THE REGULATORY ROLES OF GAMMA INTERFERON INDUCIBLE LYSOSOMAL THIOL REDUCTASE (GILT) IN CELLULAR REDOX HOMEOSTASIS

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Microbiology and Immunology

By

Hao-Sen Chiang, M.S.

Washington, DC
April 7, 2011
THE REGULATORY ROLES OF GAMMA INTERFERON INDUCIBLE LYSOSOMAL THIOL REDUCTASE (GILT) IN CELLULAR REDOX HOMEOSTASIS

Hao-Sen Chiang, M.S.

Thesis Advisor: Maja Maric, Ph.D.

ABSTRACT

Redox regulation is critical for a number of cellular functions and has been implicated in the etiology and progression of cardiovascular diseases, neurodegenerative diseases, and cancer. It has been shown that in the absence of Gamma-interferon Inducible Lysosomal Thiol reductase (GILT), cells experience moderate oxidative stress indicated by increased production of reactive oxygen species and reduced expression of mitochondrial manganese superoxide dismutase (SOD2). In the current study, we further elucidate the role of GILT in homeostatic regulation in response to oxidative stress. We show that GILT-deficient fibroblasts exhibit a decreased level of reduced glutathione, an increase in the ratio of oxidized (GSSG) vs. reduced (GSH) forms of glutathione and an accumulation of dysfunctional mitochondria. Redox-sensitive pathways involving Erk1/2 activation and high mobility group box 1 (HMGB1) protein cytosolic translocation are both activated and associated with an increased autophagy in GILT -/- fibroblasts. We hypothesize that the absence of GILT triggers these intracellular changes that ultimately result in the degradation of the damaged mitochondria and mitochondrial SOD2 in the absence of GILT. To our knowledge, this is the first time that a lysosomal enzyme has been implicated in maintenance of cellular redox homeostasis.
ACKNOWLEDGEMENT

The research and writing of this thesis would not have been possible unless the guidance, support, and encouragement of the following people.

My deepest thanks and appreciation are to my advisor, Maja Maric, Ph.D. She taught me how to question thoughts and express ideas. I have been fortunate to have an advisor who gave her exceptional support and caring throughout the five years of my doctoral studies. I hope that one day I would become as good an advisor to my students as she has been to me.

The members of my dissertation committee, Michael Cole, Ph.D., William Fonzi, Ph.D., Steven Singer, Ph.D., and Mark Williams, Ph.D., have generously given their expertise and time to better my work. I am thankful for their guidance and inspiration on my research. It is a great journey for me to complete this doctoral training.

I thank my friends and colleagues, Branka Bogunovic, Ph.D., Leonard Chen, Ph.D., and Priya Srinivasan, Ph.D., for their help, support, advice, and friendship.

I also thank my parents, Wen-Ming Chiang and Chao-Bi Lin, who have always supported, encouraged, and believed in me. Without them I would not have a doctoral degree.

Last but not least, a biggest thank you to my fiancé, Kuan-Yu Kuo. Your love and support have taught me so much about compromise and sacrifice. Without her this effort would have been worth nothing.

The dissertation is dedicated to Kuan-Yu and my parents.

Many thanks,

Hao-Sen Chiang
# TABLE OF CONTENTS

INTRODUCTION ........................................................................................................................................... 1

1. Gamma-interferon-inducible lysosomal thiol reductase ................................................................. 1

   1.1 Synthesis and maturation .................................................................................................................. 1

   1.2 Mechanism of action ......................................................................................................................... 2

   1.3 Biological roles of GILT .................................................................................................................. 4

      1.3.1 The role of GILT in MHC class II antigen processing and presentation ...... 4

      1.3.2 The regulatory roles of GILT in cell activation ................................................................. 4

2. Reactive oxygen species ....................................................................................................................... 7

   2.1 Sources of ROS within the cell ....................................................................................................... 7

   2.2 Antioxidants .................................................................................................................................. 10

      2.2.1 Enzymatic antioxidants ........................................................................................................... 10

      2.2.2 Nonenzymatic antioxidants .................................................................................................... 12

   2.3 Oxidative stress ............................................................................................................................... 13

3. Removal of damaged proteins and organelles ................................................................................. 14

   3.1 Autophagy ....................................................................................................................................... 14

   3.2 Cellular and molecular machinery of autophagy ......................................................................... 17

   3.3 Mitophagy ....................................................................................................................................... 21

4. Basis of proposed studies ................................................................................................................... 21

HYPOTHESIS AND SPECIFIC AIMS ......................................................................................................... 23
Specific aim 1. To test the hypothesis that the absence of GILT alters glutathione synthesis and leads to decreased SOD2 levels ................................................................. 23

Specific aim 2. To test the hypothesis that autophagy affects SOD2 levels in GILT -/- fibroblasts .................................................................................................................. 28

Specific aim 3. To identify the signaling pathways involved in the induction of autophagy in WT and GILT -/- fibroblasts .................................................................................. 29

Materials and Methods ........................................................................................................... 30

1. REAGENTS .......................................................................................................................... 30

2. CELL CULTURE .................................................................................................................. 30

3. REDUCED GLUTATHIONE ASSAY .................................................................................. 31

4. ANALYSIS OF GSSG/GSH RATIO ................................................................................... 31

5. MEASUREMENT OF REACTIVE OXYGEN SPECIES ...................................................... 32

6. WESTERN BLOTTING AND ANTIBODIES ..................................................................... 32

7. MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT ..................................... 33

8. ANALYSIS OF HMGB1 TRANSLOCATION ..................................................................... 34

9. STATISTICAL ANALYSIS ................................................................................................ 34

Results .................................................................................................................................... 35

GILT deficiency in fibroblasts leads to decreased glutathione levels ............................... 35

Increased GSSG/GSH ratio in GILT -/- fibroblasts ............................................................. 37

Modulation of intracellular glutathione levels alters SOD2 expression ......................... 41

Mitochondrial membrane potential is diminished in GILT -/- fibroblasts ....................... 46

Autophagy is up-regulated in GILT -/- fibroblasts .............................................................. 50
INCREASED MITOPHAGY IS DETECTED IN GILT -/- FIBROBLASTS ................................................................. 55

3-MA TREATMENT RESULTS IN INCREASED OXIDATIVE STRESS ................................................................. 55

MODULATION OF REDUCED GLUTATHIONE LEVELS LEADS TO THE CHANGES IN AUTOPHAGY

ACTIVITIES ......................................................................................................................................................... 60

INHIBITION OF ERK1/2 PHOSPHORYLATION LEADS TO THE DOWN-REGULATION OF AUTOPHAGY AND INCREASED SOD2 EXPRESSION ........................................................................................................ 61

HMGB1 TRANSLOCATION FROM THE NUCLEUS TO THE CYTOPLASM IS INCREASED IN GILT -/- FIBROBLASTS .............................................................................................................................................. 74

HMGB1 CYTOSOLIC TRANSLOCATION IS SENSITIVE TO THE CHANGES OF GSH LEVELS .................. 76

DISCUSSION ....................................................................................................................................................... 79

SPECIFIC AIM 1. TO TEST THE HYPOTHESIS THAT THE ABSENCE OF GILT ALTERS GLUTATHIONE SYNTHESIS AND LEADS TO DECREASED SOD2 LEVELS ........................................................................................................ 79

GSSG VS. GSH ...................................................................................................................................................... 80

SPECIFIC AIM 2. TO TEST THE HYPOTHESIS THAT AUTOPHAGY AFFECTS SOD2 LEVELS IN GILT -/- FIBROBLASTS .............................................................................................................................................. 81

MITOCHONDRIAL MEMBRANE POTENTIAL ........................................................................................................ 81

AUTOPHAGY ......................................................................................................................................................... 82

MITOPHAGY ......................................................................................................................................................... 84

SPECIFIC AIM 3. TO IDENTIFY THE SIGNALING PATHWAYS INVOLVED IN THE INDUCTION OF AUTOPHAGY IN WT AND GILT -/- FIBROBLASTS ........................................................................................................ 85

ERK1/2 SIGNALING PATHWAY .......................................................................................................................... 86

HMGB1 ................................................................................................................................................................. 87

vii
LIST OF FIGURES

Figure 1 Mechanism of reduction by GILT ................................................................. 3
Figure 2 The phylogenetic tree of GILT proteins from 19 representative species .......... 6
Figure 3 Mitochondrial ROS production .................................................................... 8
Figure 4 Intracellular antioxidant enzymes .............................................................. 11
Figure 5 Main forms of autophagy ........................................................................... 15
Figure 6 Cellular and molecular machinery of autophagy ........................................ 18
Figure 7 The lack of GILT leads to increased oxidative stress and the induction of the autophagy ........................................................................................................ 24
Figure 8 Regulation of the glutathione biosynthesis ................................................ 27
Figure 9 Levels of reduced glutathione (GSH) are decreased in GILT -/- fibroblasts .......... 36
Figure 10 Basal ratio of oxidized glutathione (GSSG) vs. reduced glutathione (GSH) is increased in GILT -/- fibroblasts ................................................................. 38
Figure 11 Shift of GSSG/GSH ratio toward the oxidized form of glutathione is greater in GILT -/- fibroblasts ............................................................................................ 40
Figure 12 Altered GSH levels lead to increased superoxide anion levels ..................... 44
Figure 13 Increase in GSH is followed by up-regulated SOD2 expression .................... 46
Figure 14 Mitochondrial membrane potential is reduced in GILT -/- fibroblasts .......... 49
Figure 15 Chloroquine inhibits the fusion between the autophagosomes and the lysosomes .... 52
Figure 16 Autophagy is increased in GILT -/- fibroblasts .......................................... 53
Figure 17 Inhibition of autophagy results in increased SOD2 expression.................... 54
Figure 18 The level of mitochondrial COX IV is reduced in GILT -/- fibroblasts .......... 56
Figure 19 Treatment of 3-methyladenine (3-MA) results in increased oxidative stress.............. 59
Figure 20 Altered glutathione levels lead to changes in autophagy ........................................ 63
Figure 21 Erk1/2 signaling is altered in GILT -/- fibroblasts ..................................................... 65
Figure 22 Modulation of glutathione levels correlates with changes in Erk1/2 phosphorylation and MKP3 expression ......................................................... 69
Figure 23 MEK1/2 inhibitors inhibit the Erk1/2 signaling pathway ............................................. 70
Figure 24 Erk1/2 signaling is involved in autophagy and has an effect on SOD2 expression ......... 74
Figure 25 HMGB1 cytosolic translocation promotes autophagy ............................................... 75
Figure 26 Translocation of HMGB1 into the cytoplasm is increased in GILT -/- fibroblasts ...... 77
Figure 27 The antioxidant L-NAC inhibits the translocation of HMGB1 from the nucleus to the cytoplasm in GILT -/- fibroblasts .......................................................... 78
INTRODUCTION

1. Gamma-interferon-inducible lysosomal thiol reductase

1.1 Synthesis and maturation

Gamma-interferon-inducible lysosomal thiol reductase (GILT) was initially identified by Luster et al. and named IP-30 (registered as IFI-30 in the Human Gene Nomenclature Data Base), a novel \( \gamma \) interferon-inducible protein with a vesicular subcellular location in the monocytic cell line U937 (1). GILT is constitutively expressed at high levels in professional antigen-presenting cells (APCs) and is detectable in lower amounts in other cell types, such as T cells, fibroblasts, endothelial cells, and keratinocytes, but can be up-regulated by pro-inflammatory cytokines (e.g. interleukin 1\( \beta \), tumor necrosis factor \( \alpha \), and \( \gamma \) interferon) (1, 2). It is synthesized as a soluble 35-kDa precursor glycoprotein, which is transported into the endosomal/lysosomal compartments by the mannose-6-phosphate receptor pathway, and processed to the mature 30-kDa form via proteolytic cleavage of its N- and C-terminal propeptides mediated by the lysosomal proteases, cathepsins B, D, L, and S (2, 3). Both pro-form and mature form of GILT show reductase activity (4). Mature GILT is present largely in late endosomes and lysosomes, while precursor GILT is primarily detected in early endosomes (2). In addition to the endosome/lysosome, GILT is also found extracellularly in the tissue culture supernatants of GILT-expressing B-cell lines (5, 6), human (7) and mouse serum (M. Maric,
unpublished observation). It is still unknown whether the secreted GILT is re-internalized into endosome/lysosome or has functions in extracellular space.

