down and mass analysis: after click reaction with biotin-alkyne, glutathionylated proteins enriched by streptavidin-agarose were eluted for silver staining or directly digested on-beads for LC-MS/MS analysis. B) Analysis of enriched proteins by silver staining.

Biotinylated proteins were then incubated with streptavidin agarose beads and eluted by a SDS loading dye. Eluted samples were analyzed by silver staining. Glutathionylated proteins were significantly enriched in a condition of glucose starvation with incubation of AMA. In contrast, enrichment of glutathionylated proteins were minimum with high glucose (25 mM) and lack of AMA treatment (Figure 3.16 B lane 4 against lane 1-3).
Figure 3.17 – Proteomic analysis of glutathionylated proteins. A) The number of identified glutathionylated proteins by LC-MS/MS analysis are displayed as a venn diagram. ‘Positive’ and ‘negative’ indicate proteins enriched in Figure 3.16 B lane 4 and 1, respectively. B) Comparison of identified proteins according to the spectral count in positive and negative samples.

We further analyzed the samples prepared for lane 1 and lane 4 (Figure 3.16 B) by mass spectrometry. After enrichment of glutathionylated proteins on streptavidin beads, proteins were digested with trypsin on bead. Then eluted samples were subjected to LC-MS/MS analysis. From mass analysis, we identified 1249 candidate proteins in the positive sample (lane 4 in Figure 3.15 B) and 278 proteins in the negative sample (lane 1 in Figure 3.15 B). Relatively smaller number of proteins (244) were found in both positive and negative samples (Figure 3.17 A). More importantly, 1291 proteins had 10-fold or higher enrichment in the positive sample over negative sample (Figure 3.17 B).
We analyzed identified proteins using the DAVID gene ontology program. This analysis allows us to categorize proteins depending on their localization, molecular function and cellular process. The majority of glutathionylated proteins were found in cytosol. Proteins were involved in many different cellular processes, such as RNA processing, cell cycle, DNA repair, aging and stress sensing (Figure 3.18 C, D, E)). In addition, we compared identified glutathionylated proteins with previously known glutathionylated proteins (figure 3.19 A). It was found that a half of candidate proteins that we identified were previously reported for glutathionylation, but there were 800 new glutathionylated proteins in our LC-MS/MS analysis. Also, we compared our data with sulfenylated proteins reported previously (figure 3.18 B). Approximately a half of sulfenylated proteins were found as potential candidate proteins for glutathionylation in our analysis, indicating a close interconnection between sulfenylation and glutathionylation. In addition, top 120 proteins that were found in our mass analysis with high spectral counts were analyzed by DAVID Program for their localization, cellular function and processes, which is listed in a table. Also, these proteins were compared with databases of previously reported glutathionylated and sulfenylated proteins (Table B.3.1). These data provide evidences for the sensitivity and the versatility of clickable GSH in studying protein glutathionylation.
Figure 3.18 – Gene ontology analysis of identified glutathionylated proteins. A) Comparison of glutathionylated proteins in glucose starvation/antimycin A with previous proteomic data of glutathionylome B) or sulfenylome by treatment of exogenous H$_2$O$_2$. C) DAVID gene ontology (GO) analysis of identified glutathionylated proteome. Identified proteins were annotated to depending on subcellular location, D) molecular function and E) cellular processes.
3.3.11 Validation of glutathionylated proteins by biotin pull-down and Western blotting

Next, we sought to validate the candidate proteins identified from mass analysis by pull-down and Western blotting. After inducing glutathionylation by incubating AMA under glucose starvation, cells were lysed. After cell lysis, click reaction was performed with biotin-alkyne, followed by enrichment of glutathionylated proteins by biotin pull-down. Enriched proteins were eluted and probed with primary antibodies against different individual proteins. Cell lysates were probed with same antibodies before and after streptavidin pull down. We confirmed several proteins for glutathionylation: Hsp90, actin and catalase were glutathionylated within 1 h of induction while other proteins such as PTP1B, PP2Cα and AMPK are found to be glutathionylated at 2 h (Figure 3.19 A). Importantly, we identified several new glutathionylated proteins including catalase, SMYD2 and PP2Cα (Figure 3.19 A).

We further evaluated the reversibility of several individual glutathionylated proteins upon incubation of pyruvate. It was found that PTP1B, catalase and PP2Cα are readily undergoing deglutathionylation within 30 minutes of pyruvate treatment (Figure 3.19 B). In contrast, actin and catalase did not show significant deglutathionylation upon incubation of pyruvate within 1 h, indicating that there may be differential selectivity of deglutathionylation in a time-dependent manner (Figure 3.19 B).
Figure 3.19 - Validation of glutathionylated proteins by pull-down and Western blotting. A) After incubation of azido-Ala to HEK293-GS M4 cells for 20 h, cells were subjected to glucose starvation with antimycin A in a time-dependent manner. B) After glucose starvation with treatment of antimycin A for 2 h, pyruvate was added to cells. After click reaction with biotin-alkyne, the individual proteins were probed by specific antibodies before and after the pull-down.

3.4 Discussion

Glutathionylation is a protein cysteine oxidation and taking place under elevated ROS conditions, found in many pathological conditions (173). Although many proteins are found to be glutathionylated in response to exogenous ROS stimulus, protein glutathionylation in response to glucose metabolism and/or induction of mitochondrial ROS is not systematically analyzed. In this chapter, we studied glutathionylation in response to mitochondrial dysfunction using clickable glutathione approach (211).
We prepared a stable cell line HEK293-GS M4 that express GS M4. It was found that stable expression of GS M4 in HEK293 cells did not significantly alter the total free thiol content and expression levels of redox enzymes. LC-MS analysis showed that a significant amount of azido-GSH was synthesized in HEK293/GS M4 cells. Overall, our data support that clickable glutathione is well-tolerated in cells. Therefore HEK293-GS M4 cells behave similar to normal HEK293 in terms of intracellular redox state.

Next, to evaluate glutathionylation under mitochondrial dysfunction, we used pharmacological inhibitors of the ETC, such as rotenone, AMA and myxothiazol (261). AMA and myxothiazol are complex III inhibitors while rotenone is a specific inhibitor of complex I. Because ETC inhibitors are known to generate mROS, these inhibitors may mimic mitochondrial dysfunction. Cellular metabolism is an important factor that associated in regulating redox homeostasis (229). Specially, glucose metabolism plays an important role by generating two important metabolic products, including pyruvate and NADPH (230). While pyruvate acts as free radical scavenger, NADPH is the major reducing source which used antioxidant machinery (Figure 3.20) (122,130). Indeed, glutathionylation was readily induced in response to AMA treatment under physiological glucose concentrations and this effect was significantly increased when cells exposed to glucose deprivation. Moreover, in agreement with glutathionylation data, AMA treatment increased ROS production, reduced GSH levels and increased GSSG levels. Therefore, we hypothesized that ETC inhibitor induced mROS were detoxified by high glucose. Interestingly, AMA did not induce glutathionylation when cells incubated in high glucose, suggesting an important regulatory role for glucose metabolism in glutathionylation.
We further confirmed the significance of glucose availability in glutathionylation by evaluating both global and individual protein deglutathionylation. After induction, cells were treated with either glucose or its metabolic intermediate pyruvate. Addition of these metabolites could induce rapid deglutathionylation. Our data for glutathionylation and deglutathionylation demonstrate an important role of glucose metabolism in controlling protein glutathionylation.

Figure 3.20 – Schematic representation of glucose and mitochondrial metabolism in redox homeostasis and glutathionylation.

Since glucose availability is an important determinant of protein glutathionylation and glucose deprivation is known to induce ROS, we next evaluated protein glutathionylation under glucose deprivation (262). When stable cells were exposed to glucose deprivation for longer incubation (20 h), glutathionylation was significantly induced compared to shorter time incubation (2 h and 5 h). Also, decreasing
concentrations of glucose induces glutathionylation in a dose dependent manner within 20 h.

In the ETC, ROS are generated by reduction of molecular oxygen. Because there are reports showing that hypoxia induces ROS, we examined protein glutathionylation in response to O₂ availability (Figure 3.20) (250). When stable cells incubated in a low oxygen environment, glucose deprivation did not induce glutathionylation even under a long incubation, indicating that glucose deprivation induced-ROS levels are low or not enough to induce glutathionylation in a hypoxic condition compared to a normoxic condition. On the other hand, when cells were exposed to increasing concentrations of oxygen, glucose deprivation could induce glutathionylation significantly. These data were further supported by reoxygenation experiments. As our data show, re-oxygenation could induce glutathionylation after hypoxic incubation of stable cells. Overall, a systematic evaluation of glutathionylation in response to glucose and oxygen deprivation demonstrated that oxygen availability as an important factor for inducing glutathionylation.

In addition to sensitive detection of glutathionylation in both global and individual proteins, clickable glutathione method is compatible with mass spectrometry to identify glutathionylated proteins under different physiological and pathological conditions. Therefore, we employed our clickable glutathione approach to find glutathionylated proteins under mitochondrial dysfunction and glucose deprivation. Click reaction with biotin-alkyne and pull-down with streptavidin agarose beads enriched numerous glutathionylated proteins. Trypsin digestion of enriched proteins and LC-MS/MS analysis identified more than 1400 glutathionylated proteins. Among identified proteins, 434 and 285 proteins were previously known for glutathionylation and sulfenylation respectively.
Also, GO analysis by DAVID program indicated that many identified proteins are involved in various cellular processes, indicating importance of glutathionylation in range of cellular events. We further validated the identified proteins by pull-down and Western blotting, including PP2Cα, which is a metal dependent serine/threonine protein phosphatase. PP2Cα is known to be involved in different cellular processes such as proliferation, apoptosis, cell cycle and cell migration by directly acting on several mitogen activated protein kinases (MAPK), including p38, JNK (263-265). Mostly PP2Cα acts as a negative regulator of stress sensing pathways. Therefore, further characterization and functional studies on PP2Cα glutathionylation is sought and described in Chapter 4.

3.5 Experimental procedure

3.5.1 HEK293-GS M4 Stable cell preparation

To determine the minimum hygromycin B concentration that required for killing HEK293 cells, different concentrations of hygromycin B were incubated with HEK293 cells and monitored over 10-14 days. First, a serial dilution of hygromycin B was carried out as listed in Table 3.1. After trypsinization, 1 X 10^5 cells, per well, were seeded on a six-well plate and incubated at 37 °C for 24 h. Then cell culture medium was replaced with fresh medium containing different concentrations of hygromycin B (Table 2.1). Cells were monitored over two weeks and minimum concentration of 200 µg/mL of hygromycin B was selected for the stable cell preparation. HEK293 cells were transiently transfected with pCDNA3.1-GS M4 plasmid using lipofectamine 2000 as a transfecting agent. Another 10 cm cell culture dish was maintained without transfection as the control. After 24 h of transfection, cells were trypsinized and maintained in a 12-well plate in fresh complete medium for 24 h. Then cells culture medium was replaced with fresh complete
medium with or without 200 µg/mL hygromycin B for two to three weeks. Cell culture medium was replaced every day until most of the cells are dead. Once all cell debris are washed away with warm PBS, stable cells containing cell colonies were picked using a 200 µL pipet tip and transferred to a well of 96-well plate. Cells were maintained in 200 µg/mL of hygromycin B containing medium until cells become confluent. Then cells were split in to a 24-well plate. When cells become confluent, cells were transferred to a 6-well plate. After 24 h, cells were lysed and lysates were analyzed for GS M4 overexpression by probing with anti-flag antibody. Rest of the cells were maintained in 10 cm cell culture dishes and stable cell stocks were prepared and stored in liquid nitrogen vapor phase for long term usage.

3.5.2. PEI-max transfection

General transfection procedure using PEI-max was described in Chapter 2, section 2.5.13.

3.5.3 Cell lysis

Detailed cell lysis procedure was described in Chapter 2, section 2.5.14.

3.5.4 Cell culture and assays

HEK293-GS M4 cell line, which is stably overexpressing glutathione synthetase mutant 4 (GS M4), was prepared according to a stable cell generation protocol. Briefly, after transfection of GS M4, HEK293 cells expressing GS M4 was selected and grown in the presence of hygromycin B (100 µg/mL). FLAG-tagged GS M4 expression in HEK293-GS M4 cells was confirmed by Western blotting with anti-FLAG antibody. Cells were maintained in DMEM (high glucose) with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 µg/mL), and hygromycin B (100 µg/mL) at 37°C in 5% CO₂
humidified incubator. PP2Cα wild type and mutant plasmids were transfected using PEI-max at 80% confluency in DMEM without FBS/antibiotics. Cells with or without transfection were incubated with 0.5 mM L-azido-alanine (azido-Ala) for 20 h. Cells were then washed with PBS once, and incubated for serum-starvation for 4 h in the presence of azido-Ala (0.25 mM). After washing with PBS, cells were incubated with and without antimycin A or rotenone in glucose-free (glucose starvation) or glucose-containing DMEM for different time points. For de-glutathionylation, glucose (5 mM), pyruvate (5 mM), or lactate (10 mM) was added for another 2 h. Cells were lysed using a lysis buffer [Tris HCl 100 mM, NaCl 150 mM, pH 7.4, 0.1% Tween 20, a protease inhibitor cocktail tablet, 100 μM PMSF and 50 mM N-ethylmaleimide (NEM)]. After incubation for 0.5 h at 4°C and centrifugation at 14,000 rpm, the cell lysates were collected and protein concentration was measured by Bradford assay.

