

REDOX HOMEOSTASIS AND STRESS IN MOUSE LIVERS
LACKING THE NADPH-DEPENDENT
DISULFIDE REDUCTASE SYSTEMS

by

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DEDICATION

I would like to dedicate this thesis to my dear friends Adrienne Arnold, Jacob Artz, Aoife Casey, Michael Christman, Phil Hartman, Ky Mickelsen, Sarah Partovi, Greg Prussia and Danica Walsh. Your support has been indescribably generous and completely invaluable. My quality of life, especially during graduate school, has been immeasurably improved by you all and I count myself incredibly lucky to call you my friends and colleagues.

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TABLE OF CONTENTS

1. INTRODUCTION PART ONE: NADPH-DEPENDENT AND –INDEPENDENT DISULFIDE REDUCASE SYSTEMS	1
Contribution of Authors and Co-Authors	1
Manuscript Information	2
1. Redox reactions and reducing power	4
2. NADPH: A “universal currency” for anabolic reduction	4
3. Early evolution of biological disulfide reduction	7
4. Disulfide reductase-driven reactions fuel DNA precursor biosynthesis.....	11
5. NADPH-dependent disulfide reductases	12
6. NADPH-independent disulfide reductase systems capable of sustaining DNA replication in microbes or plants.....	15
7. Sources of disulfide reducing power in eukaryotic organelles	19
8. The identification of a constitutive NADPH-independent cytosolic disulfide reductase system in mammals.....	21
9. Collateral impacts of the mammalian NADPH-independent disulfide reductase system	26
10. Is the Met-driven cytosolic disulfide reductase system truly NADPH-independent?	27
11. Evolution of the Met-dependent disulfide reductase system	28
12. Flux-direction in the transsulfuration pathway	30
13. Similar phylogenetic distributions of TrxR-“type” and transsulfuration-“direction”	35
14. Roles of the metazoan TrxR1 system in signaling.....	36
15. Closing perspectives: Balancing antioxidant activities and redox signaling	38
Chapter 1 References:	41
2. INTRODUCTION PART TWO: DISULFIDE REDUCTASE SYSTEMS IN THE LIVER.....	58
Contribution of Authors and Co-Authors	58
Manuscript Information	59
Abstract	61
The redox biology of sulfur and the need for disulfide reductase systems.....	62
The NADPH-dependent disulfide reductase systems	65
Disulfide/dithiol redox in the cytosol and nucleus	67
Disulfide/dithiol redox in mitochondria.....	70
Disulfide/dithiol redox in the endoplasmic reticulum	72

TABLE OF CONTENTS CONTINUED

Genetic disruptions of the cytosolic NADPH-dependent disulfide reductase systems in mice	74
NADPH-independent disulfide reduction in the liver	76
Cross-trafficking of disulfide reducing power between cytosolic systems	77
Redox perspective on liver physiology	78
Conclusions and perspectives	86
Chapter 2 References:	87
 3. HEPATOCYTE HYPERPROLIFERATION UPON LIVER -SPECIFIC CO-DISRUPTION OF THIOREDOXIN-1, THIOREDOXIN REDUCTASE-1, AND GLUTATHIONE REDUCTASE	 95
Contribution of Authors and Co-Authors	95
Manuscript Information	98
Summary	100
Introduction	100
Results	103
Development and Survival of Mice with Trx1-, Trx1/TrxR1-, Trx1/Gsr and Trx1/TrxR1/Gsr-null livers	103
Redistribution of Cytosolic Disulfide-Reducing Power	109
Oxidative Stress and Xenobiotic Tolerance in Disulfide Reductase-Deficient Livers	112
Hyperproliferation in Hepatocytes Having Disulfide Reductase System Deficiencies	119
Discussion	121
Experimental Procedures	125
Mice, Surgeries, and Harvests	125
Immunoblotting and Enzymatic Assays	125
Primary Fibroblast Systems	126
Statistical Analyses and Resource Sharing	126
References	128
 4. TRXR1, GSR, AND OXIDATIVE STRESS DETERMINE HEPATOCELLULAR CARCINOMA MALIGNANCY	 132
Contribution of Authors and Co-Authors	132
Manuscript Information	135
Significance	137
Abstract	137
Introduction	138
Results	141