1.2 MECHANISM OF ACTION

GILT has a –CXXC- motif similar to the -WCGH/PCK- active motif found in the thioredoxin family members (2, 8). Thioredoxin family proteins carry out reduction, oxidation, and isomerization of protein disulfide bonds in the nucleus, cytosol, endoplasmic reticulum, and mitochondria (9-12). Disulfide bond reduction also occurs in the lysosome. Unlike other thioredoxin family members that are capable of reducing disulfide bonds at neutral pH, the optimal enzymatic activity of GILT is unique at pH 4.5-5.5 (3). Both precursor and mature GILT reduce disulfide bonds with an acidic pH optimum (4). The diagram in Figure 1 shows the mechanism of reduction by GILT. The N-terminal cysteine in the active site is deprotonated and initiates nucleophilic attack on the substrate disulfide bond, generating an enzyme-substrate mixed intermediate. The formation of enzyme-substrate intermediate is then followed by intramolecular nucleophilic attack by the second deprotonated cysteine in the active site, generating an oxidized enzyme and a reduced substrate (3). In vitro studies have shown that GILT requires a reducing agent, such as dithiothreitol (DTT) or cysteine to regenerate and regain its activity (2). Although the process of regeneration of the reduced active site of GILT in vivo has not been elucidated, Phan et al. have suggested that the estimated lysosomal concentration of both cysteine and cysteinyl-glycine are capable of mediating this activity (2, 3).
To reduce a substrate, the N-terminal cysteine of the active site of GILT is deprotonated and initiate a nucleophilic attack on the substrate disulfide bond, resulting in the formation of a mixed disulfide - GILT-substrate intermediate. Subsequent intamolecular nucleophilic attack by the second deprotonated C-terminal cysteine within the active site enables the release of reduced substrate and oxidized enzyme.
1.3 Biological roles of GILT

1.3.1 The role of GILT in MHC class II antigen processing and presentation

Antigen-presenting cells, such as macrophages or mature dendritic cells activate CD4⁺ T lymphocytes by presentation of peptide epitopes associated with major histocompatibility complex (MHC) class II molecules. Exogenous antigens are internalized through the processes of endocytosis, phagocytosis, or macropinocytosis and further directed into the early endosomes, the late endosomes and ultimately fusing with the lysosomes (13). The pH of the endosomes containing protein antigens progressively decreases, causing the activation of the proteases that reside within the vesicles. The proteases degrade the endocytosed material and generate short peptides that can bind to the MHC class II molecules (14). Disulfide bonds, particularly intramolecular disulfide bonds, need to be reduced before proteins can be further processed in the endosomes. Using GILT -/- mice as a model, GILT has been shown to facilitate protein unfolding in this pathway by reducing disulfide bonds (6). The presence or absence of GILT can affect immune responses to viral (15), bacterial (16) or tumor antigens (17).

1.3.2 The regulatory roles of GILT in cell activation

GILT expression is abundant in mouse tissues that are rich in antigen-presenting cells, such as lymph nodes, spleen, and lung (6), but GILT is also present in tissues containing fewer antigen-presenting cells such as kidneys and testes. Around 10-20% of precursor GILT is
secreted as disulfide-linked dimers into the culture supernatant of human B cell lines (5, 6). It can also be detected in human (7) and mouse serum (M. Maric, unpublished observations). These expression patterns raise the possibility that GILT has additional functions not related to MHC class II antigen processing. Employing bioinformatic tools, proteins with various degrees of similarity to human and mouse GILT were identified in evolutionary distant species from unicellular organism like protozoa to insects, fish, amphibians, rodents, cattle and even plants (Figure 2). The finding of proteins similar to GILT in evolutionary distant species that do not have sophisticated immune systems and therefore lack MHC class II molecules, suggests that GILT may have additional functions, possibly evolutionarily older than the involvement in antigen processing.

Barjaktarevic et al. have shown that GILT-deficient T cells have a higher proliferation rate and cytotoxic activity in response to antigen-independent, anti-CD3 stimulation than wild type T cells (18), suggesting that GILT may have an inhibitory role in T cell activation. Furthermore, it has been found that GILT-deficient fibroblasts also have increased levels of proliferation. In addition GILT -/- fibroblasts show decreased expression, stability, and function of mitochondrial manganese superoxide dismutase (SOD2) (19). In the absence of GILT the levels of reactive oxygen species, particularly superoxide anion are increased as well (19). These observations suggest that GILT may have a role in a more fundamental cellular process than reduction of antigens in the MHC class II antigen-processing pathway. More specifically, the increased oxidative stress in GILT -/- cells indicates that GILT may have a regulatory role in redox homeostasis.
The phylogenetic tree of the GILT proteins was constructed by the neighbor-joining method with 1000 bootstrap replicates using the PHYLIP package in Tree families database (http://www.treefam.org).
2. Reactive oxygen species

Reactive oxygen species (ROS) are generally small, short-lived and highly reactive molecules generated as a byproduct of aerobic metabolism. The term “reactive oxygen species” is generally used to broadly describe O\textsubscript{2}-derived free radicals such as superoxide anion (O\textsubscript{2}•⁻), hydroxyl radical (HO‘), peroxyl radical (RO\textsubscript{2}•⁻) and alkoxyl radical (RO‘), as well as O\textsubscript{2}-derived non-radical species such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (20).

2.1 Sources of ROS within the cell

Mitochondria are considered to be a major source of ROS within the living cell as an inevitable consequence of oxidative metabolism and ATP synthesis (21). The machinery of mitochondrial metabolism involves oxidation of metabolites in the tricarboxylic acid cycle that ultimately results in the formation of NADH and FADH\textsubscript{2}. The mitochondrial respiratory chain, comprised of four enzyme complexes (I-IV), the F\textsubscript{1}F\textsubscript{0}-ATPase (complex V), ubiquinone and cytochrome c, transfers electrons derived from NADH and FADH\textsubscript{2} to molecular oxygen (Figure 3). Electron transfer is driven by the redox potential of the individual components of the respiratory chain. Protons are translocated across the inner membrane by complexes I, III, and IV that function as proton pumps, thus generating the mitochondrial membrane potential (ΔΨm), which drives the synthesis of ATP by the F\textsubscript{1}F\textsubscript{0}-ATPase. Of the total mitochondrial molecular oxygen consumed, 1-2% is reduced only partially, mainly at complexes I and III, and this one-electron reduction results in the formation of superoxide (22), which can then form H\textsubscript{2}O\textsubscript{2}. 
Figure 3 Mitochondrial ROS production

The machinery of mitochondrial metabolism involves oxidation of metabolites in the tricarboxylic acid cycle (TCA cycle), and eventual formation of NADH and FADH₂. The mitochondrial respiratory chain, which comprises of four enzyme complexes (I-IV), the F₁F₀-ATPase (complex V), ubiquinone (Q) and cytochrome c (C) transfers electrons derived from NADH and FADH₂-reducing equivalents to molecular oxygen. The redox potential of each components of the respiratory chain drives the electron transfer. Complex I, III, and IV act as proton pumps to translocate protons across the mitochondrial inner membrane. During the electron transfer through the respiratory chain, the drop in redox potential generates the
mitochondrial membrane potential (ΔΨm) that drives the ATP synthesis by the complex V. The process is not completely efficient, so the electron transfer to molecular oxygen may occurs at complex I and III, which results in the superoxide (O$_2^-$) generation. Superoxide is not membrane permeable but can be transported by ion channels, such as inner membrane ion channel (IMAC) and voltage dependent anion channel (VDAC). Superoxide can be dismutated to hydrogen peroxide (H$_2$O$_2$) by matrix SOD2 or SOD1 present in the intra-membrane space and the cytosol.

Superoxide anion is not membrane permeable but can be transported by the inner membrane ion channel (IMAC) and voltage dependent anion channel (VDAC) (23). In addition to the respiratory chain, the tricarboxylic acid cycle enzyme complexes, such as α-ketoglutarate dehydrogenase (α-KGDH), pyruvate dehydrogenase (24), and monoamine oxidases (MAOs) (25) located in the outer membrane of mitochondria, have been implicated as significant sources of mitochondrial O$_2^-$ and H$_2$O$_2$.

ROS are also synthesized outside mitochondria. Peroxisomes are sources of cytosolic H$_2$O$_2$ under physiological conditions (26). Endoplasmic reticulum (ER) monooxygenase cytochrome P450 contributes to increased cellular H$_2$O$_2$ and O$_2^-$ (27), whereas NADPH oxidase (28) oxidizes NADPH and reduces oxygen across the plasma membrane to generate O$_2^-$, which could eventually form H$_2$O$_2$ (29).
2.2 ANTI-OXIDANTS

Low levels of ROS are maintained by enzymes superoxide dismutase (SOD) (30), catalase (31), peroxidases (32), thioredoxin (33) and non-enzymatic antioxidants, such as glutathione (34), ascorbic acid (35) and vitamin E (36).

2.2.1 ENZYMATIC ANTI-OXIDANTS

The superoxide dismutase (SOD) family of enzymes is critical in protecting oxygen-utilizing cells from the toxicity of reactive oxygen species produced during normal metabolism. These enzymes are also key components of signaling pathways that regulate cell physiology. The SOD family includes cytoplasmic copper and zinc superoxide dismutase (SOD1, CuZn-SOD), mitochondrial manganese superoxide dismutase (SOD2, Mn-SOD), and extracellular copper and zinc-containing superoxide dismutase (SOD3, EC-SOD). SODs catalyze the dismutation of superoxide anion radical (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and molecular oxygen (O$_2$). Hydrogen peroxide is further detoxified to water and molecular oxygen by the major H$_2$O$_2$ detoxifying antioxidant enzymes, such as catalases and glutathione peroxidases (37, 38) (Figure 4).

Catalase is located within the cytosol and peroxisomes of the cell and decomposes hydrogen peroxide to water and molecular oxygen (39).

Glutathione peroxidase is a selenium-containing metalloenzyme, partially located within the cellular membrane, which removes hydrogen peroxide by converting reduced glutathione
Figure 4 Intracellular antioxidant enzymes

There are three major intracellular antioxidant enzymes present in mammalian cells: superoxide dismutases (SOD), catalases (158), and glutathione peroxidase (GP\textsubscript{X}). The SODs convert O\textsubscript{2}^{-} into H\textsubscript{2}O\textsubscript{2}, whereas CAT and GP\textsubscript{X} convert H\textsubscript{2}O\textsubscript{2} into water. GP\textsubscript{X} requires several secondary enzymes, including glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD), and cofactors, including glutathione (GSH), NADPH and glucose-6-phosphate, to function at high efficiency. GSH is synthesized from L-glutamate with the help of glutathione synthetase (GS) and \(\gamma\)-glutamyl cysteine synthetase (\(\gamma\)-GCS). GSH is then oxidized to glutathione disulfide (GSSG) by GP\textsubscript{X}. H\textsubscript{2}O\textsubscript{2} can be also detoxified by thioredoxin (reduced thioredoxin, (TRx\textsubscript{red}) and
oxidized thioredoxin (TR\textsuperscript{ox}) / thiolredoxin peroxidase (TPx) / thioredoxin reductase (TR) system.

\textsuperscript{40} Glutathione peroxidase requires several secondary enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase) and cofactors (GSH, NAPDH and glucose 6-phosphate) to function efficiently.

Finally, thioredoxin is similar to glutathione in its ability to maintain a reducing environment and to detoxify ROS \textsuperscript{41}. Thioredoxin has been found to act as an antioxidant by reducing ROS molecules including H\textsubscript{2}O\textsubscript{2} \textsuperscript{42}. However, the low concentration of thioredoxin in the cell (micromolar range) suggests that thioredoxin is perhaps not so significant as an antioxidant \textsuperscript{43}.

\textbf{2.2.2 Nonenzymatic Antioxidants}

Glutathione is the most abundant free thiol that is responsible for the maintenance of an optimal intracellular redox environment necessary for normal function of cellular proteins in eukaryotic cells \textsuperscript{41}. GSH levels are regulated by glutathione reductase and glutathione peroxidase. GSH serves several vital intracellular functions: detoxifying electrophiles, scavenging free radicals, maintaining the essential thiol status of proteins, and providing a reservoir of cysteine \textsuperscript{44}. GSH is synthesized in the cytosol from its precursor amino acids,
glutamate, cysteine, and glycine. After synthesis, a portion of GSH is transported to the mitochondria, endoplasmic reticulum and nucleus (45). Within the cell, glutathione exists mainly in the reduced form (GSH), but is also present as glutathione disulfide (GSSG). Under physiological conditions, the intracellular GSH concentration is in the millilmolar range and far exceeds the concentration of GSSG, which is in the micromolar range (46).

In addition to glutathione, there are several low molecular weight compounds considered as non-enzymatic antioxidants, including ascorbic acid and vitamin E (47). Ascorbic acid, a water-soluble antioxidant prevents lipid hydroperoxide formation in plasma low-density lipoprotein (LDL) and therefore has an important role in the prevention of atherosclerotic plaque formation (48). Vitamin E is a hydroxyl group containing lipid-soluble vitamin, which reduces peroxyl radicals and inhibits lipid peroxidation in biological membranes (49).

### 2.3 Oxidative Stress

Oxidative stress presents as the imbalance between the productions of ROS and the ability of various cellular antioxidant defenses to neutralize them which eventually can lead to cellular damage (DNA mutation, protein aggregation, and lipid peroxidation) (50, 51). ROS are formed as normal byproducts of aerobic metabolism but are elevated under pathophysiological conditions (20). ROS lead to chemical modifications of DNA, such as cleavage and oxidation of purines (52). A mutation results from inappropriate base pairing of oxidized purines during replication that is not repaired immediately by DNA-repair systems (52). Oxidation of DNA can lead to various pathologies, including cancer, neurodegenerative diseases and cell death (53).
addition to DNA, ROS can react with amino acid residues to generate modified and less active enzymes, or denatured and nonfunctioning proteins (27). Lipid constituents of the cell and/or organelle membranes are also targets for ROS damage. Accumulation of lipid peroxidation has been linked to several pathological processes, such as ageing and vascular diseases (54).