3.5.5 SDS-PAGE

General SDS-PAGE procedure was described in Chapter 2, section 2.5.15

3.5.6 Western blotting

General Western blotting procedure was described in Chapter 2, section 2.5.17

3.5.7 Click reaction and biotin pull-down

Concentrations of cell lysates were measured using Bradford assay as described in 2.5.15. Lysates (100 μg), prepared by addition of NEM during cell lysis, were precipitated by adding cold acetone (1:4 by volume) for 30 min in -20°C. Samples were then centrifuged at 14,000 rpm for 3 min, and the pellet was redissolved in click buffer (0.1 M Tris-HCl pH 7.4) by sonicating for 5 seconds. Click reaction was performed as described in the previous study.(266) Briefly, click reaction was carried out for 1 h at room
temperature with biotin-alkyne (0.2 mM) or rhodamine alkyne (0.2 mM) in the presence of CuBr (1 mM) and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (0.4 mM). Click reaction was terminated by adding a SDS-loading buffer and proteins were separated by SDS-PAGE. Glutathionylated proteins were visualized by in-gel fluorescence or Western blotting using streptavidin-HRP. For pull down experiments, after click reaction, all the proteins were precipitated by cold acetone, centrifuged, and washed once with cold methanol. The precipitate was completely dissolved in PBS containing 1.2% SDS, and further diluted ten times with PBS before incubating with streptavidin agarose beads (100 µL) at room temperature for 3 h. Beads were washed extensively with PBS and finally with water. Proteins on beads were eluted by a SDS-loading buffer, separated by SDS-PAGE, transferred to PVDF membrane, and probed with various primary antibodies. Alternatively, proteins on beads were digested by trypsin, and eluted for LC-MS/MS analysis.

3.5.8 LC/LC-MS/MS analysis

The streptavidin agarose beads after enrichment were pelleted and washed twice by Dulbecco’s phosphate-buffered saline (DPBS). After resuspending in 50 µL of 6 M urea/PBS, the beads were incubated at room temperature for 1 h. 100 µL DPBS was then added to dilute urea to a final concentration of 2 M. On-bead trypsin digestion was performed overnight at 37 °C with sequencing-grade trypsin (2 µg) and 1 mM CaCl₂ (100 mM stock in water). The peptide digests were centrifuged and the supernatants were collected. The beads were washed twice with water (2 × 75 µL). All supernatants and washes were combined and acidified with 15 µL formic acid. The samples were stored at -20 °C until mass spectrometry analysis. LC/LC-MS/MS analysis was performed on an
Agilent 1200 series HPLC coupled to an LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher). Peptide digests were pressure loaded onto a 250 µm fused silica desalting column packed with 4 cm of Aqua C18 reverse-phase resin (Phenomenex). The peptides were eluted onto a biphasic column (100 µm fused silica with a 5 µm tip, packed with 10 cm Aqua C18 reverse-phase resin (Phenomenex) and 3 cm Partisphere strong-cation-exchange resin (SCX, Whatman) using a gradient of 5-100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The peptides were then eluted from the SCX onto the C18 resin and into the mass spectrometer using four salt steps as previously described.(267)

The flow rate through the column was set to ~0.25 L/min and the spray voltage was set to 2.75 kV. One full MS scan (FTMS) (400-1800 m/z) was followed by 18 data dependent scans (ITMS) of the n th most intense ions with dynamic exclusion enabled. The generated tandem MS data was searched using the SEQUEST algorithm against the human UniProt database. SEQUEST output files were filtered using DTASelect 2.0.(267) Glutathionylated proteins with two or higher fold enrichment were selected and analyzed using DAVID functional annotation tool.

3.5.9 Cell viability assay

HEK 293 cells were grown on a six-well plate by adding same number of cells into each well. After 24 h, cells were incubated in DMEM only for 3 h. Cells were then subjected to different conditions for 24 h: 1) no treatment (25 mM glucose, 0 µg/mL antimycin A), 2) glucose starvation (0 mM glucose), 3) antimycin A (1 µg/mL) 4) glucose starvation (0 mM glucose) with antimycin A (1 µg/mL antimycin A), and 5) 0.01% DMSO (amount used to dissolve antimycin A). Antimycin A was dissolved in DMSO. Cells were
subjected to trypan blue cell viability assay. Briefly, cells were detached by treating with 0.05% trypsin, and diluted with the medium. Then 100 µl of trypan blue reagent was added to the 100 µl of medium with the cells and 20 µl of sample was loaded on to the slide and analyzed the percentage of viable cells by TC 20 automated cell counter (Biorad). The data was shown after duplication of two independent experiments.

3.5.10 ROS assays

HEK293 cells (10,000 cells per well) were seeded and incubated at 37 °C, 5% CO₂ for 24 h for attachment on 96-well black plates with transparent flat bottom. Cells were then washed with warm PBS, and incubated in serum-free medium for 3 h. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, 25 µM) was then added, and cells were incubated in the same conditions for another 1 h. Cells were then washed with glucose-free medium without phenol red, followed by treatment at the indicated conditions and incubated for 2 h. Untreated cells were used as a negative control. Fluorescence emission scan was measured at 528 nm after excitation at 485 nm. ROS levels were normalized using a negative control (25 mM glucose without antimycin A).

3.5.11 Determination of thiol concentration

HEK293-GS M4 cells were treated with 0.5 mM azido-Ala and cells were washed with PBS after 20 h. Cells were induced for glutathionylation for 0.5 h and 2 h as mentioned in the main text. After induction, cells were harvested and lysed in PBS by 3 times of freeze-thaw cycles. The cell lysates were collected after centrifugation. Proteins were removed by 3 kDa centrifugal filter device, and the filtrate (FIL) that contains small molecules (including glutathione) was collected. The total and reduced form of thiol contents (mostly glutathione) were analyzed by fluorescence assay using bromobimane
(excitation - 390 nm, emission - 478 nm). Bromobimane assay was performed in 0.1 M Phosphate buffer, 0.5 mM bromobimane, and 10 µL of protein free lysates (FIL). The fluorescence signal was measured by using a plate reader. To calculate total GSH levels, protein free lysates (FIL) were incubated with 1 mM TCEP at 37 °C for 30 minutes before the bromobimane assay. All the data was duplicated and the background signal was corrected before the analysis by GraphPad Prism (version 5.01).
4.1 Introduction

Glutathionylation is implicated in regulation of various signaling pathways that control cell survival, apoptosis and cancer metastasis (185). Cancer cells are adapted to an increased uptake of glucose to compensate high energy demand and to maintain redox homeostasis. While glucose metabolism provides necessary metabolic intermediates for a sustained energy production in cancer cells, glucose deprivation induces apoptosis, inflammation, and migration (268-270). For example, glucose deprivation increases the expression levels of monocarboxylate transporter 1 (MCT1) in cervical cancer cells and induces migration through mitochondrial ROS production (262).

Protein phosphatases are a class of enzymes that act on phosphoproteins by removing phosphate group from different amino acid residues thereby regulating important cellular processes (271-273). Human genome encodes for more than 140 phosphatases that are divided into two major categories, including Ser/Thr protein phosphatases and protein Tyr phosphatases. Protein Ser/Tre phosphatases consists of phosphoprotein phosphatases (PPPs) and metal ion-dependent protein phosphatases (PPM's) (274). PPM’s are monomeric and structurally distinct from phosphoprotein phosphatases (PPP’s). PPPs are heteromeric and consist of both catalytic and regulatory subunits while PPMs are only made of a catalytic subunit (264).

Among different isoforms of PPMs, PP2Cα is the most studied phosphatase and known to acts as a negative regulator of stress response pathways, such as mitogen-activated protein kinases (MAPK’s) and transforming growth factor-β (TGF-β) signaling
PP2Cα is known to directly interact and dephosphorylate several key-signaling proteins, including p38-MAPK, extra cellular regulated kinase (ERK), c-Jun N-terminal kinase 1 (JNK1) and SMAD2/3 (263,265,275). PP2Cα acts as a tumor suppressor and it is implicated in cancer migration and invasion through antagonizing a TGF-β-activated SMAD2/3 signaling in bladder cancer and breast cancer (263,276). Numerous evidences support that MAPK are also associated in cell migration and invasion (277). p38 and ERK are known to directly act on several substrate proteins that are involved in migration (278-281). Inhibition or overexpression of a dominant negative mutant of MAPK has been shown to inhibit p38 or ERK-dependent cell migration (278,282).

In addition to its more common roles in apoptosis, inflammation and differentiation, JNK is an important MAPK family member associated with cell migration. (283-285). Activation of JNK by EGF, ephrin B1, upstream MEK kinase, constitutively active MKK4 or environmental stress are shown to increase migration in various cell types (286-288). The importance of active JNK in cell migration has also been documented in several different studies, by using an inhibitor (SP600125) of JNK, overexpression of a dominant negative mutant, JNK1AF, and a JNK knockout method (289-291). We found that glucose metabolism is known to regulate protein glutathionylation, and Ser/Thr protein phosphatase (PP2Cα) was identified to undergo glutathionylation in response to glucose deprivation (292). Migration towards nutrients would be important for cancer cells during a tumor growth. To our knowledge, there is no direct study correlating the expression of PP2Cα with cancer cell migration. However, there is one proteomic study that measures expression profiles of PP2Cα among noninvasive and invasive cancer cells, suggesting a potential positive correlation between cancer cell invasiveness and the expression level
of PP2Cα availability (293). For instance, invasive MDA-MB-231 cells have a low expression level of PP2Cα when compared to noninvasive MCF7 and A431 cells (294). Rapidly proliferating solid tumor cells experience metabolic stress, due to the lack of oxygen and glucose availability (295). Metabolic stress in primary tumor cells induces initiation of metastasis, thereby invading other organs, such as lung, brain and bones (296,297). Although, PP2Cα and MAPK’s are involved in tumor invasion and metastasis, regulation of PP2Cα function in cancer cell invasion under metabolic stress is not explored, thus motivated us to study PP2Cα glutathionylation under metabolic stress.

In this study, we found PP2Cα undergoes glutathionylation at C314 in a C-terminal domain in response to metabolic stress. we further investigated functional role of PP2Cα glutathionylation under metabolic stress, i.e. limited nutrient availability. The response of glutathionylation varied depending on cancer cell types and glucose availability. We further demonstrate that PP2Cα glutathionylation in response to metabolic alterations enhances cancer cell migration and invasion potentially through activating JNK-MAPK pathway.

4.2 Results

4.2.1 S-Glutathionylation of serine/threonine protein phosphatase 2Cα (PP2Cα)

PP2Cα is a protein phosphatase which utilizes metal coordinated water molecule to catalyze the dephosphorylation of several critical proteins including JNK, p38, MAPK, AMPK, Smad2/3 and NF-Kb (265). Also, a recent study showed that PP2Cα directly interact with metabotropic glutamate receptor 3 (mGluR3) and dephosphorylates serine 845 (298). mGluR3 is a glutamate receptor which is important for the protection of neuronal cells against glutamate excitotoxicity (299). Considering the importance of
PP2Cα in different signaling pathways, we started to further evaluate the consequences of glutathionylation of PP2Cα.

PP2Cα carries 11 cysteine residues from the available crystal structure (PDB: 4RA2), we predicted Cys204 and Cys314 are potential cysteine for glutathionylation because of their solvent accessibility and charged lysine residues around Cys314. In addition, Cys238 was also considered as a potential modification site due to its proximity to a metal ion in the active site. (Figure 4.1).

We generated all three cysteine-to-serine mutants of PP2Cα and transfected to HEK293-GS M4 stable cells. After 24 h of transfection, azido-Ala was incubated and cells were induced for glutathionylation with AMA in glucose-free medium for 2 h. Cells were then lysed, and lysates were subjected to click reaction with biotin-alkyne. Biotinylated proteins were captured by streptavidin beads and analyzed in a gel.
**Figure 4.1 – Crystal structure of PP2Cα.** Three potential cysteine residues (Cys204, Cys238 and Cys314) out of 11 cysteine residues in PP2Cα are predicted for glutathionylation. Two manganese atoms (purple) and a phosphate (red) in active site of the crystal structure of PP2Cα (PDB:4RA2) are shown in space-filling model.

Eluted proteins were probed with anti-HA antibody. As shown in Figure 4.2 A, PP2Cα WT, Cys204 and Cys238 are significantly glutathionylated. In contrast, PP2Cα Cys314 mutant showed a reduced signal for glutathionylation (Figure 4.2 A lane 5). Under similar conditions, there were many proteins, including PTP1B, that are glutathionylated in addition to PP2Cα (Figure C.4.1).

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-PP2Cα</td>
<td>WT</td>
<td>WT</td>
<td>C204S</td>
<td>C238S</td>
<td>C314S</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antimycin A (µg/mL)</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>After Strep-beads</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: α-HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before Strep-beads</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: α-HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.2 – In cellulo and in vitro glutathionylation studies for identification of modified cysteine residue in PP2Cα.** A) PP2C WT, and cysteine mutants, C204S, C238S and C314S
transiently transfected to HEK293-GS M4 cells and glutathionylation was induced by treating AMA in the presence and the absence of glucose. After performing click reaction with biotin alkyne and subsequent biotin pull-down, eluted proteins were probed with ant-HA antibody to detect glutathionylation levels of PP2Cα. B) Purified PP2Cα WT and C314S mutant subjected to in vitro glutathionylation by incubating azido-GSH and diamide. Glutathionylation was detected by in-gel fluoresce.

S-Glutathionylation of PP2Cα was further confirmed by an in vitro glutathionylation assay. Briefly, purified PP2Cα WT and C314S mutant (50 μg each) were incubated with azido-GSH (1 mM). Diamide (50 μM) was then added to induce glutathionylation. Glutathionylation levels were analyzed by in-gel fluorescence after click reaction with Cy5-alkyne. In vitro glutathionylation studies showed that PP2Cα WT is strongly glutathionylated while glutathionylation was significantly decreased with PP2Cα Cys314, suggesting that Cys314 is the major cysteine for PP2Cα glutathionylation (Figure 4.2 B). Interestingly, Cys314 has a Lys310 in a close proximity that can enable hydrogen bonding.

Proximity ligation assay (PLA) has been used to study protein-protein interaction, localization and posttranslational modifications of individual proteins in fixed cells (300,301). We sought to couple a clickable-GSH approach with PLA technology to detect PP2Cα glutathionylation and its localization in cells by fluorescence imaging. Briefly, after transfection of PP2Cα WT and C314S, cells were treated with 1mM H₂O₂ for 15 minutes to induce glutathionylation. Then cells were fixed and permeabilized before the click reaction with biotin-alkyne. Primary antibodies against PP2Cα and biotin were incubated for 16 h at 4°C followed by incubation of secondary anti-rabbit and anti-mouse that are
conjugated with PLA probes for 1 h at 37 °C. After ligation and amplification, Images were captured using confocal microscope.