TABLE OF CONTENTS CONTINUED

TrxR1/Gsr-Null Livers Have Chronically Elevated DNA Damage Markers Yet Rarely Develop Spontaneous Cancer	141
TrxR1/Gsr-Null Livers Are Susceptible to Chemically Induced Cancer	142
Cancer Resistance in TrxR1-Null Livers Correlates with Altered Metabolite Profiles	144
WT and TrxR1-Null Liver Tumors Progress Similarly Under Normal Care, but TrxR1-Null Tumors Are More Aggressive Under Chronic Stress	145
Nrf2 Activity in TrxR1-, Gsr-, and TrxR1/Gsr-Null Livers	149
Phenotypic Heterogeneity in DEN-Induced Liver Tumors	153
Discussion	154
ROS and Cytosolic Disulfide Reductase Systems in Liver Carcinogenesis	154
Cytosolic Disulfide Reductase Systems in Liver Cancer Persistence or Progression	157
Interplay of Cytosolic Disulfide Reductase Systems and Nrf2 in Liver Cancer	159
Materials and Methods	161
Mice	161
Transcriptome Analysis, qRT-PCR, Histology, and Immunohistochemistry	161
Statistical Analyses	162
Acknowledgements	162
References	164
 5. SUPPLEMENTAL ASCORBATE COMPROMISES HEPATOCYTE SURVIVAL AND INCREASES RISK OF ACUTE LIVER FAILURE DURING SEVERE OXIDATIVE STRESS	169
Contribution of Authors and Co-Authors	169
Manuscript Information	170
Abstract	172
Introduction	172
Materials and Methods	178
Mice, Supplementations, and Harvests	178
Total Glutathione	179
Statistical Analyses	179
Results	180
Ascorbate Supplementation Exacerbates Acute Liver Failure Frequencies in Mice with TrxR1/Gsr-null Livers	180

TABLE OF CONTENTS CONTINUED

Ascorbate Causes Severe Histopathology and Hepatocyte Necrosis in TrxR1/Gsr-null Livers	181
Ascorbate Treatment Modestly Depletes GSH in TrxR1/Gsr- But Not in Gsr-null Livers	183
DHA Treatment Strongly Depletes GSH in Both Gsr- and TrxR1/Gsr-null Livers	185
Discussion	187
References	191
 6. L-METHIONINE AND L-CYSTINE WITH STABLE S ISOTOPE, ³⁴ S, USED TO DETECT AND FOLLOW S-METABOLITES	196
Introduction	196
Results	203
Materials and Methods	207
Materials	207
CysK Expression and Purification	208
Synthetic Procedures.....	208
L-(³⁴ S)Met	208
Na ₂ ³⁴ S	209
O-acetyl-L-serine.....	210
L-(³⁴ S)cystine	210
Cell Culture	211
Discussion	211
References	215
 7. CLOSING REMARKS.....	218
 8. REFERENCES CITED.....	221
 APPENDIX A: Spectra.....	247
L-(³⁴ S)Met ¹³ C-NMR	248
L-(³⁴ S)Met HRMS	249
O-acetyl-L-serine ¹ H-NMR.....	250
O-acetyl-L-serine ¹³ C-NMR.....	251
O-acetyl-L-serine HRMS	252
L-(³⁴ S)cystine ¹ H-NMR.....	253
L-(³⁴ S)cystine ¹³ C-NMR.....	254

LIST OF TABLES

Table	Page
3.1 Serum Markers of Hepatic Health and Function	110
4.1 Spontaneous tumor frequencies in mice after 9-14 mo under standard care.....	145

LIST OF FIGURES

Figure	Page
1.1 Chemical structure and activity of the [NADPH; NADP ⁺] redox couple	7
1.2 Oxidation states of common inorganic and organic S-compounds	10
1.3 Alternative pathways of <i>E. coli</i> disulfide reduction.....	19
1.4 Met metabolism and transsulfuration pathway activities.....	21
1.5 Cellular metabolism of sulfur	26
1.6 Phylogeny of TrxR and Gsr enzyme families, and of Met auxotrophy	35
2.1 Oxidative modifications of S amino acids	63
2.2 Disulfide reductases of the eukaryotic cell	70
2.3 Prxs in catalysis, over-oxidation and signalling	83
2.4 Hepatic transsulfuration and H ₂ S generation.....	85
3.1 Development of Mice with Liver-Specific Co-disruptions of Trx1, TrxR1, and Gsr	107
3.2 Redistribution of Reducing Power among Cytosolic Disulfide-Reducing Systems in Primary Fibroblasts.....	111
3.3 Accumulation of Oxidative Damage in Resting Adult Liver.....	114
3.4 Hepatocytic Mitochondrial Ultrastructure	116
3.5 Impacts of Acute Oxidation or Hepatotoxic Stress.....	118
3.6 Hepatocyte Morphometry and Proliferation	120
4.1 TrxR1/Gsr-null livers have high basal levels of DNA damage	142