3. Removal of Damaged Proteins and Organelles

Various defense mechanisms have been developed to prevent serious cellular damage in response to oxidative stress. The most important protective responses are an increase of intracellular antioxidants and the removal of damaged proteins/organelles (55). There are two major intracellular proteolytic systems contributing to the removal of proteins, the ubiquitin-proteasome system (56) and autophagy (57). The ubiquitin-proteasome system eliminates polyubiquitinated proteins located in the cytosol, involving two steps: 1. conjugation of multiple ubiquitins to the substrate; 2. degradation of the tagged protein by the 26S proteasome complex (58, 59). In contrast to autophagy, ubiquitin-proteasome degradation is considered to be highly selective.

3.1 Autophagy

Autophagy is an evolutionary conserved catabolic pathway for degradation of intracellular proteins and organelles via lysosomes (55). There are three types of autophagy: 1).
chaperone-mediated autophagy (CMA), 2). microautophagy, and 3). macroautophagy (60) (Figure 5).

**Figure 5 Main forms of autophagy**

Three different modes of autophagy are: 1). chaperone-mediated autophagy (CMA), 2). microautophagy, and 3). macroautophagy. CMA selectively degrades cytosolic proteins containing the KFERQ motif by translocation of unfolded proteins into the lysosome through interaction with the Lamp-2a receptor and hsc70 chaperone. In Microautophagy, cytosolic constituents are directly engulfed by the lysosomal membrane. Finally, macroautophagy involves the internalization of cellular portions into a double-membrane autophagosome that fuses with
lysosomes. Macroautophagy is initiated by the formation of the phagophore but the origins of this membrane and mechanisms of its growth are not well understood.

1). CMA is a selective pathway, which is restricted to cytosolic proteins that contain a particular pentapeptide motif, KFERQ (61). Furthermore, the KFERQ motif is recognized by a hsc70-containing chaperone, which translocates the unfolded proteins across the lysosomal membrane through interaction with the Lamp2a receptor (62). Therefore, CMA is not used to degrade large protein aggregates or organelles. Unlike CMA, both microautophagy and macroautophagy involve membrane rearrangements that eventually engulf large cytosolic components and organelles and target them to the lysosomes for degradation (55). 2). Microautophagy refers to the engulfment of cytosolic constituents through invaginations of the lysosomal membrane (63). However, our understanding of microautophagy is less well characterized, especially in mammalian cells.

3). Macroautophagy is the best-characterized type of autophagy. It is initiated by the engulfment of cytosolic proteins or organelles by a crescent-shaped isolation phagophore. The phagophore forms a closed double-membrane autophagosome to sequester target cellular constituents. Then the outer membrane of the autophagosome fuses with a lysosome to form an autolysosome and its content is degraded by lysosomal enzymes. The origin of the autophagosomal membrane is not well understood. However, recently, several articles suggested that both mitochondrial and ER membranes might serve as a source (64, 65).
3.2 **CELLULAR AND MOLECULAR MACHINERY OF AUTOPHAGY**

The basal level of macroautophagy (referred to as autophagy hereafter) is very low under normal conditions. Autophagy is activated during periods of cellular stress, including nutrient starvation, pathogen infection and under pathological conditions, such as vascular diseases, neurodegenerative diseases and cancer. The role of autophagy under such stress is to maintain metabolism and ATP levels essential for cell survival (66, 67). Therefore, in order to adapt to stress, it is critical for cells to have an efficient mechanism to induce autophagy. Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is the central inhibitor of autophagy (68). Under normal conditions, mTOR interacts and negatively regulates two other serine/threonine protein kinases, the uncoordinated (unc) 51-like kinase 1 and 2 (ULK1/2) complex by its kinase activity (69). Upon mTOR inhibition by starvation or rapamycin treatment, mTOR dissociates from the ULK1/2 complex and leads to ULK1/2 complex activation, which promotes the formation of a phagophore to initiate autophagosome formation (69, 70) (Figure 6A).

mTOR is a negative regulator of autophagy, whereas the class III phosphatidylinositol-3-kinase (PI3K) complex stimulates the process (71). Class III PI3K complex is composed of the PI3K vacuolar protein sorting 34 (Vps34) and Beclin 1 (72). The function of Beclin 1 in autophagy is regulated by an antiapoptotic protein, B-cell lymphoma/leukemia-2 (Bcl-2) through an inhibitory interaction between the Bcl-2’s BH3 binding groove and the BH3 domain of Beclin 1 (73). Autophagy-inducing signals can disrupt the inhibitory interaction and lead to the formation of the class III PI3K complex, which produces phosphatidylinositol 3-phosphate
(PI3P) to mediate the initial stages of vesicle nucleation and autophagosome formation (74) (Figure 6A).

**Figure 6 Cellular and molecular machinery of autophagy**

**A. Signaling transduction pathways regulate autophagy during phagophore formation and vesicle nucleation.** Autophagy inducers, such as starvation and chemical rapamycin modulate the inhibitory interaction of mTOR with ULK1/2 complex. mTOR dissociates from ULK1/2 complex and leads to ULK1/2 complex activation, which promotes the formation of phagophore to initiate autophagosome formation. Stress disrupts the inhibitory interaction between Bcl-2 and Beclin-1 and leads to the formation of the class III PI3K complex (Vsp34 and Beclin-1). This interaction produces phosphatidylinositol 3-phosphate (PI3P) and mediates the initial stages of vesicle nucleation and autophagosome formation.
In addition to mTOR and class III PI3K complexes, autophagosome formation requires two ubiquitin-like conjugation systems (Atg12-Atg5-Atg16 and LC3-II) in order to facilitate the elongation process (75). The first ubiquitin-like molecule is autophagy-related protein 12 (Atg12). Atg12 is covalently conjugated to Atg5 through a series of ubiquitination-like reactions that involves Atg7 and Atg10 (76). The Atg12-Atg5 conjugate then associates with Atg16 to form the multimeric Atg12-Atg5-Atg16 complex (77). The second ubiquitin-like molecule is microtubule-associated protein 1 light chain 3 (LC3). The soluble form of LC3 (named LC3-1) conjugates to phosphatidylethanolamine (PE) by a series of ubiquitination-like reactions that involves Atg4, Atg7, and Atg3 (78). The Atg12-Atg5-Atg16 complex preferentially localizes on the outer membrane of the forming autophagosome and functions as an E3 ligase of the lapidated form of LC3, LC3-2 to promote the formation of the autophagosome (79-81). Upon vesicle completion, most of the Atg/LC3 proteins are dissociated from the autophagosome, allowing the fusion of autophagosome and lysosome and cargo degradation by lysosomal proteases (82) (Figure 6B).

Although autophagy was initially considered nonspecific, it is now accepted that preferential autophagy of damaged or excess organelles, such as mitochondria (termed mitophagy) (83), peroxisomes (termed pexophagy) (84), ribosomes (termed ribophagy) (85), and endoplasmic reticulum (termed reticulophagy) (86) occurs to mediate the removal of superfluous or damaged organelles. Among these cargo-specific autophagic processes, mitophagy is the most studied.
**Figure 6B. Molecular aspects of autophagy.** Atg12-Atg5-Atg16 and LC3-2 (LC3-1 + PE) are recruited to the phagophore, facilitating the autophagosome membrane expansion step. Upon vesicle completion, most of the Atg/LC3 proteins are dissociated from the autophagosome, allowing the fusion of autophagosome and lysosome and consequently the degradation of the cargo by lysosomal proteases.
3.3 Mitophagy

As a major source of ROS, mitochondria are prone to damage by ROS. Under oxidative stress, opening of the mitochondrial permeability transition pore (MPTP) leads to the loss of mitochondrial membrane potential and release of ROS (87). At the same time, dysfunctional mitochondria produce more ROS leading to increased damage. The selective autophagy of damaged mitochondria, termed mitophagy is part of mitochondrial quality control and/or a cytoprotective response (87). Mitophagy is also linked with mitochondria turnover to regulate mitochondrial numbers to match metabolic demands at different developmental stages, such as during red blood cell differentiation (88). Recently, the molecular mechanisms regulating mitophagy have been identified (89). When mitochondria are damaged by increased cellular ROS and lose the mitochondrial membrane potential, the phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1 (PINK1) recruits the E3 ligase parkin specifically to damaged mitochondria, which then ubiquitylates mitochondrial protein voltage-dependent anion channel (VDAC) and subsequent targeting to autophagosomes by interaction with LC3-2 (89).

4. Basis of proposed studies

An abnormal production of reactive oxygen species (ROS) and the subsequent redox imbalance have long been proposed to be common pathogenic mechanisms in several diseases, such as atherosclerosis, diabetes, hypertension and cancer (90). Increased ROS can oxidize DNA, proteins, and lipids, events that could induce cell death (53, 91, 92). Our bodies are
equipped with defense mechanisms against oxidative stress, including enzymatic antioxidants, superoxide dismutase (SOD), catalase and glutathione peroxidase, along with the major non-enzymatic antioxidant glutathione (GSH). In addition, there is a growing evidence that several members of the thiol reductase family regulate cellular redox homeostasis through the oxidoreduction of protein thiols (93-95). Most of these thiol reductases are localized primarily in the cytosol. However, our group has demonstrated that ablation of the unique endosomal thiol reductase, Gamma-interferon Inducible Lysosomal Thiol reductase (GILT) leads to an increase in intracellular levels of ROS, particularly the superoxide anion. Expression, stability and activity of manganese superoxide dismutase (SOD2) are decreased in GILT -/- cells (19, 96). Reconstitution of GILT leads to increased SOD2 levels and normalized/decreased ROS levels, revealing that GILT contributes to the maintenance of redox status of the cell. However, the correlation between the deficiency of GILT and the decreased SOD2 levels has not been closely examined. Therefore, in this dissertation I studied the possible GILT-dependent mechanisms involved in the regulation of SOD2 levels.
HYPOTHESIS AND SPECIFIC AIMS

The overall goal of my research is to determine the regulatory role(s) of GILT in cellular redox homeostasis. More specifically, this study addressed the possible GILT-dependent mechanisms involved in the regulation of SOD2 levels. Given the fact that GILT mainly resides in the lysosomes and SOD2 is sequestered in the mitochondria, a functional communication between these proteins is more likely than a direct physical interaction. Based on the previous findings of the presence of oxidative stress in GILT -/- cells (19), we hypothesized that GILT has influence on the levels of the major non-enzymatic antioxidant, glutathione by limiting the availability of cysteine, and therefore contributes to the regulation of redox homeostasis. The oxidative stress caused by the deficiency of GILT may damage mitochondrial integrity, activate autophagy and eventually lead to the degradation of the damaged mitochondria, hence decreasing the SOD2 levels (Figure 7).

SPECIFIC AIM 1. TO TEST THE HYPOTHESIS THAT THE ABSENCE OF GILT ALTERS GLUTATHIONE SYNTHESIS AND LEADS TO DECREASED SOD2 LEVELS

Glutathione is the key player in the maintenance of the cellular redox status and has an important role as a free radical scavenger and detoxifying agent in the cellular defense against oxidative stress (97). Glutathione is a tripeptide consisting of cysteine, glycine, and glutamic acid (98). In the biosynthesis of glutathione, cysteine is the critical rate-limiting precursor (43). Therefore, the supply and availability of cysteine are crucial for the synthesis of glutathione.
Figure 7 The lack of GILT leads to increased oxidative stress and the induction of the autophagy

The deficiency of GILT may limit the source of cysteine, which is the rate-limiting factor of the glutathione synthesis. The decreased glutathione levels and the increased ROS production may damage the mitochondria integrity (as shown by the decreased mitochondrial membrane potential ($\Delta\Psi_m$)). In order to remove the damaged mitochondria, GILT -/- cells may employ compensatory mechanisms, such as autophagy. Autophagy removes the damaged mitochondria and therefore leads to the decreased SOD2 levels.
It has been shown that both the extracellular environment and the lysosome are the source of cysteine for glutathione and protein synthesis (99). Cysteine is a highly reactive amino acid due to the presence of a thiol group (SH) and therefore is easily oxidized into cystine (Cys-S-S-Cys) in the extracellular environment (100). Extracellular cystine is imported by the Xc\(^{-}\) antiporter into the cytoplasm (101). After uptake, cystine is reduced to cysteine in the presence of the GSH/GSSG pair to provide the cytosolic cysteine reservoir for glutathione synthesis (100). A portion of cytosolic cysteine can be imported into the lysosomes through the action of a presumed cysteine-specific transporter, which has not been identified (102). In addition to the transported cysteine, endocytosed proteins in the lysosomes can be degraded into peptides and amino acids, and therefore also serve as a source of cysteine (103). In the lysosomes, cysteine oxidation occurs either spontaneously as a result of the acidic/oxidizing environment or is facilitated by an unknown enzymatic action (102). In either process, cysteine is oxidized into cystine and exported back to the cytoplasm via the cystine transporter, cystinosin.