![Diagram of PP2Cα glutathionylation](image)

**Figure 4.3** – Schematic representation of visualization of PP2Cα glutathionylation by a proximity ligation assay.

The PLA signal that represents glutathionylation was significantly detected with PP2Cα WT versus PP2Cα C314S mutant. Glutathionylated PP2Cα appear to be in the cytoplasm (Figure 4.4). This method may be useful to study the effect of PP2Cα glutathionylation on cellular localization and protein-protein interactions.
Figure 4.4 – Visualization of PP2Cα glutathionylation by proximity ligation assay. WT PP2Cα and mutant PP2Cα transiently transfected and induced for glutathionylation. Click reaction was performed to fixed cells followed by PLA and subsequent visualization of PP2Cα glutathionylation by confocal microscopy.

4.2.2 Functional effects of glutathionylation on PP2Cα

PP2Cα WT and C314S mutant proteins were purified using His-tag purification. It was found that there are two different sizes of PP2Cα: one matches with a full-length size (50 kDa) while another one appears to be 10-15 kDa smaller than a full-length mass (~35 kDa). Because His-tag is present at a N-terminus and PP2Cα has a small C-terminal domain with about 15 kDa, we concluded that a smaller size of purified protein is likely to be truncated PP2Cα lacking the C-terminal domain. (Figure 4.5 A). First, we evaluated phosphatase activity of PP2Cα WT and C314S mutant using a simple colorimetric assay. After incubating PP2Cα WT and mutant enzyme with GSSG, phosphatase substrate
para-nitrophenyl phosphate (pNPP) was added to the reaction mixture. Phosphatase activity was quenched by adding NaOH (302). Enzyme activities were determined by measuring para-nitrophenol absorbance at 405 nm. Our in vitro assay showed that PP2Cα glutathionylation did not significantly inhibit its phosphatase activity. Although PP2Cα C314 mutant exhibited a lower catalytic activity compared to PP2Cα WT, it is unclear yet if this is due to a mutation at C314 Figure 4.5 B). But it should be noted that pNPP is a nonspecific small molecule substrates for alkaline phosphatases, which may not represent enzymatic activity on protein substrates that are often mediated by protein-protein interaction. To examine the functional consequences of glutathionylation in the context of cellular environment, we tested the phosphorylation levels of AMPK that is a downstream target of PP2Cα. Under glucose depletion, AMPK is phosphorylated, which activates downstream signaling pathways as a stress response to metabolic changes. PP2Cα directly interact and inhibits AMPK activation (Figure 4.5 C) (303). Therefore, we evaluated phosphorylation level of AMPK in cells expressing PP2Cα WT and C314S in response to ROS. After transfection of WT and Cys314 mutant, cells were induced for glutathionylation. Then cell lysates were analyzed for phosphorylation levels of AMPK using antibodies against p-AMPK and AMPK. Glucose starvation and AMA treatment significantly induced the phosphorylation of AMPK. However, the level of AMPK phosphorylation did not change in any of PP2Cα mutants compared to WT (Figure 4.5 D). Because Cys314 is in a C-terminal tail of PP2Cα and C-terminal domain was suggested to be involved in the substrate specificity and protein-protein interaction (304), we tested whether glutathionylation can disrupts the interaction with its substrates. PP2Cα directly interacts and acts on phosphorylated metabotropic glutamate receptor 3
(mGluR3) (298). Therefore, we carried out co-immunoprecipitation study to investigate the effect of PP2Cα glutathionylation on mGLUR3 interaction with PP2Cα. After transfection of HA-tagged PP2Cα WT and Cys314 mutant, cells were induced for glutathionylation.

Figure 4.5 – Functional effects of PP2Cα glutathionylation. In vitro analysis of PP2Cα enzymatic activity upon glutathionylation. (A) A gel after purification of His-PP2Cα, which gave a full-length (FL) and a truncated form (TR) that appear to have excluded about 10 kDa size of the C-terminus domain. His-tag is on the N-terminus. (B) PP2Cα dephosphorylation assay with p-nitrophenyl phosphate (pNPP): PP2Cα (50 ng/uL) and pNPP (20 mM) were incubated with and without oxidized glutathione (GSSG) (1 mM) for 30 min at 37 °C, and absorbance was measured at 405 nm.
Then cells were lysed, and lysates were subjected to immunoprecipitation using an antibody for mGluR3 eluted proteins were analyzed using antibodies against HA-tag and mGluR3. PP2Cα WT decreased its interaction with mGluR3 under stress condition. On the other hand, PP2Cα C314 mutant retained the interaction with mGluR3 under the same stress condition. In agreement with these data, endogenous PP2Cα lost it interaction with mGluR3 under stress condition (Figure 4.6 lane 1 and 2). These data showed that PP2Cα glutathionylation disrupts PP2Cα interaction with mGluR3.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-PP2Cα</td>
<td></td>
<td></td>
<td>WT</td>
<td>WT</td>
<td>C314S</td>
<td>C314S</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>25</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Antimycin A (μg/mL)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 4.6 – Co-immunoprecipitation assay for mGLUR3 and PP2Cα.** After transfection of PP2Cα WT and C314S mutant, HEK293-GS M4 cells were induced for glutathionylation with AMA and glucose deprivation. After ci-immunoprecipitation, eluted samples were probed with antibodies against PP2Cα and mGLUR3.

**4.2.3 Glutathionylation in response to glucose depletion in different cell lines**

PP2Cα is known to have regulatory function in controlling cancer cell migration by antagonizing a TGF-β-induced SMAD signaling pathway. The correlation between PP2Cα expression and metastatic properties of several cancer cells are previously reported (263). PP2Cα expression levels vary by the cell type. For example, invasive breast cancer
cell line MDA-MB231 contains a lower expression level of PP2Cα when compared to noninvasive MCF7 and skin cancer cell line A431 (Figure 4.14 B). Therefore, to understand the significance of PP2Cα glutathionylation in cancer cells, we evaluated glutathionylation in two different cell lines, MCF7 and MDA-MB-231 cancer cells. Cancer cells rely on glucose metabolism to provide enough energy and biomass required to maintain high proliferation rate and to maintain the redox homeostasis (183). Therefore, we evaluated glutathionylation in response to metabolic changes in MCF7 and MDA-MD-231 cells and cardiomyocyte H9c2 cells.

![Figure 4.7 - Glutathionylation in different cell lines. Adenovirus transduced to MCF-7, MDA-MB-231 and H9c2 cells and incubated with 0.6 mM L-AzAla for 20 h. Then cells were maintained in different concentrations of glucose containing serum free medium for 5.5 h. Lysates were subjected to click with Cy-5Alkyne and glutathionylation was analyzed by in-gel fluorescence. A) H9c2 cells were differentiated using retinoic acid for four days followed by adenovirus transduction. After inducing glutathionylation detected by in-gel fluorescence. B) Induced MCF7](image-url)
cells subjected to click reaction and visualized by in-gel fluorescence. C) Dose dependent glutathionylation in MDA-MB-231 cells.

Differentiated H9c2 cells, MCF7 and MDA-MB-231 cells were transduced with adenovirus expressing a GS M4 mutant. After 24 h of virus transduction, cells were incubated with L-AzAla for another 20 h, followed by treatment of different glucose concentrations for approximately 5.5 h. Under these conditions, GS M4 stable cells and differentiated cardiomyocytes H9c2 did not induce protein glutathionylation (Figure 3.4 and 4.7 A). However, Interestingly, there was a low but increase level of glutathionylation in MCF-7 cell under a low concentration of glucose (3 mM). MDA-MB-231 cells displayed a significant increment of glutathionylation in response to low glucose (3 mM) (Figure 4.7 B and C). These data indicated that metastatic MDA-MB-231 cells are more sensitive for inducing glutathionylation in response to glucose availability than non-invasive MCF7.

We also analyzed glutathionylation in both MDA-MB-231 and MCF7 cells with a deceasing concentration of glucose. We found that glutathionylation was significantly induced at 3 mM of glucose concentration in MDA-MB-231 cells (Figure 4.8 B). Consistent with glutathionylation status, ROS levels were higher in MDA-MB-231 cells compared to MCF7 under glucose depletion (Figure 4.8 A). We confirmed that levels of GS M4 and a few redox enzymes are similar between MCF7 and MDA-MD-231 cells, suggesting that glutathionylation signals are likely to represent the different redox states between two cell lines. (Figure 4.8 C).
Figure 4.8 – Different redox responses in MCF7 and MDA-MB-231 cells. A) ROS levels were determined by DCF assay under different glucose concentrations. B) After adenovirus transduction, cells were induced for glutathionylation under different glucose concentrations for 5 h. Glutathionylation was detected by in-gel fluorescence after performing click. In similar conditions, C) GS M4 and redox enzyme expression levels were analyzed by Western blotting. D) PP2Cα glutathionylation levels were determined by biotin pull-down and subsequent elution and probing with HA antibody.
Importantly, we confirmed PP2Cα glutathionylation in MDA-MB-231 cells under glucose depletion (Figure 4.8 C). When cells were incubated with decreasing concentrations of glucose, PP2Cα glutathionylation was increased in a dose dependent manner: PP2Cα glutathionylation was significantly increased in response to low glucose concentrations (3-5 mM) compared to a high concentration of glucose (25 mM) (Figure 4.8 C).

In addition to glucose deprivation, MDA-MB-231 cells displayed higher sensitivity towards oxygen and glucose deprivation (ODG). After OGD condition, cells were incubated in normal oxygen atmosphere (21%) for 30 minutes for reoxygenation. Glutathionylation was analyzed after click reaction with fluorophore-alkyne. Consistently, glutathionylation was induced in response to GD or OGD within 5 h (Figure C.4.3). Further, MDA-MB-231 cells slightly induced glutathionylation after 30 minutes of reoxygenation (Figure C.4.4). Altogether, data show that MDA-MB-231 cells are more sensitive to metabolic alterations.

4.2.4 Glutamate availability and the metabolic stress in MDA-MB-231 cells

Glutamate availability and the metabolic stress in MDA-MB-231 cells. We further evaluated glutathionylation upon addition of different metabolites, including pyruvate, glutamine and glutamate. MDA-MB-231 cells were incubated in a low glucose medium, but with addition of pyruvate, glutamine or glutamate for 5.5 h. Then cells were lysed and analyzed for glutathionylation by in-gel fluorescence. In agreement with an earlier study, glutathionylation was decreased by the addition of 5 mM pyruvate (Figure 4.9 A).
Figure 4.9 - Rescue of metabolic stress by pyruvate, glutamine and glutamate. A) MDA-MB-231 cells were treated with low glucose in the presence of pyruvate, glutamine and glutamate for indicated time. B) Cells were supplemented with different concentrations of glutamate (0-8 µM) for 5.5 h. After induction, cells were lysed and subjected to click reaction before analyze by in-gel fluorescence.

Interestingly, we found that glutathionylation was dramatically suppressed by glutamate. In contrast, an addition of glutamine did not change the level of glutathionylation. The gradual decrement of glutathionylation signal was observed upon addition of increasing concentrations of glutamate (Figure 4.9 B). Glutamine can be converted to glutamate by glutaminase (GLS). Glutamate can be fluxed to produce pyruvate and NADPH in cells. (Figure C.4.5). GLS enzyme activity may be inhibited to prevent glutaminolysis in our condition (305). Overall, supplementation of glutamine did
not rescue the cells from metabolic stress while glutamate supplementation rescued cells from metabolic stress (Figure 4.9 A).

4.2.5 PP2Cα glutathionylation in cancer cell migration (wound healing assay)

Next, we investigated the functional importance of PP2Cα glutathionylation in MDA-MB-231, an invasive breast cancer cell line. Since PP2Cα is implicated in cell migration through SMAD and MAPK signaling pathways, we speculated that PP2Cα glutathionylation might be playing an important role in cell migration and invasion. To probe this hypothesis, we first investigated cell migration of MDA-MD-231 cells after expressing PP2Cα WT or C314S. Cells were evaluated for a wound-healing assay in high and low glucose conditions for 36 h. After making a wound across cells using pipet tip cells were monitored using a light microscope connected to a camera. Non-transfected MDA-MD-231 cells displayed a higher wound healing capacity in both high glucose (25 mM) and low glucose concentrations (10 mM). Overexpression of PP2Cα WT completely attenuated the wound healing ability of MDA-MB-231 cells under a high glucose condition. The same pattern was observed in cells with PP2Cα C314S, indicating an inhibition of cell migration by PP2Cα (Figure 4.10).

Interestingly, MDA-MD-231 cells with PP2Cα WT restored the wound healing capacity or increased the migration of cells when incubated in a low glucose condition (Figure 4.10). On the other hand, migration or wound healing did not increase for MDA-MD-231 cells expressing PP2Cα C314 in a low glucose condition (Figure 4.10). These data suggest that PP2Cα may lead to a loss of its activity in a low glucose condition where PP2Cα can be glutathionylated (Figure 4.10).
Figure 4.10 – Wound healing assay with MDA-MB-231 cells. Similar number of PP2C WT and C314S mutant transfected cells were seeded in a 12-well plate and incubated in 25 mM and 10 mM glucose containing medium for 36 h after creating a wound. After, 36 h, images were captured in the wounded area and analyzed cell migration by quantifying invaded area. Percent invasion or migration was represented as a bar graph, (p<0.05).
Next, we evaluated whether glutathionylation is induced under low glucose conditions. Glutathionylation was readily induced at low glucose conditions (5-10 mM) (Figure 4.11 A). We also confirmed PP2Cα is glutathionylated in 10 mM glucose (Figure 4.11 B). Overall, these data support that glutathionylation of PP2Cα leads to a loss of a negative activity of PP2Cα in cell migration.