LIST OF FIGURES CONTINUED

Figure	Page
4.2 Den induces cancer in TrxR1/Gsr-null livers	143
4.3 Marked-mosaic analysis of cancer progression by WT vs. TrxR1-null Hepatocytes	147
4.4 Chronic post-DEN initiation PB stress promotes liver cancer in TrxR1-null livers	149
4.5 The Nrf2 response predominates in the transcriptome of TrxR1/Gsr-null livers compared to WT livers	151
4.6 Nrf2 activity in WT, TrxR1-null, and TrxR1/Gsr-null livers.....	153
4.7 Nrf2 activity in tumors.....	154
4.8 Roles of the endogenous antioxidant network in cancer initiation and progression.....	160
5.1 Asc lowers survival of mice with TrxR1/Gsr-null livers during crisis period	181
5.2 H&E stained histology of Gsr-null and TrxR1/Gsr-null livers treated daily with Asc	183
5.3 Hepatic levels of total glutathione in mice given IP Asc.....	185
5.4 Hepatic levels of total glutathione in mice given IP DHA	186
6.1 Energy and amino acid requirements of Met-driven H ₂ O ₂ reduction	198
6.2 S-efficient oxidation pathways of Cys	201
6.3 Synthesis and characterization of L-(³⁴ S)Met.....	202
6.4 Identifying metabolites of L-(³⁴ S)Met -treated HEK-293 cells	204
6.5 Synthesis and characterization of L-(³⁴ S)cystine.....	207

LIST OF FIGURES CONTINUED

Figure	Page
A1. L-(³⁴ S)Met ¹³ C-NMR	248
A2. L-(³⁴ S)Met HRMS.....	249
A3. O-acetyl-L-serine ¹ H-NMR.....	250
A4. O-acetyl-L-serine ¹³ C-NMR	251
A5. O-acetyl-L-serine HRMS	252
A6. L-(³⁴ S)cystine ¹ H-NMR	253
A7. L-(³⁴ S)cystine ¹³ C-NMR	254

CHAPTER ONE

INTRODUCTION – NADPH-DEPENDENT AND –INDEPENDENT DISULFIDE REDUCTASE SYSTEMS

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CHAPTER ONE – INTRODUCTION – NADPH-DEPENDENT AND –
INDEPENDENT DISULFIDE REDUCTASE SYSTEMS

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1. Redox reactions and reducing power

Reduction and oxidation (redox) reactions involve the transfer of electrons between molecules. Reducing agents (reductants) carry electrons that can be transferred to a more oxidized substrate. Oxidizing agents (oxidants) are electrophilic and will readily accept electrons from a more reduced substrate. All reduction reactions are also oxidation reactions, and vice versa, in that the reduction (gaining of electrons) of one compound requires the oxidation (loss of electrons) of another. Thus, the concept of “reducing power” can be established to describe the propensity of one compound to donate its electrons to another compound, and has an associated value, expressed in volts, that describes how readily those electrons will be donated, or their “redox potential.” Electrons are often transferred as pairs (2-electron transfers), but single electron transfers also occur in biology. Molecules with unpaired electrons are free radicals, which are typically unstable and highly reactive. Some free radicals are biologically relevant as damaging agents. Others are essential for biological functions, in particular as critical intermediates within certain enzymatic reactions. There is an extensive body of knowledge regarding the electron transfer mechanisms within enzymatic reactions, including single- and paired-electron transfers; however coverage of this literature is beyond the scope of this review.

2. NADPH: A “universal currency” for anabolic reduction reactions

Inorganic carbon, for example within CO_2 , is usually in an oxidized state, whereas the carbon in organic molecules is highly reduced. Organic molecules are generated via

the reduction of oxidized inorganic carbon sources by autotrophs, which include both photosynthetic and chemolithotrophic organisms. To generate organic molecules, autotrophs use energy from photons of light or from oxidation of inorganic minerals to obtain electrons, and these are used to chemically reduce the CO₂ by forming carbon-carbon or other bonds. The bonds of organic molecules store most of Life's energy, which is released under hetero-trophic conditions by oxidation (i.e., extraction of the electrons) of these bonds. Most of the energy that drives Life's processes likely entered the biological world via photosynthesis. In the light-dependent reactions of photosynthesis, energy from photons is used to extract electrons from a low energy source, usually water. Typically four photons are used to cleave two molecules of water, yielding one molecule of molecular oxygen (O₂), four protons (H⁺), and four electrons of reducing power. A small amount of the energy from this process is also often used to generate three phosphodiester bonds within adenosine triphosphate (ATP). The four electrons are used to reduce two molecules of oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺) into their reduced form (NADPH; Fig. 1), thereby capturing the reducing power (Eq. (1)).