We hypothesized that GILT may facilitate the process of cysteine oxidation/cysteine reduction. When the active site (CXXC) of GILT is oxidized upon interaction with disulfides of the substrate within the lysosome, in order to regenerate its SH groups and therefore restore its reductase function GILT may be re-activated by cysteine (102). Therefore, in the absence of GILT, the rate of oxidation of cysteine into cystine may be slowed down, thus making less cysteine available for glutathione biosynthesis (Figure 8).
WT cells

GILT -/- cells

Figure 8A

Figure 8B
Figure 8 Regulation of the glutathione biosynthesis

A. Both the extracellular environment and the lysosomes provide the source of cysteine for the glutathione and protein synthesis. Cystine, the oxidized form of cysteine is imported by the $X_c$-antiporter into the cytoplasm and then is reduced to cysteine in the presence of the glutathione (GSH)/glutathione disulfide (GSSG) pair. A portion of cytosolic cysteine can be imported into the lysosomes through a specific cysteine transporter. Lysosomes also provide the source of cysteine through the enzymatic protein degradation. In the lysosomes, cysteine oxidation occurs either spontaneously or is facilitated by an unknown enzymatic action (we hypothesize GILT may contribute to this process). In either process, cysteine is oxidized into cystine and exported back to the cytoplasm via the cystine transporter, cystinosin. B. In GILT deficient cells, the rate of the balance of cysteine/cystine pair may be altered thus making less cysteine available for glutathione biosynthesis.

SUBAIMS

1.1 To determine the intracellular reduced glutathione levels in WT vs. GILT -/- fibroblasts.

1.2 To determine the ratios of oxidized glutathione (GSSG) vs. reduced glutathione (GSH) in WT and GILT -/- mouse fibroblasts.

1.3 To determine whether the modulation of reduced glutathione levels alters SOD2 expression in WT and GILT -/- mouse fibroblasts.
Specific Aim 2. To test the hypothesis that autophagy affects SOD2 levels in GILT -/- fibroblasts

Mitochondria are one of the major sources of ROS (21). Mitochondrial ROS are detoxified by enzymatic (e.g. SOD2) and non-enzymatic (e.g. glutathione) antioxidants (51). When the balance between production and elimination of ROS is disrupted, mitochondria are especially prone to ROS damage (83). Thus, such damage could lead to the mitochondrial permeability transition and loss of mitochondrial membrane potential (104). Increased oxidative stress usually leads to cell death (105). However, there are studies that show beneficial effects of increased ROS (106, 107). Indeed, GILT -/- fibroblasts are able to survive and show an increased proliferation rate (19). Therefore, we hypothesized that GILT -/- cells might employ compensatory mechanisms, such as autophagy to neutralize the damage caused by increased oxidative stress (55, 108).

Subaims

2.1 To determine whether mitochondria are damaged in WT and GILT -/- fibroblasts.

2.2 To determine the magnitude extent of autophagy act in WT vs. GILT -/- fibroblasts.

2.3 To determine how autophagy affects SOD2 levels in WT vs. GILT -/- fibroblasts.
Specific aim 3. To identify the signaling pathways involved in the induction of autophagy in WT and GILT -/- fibroblasts

It has been reported that at low to moderate levels, ROS can act as signaling molecules to control various cellular processes, such as proliferation, autophagy, immunity and defense against pathogens (82, 109-111). Therefore, we hypothesized that the oxidative stress in GILT -/- fibroblasts alters the redox-sensitive signaling pathways involved in the induction of autophagy (112). Among the signaling pathways leading to autophagy induction, MAPK Erk1/2 activation (113, 114) and HMGB1 translocation from the nucleus to the cytoplasm (115-117) are the most studied pathways.

Subaims

3.1 To determine whether the Erk1/2 signaling pathway regulates the induction of autophagy in WT and GILT -/- fibroblasts.

3.2 To determine whether the nuclear HMGB1 cytosolic translocation is promoted by oxidative stress in WT and GILT -/- fibroblasts.
MATERIALS AND METHODS

1. REAGENTS

2-Mercaptoethanol (2-ME) (catalog number: M7522), N-Acetyl-L-cysteine (L-NAC) (catalog number: A9165), and L-Buthionine-sulfoximine (BSO) (catalog number: B2515) were obtained from Sigma. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (catalog number: M34152) was purchased from Invitrogen. Chloroquine (catalog number: 193919) was obtained from MP Biomedicals. 3-methyladenine (3-MA) (catalog number: 37979) was purchased from Acros Organics. PD98059 (catalog number: 9903) and U0126 (catalog number: 9900) were obtained from Cell Signaling Technology. Rapamycin (catalog number: R-5000) was purchased from LC Laboratories.

2. CELL CULTURE

WT and GILT -/- SV40 large T antigen immortalized mouse fibroblast cell lines were generated from WT and GILT -/- C57BL/6 mice in Dr. Peter Cresswell’s laboratory at Yale University as previously described (118). A stable transfection of GILT -/- fibroblasts with plasmid mGILT-pcDNA3.1(-) was made using Lipofectamine 2000 transfection reagent (Invitrogen) and standard protocols (Dr. Maja Maric’s laboratory, Georgetown University). Fibroblasts were propagated in the RPMI 1640 medium (catalog number: 11875, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (catalog number: SH3007003HI, Thermo Scientific).
3. **Reduced Glutathione Assay**

Reduced glutathione was measured using the Quantichrom glutathione assay kit (catalog number: DIGT-250, Bioassay Systems). The assay is based on the reduction of 5,5’-dithiobis(2-nitrobenzoic acid) by reduced glutathione to form a yellow product. The optical density was measured at 412nm and was proportional to the reduced glutathione concentration in the samples. One million cells were lysed using a dounce homogenizer in 500µl cold PBS with 1mM EDTA. 20µg protein of each total cell lysate were used for the assay.

4. **Analysis of GSSG/GSH Ratio**

The intracellular GSSG/GSH ratio was assessed by the method of Gutcher *et al.* (119). WT and GILT -/- fibroblasts were transiently transfected with Grx1-roGFP2 or Mito-Grx1-roGFP2 (kind gift of Dr. Tobias Dick, German Cancer Research Center, Heidelberg, Germany) using Lipofectamine LTX (catalog number: 15338, Invitrogen). Cells were seeded and imaged in FD-35 FluoroDishes (catalog number: FD35-100, World Precision Instrument) at 37°C on a Zeiss LS510 confocal microscopy system. Cells were excited with 405 and 488nm lasers. The ratio of emissions in the green channel (505-550nm) was calculated. Raw data were exported to ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009) as 16-bit TIF for analysis.
5. MEASUREMENT OF REACTIVE OXYGEN SPECIES

Intracellular superoxide anions were determined by using the probe dihydroethidium (DHE) (catalog number: D11347, Invitrogen). 2µM DHE was added to cells and incubated for 30 minutes at 37°C, 5% CO₂. Cells were then washed twice in PBS, resuspended in PBS with 0.5% bovine serum albumin, and fluorescent intensity (FL-2 channel) was measured by a FACS Scan flow cytometer (FACScan, Becton Dickinson).

6. WESTERN BLOTTING AND ANTIBOIES

Cells were lysed in Tris-Saline (pH7.5) containing 1% Triton X-100, 200µM Na₃VO₄, 1mM NaF, 10µM β-glycerophosphate and protease inhibitor cocktail tablet (catalog number: 04693124001, Roche). Protein preparations were fractionated by electrophoresis on standard SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (catalog number: IPVH00010, Millipore). PVDF membranes were incubated with the following primary antibodies in PBS, 0.1% Tween 20, 5% BSA at 4°C overnight as indicated: mouse anti-SOD2 (catalog number: ab16956, Abcam), rabbit anti-LC3 (catalog number: 2775, Cell Signaling Technology, or catalog number: PA1-16930, Thermo SCIENTIFIC), rabbit anti-COX IV (catalog number: 4844, Cell Signaling Technology), rabbit anti-phosphoErk1/2 (catalog number: 4376, Cell Signaling Technology), rabbit anti-totalErk1/2 (catalog number: 4695, Cell Signaling Technology), rabbit anti-MKP3 (catalog number: 2138-1, Epitomics). Following incubation the appropriate horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse or anti-rabbit IgG antibodies (catalog number: 115-035-003; 111-035-003, Jackson
ImmunoResearch Laboratories) were incubated with the PVDF membranes in PBS, 0.1% Tween 20, 1% BSA at room temperature for 40 minutes. Proteins were detected with enhanced chemiluminescence Western Lightning (catalog number: NEL103001EA, Perkin Elmer Life Science). In some cases membranes were stripped by Restore western blot stripping buffer (catalog number: 21059, Pierce) and incubated at room temperature for 40 minutes, followed by incubation with rabbit anti-β-actin (catalog number: A2066, Sigma), and rabbit anti-GAPDH (catalog number: sc-25778, Santa Cruz) as a loading control and the appropriate secondary HRP-conjugated antibody. Densitometry analysis was done with the Gel Logic 100 Imaging system and Molecular imaging software (Kodak).

7. MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT

Mitochondrial membrane potential was assessed using the MitoProbe JC-1 Assay kit (catalog number: M34152, Invitrogen). Cells were trypsinized, washed once with phosphate-buffered saline (PBS) and incubated with 2µM JC-1 dye for 15 minutes at 37°C, 5% CO₂ in RPMI 1640 medium. Cells were then washed twice with PBS. Twenty thousand cells were examined per sample for the presence of JC-1 aggregates (FL-2) vs. monomers (FL-1) using a FACS Scan flow cytometer (FACScan, Becton Dickinson). Change in mitochondrial membrane potential was determined by the ratio of JC-1 red fluorescence intensity (aggregates) to JC-1 green fluorescence intensity (monomers).
8. Analysis of HMGB1 Translocation

WT, GILT -/-, and GILT-reconstituted fibroblasts were seeded in 8-well chamber slides (catalog number: 155411, Lab-Tek, Thermo SCIENTIFIC) and cultured in RPMI 1640. Cells were then fixed with freshly prepared 3% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with rabbit anti-HMGB1 antibody (catalog number: 3935, Cell Signaling Technology) followed by goat anti-rabbit Alexa Fluor 594 (catalog number: A11012, Invitrogen) and Hoechst 33342 (catalog number: 4082, Cell Signaling Technology). Images were taken using an Olympus Fluoview-FV300 Laser Scanning Confocal System and analyzed by MetaMorph (Molecular Devices) for the nuclear and cytosolic HMGB1 intensity measurement. At least 300 cells of each group were analyzed.

9. Statistical Analysis

Statistical analysis was performed by analysis of variance with post hoc Student’s t test of GraphPad Prism version 5.01 (GraphPad Software). Data were reported as means ± S.E., where \( n \) = at least 3. A \( p \) value of \( \leq 0.05 \) was considered significant.
RESULTS

GILT deficiency in fibroblasts leads to decreased glutathione levels

Based on our previous finding that GILT -/- cells have decreased expression and function of SOD2 and increased superoxide anion we hypothesized that these cells are under increased oxidative stress (19). While it has been shown that in the absence of GILT, the SOD2 levels and activity were significantly decrease the levels of SOD1 were unaffected (19).

Aerobic metabolism results in naturally-occurring superoxide radicals that are catalytically converted into molecular oxygen and hydrogen peroxide by a family of enzymes called superoxide dismutases. Within the cell SOD1 is located in the cytoplasm and SOD2 resides in mitochondria, while SOD3 is an extracellular enzyme (30, 120).

In addition to enzymatic antioxidants, cells also have non-enzymatic antioxidant systems for the removal of reactive oxygen species (ROS). Among non-enzymatic antioxidants, glutathione is the major, well-recognized non-protein thiol and is required for many critical cellular processes, such as cell proliferation and differentiation (45). Therefore, we examined whether GILT affects the level of glutathione (Figure 9). Reduced glutathione (GSH) levels were decreased by 25% in GILT -/-, relative to wild type (WT) fibroblasts. These data indicate that GILT may regulate levels of GSH in the cytosol and also suggested that GILT might have a more global effect on intracellular redox balance.
Reduced glutathione (GSH) was measured using Quantichrom glutathione assay kit of Bioassay Systems. The assay is based on the reduction of 5,5’-dithiobis(2-nitrobenzoic acid) by reduced glutathione to form a yellow colored product. The optical density was measured at 412nm and was proportional to GSH concentration in samples. One million cells were lysed using a dounce homogenizer in 500μl cold PBS with 1mM EDTA. 20μg of each total cell lysate were used for the assay. Mean ± S.E. for three separate experiments are shown. *, p < 0.05 vs. WT. Statistical analysis was performed by analysis of variance with post hoc Student’s *t* test.