Figure 4.11 – Glutathionylation in MDA-MB-231 cells in response to a low glucose concentration. A) MDA-MB-231 cells were infected with adenovirus and glutathionylation was induced by incubating 10 mM and 5 mM glucose for 24 h. Glutathionylation levels were analyzed by performing click reaction with fluorophore alkyne. B) Induced cell lysate subjected to click reaction with biotin alkyne and subsequent biotin pull-down. Cell lysate and eluted proteins from biotin pull-down, were analyzed by HA antibody.

In solid tumor, most cancer cells are lacking nutrients and oxygen supplementation due to the poor vascularization. Glucose and oxygen deprivation are the most common challenges that solid tumors encounter during later stages of cancer development.
Therefore, cancer cells adapt to overcome these challenges by growing towards the vasculature or gaining epithelial-to-mesenchymal transition (EMT) to migrate to other parts of the body (306). Hence, these data provide evidences on regulatory effects of PP2Cα glutathionylation in cancer cell invasion under glucose limiting condition.

4.2.6 PP2Cα glutathionylation in response to metabolic stress promotes MDA-MB-231 cell invasion: transwell invasion assay

We further evaluated the role of PP2Cα glutathionylation in a transwell invasion assay. Transwell invasion assay consists of two chamber systems where an upper chamber holds cells and a lower chamber has the chemoattractant as a stimulant for invasion (Figure 4.12). Transwell inserts having a basement membrane that allows for cells to penetrate or invade. A similar number of MDA-MB-231 cells non-transfected or transfected with PP2Cα WT and C314S) were seeded on a transwell-insert containing 10 mM glucose medium. A Lower chamber was filed with a medium containing 10% FBS. Cells were then incubated in the incubator for 24 h.

Figure 4.12 – Diagram of transwell set up in a 24-well plate for investigating cancer cell invasion.

Then invaded cells were fixed and permeabilized before staining with crystal violet. Images were captured using a camera attached to alight microscope. When cells were incubated in 25 mM glucose, the number of invaded MDA-MB-231 cells was significantly
decreased in cells with PP2Cα WT or C314 when compared non-transfected cells, indicating that PP2Cα WT and C314 showed a negative role in cancer cell invasion under a high glucose condition (25 mM). In contrast, the number of invaded cells were significantly increased within 24 h, when cells with PP2Cα WT were incubated in low glucose (10 mM). However, cells expressing PP2Cα C314 did not show any increased number of invaded cells. (Figure 4.13). These data further support that PP2Cα glutathionylation at C314 enhances cancer cell invasion in response to metabolic stress.
Figure 4.13 – Transwell invasion assay with MDA-MB-231 cells. Transfected and non-transfected cells were incubated in transwell inserts. Low glucose containing medium was added to upper chamber and 10% FBS containing medium was added lower chamber. After 24 h,
invaded cells were fixed and analyzed after crystal violet staining. Migrated cells were quantified after extracting crystal violet with 33% acetic acid and showed as a bar graph, (p<0.05).

4.2.7 PP2Cα glutathionylation in A431 cells

In order to test the role of endogenous PP2Cα glutathionylation in cancer cell migration, we carried out a transwell invasion assay in A431 cells. A431 cells have a high expression level of endogenous PP2Cα compared to other cell lines (294). We confirmed expression levels of PP2Cα in three different cell lines, including MDA-MB-231, MCF7 and A431. MDA-MB-231 cells displayed lower expression levels of PP2Cα compared to both MCF7 and A431 cells (Figure 4.14 B). In similar to MDA-MB-231, A431 cells showed a significantly increased level of glutathionylation in response to low glucose (Figure 4.14 A), indicating susceptibility of A431 cells to metabolic alterations.

A Transwell invasion assay was performed to further investigate about PP2Cα glutathionylation in A431 cell migration under low glucose supplementation. In a similar manner to MDA-MB-231 cells, A431 cells gained more invasive properties in a low glucose condition compared to a high glucose condition. (Figure 4.15 A). Under the same conditions, global and PP2Cα glutathionylation were induced (Figure 4.15 B and C). Next, we sought to understand the potential mechanism of PP2Cα glutathionylation in enhancing cancer cell migration
Figure 4.14 – Comparison of glutathionylation among MCF7, MDA-MB-231 and A431 cells.

A) A431 cells induced for glutathionylation by low glucose for 5 h. Cell lysates from MCF7, MDA-MB-231 and A431 were subjected to click reaction and analyzed by in-gel fluorescence. B) In similar experimental conditions, expression levels of GS M4 (flag), PP2Cα and actin were probed using appropriate antibodies.
Figure 4.15 – Effect of PP2Cα glutathionylation on A431 cell migration. A) Transwell invasion assay for A431 cells was performed under low glucose concentration. After seeding approximately similar number of cells in transwell insert, invaded cells were stained and captured using a light microscope. B) Glutathionylation of PP2Cα in A431 cells in response to different glucose concentrations. After pull down, eluted proteins were probed with anti-PP2Cα antibody. C) Global glutathionylation levels in A431 cells in response to different glucose concentrations. After induction, lysates were subjected to click reaction and analyzed by in-gel fluorescence.
4.2.8 PP2Cα glutathionylation activates c-Jun N-terminal kinase (JNK) in MDA-MB-231 cells

PP2Cα acts as a negative regulator of stress responsive pathways and controls different signaling pathways that are involved in apoptosis and cell migration. Specially, PP2Cα acts on MAPK pathways by dephosphorylating p38 and JNK. p38 and JNK are activated in response to cellular stress, such as metabolic stress, and Activation of p38 and JNK is associated with an increased migratory property of cells. Therefore, we hypothesized that PP2Cα glutathionylation might interfere with dephosphorylation of p38, JNK, SMAD2 and SMAD3, which may lead to enhance cancer cell migration. To study the effect of PP2Cα glutathionylation of these proteins, MDA-MB-231 cells were transfected with PP2Cα WT and C314S. Cells were then induced for glutathionylation under a low glucose condition (5 mM) for 5 h. Cell lysates were then analyzed by Western blotting using appropriate antibodies (Figure 4.16). phosphorylation levels of SMAD2 or SMAD3 were not detectable in a basal level or under a low glucose condition, suggesting that SMAD2/SMAD3 may not play a crucial role for PP2Cα glutathionylation induced cell migration (Figure 4.16 C and D). p38 phosphorylation remains unchanged upon expression of PP2Cα WT or C314 or under both high glucose and low glucose conditions, suggesting a minimal involvement of PP2Cα in p38 dephosphorylation in MDA-MD-231 cells (Figure 4.16 B).
Figure 4.16 – Evaluation of phosphorylation levels of several PP2Cα substrates that are involved in cell migration. After transfection of PP2C WT and C314S, MDA-MB-231 cells were induced for glutathionylation by incubating low glucose containing medium for 5 h. A) Lysates were analyzed for phosphorylation levels and total protein levels for A) JNK B) p38 C) SMAD2 D) SMAD3 using relevant antibodies.

On the other hand, overexpression of PP2Cα WT and C314S mutant strongly inhibited JNK activation compared to non-transfected cells, indicating that PP2Cα WT and C314S are involved in dephosphorylating JNK in MDA-B-231 cells. More importantly, when cells expressing PP2Cα WT showed an increase level of JNK phosphorylation in a low glucose condition. In contrast, cells with PP2Cα C314S did not increase JNK phosphorylation under a low glucose condition. These data support that PP2Cα C314 glutathionylation leads to a loss of PP2Cα dephosphorylation activity for JNK under a low glucose condition. Because an increased JNK phosphorylation is associated with
enhance migration and invasion of cells, our data support that PP2Cα glutathionylation promotes cancer cell migration potentially by increasing JNK activation.

4.3 Discussion

PP2Cα is a Ser/Thr protein phosphatase that acts as a negative regulator in various stress induced cellular pathways (264). Among many substrates, PP2Cα inactivates SMAD2, SMAD3, p38 and JNK MAPK’s (265,275). Several studies have been reported to have regulatory function and different expression pattern of PP2Cα in inflammatory homeostasis and wound healing (307). Previously, we identified PP2Cα as a potential candidate for S-glutathionylation in response to mitochondrial dysfunction. In this study, we further determined the role of PP2Cα glutathionylation in in cancer cell migration.

PP2Cα has 11 cysteine residues and several cysteines are buried inside the structure, thus not directly accessible for ROS or GSH (308). According to the available crystal structure of PP2Cα, we predicted three potential cysteine residues for glutathionylation, including Cys204 and Cys314. Both cysteine residues are solvent exposed as seen in crystal structure (308). In addition, Cys314 is in close proximity with a positively charged amino acid Lys310. Therefore, it is possible that Cys314 thiol can be more nucleophilic or reactive. Because Cys238 is in a close distance to catalytic metal ion in the active site of PP2Cα, we also considered Cys238 as a potential candidate for glutathionylation. Our study showed that Cys314 is the major glutathionylated cysteine residue in response to mitochondrial dysfunction. This modification site is further confirmed by in vitro glutathionylation assay with purified PP2Cα WT and C314S mutant proteins.
protein phosphatase 2A (PP2A) is a heterotrimeric protein where a B-regulatory subunit seems to govern the substrate specificity (309). However, PP2Cα does not have a regulatory subunit, its c-terminal α-helix may be involved in substrate specificity (310). Also, our data confirmed that Cys314 glutathionylation disrupts interaction with mGLUR3, indicating PP2Cα glutathionylation potentially regulates its function under cellular stress conditions. On the other hand, PP2Cα glutathionylation did not impact on AMPK activation, suggesting that the effect of PP2Cα glutathionylation can depend on individual substrate proteins.

PP2Cα is known to regulate cancer cell migration through dephosphorylation of TGF-β induced SMAD signaling (263). Also, other substrates of PP2Cα, such as p38 and JNK are important players of cancer cell migration (311,312). Moreover, in solid tumor, cells are rapidly proliferating. Cells that grow away from vasculature may encounter with oxygen and nutrient deprivation (313). Some cancer cells appear to be more aggressive and invasive under metabolic stress (314). Therefore, we have investigated the role of PP2Cα glutathionylation in cancer cell migration under metabolic stress.

First, we evaluated global glutathionylation status in different cell lines, including two breast cancer cell lines, A431, MCF7 and MDA-MB-231. We used adenovirus expressing GS M4, which allow to express GS M4 in many cell lines with a high transfection efficiency. Using adenovirus transduction, we could evaluate glutathionylation in differentiated cardiomyocyte, A431, MCF7 and MDA-MB-231 cells. Glutathionylation studies revealed that MDA-MB 231 and A431 cells are more sensitive for inducing glutathionylation under metabolic alterations. Also, levels of a few redox enzymes and GS M4 were not significantly different between MDA-MB-231 and MCF7
cells, indicating the glutathionylation difference is due to higher metabolic stress in MDA-MB-231 cells. We also noticed that MDA-MB-231 cells are associated with a higher ROS level, and a higher sensitivity to low glucose condition when compared to MCF7 cells.

**Figure 4.17 – Cell migration induces by PP2Cα glutathionylation under metabolic stress.**

Further, glutamate supplementation could rescue MDA-MB-231 cells from metabolic stress resulting from a low glucose condition. But, glutamine addition did not recover cells from metabolic stress. It may be explained by the fact that the enzymatic activity of GLS depends on the availability of phosphate, acetyl-CoA and succinyl-CoA, and metabolic state of the cells (315,316). Also, in many cancer cells, sirtuin 5 is overexpressed and involved in desuccinylating GLS to inhibits its catalytic activity (315,316). Therefore, glutamate availability rather than glutamine may be important for maintaining redox homeostasis under low glucose.
Next, we evaluated the functional roles of PP2Cα glutathionylation in cell migration using MDA-MB-231 cells. Wound healing experiments and transwell invasion assay revealed that overexpression of PP2Cα WT or C314 significantly inhibited cell migration under a high glucose concentration. Interestingly, cells expressing PP2Cα WT could recover its wound healing capacity under a low glucose condition. On the other hand, cells with PP2Cα C314S could not recover wound healing ability. We also found that PP2Cα is glutathionylated at C314 position in response to low glucose, indicating PP2Cα glutathionylation promotes cancer cell migration under metabolic stress. When tumor is growing away from vasculature, cells are always encounter glucose-oxygen deprivation. There are number of studies suggesting that cancer cells are more invasive under metabolic stress. In agreement with other studies, our data collectively provide evidences that cancer cells are gaining more invasive properties through PP2Cα glutathionylation. We also found that PP2Cα C314 glutathionylation inhibits its dephosphorylating activity toward JNK, suggesting that PP2Cα glutathionylation increases cancer cell migration potentially via activating JNK and subsequent activation of downstream signaling associated with cell migration.

4.4 Experimental procedure

4.4.1 Cloning and mutagenesis

Human PP2Cα cDNA clone was purchased from OriGene and the gene was subcloned into pcDNA3.1/hygro (+) mammalian vector using BamHI and XhoI restriction sites. PCR reaction was performed using a forward primer (5'-GGT GGT GGA TCC GCC ACC ATG GGA GCA TTT TTA GAC AAG C-3') containing Kozak sequence and BamHI restriction site and a reverse primer containing HA tag and XhoI restriction site (5'-GGT
GGT CTC GAG CTA AGC GTA ATC TGG AAC ATC GTA TGG GTA CCA CAT ATC ATC TGT TGA TGT AGA G-3'). Both PCR product and the empty pcDNA3.1 vector were double digested with above two restriction enzymes and ligated using T4 DNA ligase. All the mutants were generated using pcDNA3.1-PP2C<sub>α</sub> clone as the template. QuickChange mutagenesis was performed using following mutagenic primers. PP2C<sub>α</sub> mutant C204S (forward: 5'-GCC CTT GGG GAT TTT GAT TAC AAA AGT GTC CAT GGA AAA GGT CC-3' and reverse: 5'-GGA CCT TTT CCA TGG ACA CT TTT TG TAA TCA AAA TCC CCA AGG GC-3'), C238S (forward: 5'-CAG TTC ATT ATC CTT GCA AGT GAT GGT ATC TGG GAT GTT ATG G-3' and reverse: 5'-CCA TAA CAT CCC AGA TAC CAT CAC TTG CAA GGA TAA TGA ACT TGT CC-3') and C314S (forward: 5'-GGA CAA GTA CCT GGA AAG CAG AGT AGA AGA AAT CAT AAA GAA GC 3' and reverse: 5'-GCT TCT TTA TGA TTT CTA CTC TGC TTT CCA GGT ACT TGT CC-3'). pcDNA3.1-PP2C<sub>α</sub> and empty pET28a vectors were double digested using BamHI and XhoI. The digested PP2C<sub>α</sub> and pET28a were gel purified and ligation was performed using T4 DNA ligase at 22 °C for 1h. To generate adenovirus containing GS M4 gene, flag-GS M4 was subcloned into Dual-CCM<sup>+</sup> shuttle vector. All plasmids were confirmed by DNA sequencing of entire open reading frames.