Interestingly, chemolithotrophs also capture reducing power, albeit obtained from oxidation of inorganic compounds rather than from photons of light, as NADPH. Thus, NADPH is the organic compound to initially receive the reducing power obtained from inorganic sources in all autotrophs. In the light-independent (“dark”) reactions of photosynthesis (the Calvin cycle, which is also used in chemolithotrophs), the electrons from

NADPH are used to reductively “fix” CO₂ into sugar. This more stably stores the reducing power as reduced carbon, pre-dominantly within carbon-carbon bonds [1–3]. Although some NADPH-driven reductions are catabolic, more generally NADPH is the electron donor for anabolic reduction reactions and, accordingly, hundreds of reductive enzymes (reductases) are known to be NADPH-dependent. As such, NADPH can be considered a “universal currency” for anabolic reduction reactions. NADPH cannot be acquired from extracellular sources but, rather, must be generated by the reduction of NADP⁺ within each cell. To generate NADPH in heterotrophic conditions, the reducing power that was stored in the bonds of organic molecules during the Calvin cycle can be used by a few reactions to reduce $\text{NADP}^+ + \text{H}^+ + 2\text{e}^- \rightarrow \text{NADPH}$. In mammals, oxidations of several organic molecules can fuel NADPH-generating reactions. These include oxidation of glucose in the pentose phosphate pathway, oxidation of isocitrate by isocitrate dehydrogenase, oxidation of malate by malic enzyme, and oxidation of methylenetetrahydrofolate [4–8]. The [NADPH/NADP⁺] redox couple is one of two predominant nicotinamide adenine dinucleotide systems in cells, the other being [NADH/NAD⁺]. The [NADH/NAD⁺] redox couple is used primarily for biological oxidations, such as for extracting energy from organic molecules, and therefore its steady-state equilibrium in living cells favors the oxidized NAD⁺ over NADH. Conversely the reduced NADPH is about ten-times more abundant than the oxidized NADP⁺, and NADPH is used predominantly for reduction reactions, such as within anabolic processes, as described above, or for antioxidant activities. In some situations, reducing power can be transferred between NADH and NADPH by nicotinamide

nucleotide transhydrogenases (NNT) [9, 10]. In mitochondria, wherein NADH is abundant, NNT-derived NADPH is estimated to account for half of the NADPH, and NNT-knockdown alters mitochondrial redox status [11].

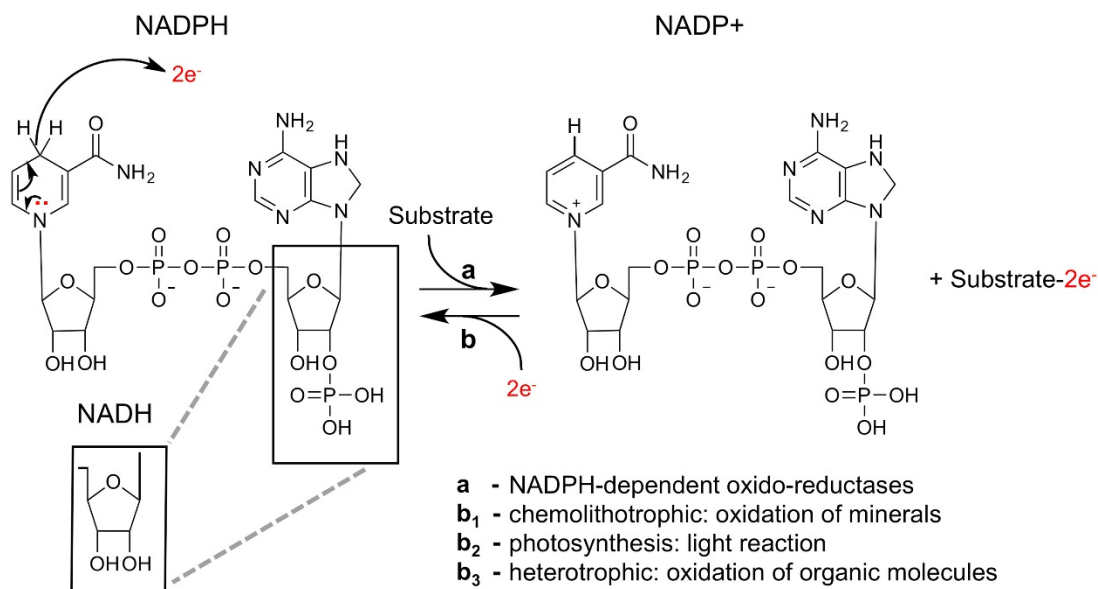


Fig. 1.1. Chemical structure and activity of the [NADPH;NADP⁺] redox couple. The redox-active electrons are denoted in red font by the symbol e^- . The boxed inset shows the structural difference between the NADP- and the NAD-family of nicotinamide cofactors.