---

**Figure 9 Levels of reduced glutathione (GSH) are decreased in GILT -/- fibroblasts**
INCREASED GSSG/GSH RATIO IN GILT -/- FIBROBLASTS

During oxidative stress, the biochemically dominant reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG). Therefore, the GSSG/GSH redox couple serves as a useful target for monitoring the intracellular oxidative status (121). Within the cell, glutathione exists mainly (>98%) in the reduced form (GSH), but a portion is also present as glutathione disulfide (GSSG) (46). Under normal physiological conditions, intracellular GSH concentration is in the millimolar range and far exceeds the concentration of GSSG, which is in the micromolar range (122). Because of the low GSSG concentration, it is difficult to accurately measure GSSG levels using a GSSG reductase-dependent enzymatic assay or an HPLC method with fluorescence detection (123). Therefore, we measured real-time intracellular redox changes by taking dynamic images of the redox sensitive biosensor, Grx1-roGFP2 (119) that rapidly equilibrates the GSSG/GSH redox couple and the GFP sensor. The oxidized and reduced forms of Grx1-roGFP2 exhibit distinct excitation peaks (119). In order to test whether GILT -/- fibroblasts have an altered intracellular GSSG/GSH ratio compared to WT fibroblasts, we transfected the cells with Grx1-roGFP2 plasmids and measured the redox response to the application of oxidative stress in the form of 50μM H₂O₂ and the subsequent recovery due to the addition of 0.5mM dithiothreitol (DTT). We took dynamic live images by confocal microscopy of the Grx1-roGFP2 fusion protein expressed in either the cytoplasm or the mitochondria and determined the ratio of GSSG/GSH. Prior to the addition of oxidizing agent (H₂O₂), the ratio of GSSG/GSH in both the cytoplasm and the mitochondria was shifted toward a more oxidized state in GILT -/- fibroblasts (Figure 10). The addition of 50μM H₂O₂ to the cell culture medium led to a shift in the cytoplasmic GSSG/GSH ratio toward the oxidized form and the shift was
Figure 10 Basal ratio of oxidized glutathione (GSSG) vs. reduced glutathione (GSH) is increased in GILT −/− fibroblasts

WT and GILT −/− fibroblasts expressing either cytoplasmic-targeted or mitochondria-targeted Grx1-roGFP2 were seeded and observed in fluoro-dishes at 37°C on a Zeiss LS510 confocal microscopy system. Cells were excited with 405 and 488nm laser. The ratio of emissions in the green channel (505-550nm) was calculated. Raw data were exported to ImageJ software as 16-bit TIF for analysis. Mean ± S.E. for at least two hundred individual cells are shown. *, p < 0.05 vs. WT. Statistical analysis was performed by analysis of variance with post hoc Student’s t test.
significantly greater in GILT -/- cells (Figure. 11A). The Grx1-roGFP2 sensor remained in the oxidized state until the addition of 0.5mM DTT, which reduced the sensor. A similar tendency in GSSG/GSH ratio shifts was observed in the mitochondria (Figure. 11B) but the difference in shift was transient and less prominent than in the cytoplasm. These data indicated that the presence of GILT influences the balance of GSH and GSSG in fibroblasts and therefore most likely the overall redox status of the cell. However, the question remains whether the altered glutathione levels can affect SOD2 expression and activity.

Figure 11A
Figure 11 Shift of GSSG/GSH ratio toward the oxidized form of glutathione is greater in GILT -/- fibroblasts

WT and GILT -/- fibroblasts expressing either cytoplasmic-targeted or mitochondria-targeted Grx1-roGFP2 were excited with 405 and 488nm laser of Zeiss LS510 confocal microscopy system. The ratio of emissions in the green channel (505-550nm) was calculated. A. Cytoplasm-targeted Grx1-roGFP2 plasmid was used to determine the GSSG/GSH ratio in the cytoplasm of WT vs. GILT -/- cells. 50 seconds upon the start of detection, cells were treated with 50µM H₂O₂ followed by the addition of 0.5mM DTT 2 minutes later. The ratio was normalized to 0sec untreated cells (initial ratio=1), quantified for at least two hundred individual cells and plotted against time. Means ± S.E are shown. *, p < 0.05 vs. WT. B. Mitochondrial-targeted Grx1-
roGFP2 was used in experiments and followed the same procedure as described previously in Figure 11A. Means ± S.E for a minimum of two hundred individual cells are shown and plotted against time. *, p < 0.05 vs. WT. Statistical analysis was performed by analysis of variance with post hoc Student’s t test.

MODULATION OF INTRACELLULAR GLUTATHIONE LEVELS ALTERS SOD2 EXPRESSION

In order to assess whether the detected decrease of GSH in GILT -/- fibroblasts alters SOD2 expression, we modulated intracellular GSH levels by treating cells with either the reducing agents β-mercaptoethanol (2ME) or N-acetyl-L-cysteine (L-NAC), the latter provides extra sources of cysteine for the glutathione synthesis, or with L-buthionine-S, R-sulfoximine (BSO) (124), a specific inhibitor of γ-glutamylcysteine synthetase (γ-GCS), an enzyme that catalyzes the rate-limiting step in GSH biosynthesis. Treatment of the cells with BSO is expected to decrease GSH levels and therefore mimic an oxidant. Following these treatments, we measured the levels of GSH (Figure 12A and B), superoxide anion (Figure 12C and D) and SOD2 expression (Figure 13A and B). In both WT and GILT -/- fibroblasts, the addition of antioxidants 2ME or L-NAC raised SOD2 levels by 30% and 60% respectively. In the same experiment we also detected increased intracellular GSH levels and lower levels of the intracellular superoxide anion.

As expected, the treatment with BSO depleted the GSH levels and decreased the SOD2 levels in WT fibroblasts by 25% (Figure 12B and 13B). Using the same samples, increased
Figure 12C

Figure 12D
Figure 12 Altered GSH levels lead to increased superoxide anion levels

WT and GILT -/- fibroblasts were grown in RPMI medium 1640 without or with the addition of antioxidants: 2ME (55µM), or L-NAC (15mM) and glutathione synthesis inhibitor BSO (150µM) for 6 hours at 37°C, 5% CO₂. A and B. Reduced glutathione (GSH) was measured using Quantichrom glutathione assay kit by Bioassay Systems. One million cells were lysed using a dounce homogenizer in 500µl cold PBS with 1mM EDTA. 20µg of each total cell lysate were used for the assy. Mean ± S.E. for three independent experiments are shown. *, p < 0.05 vs. control medium. Post hoc Student’s t test was applied. C and D. FACS analysis of intracellular superoxide anions. 2µM of dihydroethidium (DHE) was added to untreated and treated cells and incubated for 30 minutes at 37°C, 5% CO₂. Cells were washed twice in PBS, resuspended in PBS with 0.5% bovine serum albumin, and fluorescent intensity (FL-2 channel) was measured by a FACS Scan flow cytometer. Bar graphs indicate mean ± S.E fluorescence/cell measured in twenty thousand cells per sample in three separate experiments. *, p < 0.05 vs. control medium. Statistical analysis was performed by analysis of variance with post hoc Student’s t test.

-------------------------------------------------------------------------------------------------

intracellular superoxide levels were detected in both WT and GILT -/- fibroblasts (Figure 12D). These results suggested that SOD2 levels were sensitive to the changes in GSH levels in both WT and GILT -/- fibroblasts. Interestingly, the effect of the BSO treatment was less significant in GILT -/- cells, indicating that perhaps the impact of the absence of GILT on the observed parameters was the maximum possible in GILT -/- cells without causing cell death.
Figure 13 Increase in GSH is followed by up-regulated SOD2 expression

WT and GILT -/- fibroblasts were grown in RPMI medium 1640 without or with addition of A. 55µM 2ME, B. 150µM BSO, or 15mM L-NAC for 6 hours at 37°C, 5% CO₂ and SOD2 levels were determined by quantitative Western blot. 5µg per well of each cell sample was loaded into the 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with mouse anti-SOD2 antibodies at 4°C overnight. Membranes were stripped and re-probed with anti-GAPDH or anti-β-actin antibodies. Densitometry analysis for SOD2 was normalized to GAPDH or β-actin content and presented as arbitrary units relative to untreated WT cells. Means ± S.E. for three blots were analyzed. *, p < 0.05 vs. control medium. Statistical analysis was performed by analysis of variance with post hoc Student’s t test.

C, untreated; B, BSO; N, L-NAC.

Mitochondrial membrane potential is diminished in GILT -/- fibroblasts

Based on the finding that the modulation of GSH levels is associated with a corresponding change in SOD2 expression, we focused on the interface between redox homeostasis and SOD2 levels. Mitochondria are the major source of ROS production during respiratory metabolism (21). Mitochondrial ROS are detoxified by enzymatic (e.g. SOD2) and non-enzymatic (e.g. glutathione) means (51). When the balance between ROS production and elimination is disrupted, mitochondria are especially prone to ROS damage (83). Therefore, mitochondrial damage and ensuing mitochondrial permeability transition and the loss of
mitochondrial membrane potential ($\Delta \Psi_m$) could be caused by the increased GSSG/GSH ratio and the increased ROS production.

To test whether the mitochondrial membrane potential was altered in GILT -/- fibroblasts, the membrane potential was assayed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) fluorescence dye staining. JC-1 is a lipophilic cation, which can selectively enter into the mitochondria where it exhibits green fluorescence when in the form of monomers, which occurs under condition of low membrane potential. JC-1 reversibly changes emission color from green to red as it forms aggregates when mitochondrial membrane potential is high (125). At normal values of the mitochondrial membrane potential (-180~200 mV), JC-1 predominately forms red aggregates. Therefore, mitochondrial membrane depolarization can be identified by a decreased ratio of red to green fluorescence. As a positive control, cells were treated with the mitochondrial respiratory chain de-coupler, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which resulted in the loss of mitochondrial membrane potential (Figure 14A). In the absence of any treatment, GILT -/- fibroblasts exhibited increased levels of depolarized mitochondrial membranes, as indicated by a 70% decrease of the JC-1 red/green fluorescence intensity ratio. In addition, the percentage of the cells with only JC-1 aggregates (upper left quadrant) in GILT -/- cells is lower than in WT fibroblasts (51.8% vs. 84%).

We hypothesized that the modulation of GSH levels, the oxidation status of the GSSG/GSH pair and the resulting change in ROS levels could lead to a change in the mitochondrial membrane potential. Thus, we treated cells with antioxidants 2ME, L-NAC, or an inhibitor of glutathione synthesis - BSO, for 6 hours under conditions shown in Figure 12 and 13.
Figure 14B

**Figure 14 Mitochondrial membrane potential is reduced in GILT -/- fibroblasts**

A. Samples were analyzed for the presence of JC-1 aggregates and monomers. Fibroblasts were incubated in RPMI 1640 medium containing DMSO solvent (0.1%) or CCCP (50µM) for 15 minutes in the presence of 2µM JC-1 at 37°C, 5% CO₂. Twenty thousand cells were examined per sample for the presence of JC-1 aggregates (FL-2) vs. monomers (FL-1) using a FACS Scan flow cytometer. Change in mitochondrial membrane potential was determined by the ratio of JC-1 red fluorescence intensity (aggregates) to JC-1 green fluorescence intensity (monomers). Ratio was normalized to untreated WT. Means ± S.E. for three independent experiments are shown. *,
and measured the mitochondrial membrane potential in WT and GILT -/- fibroblasts (Figure 14B). The addition of BSO caused a significant depolarization of the mitochondrial membrane (30% reduction in the ratio of JC-1 aggregate to JC-1 monomer) in WT fibroblasts. In contrast, the treatment of GILT -/- fibroblasts with 2ME led to an increase of the JC-1 red/green ratio by 60%, indicating that the mitochondrial membrane was polarized. A similar tendency (increased JC-1 red/green ratio by 40%) was observed in the L-NAC treated GILT -/- fibroblasts. Our results revealed the elevated level of dysfunctional mitochondria within GILT -/- fibroblasts and the sensitivity of the mitochondrial membrane potential to the changes in GSH in both cell types.

**Autophagy is up-regulated in GILT -/- fibroblasts**

Increased oxidative stress and damaged mitochondria usually lead to cell death (105). However, GILT -/- fibroblasts are able to survive and show an increased proliferation rate (19). Therefore, we hypothesized that GILT -/- cells might employ compensatory mechanisms to neutralize the damage caused by increased oxidative stress (55, 108). Previous studies indicated
that loss of the mitochondrial membrane potential is a common feature of mitophagy (126). Mitophagy is the specific form of autophagy used by the cell for degrading unstable and potentially dangerous mitochondria. An intact mitochondrial membrane is essential to cellular homeostasis (127). We hypothesized that increased numbers of dysfunctional mitochondria may induce autophagy, or more specifically mitophagy, that would lead to the decreased SOD2 expression in GILT -/- fibroblasts.

To test this, we first examined the level of the autophagy marker, microtubule-associated protein light-chain 3 II (LC3-2) in WT and GILT -/- fibroblasts. LC3-2 is formed from LC3-1 (a microtubule associated cytosolic protein) (81) after conjugation with phosphatidylethanolamine and attachment to the autophagosomal membrane. As we were observing autophagic flux, the cells were treated with chloroquine, an inhibitor that blocks autophagosome and lysosome fusion (128) (Figure 15). After 6 and 18 hours of chloroquine treatment, the LC3-2/LC3-1 ratio was significantly increased in GILT -/- fibroblasts (30% increase in the 6 hour treatment and 90% increase in the 18 hour treatment compared with WT cells) (Figure 16) indicating that autophagy was more active in these cells.