4.4.2 Purification of PP2C<sub>α</sub>

pET28a-PP2C<sub>α</sub> was transformed in to BL21(DE3) cells and single colony was incubated overnight in LB containing 50 µg/mL kanamycin. Overnight culture (5 mL) was diluted into 1 L of LB and grown at 37 °C until OD<sub>600</sub> reaches 0.4. Protein overexpression was induced for 4 h at 37 °C with 0.5 mM IPTG. Cells were harvested, lysed and purified as described in section 3.3.
4.4.3 Enzyme assay of PP2Cα

PP2Cα enzyme (WT and C314S) activities were calculated by end point assay using para-nitrophenyl phosphate (pNPP) as the substrate. Proteins were incubated with or without 0.5 mM GSSG in 20 mM Tris, pH 7.4 for 15 minutes at 37 °C. Phosphatase assay was performed in the colorimetric assay buffer containing 20 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM ethylene glycol-bis(2-aminoethyl)ether)-N,N,N',N'-tetraacetic acid (EGTA) and 0.1 mg/mL BSA. Different amounts of each protein were mixed with 20 mM pNPP in 100 µL final volume and incubated for 30 minutes at 37 °C before quenching the reaction with 50 µL of 5 N NaOH. Enzyme activities (µmoles/min/µg) were calculated using absorbance measured at 405 nm. Data analyzed by GraphPad Prism (version 5.01) software.

4.4.4 Mammalian cell culture and transfection

General cell culture and transfection methods were discussed in section 2.5.10 and section 2.5.13.

4.4.5 Adenovirus transduction

Adenovirus production was performed by Vector BioLabs. Briefly, flag-GS M4 was cloned into dual-CCM+ shuttle vector and then transferred expression cassette to an adenovirus vector (pAd Vector). Then low-titer viral particles were produced by transfecting linearized adenoviral DNA to HEK293 cells over couple of weeks. Next, virus was amplified by growing in a 300 mL culture and then virus was concentrated to 2 mL in viral storage buffer. Concentration of viral particles was determined by performing a plaque formation assay. To find out the multiplicity of infection (MOI) of Ad-CMV-GS M4, different volumes from virus stock (corresponding to MOI- 10, 50 and 100) were infected
to MDA-MB-231 cells. After cells were reached to 85-90% confluency in 100 mm cell culture dish, growth medium was replaced with 5 mL of 2% FBS containing DMEM (without penstrep) and transferred back to the incubator. Viral particles were diluted in 500 μL of 2% FBS containing DMEM medium in the presence of 1 mg/mL Polybrene in a sterile eppendorf tube. Virus and polycationic transfecting agent (Polybrene) complex was incubated for 20 minute at room temperature before adding to the cells. After 5-8 h of virus infection, virus containing medium was replaced with complete growth medium and incubated for 24 h or 48 h. During the use of virus in cell culture, extreme safety precautions must be followed. Cell culture dishes, pipette tips, and all other material should be touched with 10% regular bleach for at least 30 minutes. Cell culture medium containing virus should not aspirate into regular aspirator setup, instead, all contaminated medium and other liquids should be disposed in to bleach and final concentration of bleach should be 10%. Avoiding generation of splashes while transferring medium and cross contamination with other sterile cell culture medium or other equipment should be considered. Virus containing liquids on metal surfaces should disinfect with spore-Klenz for at least 30 minutes. Switching gloves is a must before leaving the cell culture room and appropriate waste disposal to a trash bin labeled with biohazard sticker for virus. Regular autoclaving of virus disposals must be maintained at least once a week.

4.4.6 Cell lysis

General mammalian cell lysis was described in section 2.5.14.

4.4.7 Click reaction and biotin pull-down

Click reaction and biotin pull-down experiments were described in section 2.5.18 and section 2.5.23.
4.4.8 Proximity ligation assay

HEK293-GS M4 stable cells were transfected with PP2C WT and C314S mutant using PEI-max as the transfecting agent. After 24 h of transfection, 0.6 mM of L-AzAla was incubated for another 20 h and induced for glutathionylation using 1 mM H₂O₂ for 15 minutes. After induction, cells were washed several times with cold PBS, and fixed for 15 minutes using ice cold methanol at -20 °C. After fixation, cells were then permeabilized with 1X PBST (pH 7.4, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 150 mM NaCl, 0.1% Trion-X100) for 10 minutes at room temperature. Cells were washed three more times with PBS and subjected to click reaction with biotin-alkyne. Briefly, click reaction mixture was prepared by using the same protocol described in section 2.5.18. In this procedure, TBTA was replaced with THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine) because of water solubility. Individual cover slips were incubated with 100 μL of click reaction mixture at 37 °C humidified incubator. Cover slips were then washed at least three times with 10-minute incubation at room temperature. Then, cells were incubated in the blocking buffer (pH 7.4, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 150 mM NaCl, 0.3% Trion-X100, 5% BSA) for 1 h at room temperature. Primary antibodies against PP2Cα (mouse anti-HA, 1:100 dilution) was prepared in the blocking buffer and incubated with cells for 16 h (overnight) at 4 °C. Cells were washed three times with PBS and primary antibodies against biotin (rabbit anti-biotin, 1:100 dilution) was incubated for 1 h at room temperature followed by three washings with PBS. PLA probes were diluted in 1:5 ratio with blocking buffer (for 40 μL of final antibody solution, 8 μL from each minus and plus PLA probes were mixed with 24 μL of blocking buffer) and incubated with individual slides for 1 h in a humidified incubator at 37 °C. After incubation of PLA probes, cover slips were washed two times with Duolink
wash buffer A for 10 minutes. Ligation mixture was prepared by diluting ligation buffer in 1:5 ratio with distilled water and 1 μL of ligase (1:40) was added just before the incubation. Ligation step was carried out at 37 °C in a humidified incubator for 30 minutes. Amplification-polymerase reaction mixture was prepared by diluting amplification stock 1:5 in distilled water and 0.5 μL of polymerase enzyme (1:80) was mixed at the time of incubation. After 30 minutes of ligation, cells were washed twice with 1X Duolink wash buffer A or 5 minutes. Soon after washings, cells were incubated with amplification-polymerase reaction mixture for 100 minutes at 37 °C. After amplification, cells were washed twice with 1X Duolink wash buffer B for 15 minutes and anti-mouse secondary antibody conjugated to Alexa-488 was incubated for 1 h at room temperature to detect total PP2Cα levels. After three times washings with 1X Duolink wash buffer B, cells were washed once with 0.01X wash buffer B and mounted on microscope slides with a mounting solution containing DAPI. Fluorescence images were captured using Leica DMi8 inverted confocal microscope and images were processed by LAS AF analysis software. Microscope filters were set for nucleus staining with a DAPI channel (λ<sub>ex</sub> 350nm, λ<sub>em</sub> 470nm), for proximity ligation signal with Texas Red channel (λ<sub>ex</sub> 594 nm, λ<sub>em</sub> 624 nm) and for total PP2Cα with Alexa-488 (λ<sub>ex</sub> 490 nm, λ<sub>em</sub> 525 nm).

4.4.9 Co-immunoprecipitation

After transfection of PP2Cα WT and C314S mutant, cells were induced for glutathionylation with 2 μg/mL AMA treatment in the absence of glucose for 2 h. Co-immunoprecipitation was performed after lysing cells using the protocol described in section 4.4.7. HEK293-GS M4 cells were transfected with PP2Cα WT and C314S mutant and incubated for glucose starvation with antimycin A. Cells were then lysed using a lysis
buffer PBST (pH-7.4, 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) without NEM. Lysates (1 mg/0.5 mL) were incubated with mouse mGluR3 primary antibody (3 µg) at 4°C for 1 h, mixed with pre-washed protein G agarose beads (30 µL), and incubated overnight at 4°C. Beads were then washed three times with PBST (pH-7.4, 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) for 10 min each time. Beads were eluted with a SDS-loading buffer in the presence of β-mercaptoethanol. Eluted proteins were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with primary mouse mGluR3 or rabbit PP2Cα antibodies in a TBST buffer (Tris HCl 50 mM, NaCl 150 mM, 0.1% Tween 20, 3% BSA) overnight at 4°C. HRP-conjugated light chain specific secondary mouse and rabbit antibodies were incubated for 1 h at room temperature. After washing 3 times with a TBST buffer, immunoprecipitated proteins were visualized by chemiluminescence.

4.4.10 SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were conducted using the protocol described in section 2.5.15 and 2.5.17.

4.4.11 DCF assay for ROS detection

HEK293 cells (10,000 cells per well) were seeded and incubated at 37 °C, 5% CO₂ for 24 h for attachment on 96-well black plates with transparent flat bottom. Cells were then washed with warm PBS, and incubated in serum-free medium for 3 h. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, 25 µM) was then added, and cells were incubated in the same conditions for another 1 h. Cells were then washed with glucose-free medium without phenol red, followed by treatment at the indicated conditions and incubated for 2 h. Untreated cells acted as a negative control. Fluorescence emission
scan was measured at 528 nm after excitation at 485 nm. ROS levels were normalized using a negative control (25 mM glucose without antimycin A).

4.4.12 Wound healing assay

MDA-MB-231 cells were transfected with PP2Cα WT and C314S using lipofectamine 3000 (ThermoFisher, catalog number L3000015) transfecting agent according to the manufacturer’s instructions. After 24 h of transfection, approximately similar number of non-transfected and transfected cells (1X10⁷) were seeded on a 12-well plate and incubated for another 24 h. Next, an even wound was created using a sterile 10 L pipette tip across the well and washed with warm PBS twice to remove any floating cells. This time point was considered as the 0 h and cells were incubated in 25 mM and 10 mM glucose containing medium for 36 h. Images of wounded area were captured at 0 h and after 36 h using a camera attached to a microscope. Images were analyzed by ImageJ software using MRI Wound Healing Tool. Briefly, an image can be opened by clicking on file and then hitting on open button. Once image is uploaded to the program, right click on “m” button and set the method to find edges, variance filter radius to 10, threshold to 50, radius open to 4 and adjust min.size according to the picture quality or use default setting. Before analyzing the images, option black background was selected by following process>binary>options. Then, images analyzed by clicking on “m”. The area of the open wound will be calculated and give as number with arbitrary units. By subtracting the area of the wound at 36 h from the area of the wound at 0 h, percent migration was calculated and data from three independent trial were showed as a bar graph using GraphPad Prism software.

4.4.13 Transwell invasion assay
On the first day, MDA-MB-231 cells were transfected with PP2Cα WT and C314S using Lipofectamine 3000 according to the manufacturer’s protocol and incubated for 24 h. On the same day, 0.5X Basement Membrane Extract (BME) was prepared by diluting 5X BME stock solution in 1X Travigen Inc coating buffer. Briefly, 100 μL of 10X coating buffer was diluted in 900 μL of sterile water. Then 900 μL of 1X coating buffer was mixed with 100 μL of 5X BME to make working 0.5X BME solution. Six Corning transwell permeable inserts were placed on six well of 24-well plate separately. Then 100 μL of 0.5X BME solution was added to each transwell insert and incubated overnight. Following day, transfected cells were trypsinized and similar number of non-transfected and transfected cells (~3x10⁵ cells) were seeded on all six transwell inserts. Trypsinized cells were mixed with glucose free medium and approximately 100 μL from each cell suspension was added to appropriate transwell inserts followed by another 100 μL from 50 mM and 20 mM glucose containing, but FBS free medium to get final 25 mM and 10 mM glucose concentrations respectively. Lower chamber was filled with 10% FBS containing medium without glucose. Whole setup was incubated in mammalian cell culture incubator for 24 h. After 24 h, cell culture medium was removed from both lower and upper chambers and washed three times with PBS. Non-invasive cells were removed using a cotton swab and bottom side of the membrane of transwell inserts were fixed with 4% formaldehyde for 10 minutes at room temperature followed by permeabilization with PBST (pH-7.4, 50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton-X100) for another 10 minutes. Inserts were washed once with PBS and stained with 0.2% (W/V) crystal violet solution for 20 minutes at room temperature. Inserts were then extensively washed with PBS to remove all excess dye. Imagers were captured using a camera attached to a microscope.
After taking pictures, stained dye was extracted with 300 µL of 33% acetic acid and absorbance was measured at 560 nm using a plate reader. Similar invasion assay was performed to A431 cells without transfection of PP2Cα.
Figure A.2.1 – SDS-PAGE analysis of the purity of GS WT and GS mutants. A) Purified proteins were diluted to 1 μg/mL working concentrations and boiled with SDS loading dye in the presence of reducing agents. Protein samples were separated from SDS-PAGE and stained with Coomassie blue and captured. B) After overexpression lysis, GS M4 was purified by Ni-NTA beads. A portion of 5 μL from lysate, flow through (FT), washings (W1, W2) and elution (E1, E2, etc) was loaded to SDS-PAGE and analyzed by Coomassie staining. C) To measure the concentrations of purified GS mutants, protein band intensities were compared to BSA standards. According to gel comparison and Bradford assay, purified GS M4 concentration was ~ 8 mg/mL.
Figure A.2.2 – Kinetic analysis of GS WT and GS M4 mutant. Different concentrations of each substrate were analyzed by measuring the absorbance at 340 nm. To calculate initial rates for each concentration, initial absorbance data was plotted against time and slope was calculated. Then Michaelis-Menten constants were calculated by plotting initial rates against different substrate concentrations.
Figure A.2.3 – LC-MS analysis of *in vitro* GS enzyme assay reaction mixture. A) Ion extraction for FL-IA modified endogenous GSH and mass spectra showing m+1 for FL-IA modified GSH. B) Ion extraction for different GSH derivatives and m+1 for GSH, azido-GSH and Met-GSH.
Figure A.2.4 – LC-MS analysis of L-AzAla dose dependent azido-GSH biosynthesis. After transient transfection of GS M4, HEK293 cells were incubated with increasing concentrations of L-AzAla for 20 h. Protein free lysates were analyzed by LC-MS and masses corresponding to different glutathione derivatives were extracted and overlaid.
Figure A.2.5 – Glutathionylated proteome visualized by rhodamine-alkyne or biotin alkyne.

Different concentrations or 1 mM of L-AzAla incubated with HEK293 stable cells with or without overexpression of GS M4, for 20 h. Cells were then induced for glutathionylation with or without H₂O₂. After induction, cell lysates were subjected to click reaction either with biotin-alkyne or rhodamine-alkyne. Glutathionylation was analyzed by in-gel fluorescence or by Western blotting.
**Figure A.2.6 – Analysis of glutathionylation in different cell lines using azido-GSH.** GS M4 transfected using PEI-max. After 24 h, 1 mM L-AzAla was incubated for 20 h and glutathionylation was induced with H2O2 for 15 minutes. Glutathionylation levels analyzed in different cells by analyzing in-gel fluorescence after performing click reaction with fluorophore-alkyne.
**Figure B.3.1 – Glutathionylation in response to 13 h glucose deprivation.** L-AzAla incubated with HEK293-GS M4 cells and induced for glutathionylation by incubating glucose free medium for 13 h. Lysates were analyzed for glutathionylation after performing the click reaction with biotin-alkyne.
Figure B.3.2 – Glutathionylation in response to CoCl2 induced hypoxia and glucose deprivation. L-AzAla incubated with HEK293-GS M4 for 20 h. Cells were washed with PBS and treated with different concentrations of CoCl2 with or without glucose for 6 h. After cell lysis, cell lysates were subjected to click reaction with Cy5-alkyne and analyzed by in-gel fluorescence.
Figure B.3.3 – Analysis of glutathionylation in response to oxygen-glucose deprivation and reoxygenation (OGD/R). HEK293-GS M4 cells were induced for glutathionylation by incubating glucose free medium under high (21%) and low (1%) oxygen concentrations for 13 h. After hypoxic condition, cells were incubated under high oxygen (21%) environment for 15 minutes and 60 minutes or immediately lysed. After lysis, cell lysates were subjected to click reaction and analyzed for in-gel fluorescence.
Table B.3.1 - A list of glutathionylated proteins identified in mass analysis that have a high spectral count and a high enrichment in the positive over negative sample.

\(^a\text{Neg and Pos = spectral count of the negative and positive samples, respectively.}\)

\(^b\text{Loc = Localization (C: cytosol, N: nucleus, EC: extracellular, L: lysosome, M: mitochondrion)}\)

\(^c\text{Function = molecular function determined by DAVID GO analysis}\)

\(^d\text{Process = biological processes determined by DAVID GO analysis}\)

\(^e\text{G = glutathionylation proteomic result by exogenous H}_2\text{O}_2\)