Next, we tested whether the inhibition of autophagy would lead to a recovery of SOD2 expression in GILT -/- fibroblasts. Cells were treated with 3-methyladenine (3-MA) (129), a class III phosphoinositide 3-kinase (PI3K) inhibitor, which inhibits autophagy at the sequestration stage when the autophagosome is formed (130). Treatment with the autophagy inhibitor increased SOD2 levels in both WT and GILT -/- fibroblasts by 50% and 100%, respectively (Figure 17). The levels of SOD2 in 3-MA treated GILT -/- cells were even higher
than the basal SOD2 levels in WT cells. These results suggested that autophagy is responsible, at least in part, for the decreased SOD2 levels in GILT -/- fibroblasts.

Figure 15 Chloroquine inhibits the fusion between the autophagosomes and the lysosomes

Chloroquine is a weak base and tends to accumulate in acidic compartments, such as lysosomes. Treatment of chloroquine raises pH value in lysosomes, disrupts lysosomal hydrolases, and inhibits the fusion of autophagosomes and lysosomes. The autophagy marker, microtubule-associated protein light-chain 3 II (LC3-2) is formed from the cytosolic LC3-1 after conjugation with phosphatidylethanolamine.
Figure 16 Autophagy is increased in GILT -/- fibroblasts

WT and GILT -/- fibroblasts were grown with RPMI 1640 medium in the presence or absence of 20μM chloroquine at 37°C, 5% CO₂ to inhibit autophagosome fusion to lysosomes and subsequent degradation. After 6 or 18 hours of treatments, 5μg of each cell lysate was analyzed for LC3 by Western blot. The blots were stripped and re-probed for β-actin as a loading control. Densitometry analysis for LC3-2/LC3-1 is presented in the graph form. Means ± S.E. for three blots are shown. *, p < 0.05 vs. WT. Post hoc Student’s t test was applied.
Figure 17 Inhibition of autophagy results in increased SOD2 expression

WT and GILT -/- fibroblasts were grown in RPMI 1640 medium without or with addition of 1μM 3-MA, an inhibitor of autophagy for 18 hours at 37°C, 5% CO₂. The effect of 3-MA on SOD2 levels was determined by quantitative Western blot. Densitometry analysis for SOD2 was normalized to GAPDH content and presented as arbitrary units relative to untreated WT cells. Means ± S.E. for three blots are shown. *, p < 0.05 vs. untreated control. Post hoc Student’s t test was applied.
**INCREASED MITOPHAGY IS DETECTED IN GILT -/- FIBROBLASTS**

Our results suggest that up-regulated autophagy is at least partially responsible for eliminating the damaged mitochondria and mitochondrial SOD2 in GILT -/- fibroblasts. It is of interests to evaluate whether this is the consequence of mitophagy or possibly SOD2 “leaking” into the cytosol and its removal by regular autophagy and/or other mechanisms. Therefore, we tested the expression of cytochrome c oxidase IV (COX IV) in WT and GILT -/- fibroblasts. COX IV is one of the 13 subunits of the cytochrome c oxidase complex, the terminal enzyme complex in the mitochondrial respiratory chain (131). It is expressed at a constitutively high level and is located in the inner mitochondrial membrane (132). The level of COX IV in GILT -/- cells is 25% lower than in WT fibroblasts (Figure 18). Similar results were observed in the treatment with the autophagy inhibitor – 3-MA. COX IV levels are increased in both WT and GILT -/- fibroblasts by 45% and 65%, respectively upon treatment with 3-MA. Taken together, these results suggest that mitophagy, is the most likely mechanism responsible for the degradation of mitochondrial proteins in GILT -/- fibroblasts.

**3-MA TREATMENT RESULTS IN INCREASED OXIDATIVE STRESS**

Treatment with 3-MA inhibits autophagosome formation and leads to an increase of mitochondrial proteins (SOD2 and COX IV) in both WT and GILT -/- fibroblasts. However, it is not clear whether 3-MA treatment affects the redox status within the cells. Therefore, we measured the cellular levels of GSH and ROS production. In order to examine autophagy activity, chloroquine was added into control (untreated) and 3-MA treated cells for 6 hours.
Figure 18 The level of mitochondrial COX IV is reduced in GILT -/- fibroblasts

WT and GILT -/- fibroblasts were grown in RPMI 1640 medium without or with the addition of 1µM 3-MA, an inhibitor of autophagy for 18 hours at 37°C, 5% CO₂. The effect of 3-MA on COX IV levels was determined by quantitative Western blot. 1µg per well of each sample was loaded into SDS PAGE gels. Densitometry analysis for COX IV was normalized to GAPDH content and presented as arbitrary units relative to untreated WT cells. Means ± S.E. for three blots are shown. *, p < 0.05 vs. untreated control. #, p < 0.05 vs. WT. Post hoc Student’s t test was applied.
The ratio of LC3-2/ LC3-1 was decreased by 60% in both WT and GILT -/- cells treated with 3-MA (Figure 19A). Furthermore, 3-MA significantly decreased GSH levels by 25% and elicited 20% more superoxide production in WT and GILT -/- fibroblasts suggesting that the oxidative stress was increased (Figure 19B and C). Interestingly, the inhibition of autophagy by 3-MA resulted in an obvious reduction in the ratios of JC-1 red to green fluorescence, indicating a large-scale mitochondrial depolarization in both WT and GILT -/- fibroblasts (Figure 19D). These results could be a consequence of decreased autophagy and therefore prolonged presence of damaged mitochondria as an additional source of ROS that worsened the redox status in the cells.

Figure 19A
Figure 19B

Figure 19C
Figure 19 Treatment of 3-methyladenine (3-MA) results in increased oxidative stress

WT and GILT -/- fibroblasts were grown in RPMI 1640 medium without or with the addition of 1μM 3-MA, an inhibitor of autophagy for 18 hours at 37°C, 5% CO₂. A. In order to measure the LC3-2/LC3-1 ratio, 20μM chloroquine was added into 3-MA untreated or treated cells for additional 6 hours at 37°C, 5% CO₂. 5μg of each cell lysate was analyzed for the expression of LC3 by Western blot and the blots were stripped and re-probed for β-actin as a loading control. Densitometry analysis for LC3-2/LC3-1 was analyzed. Ratio was normalized to 3-MA untreated WT. Means ± S.E. for three blots are shown. *, p < 0.05 vs. 3-MA untreated control. Post hoc Student’s t test was applied. B. Reduced glutathione (GSH) was measured using Quantichrom glutathione assay kit by Bioassay Systems. One million cells were lysed using a dounce
homogenizer in 500μl cold PBS with 1mM EDTA. 20μg of each total cell lysate were used for the assay. Mean ± S.E. for three independent experiments are shown. *, p < 0.05 vs. control medium. Post hoc Student’s t test was applied. C. FACS analysis of cellular superoxide anions. 2μM of DHE was added to untreated and treated cells and incubated for 30 minutes at 37°C, 5% CO₂. Cells were washed twice in PBS, resuspended in PBS with 0.5% bovine serum albumin, and fluorescent intensity (FL-2 channel) was measured by FACS Scan flow cytometer. Bar graphs indicate mean ± S.E fluorescence/cell measured in twenty thousand cells per sample in three separate experiments. *, p < 0.05 vs. control medium. Post hoc Student’s t test was applied. D. Twenty thousand cells were examined per sample for the presence of JC-1 aggregates (FL-2) vs. monomers (FL-1) using a FACS Scan flow cytometer. The change in mitochondrial membrane potential was determined by the ratio of JC-1 red fluorescence intensity to JC-1 green fluorescence intensity. Ratio was normalized to untreated WT. Means ± S.E. for three independent experiments are shown. *, p < 0.05 vs. control medium. Post hoc Student’s t test was applied

-------------------------------------------------------------------------------------------------

**Modulation of Reduced Glutathione Levels Leads to the Changes in Autophagy Activities**

We have shown that SOD2 levels (Figure 13) and mitochondrial membrane potential (Figure 14) were sensitive to the changes in GSH in WT and GILT -/- fibroblasts. Increased
autophagy/mitophagy is also indicated to be responsible for the decreased SOD2 expression in GILT -/- fibroblasts (Figure 16-18). However, it is not clear whether autophagy is sensitive to the modulation of GSH levels. In order to examine the relationship between glutathione / ROS levels and autophagy in WT and GILT -/- fibroblasts, we modulated intracellular glutathione levels. Modification of glutathione levels was achieved by the treatment of the cells with 2ME and L-NAC or with glutathione synthesis inhibitor – BSO. Expression of the autophagy marker LC3-2 was determined by a semi-quantitative Western blot. 2ME (Figure 20A) and L-NAC (Figure 20B) treatments decreased the autophagy response by 15% and 25% respectively in GILT -/- cells. On the contrary, treatment with BSO significantly elevated the LC3-2/LC3-1 ratio in WT fibroblasts (Figure 20C). These results are in accordance with the effects of glutathione levels on the mitochondrial membrane potential and SOD2 levels in WT and GILT -/- cells, suggesting that the diminished expression of glutathione-sensitive SOD2 is the consequence of up-regulated autophagy/mitophagy in GILT -/- fibroblasts.

**Inhibition of Erk1/2 phosphorylation leads to the down-regulation of autophagy and increased SOD2 expression**

Thus far, our results suggested that the absence of GILT in the cells results in increased oxidative stress and decreased mitochondrial integrity. In addition, GILT -/- cells show increased autophagy, which is a common cellular response against increased oxidative stress. Given the fact that ROS production is elevated and autophagy is increased in GILT -/- fibroblasts, it was of
Figure 20A

WT  GILT -/-
C 2ME  C 2ME chloroquine
LC3-1
LC3-2
GAPDH

LC3-2/LC3-1 (arbitrary units)

WT  GILT -/-
control 2ME control 2ME

Figure 20B

WT  GILT -/-
C NAC  C NAC chloroquine
LC3-1
LC3-2
GAPDH

LC3-2/LC3-1 (arbitrary units)

WT  GILT -/-
control L-NAC control L-NAC

*
Figure 20 Altered glutathione levels lead to changes in autophagy

WT and GILT -/- fibroblasts were grown in RPMI 1640 medium without or with the addition of A. 55μM 2ME, B. 150μM BSO, or C. 15mM L-NAC for 6 hours followed by the incubation of RPMI 1640 medium with 20μM chloroquine at 37°C, 5% CO2 to inhibit autophagosomes fusion to lysosomes and subsequent degradation. After 18 hours of chloroquine treatments, 5μg of each sample was analyzed for the LC3 expression by Western blot and the blots were stripped and re-probed for β-actin as a loading control. Densitometry analysis for LC3-2/LC3-1 was analyzed. Means ± S.E. for three blots are shown. *, p < 0.05 vs. control. Post hoc Student’s t test was applied.
interest to evaluate whether redox-sensitive signaling pathways regulate autophagy in our system.

An increasing number of studies have indicated that Erk1/2 activation modulates autophagy (133-135). Therefore, the level of Erk1/2 phosphorylation in WT and GILT -/- fibroblasts was examined. As shown in Figure 21, phosphorylation of Erk1/2 in GILT -/- cells was increased while the expression of the dual-specificity protein phosphatase, MAP Kinase Phosphatase 3 (MKP3), which specifically inactivates Erk1/2, was decreased (136). It has been shown that ROS-sensitive MKP3 is regulated by oxidation and/or reduction of the cysteines located in its active site (137). To test whether redox status in our cells affected Erk1/2 pathway, we modulated the redox status by adding either antioxidants (2ME, L-NAC) or the glutathione synthesis inhibitor BSO, and examined their effects on Erk1/2 phosphorylation and MKP3 levels. Treatment of WT or GILT -/- fibroblasts with 2ME led to reduced Erk1/2 phosphorylation and increased MKP3 expression (Figure 22A). Similar results were observed when the cells were treated with L-NAC (Figure 22B). Conversely, phosphorylation of Erk1/2 was increased and MKP3 expression reduced in the presence of BSO (Figure 22C).
Figure 21 Erk1/2 signaling is altered in GILT -/- fibroblasts

5μg of cell extract from either WT and/or GILT -/- fibroblast lysates were analyzed by Western blot for the expression of A. phospho-Erk1/2 and B. MKP3. Densitometry analysis for phospho-Erk1/2 or MKP3 was normalized to GAPDH or β-actin content and presented as arbitrary units relative to WT cells. Means ± S.E. for three blots are shown. *, p < 0.05 vs. WT. Post hoc Student’s t test was applied.
Figure 22A
Figure 22B
Figure 22C

WT  | GILT -/-
---  |--------
C    | BSO C  | BSO

phoErk1/2
GAPDH
MKP3
GAPDH

WT         | GILT -/-
---         |--------
control    | BSO     | control  | BSO

phoErk1/2 (arbitrary units)

WT         | GILT -/-
---         |--------
control    | BSO     | control  | BSO

MKP3 (arbitrary units)

Figure 22C
Figure 22 Modulation of glutathione levels correlates with changes in Erk1/2 phosphorylation and MKP3 expression

WT and GILT -/- fibroblasts were grown in RPMI 1640 medium untreated or treated with A. 55µM 2ME, B. 15mM L-NAC, or C. 150µM BSO for 6 hours at 37°C, 5% CO2. 5µg per lane from each fibroblast lysates was separated by SDS-PAGE, followed by Western blot and analyzed for the expression of Phospho-Erk1/2 and MKP3. Densitometry analysis for phospho-Erk1/2 or MKP3 was normalized to GAPDH content and presented as arbitrary units relative to untreated WT cells. Means ± S.E. for three blots are shown. *, p < 0.05 vs. untreated control. Post hoc Student’s t test was applied.