\(^f\text{S = sulfenylation proteomic result by exogenous H}_2\text{O}_2\)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Neg</th>
<th>Pos</th>
<th>Loc</th>
<th>Function</th>
<th>Process</th>
<th>G</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
<td>0.0</td>
<td>79.8</td>
<td>EC</td>
<td>Peptidase</td>
<td>Cell proliferatio</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TBA1A</td>
<td>Tubulin alpha-1A chain</td>
<td>0.0</td>
<td>59.5</td>
<td>C</td>
<td>GTPase</td>
<td>Cell division</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CTPS1</td>
<td>CTP synthase 1</td>
<td>0.0</td>
<td>43.5</td>
<td>C</td>
<td>ligase</td>
<td>B cell proliferatio</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RRM1</td>
<td>Ribonucleoside-diphosphate reductase large subunit</td>
<td>0.0</td>
<td>35.0</td>
<td>C</td>
<td>ATP binding</td>
<td>Cell proliferatio</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GMPS</td>
<td>GMP synthase</td>
<td>0.0</td>
<td>30.5</td>
<td>C</td>
<td>Ligase</td>
<td>Metabolic</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PPP2R1A</td>
<td>Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform</td>
<td>0.0</td>
<td>29.0</td>
<td>C</td>
<td>Phosphatase regulatory</td>
<td>Apoptosis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NPEPPS</td>
<td>Puromycin-sensitive aminopeptidase</td>
<td>0.0</td>
<td>28.8</td>
<td>C, N</td>
<td>Peptidase</td>
<td>Stress response</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DHX15</td>
<td>Putative pre-mRNA-splicing factor ATP-dependent RNA helicase</td>
<td>0.0</td>
<td>28.3</td>
<td>N</td>
<td>Helicase</td>
<td>mRNA processing</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GSPT1</td>
<td>Eukaryotic peptide chain release factor GTP-binding subunit ERF3A</td>
<td>0.0</td>
<td>24.8</td>
<td>C</td>
<td>GTPase</td>
<td>Cell cycle</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Value</td>
<td>Source</td>
<td>Function</td>
<td>Regulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>-------</td>
<td>--------</td>
<td>----------</td>
<td>------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly [ADP-ribose] polymerase 1</td>
<td>0.0</td>
<td>N</td>
<td>Glycosyltransferase</td>
<td>DNA repair</td>
<td>+ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAA15</td>
<td>N-alpha-acetyltransferase 15, NatA auxiliary subunit</td>
<td>0.0</td>
<td>C</td>
<td>Acetyltransferase</td>
<td>Angiogenesis</td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VARS</td>
<td>Valine-tRNA synthetase</td>
<td>0.0</td>
<td>C</td>
<td>Ligase</td>
<td>Protein biosynthesis</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAND1</td>
<td>Cullin-associated NEDD8-dissociated protein 1</td>
<td>0.0</td>
<td>C, N</td>
<td>Ubiquitin conjugation</td>
<td>Proteolysis</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSTB</td>
<td>Cystatin-B</td>
<td>0.0</td>
<td>C, N</td>
<td>Peptidase inhibitor</td>
<td>Proteolysis</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP7</td>
<td>Ubiquitin carboxyl-terminal hydrolase 7</td>
<td>0.0</td>
<td>C, N</td>
<td>Peptidase</td>
<td>DNA repair</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIS3</td>
<td>Exosome complex exonuclease RRP44</td>
<td>0.0</td>
<td>C, N</td>
<td>Endonuclease</td>
<td>RNA processing</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUBA3D</td>
<td>Tubulin alpha-3C/D chain</td>
<td>0.0</td>
<td>C</td>
<td>GTPase</td>
<td>Protein folding</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPP2</td>
<td>Tripeptidyl-peptidase 2</td>
<td>0.0</td>
<td>C, N</td>
<td>Peptidase</td>
<td>Proteolysis</td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBBP7</td>
<td>Histone-binding protein RBBP7</td>
<td>0.0</td>
<td>N</td>
<td>RNA binding</td>
<td>DNA replication</td>
<td>+ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPA4L</td>
<td>Heat shock 70 kDa protein 4L</td>
<td>0.0</td>
<td>C, N</td>
<td>Chaperone</td>
<td>Stress response</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP4</td>
<td>Microtubule-associated protein 4</td>
<td>0.0</td>
<td>C</td>
<td>Microtubule binding</td>
<td>Cell division</td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPH1</td>
<td>Heat shock protein 105 kDa</td>
<td>0.0</td>
<td>C</td>
<td>ATP binding</td>
<td>Stress response</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIPRL</td>
<td>TIP41-like protein</td>
<td>0.0</td>
<td>C</td>
<td>PP2A inhibitor</td>
<td>Cell cycle</td>
<td>+ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUWE1</td>
<td>E3 ubiquitin-protein ligase</td>
<td>0.0</td>
<td>C, N</td>
<td>Ligase</td>
<td>DNA repair</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPP2R2A</td>
<td>Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform</td>
<td>0.0</td>
<td>C</td>
<td>Phosphatase</td>
<td>Cell cycle</td>
<td>- +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCMBP</td>
<td>Mini-chromosome maintenance complex-binding protein</td>
<td>0.0</td>
<td>N</td>
<td>Chromatin binding</td>
<td>Cell cycle</td>
<td>+ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBA6</td>
<td>Ubiquitin-like modifier-activating enzyme 6</td>
<td>0.0</td>
<td>C</td>
<td>ATP binding</td>
<td>Protein ubiquitination</td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP5</td>
<td>Ubiquitin carboxyl-terminal hydrolase 5</td>
<td>0.0</td>
<td>L</td>
<td>Hydrolase</td>
<td>Protein catabolism</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Protein Name and Description</td>
<td>Score</td>
<td>p-value</td>
<td>Function</td>
<td>Gene Expression</td>
<td>Translation</td>
<td>Processing</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>-------</td>
<td>---------</td>
<td>-----------------------------------</td>
<td>-----------------</td>
<td>-------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>SNRNP200</td>
<td>U5 small nuclear ribonucleoprotein 200 kDa helicase</td>
<td>0.0</td>
<td>14.3</td>
<td>Helicase</td>
<td>mRNA processing</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>GSTM3</td>
<td>Glutathione S-transferase Mu 3</td>
<td>0.0</td>
<td>14.0</td>
<td>C</td>
<td>Glutathione</td>
<td>Detoxification</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DNM1L</td>
<td>Dynamin-1-like protein</td>
<td>0.0</td>
<td>14.0</td>
<td>C</td>
<td>Hydrolase</td>
<td>Apoptosis</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KHSRP</td>
<td>Far upstream element-binding protein 2</td>
<td>0.0</td>
<td>14.0</td>
<td>N</td>
<td>DNA binding</td>
<td>mRNA processing</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AIMP2</td>
<td>Aminoacyl tRNA synthase complex-interacting multifunctional protein 2</td>
<td>0.0</td>
<td>14.0</td>
<td>C, N</td>
<td>Developmental</td>
<td>Apoptosis</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PABPC4</td>
<td>Polyadenylate-binding protein 4</td>
<td>0.0</td>
<td>14.0</td>
<td>C</td>
<td>mRNA processing</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NCAPD2</td>
<td>Condensin complex subunit 1</td>
<td>0.0</td>
<td>14.0</td>
<td>N</td>
<td>Chromatin binding</td>
<td>Cell cycle</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MARS</td>
<td>Methionine--tRNA ligase, cytoplasmic</td>
<td>0.0</td>
<td>13.8</td>
<td>C</td>
<td>tRNA ligase</td>
<td>Gene expression</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>UNC45A</td>
<td>Protein unc-45 homolog A</td>
<td>0.0</td>
<td>13.8</td>
<td>C</td>
<td>Chaperone</td>
<td>Cell differentiation</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>STIP1</td>
<td>Stress-induced-phosphoprotein 1</td>
<td>0.0</td>
<td>13.5</td>
<td>C, N</td>
<td>RNA binding</td>
<td>Stress response</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CORO1C</td>
<td>Coronin-1C</td>
<td>0.0</td>
<td>12.8</td>
<td>C</td>
<td>Actin binding</td>
<td>Cytokinesis</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MSH6</td>
<td>DNA mismatch repair protein Msh6</td>
<td>0.0</td>
<td>12.5</td>
<td>N</td>
<td>DNA binding</td>
<td>DNA repair</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IARS</td>
<td>Isoleucine--tRNA ligase, cytoplasmic</td>
<td>0.0</td>
<td>12.5</td>
<td>C</td>
<td>tRNA editing</td>
<td>Gene expression</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PABPC1</td>
<td>Polyadenylate-binding protein 1</td>
<td>0.0</td>
<td>12.0</td>
<td>C, N</td>
<td>RNA binding</td>
<td>mRNA processing</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EIF4G1</td>
<td>Eukaryotic translation initiation factor 4 gamma</td>
<td>0.0</td>
<td>12.0</td>
<td>C</td>
<td>Translation factor</td>
<td>Translation</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SPAG9</td>
<td>C-Jun-amino-terminal kinase-interacting protein 4</td>
<td>0.0</td>
<td>11.5</td>
<td>C</td>
<td>Kinase binding</td>
<td>Cell differentiation</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KHDRB1S1</td>
<td>KH domain-containing, RNA-binding, signal transduction-associated protein 1</td>
<td>0.0</td>
<td>11.5</td>
<td>N</td>
<td>DNA binding</td>
<td>Cell cycle</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PSMD2</td>
<td>26S proteasome non-ATPase regulatory subunit 2</td>
<td>0.0</td>
<td>11.3</td>
<td>C</td>
<td>MAPKK activator</td>
<td>Cell proliferation</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ABCE1</td>
<td>ATP-binding cassette sub-family E member 1</td>
<td>0.0</td>
<td>11.0</td>
<td>C</td>
<td>ATPase activity</td>
<td>RNA catabolism</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Protein Name</td>
<td>Expression</td>
<td>Fold</td>
<td>Function</td>
<td>Regulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------</td>
<td>------</td>
<td>-----------------------------------------</td>
<td>------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFPT1</td>
<td>Glucosamine--fructose-6-phosphate aminotransferase 1</td>
<td>0.0</td>
<td>11.0</td>
<td>Carbohydrate binding</td>
<td>metastasis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SND1</td>
<td>Staphylococcal nuclease domain-containing protein 1</td>
<td>0.0</td>
<td>11.0</td>
<td>Nuclease</td>
<td>Transcript</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNPEP</td>
<td>Aminopeptidase B</td>
<td>0.0</td>
<td>10.8</td>
<td>Peptidase</td>
<td>Proteolysis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMC4</td>
<td>Structural maintenance of chromosomes protein 4</td>
<td>0.0</td>
<td>10.8</td>
<td>ATP binding</td>
<td>Cell cycle</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPO11</td>
<td>Importin-11</td>
<td>0.0</td>
<td>10.5</td>
<td>Protein transporter</td>
<td>Protein transport</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP9X</td>
<td>Probable ubiquitin carboxyl-terminal hydrolase FAF-X</td>
<td>0.0</td>
<td>10.5</td>
<td>Hydrolase</td>
<td>Cell cycle</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRKL</td>
<td>Crk-like protein</td>
<td>0.0</td>
<td>10.3</td>
<td>MAPKK activator</td>
<td>Cell prolifer</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBCD</td>
<td>Tubulin-specific chaperone D</td>
<td>0.0</td>
<td>10.3</td>
<td>Chaperone</td>
<td>Protein folding</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSMD1</td>
<td>26S proteasome non-ATPase regulatory subunit 1</td>
<td>0.0</td>
<td>10.3</td>
<td>MAPKK activator</td>
<td>Cell prolifer</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTF2I</td>
<td>General transcription factor II-I</td>
<td>0.0</td>
<td>10.3</td>
<td>DNA binding</td>
<td>Transcript</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LARP1</td>
<td>La-related protein 1</td>
<td>0.0</td>
<td>10.3</td>
<td>mRNA binding</td>
<td>Cell prolifer</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTLL12</td>
<td>Tubulin--tyrosine ligase-like protein 12</td>
<td>0.0</td>
<td>10.0</td>
<td>ATP binding</td>
<td>Tubulin PTM</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPRS</td>
<td>Bifunctional glutamate/proline--tRNA ligase</td>
<td>1.0</td>
<td>68.5</td>
<td>Ligase</td>
<td>Translation</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFAS</td>
<td>Phosphoribosylformylglycinamidine synthase</td>
<td>0.7</td>
<td>34.5</td>
<td>Ligase</td>
<td>Purine biosynthesis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NASP</td>
<td>Nuclear autoantigenic sperm protein</td>
<td>0.7</td>
<td>33.0</td>
<td>Protein binding</td>
<td>Cell prolifer</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBE2L3</td>
<td>Ubiquitin-conjugating enzyme E2 L3</td>
<td>0.7</td>
<td>32.8</td>
<td>Transferase</td>
<td>Transcript</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GARS</td>
<td>Glycine--tRNA ligase</td>
<td>0.7</td>
<td>32.0</td>
<td>Ligase</td>
<td>Translation</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Expression</td>
<td>Gene Function</td>
<td>Polarity</td>
<td>Polarity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
<td>------------</td>
<td>---------------------</td>
<td>----------</td>
<td>----------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRMT1</td>
<td>Protein arginine N-methyltransferase 1</td>
<td>0.7</td>
<td>Methyl transferase</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSCN1</td>
<td>Fascin</td>
<td>0.7</td>
<td>Actin binding</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNRNP K</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>1.0</td>
<td>Pre-mRNA binding</td>
<td>C, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XPO1</td>
<td>Exportin-1</td>
<td>1.0</td>
<td>Protein transporter</td>
<td>C, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPA1</td>
<td>Inorganic pyrophosphatase</td>
<td>1.0</td>
<td>Phosphatase</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAICS</td>
<td>Multifunctional protein ADE2</td>
<td>0.7</td>
<td>Decarboxylase</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PREP</td>
<td>Prolyl endopeptidase</td>
<td>0.7</td>
<td>Peptidase</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSE1L</td>
<td>Exportin-2</td>
<td>0.7</td>
<td>Protein transport</td>
<td>C, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEF2</td>
<td>Elongation factor 2</td>
<td>8.3</td>
<td>GTPase</td>
<td>C, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCMT1</td>
<td>Protein-L-isoaspartate(D-aspartate) O-methyltransferase</td>
<td>2.3</td>
<td>Methyl transferase</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPM1</td>
<td>Nucleophosmin</td>
<td>1.0</td>
<td>Chaperone</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLS3</td>
<td>Plastin-3</td>
<td>1.3</td>
<td>Calcium binding</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMPDH 2</td>
<td>Inosine-5'-monophosphate dehydrogenase 2</td>
<td>0.7</td>
<td>Oxidoreductase</td>
<td>C, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLTC</td>
<td>Clathrin heavy chain 1</td>
<td>1.7</td>
<td>Protein or RNA binding</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRKDAC</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
<td>2.3</td>
<td>kinase</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YWHA Q</td>
<td>14-3-3 protein theta</td>
<td>0.7</td>
<td>Protein binding</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP-citrate synthase</td>
<td>2.0</td>
<td>Transferase</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEF1G</td>
<td>Elongation factor 1-gamma</td>
<td>3.0</td>
<td>Transferase</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Fold</td>
<td>Ratio</td>
<td>Class</td>
<td>GO Term</td>
<td>Function</td>
<td>Effect</td>
<td>Effect</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------------</td>
<td>------</td>
<td>-------</td>
<td>------------</td>
<td>------------------------------------------</td>
<td>-----------------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>RCC2</td>
<td>Protein RCC2</td>
<td>1.3</td>
<td>25.3</td>
<td>N</td>
<td>GTPase activator</td>
<td>Cell cycle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GART</td>
<td>Trifunctional purine biosynthetic protein adenosine-3</td>
<td>5.0</td>
<td>78.8</td>
<td>C</td>
<td>Ligase</td>
<td>Purine biosynthesis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CPNE1</td>
<td>Copine-1</td>
<td>0.7</td>
<td>10.5</td>
<td>N</td>
<td>Repressor</td>
<td>Differentiation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CCT2</td>
<td>T-complex protein 1 subunit beta</td>
<td>4.3</td>
<td>66.5</td>
<td>C</td>
<td>Chaperone</td>
<td>Protein folding</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>11.0</td>
<td>165.3</td>
<td>C</td>
<td>Hydrolase, Transferase</td>
<td>Fatty acid biosynthesis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AHCY</td>
<td>Adenosylhomocysteinase</td>
<td>1.3</td>
<td>20.0</td>
<td>C</td>
<td>Hydrolase</td>
<td>One-carbon metabolism</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DYNC1</td>
<td>Cytoplasmic dynein 1 heavy chain 1</td>
<td>1.3</td>
<td>19.5</td>
<td>C</td>
<td>ATPase</td>
<td>Transport</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PTBP1</td>
<td>Polypyrimidine tract-binding protein 1</td>
<td>1.7</td>
<td>24.0</td>
<td>N</td>
<td>Pre-mRNA binding</td>
<td>mRNA processing</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TARS</td>
<td>Threonine--tRNA ligase, cytoplasmic</td>
<td>1.0</td>
<td>13.3</td>
<td>C</td>
<td>Ligase</td>
<td>Translation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein HSP 90-beta</td>
<td>10.7</td>
<td>137.3</td>
<td>C</td>
<td>Chaperone</td>
<td>Stress response</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DDX3X</td>
<td>ATP-dependent RNA helicase DDX3X</td>
<td>1.0</td>
<td>12.8</td>
<td>N</td>
<td>Helicase/ATPase</td>
<td>Transcripton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCP1</td>
<td>T-complex protein 1 subunit alpha</td>
<td>2.7</td>
<td>33.8</td>
<td>C</td>
<td>Chaperone</td>
<td>Protein folding</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IPO5</td>
<td>Importin-5</td>
<td>1.7</td>
<td>21.0</td>
<td>C, N</td>
<td>GTPase inhibitor</td>
<td>Protein transport</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GSPT2</td>
<td>Eukaryotic peptide chain release factor GTP-binding subunit ERF3B</td>
<td>1.0</td>
<td>12.5</td>
<td>C</td>
<td>GTPase</td>
<td>Cell cycle</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CFL1</td>
<td>Cofilin-1</td>
<td>4.7</td>
<td>56.8</td>
<td>N</td>
<td>Actin binding</td>
<td>Cytokinesis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CCT6A</td>
<td>T-complex protein 1 subunit zeta</td>
<td>1.0</td>
<td>11.0</td>
<td>C</td>
<td>Chaperone</td>
<td>Protein folding</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PRDX2</td>
<td>Peroxiredoxin-2</td>
<td>2.3</td>
<td>24.3</td>
<td>C, M</td>
<td>Peroxidase</td>
<td>Oxidative stress response</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PHGDH</td>
<td>D-3-phosphoglycerate dehydrogenase</td>
<td>1.3</td>
<td>13.8</td>
<td>C</td>
<td>Oxidoreductase</td>
<td>Amino-acid biosynthesis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Expression</td>
<td>Regulation</td>
<td>Function</td>
<td>Notes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>------------</td>
<td>------------</td>
<td>--------------------------------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCN1L</td>
<td>Translational activator GCN1</td>
<td>2.3</td>
<td>C</td>
<td>RNA binding</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRDX1</td>
<td>Peroxiredoxin-1</td>
<td>2.7</td>
<td>C, M</td>
<td>Peroxidase</td>
<td>Oxidative stress response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBA1</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>6.7</td>
<td>C,M</td>
<td>Ligase</td>
<td>proteolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKM</td>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>6.3</td>
<td>C, N</td>
<td>Kinase</td>
<td>Glycolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL18A</td>
<td>60S ribosomal protein L18a</td>
<td>2.0</td>
<td>C</td>
<td>RNA binding</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDX1</td>
<td>ATP-dependent RNA helicase</td>
<td>1.3</td>
<td>N</td>
<td>Helicase</td>
<td>Transcription</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNRNP F</td>
<td>Heterogeneous nuclear ribonucleoprotein F</td>
<td>1.7</td>
<td>N</td>
<td>RNA binding</td>
<td>mRNA processing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>20.7</td>
<td>C</td>
<td>Chaperone</td>
<td>Stress response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRDX6</td>
<td>Peroxiredoxin-6</td>
<td>6.0</td>
<td>C</td>
<td>Peroxidase</td>
<td>Oxidative stress response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QARS</td>
<td>Glutamine--tRNA ligase</td>
<td>1.7</td>
<td>C, M</td>
<td>Ligase</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNRNP L</td>
<td>Heterogeneous nuclear ribonucleoprotein L</td>
<td>5.0</td>
<td>C, N</td>
<td>RNA binding</td>
<td>mRNA processing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIF1A2</td>
<td>Elongation factor 1-alpha 2</td>
<td>12.3</td>
<td>N</td>
<td>GTPase</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRCC6</td>
<td>X-ray repair cross-complementing protein 6</td>
<td>2.7</td>
<td>N</td>
<td>Helicase</td>
<td>DNA repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRCC5</td>
<td>X-ray repair cross-complementing protein 5</td>
<td>6.3</td>
<td>N</td>
<td>Helicase</td>
<td>DNA repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIF5AL1</td>
<td>Eukaryotic translation initiation factor 5A-1-like</td>
<td>5.7</td>
<td>C, N</td>
<td>mRNA binding</td>
<td>mRNA transport</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEF1A1</td>
<td>Elongation factor 1-alpha 1</td>
<td>18.3</td>
<td>C, N</td>
<td>GTPase</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>CAD protein</td>
<td>2.7</td>
<td>C, N</td>
<td>Hydrolase, ligase</td>
<td>Pyrimidine biosynthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YWHA</td>
<td>14-3-3 protein epsilon</td>
<td>4.0</td>
<td>C</td>
<td>HDAC binding</td>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPA8</td>
<td>Heat shock cognate 71 kDa protein</td>
<td>3.0</td>
<td>C</td>
<td>ATPase, Chaperone</td>
<td>Transcripti</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDOA</td>
<td>Fructose-bisphosphat aldolase A</td>
<td>3.0</td>
<td>C</td>
<td>Lyase</td>
<td>Glycolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL10</td>
<td>60S ribosomal protein L10</td>
<td>4.7</td>
<td>C</td>
<td>RNA binding</td>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>K</td>
<td>K'</td>
<td>Function</td>
<td>Protein folding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
<td>---</td>
<td>----</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCT5</td>
<td>T-complex protein 1 subunit epsilon</td>
<td>6.0</td>
<td>16.0</td>
<td>ATP binding</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>30.7</td>
<td>77.3</td>
<td>Oxidoreductase/transf erase</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCM3</td>
<td>DNA replication licensing factor MCM3</td>
<td>4.0</td>
<td>10.0</td>
<td>Helicase</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NME1</td>
<td>Nucleoside diphosphate kinase A</td>
<td>7.3</td>
<td>18.3</td>
<td>Kinase</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGAM1</td>
<td>Phosphoglycerate mutase 1</td>
<td>8.0</td>
<td>17.5</td>
<td>Isomerase</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>7.7</td>
<td>15.5</td>
<td>Isomerase</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.4.1 – Glutathionylation in response to mitochondrial dysfunction in PP2Cα transfected HEK293-GS M4 cells. PP2C transfected to HEK293-GS M4 using PEI-max and after 24 h, L-AzAla was incubated with Cells. After 20 h, cells were washed with PBS and incubated with 2 μg/mL AMA with or without glucose. Lysates were subjected to click reaction with biotin-alkyne and incubated with streptavidin agarose beads. After incubation bound protein were eluted and probed with strep-HRP, anti-HA and anti-PTP1B.
Figure C.4.2 – Cellular GSH levels in MCF7 and MDA-MB-231 cells. MDA-MB-231 and MCF7 cells were infected with adenovirus for the overexpression of GS M4. After 24 h, L-AzAla incubated with cells for 20 h. Cells were induced for glutathionylation by incubating different concentrations of glucose for 5 h. Cells were trypsinized and collected by centrifugation. Cells were lysed using several freeze-thaw cycles and protein free lysates were subjected to the bromobimane assay.

Figure C.4.3 – Glutathionylation in response to oxygen-glucose deprivation (OGD) and reoxygenation (OGD/R) in MDA-MB-231 cells. MDA-MB-231 cells were infected with
adenovirus having GS M4 gene. After 24 h, L-AzAla was incubated for another 20 h and induced for glutathionylation in low glucose medium under normal oxygen (21%) or low oxygen concentration (1%) for 5.5 h. After 5.5 h, cells were immediately lysed or incubated in normal oxygen environment for 30 minutes. Cell lysates were subjected to click reaction with Cy5-alkyne and analyzed for in-gel fluorescence.

**Figure C.4.4 – Glutamine metabolism and redox homeostasis.** Glutaminase (GLS) catalyzes glutamate synthesis using glutamine as the substrate. Glutamate enters TCA cycle and takes part in NADPH and pyruvate production. Glutamate also a precursor for GSH biosynthesis.
APPENDIX D - COPYRIGHT PERMISSIONS

Title: Cellular mechanisms and physiological consequences of redox-dependent signalling
Authors: Kira M. Holmstrom, Toren Finkel
Publication: Nature Reviews Molecular Cell Biology
Publisher: Nature Publishing Group
Date: May 23, 2014
Copyright © 2014. Rights Managed by Nature Publishing Group

Order Completed
Thank you for your order.

This Agreement between Wayne State University -- Kusal Samarasinghe ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details:

License Number: 4138646410701
License Date: Jun 29, 2017
Licensed Content Publisher: Nature Publishing Group
Licensed Content Publication: Nature Reviews Molecular Cell Biology
Licensed Content Title: Cellular mechanisms and physiological consequences of redox-dependent signalling
Licensed Content Author: Kira M. Holmstrom, Toren Finkel
Licensed Content Date: May 23, 2014
Licensed Content Volume: 15
Type of Use: reuse in a dissertation / thesis
Reuseter type: academic/educational
Format: electronic
Portion: figures/tables/illustrations
Number of Figures/Tables/Illustrations: 1
High-res required: no
Figures: Figure 3: Intracellular sources of reactive oxygen species. Figure 3: Reactive oxygen species can function as mediators of intracellular signalling.

Author of this NPG article: no
Your reference number: no
Title of your thesis / dissertation: DEVELOPMENT OF CHEMICAL TOOLS TO INVESTIGATE PROTEIN GLUTATHIONYLATION IN RESPONSE TO METABOLIC ALTERATION
Expected completion date: Aug 2017
Estimated size (number of pages): 95

Reuseter Location: Wayne State University
Street: 5101 Cass Avenue
City: Detroit
State: MI
ZIP: 48201
Country: United States
Attention: Kusal

Billing Type: Divide
Billing address: Wayne State University
Street: 5101 Cass Avenue
City: Detroit
State: MI
ZIP: 48201
Country: United States
Attention: Kusal

Total: 0.00 USD

ORDER MORE  CLOSE WINDOW
Thank you for your order!

Dear Mr. Kusal Samarasinghe,

Thank you for placing your order through Copyright Clearance Center’s RightsLink® service.

**Order Summary**

- Licensee: Wayne state university
- Order Date: Jun 29, 2017
- Order Number: 4138340410701
- Publication: Nature Reviews Molecular Cell Biology
- Title: Cellular mechanisms and physiological consequences of redox-dependent signalling
- Type of Use: reuse in a dissertation / thesis
- Order Total: 0.00 USD

View or print complete details of your order and the publisher’s terms and conditions.

Sincerely,

Copyright Clearance Center

How was your experience? Fill out this survey to let us know.

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
https://myaccount.copyright.com
PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

If credit is given to another source for the material you requested, permission must be obtained from that source.
Title: A clickable glutathione approach for identification of protein glutathionylation in response to glucose metabolism

Author: Kusal T. G. Samarasinghe, Dhanushka N. P. Mankananta Godage, Yani Zhou, Ndombere Ernestie, Weerasara, Young-Hoon Ahn

Publication: Molecular BioSystems
Publisher: Royal Society of Chemistry
Date: May 10, 2016

Order Completed
Thank you for your order.

This Agreement between Wayne state university — Kusal Samarasinghe ("You") and Royal Society of Chemistry ("Royal Society of Chemistry") consists of your license details and the terms and conditions provided by Royal Society of Chemistry and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

### Licensee Details

<table>
<thead>
<tr>
<th>Licensee Number</th>
<th>0123412345678</th>
</tr>
</thead>
<tbody>
<tr>
<td>License Date</td>
<td>Jul 29, 2017</td>
</tr>
<tr>
<td>Published</td>
<td>Royal Society of Chemistry</td>
</tr>
<tr>
<td>Title</td>
<td>A clickable glutathione approach for identification of protein glutathionylation in response to glucose metabolism</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Thesis/Dissertation</td>
</tr>
<tr>
<td>Receptor type</td>
<td>academic/educational</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/images</td>
</tr>
<tr>
<td>Number of</td>
<td>3</td>
</tr>
<tr>
<td>Distribution</td>
<td>1</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Order Reference</td>
<td>No</td>
</tr>
</tbody>
</table>

### License Details

<table>
<thead>
<tr>
<th>General</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>12</td>
</tr>
<tr>
<td>Issue</td>
<td>5</td>
</tr>
<tr>
<td>Expected</td>
<td>Development of Chemical Tools to Investigate Protein Glutathionylation in Response to Metabolic Alteration</td>
</tr>
<tr>
<td>Date</td>
<td>Aug 2017</td>
</tr>
<tr>
<td>Size</td>
<td>350</td>
</tr>
<tr>
<td>Location</td>
<td>Wayne State University, 5101 Cass Ave, Detroit, MI 48202, United States</td>
</tr>
<tr>
<td>Note</td>
<td>Additional Information here</td>
</tr>
</tbody>
</table>

### Billing Details

<table>
<thead>
<tr>
<th>Company</th>
<th>Wayne State University</th>
</tr>
</thead>
<tbody>
<tr>
<td>Address</td>
<td>5101 Cass Ave, Detroit, MI 48202, United States</td>
</tr>
<tr>
<td>Note</td>
<td>Additional Address Information here</td>
</tr>
</tbody>
</table>

### Total

0.00 USD

---

This is an automatically generated report and may contain errors. Please check the original document for accuracy.
**Order Completed**

Thank you for your order.

This Agreement between Wayne state university -- Kusal Samarasinge ("You") and Thieme ("Thieme") consists of your license details and the terms and conditions provided by Thieme and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

### Printable details

<table>
<thead>
<tr>
<th>License Number</th>
<th>41.8376/681296</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Jan 29, 2017</td>
</tr>
<tr>
<td>Licensed Content</td>
<td>Thieme</td>
</tr>
<tr>
<td>Licensed Content Publisher</td>
<td>Synlett</td>
</tr>
<tr>
<td>Licensed Content Title</td>
<td>Synthesizing Clickable Glutathione by Glutathione Synthetase Mutant for Detecting Protein Glutathionylation</td>
</tr>
<tr>
<td>Licensed Content Author</td>
<td>Kusal T. G. Samarasinge, Young-Hoon Ahn</td>
</tr>
<tr>
<td>Licensed Content Date</td>
<td>Jan 1, 2015</td>
</tr>
<tr>
<td>Licensed Content Volume</td>
<td>26</td>
</tr>
<tr>
<td>Licensed Content Issue</td>
<td>03</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>author of the original Thieme publication</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/images</td>
</tr>
<tr>
<td>Number of figures/tables/images</td>
<td>3</td>
</tr>
<tr>
<td>Will you be translating</td>
<td>no</td>
</tr>
<tr>
<td>Distribution quantity</td>
<td>1</td>
</tr>
<tr>
<td>Specified additional information</td>
<td>Scheme 2 Biochemical methods for identifying protein glutathionylation.</td>
</tr>
<tr>
<td>Order reference number</td>
<td>DEVELOPMENT OF CHEMICAL TOOLS TO INVESTIGATE PROTEIN GLUTATHIONYLATION IN RESPONSE TO METABOLIC ALTERATION</td>
</tr>
<tr>
<td>Title of your dissertation/thesis</td>
<td>DEVELOPMENT OF CHEMICAL TOOLS TO INVESTIGATE PROTEIN GLUTATHIONYLATION IN RESPONSE TO METABOLIC ALTERATION</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Aug 2017</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>250</td>
</tr>
</tbody>
</table>
| Requestor Location | Wayne state university  
5101 Cass avenu  
Detroit  
MI  
DEPTOIT, MI 48201  
United States  
Attn: Kusal |
| Publisher Tax ID | DE 1492900807 |
| Billing Type | Invoice |
| Billing address | Wayne state university  
5101 Cass avenu  
Detroit  
MI  
DEPTOIT, MI 48201  
United States  
Attn: Kusal |
| Total | 0.00 USD |
Permission Not Required

Permission is not required for this type of use.
Dear Kusal,

Thank you for your email.

I am pleased to grant you permission to re-use this as long as the original work is correctly cited and reference as part of a bibliography and in text citations.

Kind Regards,

Aisha Hayat | Production Editor
Biochemical Society | Portland Press
Charles Darwin House, 12 Roger Street, London WC1N 2JU
Tel +44 (0)207 685 2450 | aisha.hayat@portlandpress.com

Follow us on Twitter: @BiochemSoc | @PPPublishing | https://www.biochemistry.org/ | https://www.portlandpresspublishing.com/

Portland Press Ltd registered in England and Wales No. 2453983.
Registered Office: Charles Darwin House, 12 Roger Street, Third Floor, London, WC1N 2JU.

VAT registration number GB 523 2902 09.

The contents of this email are for the recipient only.

If you are not this person (or not responsible for delivery to this person), then notify the sender and delete the email immediately.

Any opinions contained in this message are those of the author and are not given or endorsed by Portland Press Ltd or the division through which this message is sent unless otherwise clearly indicated in this message.

Charles Darwin House, 12 Roger Street, Third Floor, London, WC1N 2JU
REFERENCES


normally and show no increased sensitivity to hyperoxia. *The Journal of biological chemistry* **272**, 16644-16651


glutathionylated proteins in oxidatively stressed human T lymphocytes. 

*Proceedings of the National Academy of Sciences of the United States of America* **99**, 3505-3510


monocarboxylate transporter 1 (MCT1) expression and MCT1-dependent tumor cell migration. *Oncogene* **33**, 4060-4068


A., Skollermo, A., Steen, J., Stenvall, M., Sterky, F., Stromberg, S., Sundberg, M.,
Tegel, H., Tourle, S., Wahlund, E., Walden, A., Wan, J., Wernerus, H., Westberg,

an anticancer strategy. *Nature reviews. Drug discovery* 12, 931-947


and Mayo, J. C. (2015) Manganese superoxide dismutase (SOD2/MnSOD)/catalase and SOD2/GPx1 ratios as biomarkers for tumor
progression and metastasis in prostate, colon, and lung cancer. *Free radical biology & medicine* 85, 45-55

C., and Greengard, P. (2003) Protein phosphatase 2C binds selectively to and
dephosphorylates metabotropic glutamate receptor 3. *Proceedings of the National
Academy of Sciences of the United States of America* 100, 16006-16011

activation is required for long-term depression in medial prefrontal cortex and fear
extinction. *Proceedings of the National Academy of Sciences* 112, 1196-1201


ABSTRACT

DEVELOPMENT OF CHEMICAL TOOLS TO INVESTIGATE PROTEIN S-GLUTATHIONYLATION IN RESPONSE TO METABOLIC ALTERATION

by

KUSAL T. G SAMARASINGHE

August 2017

Advisor: Prof. Young-Hoon Ahn

Major: Chemistry (Biochemistry)

Degree: Doctor of Philosophy

Oxidative stress is a common characteristic of age-related diseases such as vascular diseases, diabetes and cancer. Many diseases are known to be regulated by glutathionylation. Glutathionylation is referred to as the formation of disulfide bond between a protein cysteine and a glutathione. To understand the molecular mechanisms behind the disease initiation and progression, identification of such glutathionylated proteins is important. Even though existing methods have been widely used, several limitations of these methods hinder the identification of such proteins in disease conditions. Therefore, we developed a versatile chemical method that generates clickable glutathione inside the cells. In this method, we generated a mutant form of glutathione synthetase (GS M4) to metabolically synthesize clickable glutathione for studying and identifying proteins in disease conditions. Using clickable glutathione approach, we uncovered a regulatory role of glucose metabolism in glutathionylation. Also, we have identified over 1400 of glutathionylated proteins in response to mitochondrial dysfunction and glucose deprivation, a common characteristic found in metabolic disorders. Moreover, we have identified several new glutathionylated proteins including ser/thr...
protein phosphatase 2Cα (PP2Cα). Further, we uncovered a new role for PP2Cα glutathionylation that promotes cancer cell migration through the activation of JNK in response to metabolic alterations.
AUTOBIOGRAPHICAL STATEMENT
KUSAL T. G SAMARASINGHE

EDUCATION
Ph.D., Chemistry (anticipated July, 2017)
Department of Chemistry, Wayne State University, Detroit, MI, USA
Advisor: Prof. Young-Hoon Ahn
B.Sc., Chemistry (July 2006 - August 2010)
Department of Chemistry, University of Colombo, Sri Lanka
Advisor: Prof. Rohini Perera

AWARDS AND HONORS

- Outstanding Biological Chemistry Student award, Department of Chemistry, Wayne State University for superior academic and research achievement
- Graduate school citation for excellence in teaching, Awarded by Graduate school, Wayne State University
- Knoller Fellowship, Awarded by Graduate school, Wayne State University for superior academic achievement, September 2014 - May 2015
- Graduate Student Professional Travel Award, Wayne State University
- Thomas C. Rumble fellowship: Awarded by Graduate school, Wayne State University for superior academic achievement, September 2014 - May 2015

PUBLICATIONS

- Samarasinghe, K. T. G. and Ahn, Y.H., Glutathionylation of serine/threonine protein phosphatase (PP2Ca) promotes cancer cell migration under metabolic stress. (manuscript in preparation)