Erk1/2 are positioned downstream of MAPK Kinase 1/2 (MEK1/2) (Figure 23). MEK1/2 activate Erk1/2 by phosphorylation of threonine and tyrosine residues (138, 139). To determine whether the Erk1/2 signaling pathway is involved in the induction of autophagy, we examined the effects of two MEK1/2 specific inhibitors, PD98059 and U0126, on autophagy and SOD2 expression. Inhibition of Erk1/2 signaling with MEK1/2 inhibitors abolished the autophagic response as we detected a decreased LC3-2/LC3-1 ratio (Figure 24A). In WT cells, the LC3-2/LC3-1 ratio was decreased by 30% (PD98059) or 37% (U0126), while the same treatment of GILT -/- cells decreased the LC3-2/LC3-1 ratio by 25% (PD98059) or 49% (U0126). Consistent with this inhibition, the levels of SOD2 were increased 20-25% in both WT and GILT -/- fibroblasts (Figure 24B and C). These results suggested that the induction of autophagy at least
partially depends on redox-sensitive Erk1/2 activation and likely contributes to the decreased SOD2 levels in GILT -/- fibroblasts.

**Figure 23 MEK1/2 inhibitors inhibit the Erk1/2 signaling pathway**

Ras/Raf/Erk signaling pathway has a role in regulating many cellular processes such as cell proliferation and differentiation. Upon stimulation, a sequential three-part protein kinase cascade is initiated, consisting of MAP kinase kinase kinase (MAPKKK): Raf, MAP kinase kinase (MAPKK): MEK1/2, and MAP kinase (MAPK): Erk1/2. Erk1/2 are inactivated by MAPK phosphatase 3 (MKP3), which its function is regulated by ROS. Activation of MEK1/2 occurs through phosphorylation of two serine residues by Raf. MEK1/2 activates Erk1/2 by phosphorylating its threonine and tyrosine. PD98059 and U0126 have been shown to act as highly selective inhibitors of MEK1/2 activation and the Erk1/2 pathway.
Figure 24A
Figure 24B

WT    GILT -/
C    PD   C    PD

phoErk1/2

totalErk1/2

SOD2

GAPDH

phospho/total Erk1/2

WT  GILT -/

control  PD98059  control  PD98059

SOD2 (arbitrary units)

WT  GILT -/

control  PD98059  control  PD98059

* Significant difference
Figure 24C
Figure 24 Erk1/2 signaling is involved in autophagy and has an effect on SOD2 expression

A. WT and GILT −/− fibroblasts were either left untreated or treated with MEK1/2 inhibitors: PD98059 (PD) or U0126 (U) for 6 hours and then chloroquine (20µM) was added for 18 additional hours at 37°C, 5% CO2. Cell lysates were analyzed for the expression of LC3 by Western blot and blots were stripped and re-probed for GAPDH as a loading control. Densitometry analysis for LC3-2/LC3-1 was analyzed and normalized to untreated WT. Means ± S.E. for three blots are shown. *, p < 0.05 vs. untreated control (C). Post hoc Student’s t test was applied. B and C. Cells were treated with (B) PD98059 or (C) U0126 and the expression of phospho-Erk1/2 or SOD2 were detected by quantitative Western blot. Densitometry analysis for phospho-Erk1/2 or SOD2 was normalized to total-Erk1/2 or GAPDH content and presented as arbitrary units relative to untreated WT cells. Means ± S.E. for three blots are shown. *, p < 0.05 vs. untreated control. Post hoc Student’s t test was applied.

-----------------------------------------------

HMGB1 translocation from the nucleus to the cytoplasm is increased in GILT −/− fibroblasts

A recent report by Tang et al. indicated that ROS generated by cellular stress induce High Mobility Group Box 1 (HMGB1) protein translocation from the nucleus to the cytosol, a process which induces autophagy (117) (Figure 25). HMGB1 is a highly conserved chromatin-binding protein that facilitates the access of transcriptional activities by bending DNA (140). In addition
ROS generated by cellular stress promote HMGB1 translocation to the cytoplasm, which in turn induces autophagy. Cytosolic HMGB1 interacts with the autophagy protein Beclin 1 and disrupts its interaction with Bcl-2, which suppresses autophagy. Additionally, activation of Erk1/2 pathway also induces autophagy by disrupting the formation of a Beclin 1 / Bcl-2 complex.

Figure 25 HMGB1 cytosolic translocation promotes autophagy

to its nuclear role, HMGB1 also functions as a damage-associated molecular pattern (DAMP) molecule and an extracellular signaling molecule during inflammation (141). Thus, we
investigated whether HMGB1 cytosolic translocation is promoted by the increased oxidative stress in the GILT -/- fibroblasts (Figure 26). In the absence of any treatment, the level of cytosolic HMGB1 was increased in GILT -/- fibroblasts. There was a 24% increase in cytosolic HMGB1 in GILT -/- fibroblast compared to WT fibroblasts. The increased HMGB1 amount in the cytoplasm of GILT -/- cells correlated, as expected, with increased autophagy in GILT -/- cells. Treatment of cells with rapamycin, an autophagy enhancer, induced HMGB1 translocation to the cytoplasm in both GILT -/- and WT fibroblasts. Reconstitution of GILT in GILT -/- fibroblasts resulted in a reduction of cytosolic HMGB1 strengthening the correlation of GILT’s presence and regulation of autophagy.

**HMGB1 CYTOSOLIC TRANSLOCATION IS SENSITIVE TO THE CHANGES OF GSH LEVELS**

To determine whether the increase in ROS is responsible for the enhanced HMGB1 cytosolic translocation observed in GILT -/- fibroblasts we evaluated the effects of an antioxidant (L-NAC) and a GSH synthesis inhibitor - BSO on the distribution of HMGB1 in our system. L-NAC decreased the percentage of HMGB1 localized to the cytoplasm by 20% in GILT -/- cells (Figure 27). Conversely, there is an increase (22%) in the percentage of cytosolic HMGB1 in WT cells treated with BSO. These data suggest that HMGB1 is involved in the response to oxidative stress.
Figure 26 Translocation of HMGB1 into the cytoplasm is increased in GILT -/- fibroblasts

Fibroblasts were either left untreated or treated with 1µM rapamycin for 16 hours and were immunostained with rabbit anti-HMGB1 antibody followed by goat anti-rabbit Alexa Fluor 594 (Red) and Hoechst 33342 (blue). The mean intensity of nuclear and cytosolic HMGB1 per cell was analyzed as described in Material and methods. Scale bar length, 22µM. Means ± S.E. for at least three hundreds individual cells are shown. **, $p < 0.05$ vs. untreated WT. *, $p < 0.05$ vs. untreated control. Representative images are shown. Post hoc Student’s $t$ test was applied.
Figure 27 The antioxidant L-NAC inhibits the translocation of HMGB1 from the nucleus to the cytoplasm in GILT -/- fibroblasts

Fibroblasts were either left untreated or treated with 15mM L-NAC or 150µM BSO for 6 hours and were immunostained with rabbit anti-HMGB1 antibody followed by incubation with goat anti-rabbit Alexa Fluor 594 (Red) and Hoechst 33342 (blue). The mean intensity of nuclear and cytosolic HMGB1 per cell was analyzed as described in Material and Methods. Scale bar length, 22µM. Means ± S.E. for at least two hundreds individual cells are shown. **, *p* < 0.05 vs. untreated WT. *, *p* < 0.05 vs. untreated control. Representative images are shown. Post hoc Student’s *t* test was applied.
DISCUSSION

Expression of the evolutionarily conserved GILT gene (IFI-30) homologs in different species, from unicellular organism like protozoa, to insects, fish, amphibians, rodents, cattle, human, and even plants indicates that GILT may have more fundamental cellular functions not related to MHC class II antigen processing. Indeed, our previously published study (19) has shown that the absence of GILT leads to reduced expression of mitochondrial SOD2 and increased production of ROS within GILT -/- cells, indicating that GILT -/- cells are under moderately increased oxidative stress. In addition, and perhaps as a consequence, these cells show an increased rate of proliferation. In the current study we further defined the role of GILT in the regulation of cellular redox homeostasis.

SPECIFIC AIM 1. TO TEST THE HYPOTHESIS THAT THE ABSENCE OF GILT ALTERS GLUTATHIONE SYNTHESIS AND LEADS TO DECREASED SOD2 LEVELS

A change in redox homeostasis can result in the oxidative damage of cellular constituents resulting in DNA mutation, protein aggregation and lipid peroxidation and may lead to cell death. Cells have developed various enzymatic and nonenzymatic antioxidant agents to detoxify ROS and prevent oxidative stress. These antioxidant agents include enzymes such as superoxide dismutases (SODs), catalases, peroxidases, thioredoxin and a non-enzymatic tripeptide, glutathione. SODs exist in the cytoplasm and the mitochondria. SODs catalyze superoxide anion conversion into hydrogen peroxide, which is further decomposed into water and molecular oxygen by a catalase and/or peroxidase. Thioredoxin detoxifies hydrogen peroxide in
cooperation with thioredoxin peroxidase. In addition, glutathione maintains the cellular reducing environment to detoxify ROS.

**GSSG vs. GSH**

Surprisingly, in the absence of GILT, a major intracellular antioxidant, glutathione is reduced and the ratio of oxidized (GSSG) vs. reduced (GSH) form of glutathione is shifted toward GSSG (Figure 10). Within the cell, glutathione exists mainly (>98%) in the reduced form, but some is also present as glutathione disulfide (GSSG) (46). GSH maintains an optimal redox environment for proper function of cellular proteins in aerobic conditions. Under normal physiological conditions, intracellular GSH concentration is in the millimolar range and far exceeds the concentration of GSSG, which is in micromolar range (122). It has been shown that GSH deficiency and/or an increase in GSSG/GSH ratio leads to an increased susceptibility to oxidative stress (142) and the resulting damage is thought to be involved in a number of diseases, including cancer (143), neurodegenerative diseases (144), inflammatory and immune system diseases (145, 146), and cardiovascular diseases (147). Therefore, the GSSG/GSH ratio in the cell is an important indicator of the redox environment (43).

Our data showed that the ratio of cytoplasmic GSSG/GSH in GILT -/- fibroblasts was shifted toward GSSG under the basal conditions of growth (Figure 10). Thus, the cytosol of GILT -/- fibroblasts is a more oxidative environment than the cytosol of WT fibroblasts (19). In addition, in response to the treatment with oxidizing and reducing agents, the changes in the cytosolic GSSG/GSH ratio were more significant than the changes detected in the mitochondrial GSSG/GSH ratio in GILT -/- fibroblasts (Figure 11). The observed smaller change in mitochondrial GSSG/GSH ratio upon the treatment with an oxidant can be interpreted as a
consequence of more effective antioxidant mechanisms present in the mitochondria, for example: glutathione reductase and/or NADPH.

**Specific aim 2. To test the hypothesis that autophagy affects SOD2 levels in GILT-/- fibroblasts**

**Mitochondrial membrane potential**

When the redox balance is skewed toward a more oxidative state, accumulation of ROS oxidize cellular components and eventually lead to cell death (148). However, at moderate or low levels, ROS act as signaling molecules to activate various defense mechanisms, including autophagy, for reducing oxidative damage (55, 149, 150). Autophagy acts as a survival pathway in the cells and its inhibition results in up-regulated apoptosis (151). It has been shown that the ROS have a major impact on the mitochondria (152). Although a major source of ROS, mitochondria are also sensitive to damage by ROS. Dysfunctional mitochondria produce more ROS leading to more damage. It is generally recognized that mitophagy, a specific form of autophagy targeting mitochondria is critical for the removal of damaged mitochondria and acts as a cellular quality control that helps maintain cellular homeostasis and reduce oxidative stress (83, 126). Increasing evidence indicates that mitophagy is a selective process, which removes damaged mitochondria (153). Under oxidative stress, a mitochondrial permeability transition (MPT) is induced and the mitochondrial membrane is depolarized, which results in either apoptosis or autophagy depending on the severity of the oxidative damage (149). We hypothesized that the decreased levels of reduced glutathione, along with the oxidative shift of
the GSSG/GSH ratio, and/or the increased ROS production promote mitochondrial damage and the loss of mitochondrial membrane potential. Our experimental data showed that the mitochondrial membrane potential in GILT -/- cells is altered when compared with WT cells (Figure 14A). This finding suggested that an increased portion of mitochondria present in GILT -/- cells is probably damaged.

Several studies indicated that antioxidants can inhibit the loss of mitochondrial membrane potential by elevating intracellular glutathione levels and quenching ROS (28, 154). In our experiments, supplementation with antioxidants 2ME and L-NAC increased glutathione levels, effectively prevented ROS accumulation (Figure 12) and rescued the loss of mitochondrial membrane potential (Figure 14B) in GILT -/- fibroblasts. These results suggested that oxidative stress plays a critical role in the regulation of ROS production and loss of mitochondrial membrane potential in GILT -/- fibroblasts. It also raised the possibility that GILT deficiency affects GSH synthesis. We hypothesized that GILT decreases the availability of cysteine, which is the limiting factor for GSH synthesis (155).

**AUTOPHAGY**

Decreased GSH levels may lead to the loss of mitochondrial membrane potential, and therefore increased numbers of dysfunctional mitochondria. In order to remove the increased numbers of damaged mitochondria, cells increase the rate of autophagy. In agreement with this hypothesis, our data showed that GILT -/- cells have increased expression of LC3-2, a marker of autophagy (Figure 16). LC3 is synthesized as a precursor, proLC3 and proLC3 is converted to LC3-1 by proteolysis (156) within 6 minutes of synthesis in the cytoplasm (81). In the induction of autophagy, LC3-1 is modified to the phosphatidylethanolamine (PE)-conjugated form, LC3-2,
by lipidation (81). After lipidation, LC3-2 is associated with the autophagosome membrane. Therefore, it is well accepted that the ratio of LC3-2 vs. LC3-1 detected in Western blot correlates with the autophagy flux (157, 158).

In the late stage of autophagy, LC3-2 is degraded as the autophagosomes fuse with the lysosomes. However, in order to recycle LC3-1, a portion of LC3-2 may be delipidated by a cysteine protease: autophagy-related gene 4 homolog B (Atg4B) (159). It has been shown that ROS oxidize the cysteine residue located in the active site of Atg4B to inactivate this essential enzyme (106). Therefore, we speculate that the increased ROS production in GILT -/- fibroblasts is likely to contribute to the enhanced autophagy by directly affecting LC3-2 expression through Atg4B regulation.

The increased autophagy detected in GILT -/- fibroblasts could also be due to lysosomal membrane permeabilization (LMP) (160). It is well known that lysosomes participate in the turnover of organelles and macromolecules through autophagy (161). In the autophagolysosomes, lysosomal hydrolases are responsible for the digestion of autophagic material after fusion between autophagosomes and lysosomes (161). Therefore, the lysosome is a redox-active compartment rich in cysteine and low-mass iron, the latter originating from the autophagic degradation of ferrous materials, such as mitochondrial cytochromes, ferritin, and other iron-containing metalloproteins (162). Because of the acidic environment and high thiol concentration within the lysosomes, lysosomal iron stays in the reduced state, which makes lysosomes susceptible to oxidative damage through the Fenton reaction (163, 164). In Fenton reaction intracellular hydrogen peroxide reacts with ferrous iron yielding hydroxyl radicals, which may cause damage to the lysosomal membrane and lead to lysosomal membrane
permeabilization (LMP) (160). LMP sequentially causes the release of ROS and lysosomal hydrolases from the lysosomal lumen to the cytosol which can then damage other cellular organelles, such as mitochondria (165, 166). It has been shown that the “leaking” lysosomal enzymes promote a mitochondrial permeability transition (MPT) and reduce mitochondrial membrane potential (167, 168). Under conditions of sub-lethal damage to cytoplasmic organelles, cytoprotective autophagy might increase in order to remove defective organelles for cell survival (166). Therefore, the increased ROS levels in GILT -/- fibroblasts may impact not only mitochondria but lysosomes as well. Damage to lysosomal membranes may lead to LMP and increased oxidative stress on other organelles, which promotes autophagy.

**Mitophagy**

Damage to the mitochondria and increased autophagy could also explain the lower expression, activity and decreased stability of SOD2 shown previously (19). Treatment of GILT -/- cells with an inhibitor of autophagy, 3-methyladenine (3-MA) rescued SOD2 expression (Figure 17). We also found that the expression level of another mitochondrial protein, COX IV, is reduced in GILT -/- cells compared with WT fibroblasts. Treatment with 3-MA restored the COX IV expression as well (Figure 18). Therefore, another mitochondrial resident protein is down-regulated indicating that SOD2 expression levels are most likely regulated by mitophagy in GILT -/- fibroblasts.

Recent studies have shown that parkin, an E3 ubiquitin ligase linked to Parkinson’s disease is selectively recruited to dysfunctional mitochondria where it promotes mitophagy (169). The translocation of parkin to impaired mitochondria and the induction of parkin-mediated mitophagy are mediated by the activity of phosphatase and tensin homolog (PTEN)-
induced kinase 1 (PINK1), a Parkinson’s disease-related protein (170, 171). Parkin ubiquitylates the mitochondrial protein voltage-dependent anion channel (VDAC) and binds to p62 (89). Then p62 mediates the targeting of damaged mitochondria into autophagosomes by interacting with LC3-2 (172). Therefore, in order to strengthen our hypothesis that mitophagy regulates the levels of SOD2, it would be useful to determine whether these processes (Parkin-PINK1) are enhanced in GILT -/- fibroblast.

SOD2 expression might be regulated at the transcriptional level as well. It has been shown by quantitative RT-PCR that in comparison with WT fibroblasts, SOD2 mRNA is about 35% less abundant in GILT -/- fibroblasts (19). We hypothesize that the global redox imbalance detected in GILT -/- cells may affect the activity of redox-sensitive transcription factors, such as nuclear respiratory factor 2 (Nrf2) (173-175), NFκB (176-179), and AP-1 (177) that are involved in SOD2 transcription. Most likely, both mechanisms, autophagy and redox-sensitive transcription factors, may be involved in the regulation of SOD2 expression in GILT -/- fibroblasts.

**Specific aim 3. To identify the signaling pathways involved in the induction of autophagy in WT and GILT -/- fibroblasts**

Generation of ROS through oxidative stress results in the induction of autophagy (57). It has been shown that applying a chemical ROS scavenger or overexpressing SOD2 reduces autophagy, indicating that ROS possibly act as signaling molecules to induce autophagy (180). However, few details are known about how ROS regulates autophagy. One model proposed by
Scherz-Shouval and colleagues indicates that ROS directly oxidize the critical cysteine residue near the catalytic site of Atg4B protease and thereby accelerate the production of LC3-2 (106). Other studies show that ROS activate autophagy by inhibiting mTOR activity (181, 182). In addition, the cellular response to increased ROS production often involves the activation of mitogen-activated protein kinases (MAPKs), which are downstream effectors of ROS in autophagy induction (134).

**Erk1/2 signaling pathway**

Several studies have shown that autophagy-related activities, induction of LC3 (183) and conversion of LC3-1 to LC3-II, are modulated by Erk1/2 signaling (114, 133, 134). In addition, the MAPK Erk1/2 signaling pathway also regulates autophagosome maturation (135). In GILT-/- cells, the ROS sensitive MAP Kinase Phosphatase 3 (MKP3) is down-regulated and consequently its target MAP kinase, Erk1/2, exhibits increased phosphorylation. Inhibition of the Erk1/2 pathway with MEK inhibitors (PD98059 or U0126) demonstrated that activation of Erk1/2 increases autophagy and down-regulates SOD2 levels. In our model, the treatments with U0126 showed more significant effects than PD98059 on the autophagy process and SOD2 expression because U0126 is a more potent inhibitor. U0126 targets both MEK1/2, whereas PD98059 only inhibits MEK1 (184). However, it has been shown that PD98059 (2’-amino-3’-methoxyflavone), a flavone derivative that belongs to the class of flavonoids naturally occurring in fruits and vegetables (185), increases cellular cysteine and glutathione levels in a concentration-dependent manner in cells (186, 187). If the addition of PD98059 increases glutathione levels, there is the possibility that the increased SOD2 levels and decreased autophagy are a consequence of the change of glutathione level, not solely inhibition of the
Erk1/2 pathway. In order to address this question, we measured the glutathione level after PD98059 treatment. There was no significant difference in the glutathione levels after the addition of PD98509 in fibroblasts indicating that the effects of PD98059 observed in these experiments were not related to the glutathione levels but inhibition of the Erk1/2 pathway.

**HMGB1**

A study by Wang et al. showed that the Erk1/2 pathway regulates autophagy via regulating beclin 1 (188). A recent study by Tang and colleagues has shown that in response to oxidative stress, high mobility group box 1 (HMGB1) protein is translocated from the nucleus into the cytoplasm and leads to the induction of autophagy (117). HMGB1 functions both in the nucleus as a chromatin-binding factor that enhances transcription and as an extracellular cytokine during cell migration and proliferation, inflammation, and infection (189, 190). Therefore, HMGB1 is thought to be a critical pro-autophagic protein that promotes cell survival and suppresses apoptosis. Cytosolic HMGB1 induces autophagy by the regulation of Bcl-2 phosphorylation through the Erk1/2 pathway to disrupt the interaction between Bcl-2 and beclin 1 and to form an HMGB1-beclin 1 complex (Figure 25) (117, 191). It has been shown that the intra-molecular disulfide bond (Cys23-Cys45) of HMGB1 is critical for the binding to beclin 1 and sustained autophagy (117). Under normal redox conditions, the intra-molecular disulfide bond of HMGB1 is reduced by cellular glutathione and the thioredoxin system, thus preventing the interaction with beclin 1 (192). The oxidative environment in GILT -/- fibroblasts may lead to the accumulation of oxidized HMGB1, thus promoting autophagy.

Because GILT -/- cells showed both increased oxidative stress and elevated phosphorylation of Erk1/2, we tested whether HMGB1 translocation from the nucleus to the
cytoplasm was enhanced and whether this was sensitive to the change of glutathione levels. In our experiment (Figures 26 and 27), enhanced HMGB1 in the cytoplasm correlated with increased oxidative stress and autophagy in GILT -/- fibroblasts, suggesting that HMGB1 is involved in the regulation of autophagy.

**PROPOSED MECHANISMS**

In conclusion, in our study we showed that the absence of GILT causes redox imbalance and increases oxidative stress within the cell, which leads to mitochondrial damage. This is manifest as an altered mitochondrial membrane potential and decreased expression of mitochondrial SOD2. In order to survive such damage GILT -/- cells have increased autophagy in order to remove damaged organelles and/or proteins (Figure 7). We also examined the major signaling pathways and demonstrated increased Erk1/2 phosphorylation in the absence of GILT and the enhanced cytosolic translocation of the pro-autophagic protein HMGB1, which is a prerequisite for the induction of autophagy. However, it remains to be determined how GILT prevents oxidative stress under normal circumstances.

We hypothesize that the absence of GILT alters cysteine metabolism within the lysosomes and thus restricts the availability of cysteine, which is the limiting factor in glutathione biosynthesis (Figure 8B). Interestingly, some of the phenotypes of GILT -/- cells have been seen in the human condition of cystinosis, a metabolic disease characterized by an abnormal lysosomal accumulation of cysteine in many tissues. In untreated patients, disease progression leads to chronic renal failure, hypothyroidism, myopathy, and pancreatic dysfunction, etc. (193).
Cystinosis is caused by mutations in the gene encoding cystinosin, a lysosomal transporter that is responsible for cystine export to the cytoplasm (194). Therefore, cystinotic cells generally exhibit reduced GSH levels and an increased GSSG/GSH ratio (195). In addition, cystinotic cells also exhibit abnormal mitophagy with greater expression of the LC3-2/LC3-1 autophagy marker (196, 197). Gene expression profiles in cystinosis patients were reported recently and showed that several genes, including GILT were differentially expressed between cystinosis patients and healthy controls (198). The expression of GILT gene is up-regulated in cystinotic patients indicating that the presence of GILT may be involved in the process of cysteine oxidation / cystine reduction. Interestingly, GILT -/- mice do not show symptoms similar to cystinosin defective individuals and therefore the GILT defect may cause either a mild effect and/or is compensated for by other as yet unknown mechanisms.

In addition to cystinotic cells, several studies also indicate that some malignant cells have phenotypes similar to GILT -/- cells. Malignant breast cancer cells often exhibit lower expression and activity of SOD2 than their normal cell counterpart (199, 200). The decreased expression of SOD2 leads to the accumulation of superoxide anion, which acts as a second messenger to promote cell proliferation and cancer growth (201). In the early stage of tumorigenesis, the GSSG/GSH redox couple becomes oxidized when cells are induced to differentiate by chemical agents (202). It has been found that the GSSG/GSH ratio measured in the blood of patients with breast and colon cancer is significantly increased compared to controls (34, 203). At advanced stages of tumor development, autophagy is upregulated as a protective mechanism against stressful conditions, such as low-oxygen and nutrient deprivation (204, 205).

Interestingly, GILT is absent or expressed at greatly reduced levels in human melanomas
and glioblastomas (17, 206, 207). Previous research about GILT’s function in cancer was focused on the processing and presentation of tumor antigens (17, 206, 207), as well as the molecular regulation of GILT expression in human melanomas (208). Because of the decreased SOD2 expression, superoxide over-production, increased GSSG/GSH ratio, and enhanced autophagy at different stages of tumor development, it will be interesting to examine the role of GILT in the regulation of growth and aggressiveness in those malignant cells through redox homeostasis.

The importance of our study lies in the fact that a lysosomal thiol reductase has an influence on cellular processes beyond its cellular location. Greater understanding of the function of GILT in redox homeostasis and autophagic regulation may lead to the development of new pharmacological targets for diseases associated with oxidative stress.
REFERENCE