3. Early evolution of biological disulfide reduction

Life arose in an anoxic environment and likely relied heavily on hydrogen sulfide and ferrous iron (H_2S/Fe^{2+})-based redox reactions [12, 13]. Before photosynthetic organisms arose that could harvest energy from light, all autotrophic life was chemolithotrophic, meaning that nutrients and energy were acquired from chemical reactions involving minerals. An abundant and therefore likely source of chemolithotrophic potential for supporting early life is associated with sulfur (S)-rich

hydrothermal vents, which expel large amounts of both H_2S and Fe^{2+} in an environment suitable for sustaining life (pH 3–4 and temperature/pressure conditions in which water is liquid)[14,15]. The abundant iron-sulfur (FeS) compounds found in such environments are capable of (i) catalyzing other redox reactions (e.g., N_2 to NH_3 or NO_3^-) [16]; (ii) forming planar sheets that likely became the first barriers encapsulating the biological machinery from the external environment as a primitive cell; and (iii) assembling into FeS clusters, which still serve as electron conduits in contemporary biological redox reactions. Other metal-S minerals, like ZnS and (FeNi) S, can activate C-H bonds, and thereby abiotically catalyze the formation of C-X bonds ($\text{X} = \text{C}, \text{O}, \text{or N}$) to generate simple organic molecules [17–19]. These inorganic redox reactions and simple organic molecules were likely critical to the onset of life. Because H_2S and Fe^{2+} are abundant in hydrothermal environments, primitive organisms likely extracted two electrons of reducing power from oxidation of H_2S , which then coupled with Fe^{2+} , yielding pyrite(FeS_2) [20]. However, the capacity for more efficient extraction of reducing power existed and was eventually exploited, since complete oxidation of H_2S to sulfate (SO_4^{2-}) yields eight electrons (Fig. 2). Later organisms would use intracellular reducing power, stored as organic carbon sources and transferred via NADPH, to enzymatically reduce environmental sulfate into sulfite by adenylyl-sulfate (APS) reductase and phosphoadenylyl-sulfate (PAPS) reductase. Further reduction to sulfide through three consecutive 2-electron transfers by ferredoxins would then support the biological production of sulfide. This allowed organisms to radiate out from restricted sulfide-rich hydrothermal vent ecosystems into new environments and expanded niches [21–

23]. Photosynthetic autotrophs first appeared about 2.3 billion years ago. H₂S likely also played a critical role in the evolution of photo-synthetic organisms from chemolithotrophic predecessors. Because of its high abundance in certain environments and its being similar to H₂O, yet with more easily extracted electrons (oxidation potential $-0.14 \text{ E}^\circ(\text{V})$ for H₂S as compared to $-1.78 \text{ E}^\circ(\text{V})$ for H₂O), H₂S would have provided an accessible electron source for early chlorophylls that were unable to oxidize H₂O [24, 25]. The subsequent evolution of more efficient photosystems would have led to more powerful photo reactions that could extract electrons from H₂O, and this advance would lead, eventually, to “the great oxygenation event”. Herein, the production of O₂ as a byproduct of the photolysis of water (Eq. (1)) resulted eventually in a ~105-fold increase in the concentration of atmospheric oxygen on earth [26]. This new and generally ubiquitous abundance of molecular oxygen allowed the subsequent evolution of respiration, wherein O₂ serves as the final electron acceptor for biological reductions, yielding H₂O. The abundance of O₂ in the atmosphere peaked 200 million years ago at ~33%. Subsequently the equilibrium between photosynthetic production and respiratory consumption of O₂ in the biosphere brought atmospheric O₂ levels to ~20%, which has shown only modest fluctuations thereafter [27, 28]. The great oxygenation event, coupled with biogenesis of reactive oxygen species (ROS) as a byproduct of respiration, marked the onset of biological oxidative stress. It has been speculated that early antioxidant mechanisms were only slight alterations of previous S-based redox systems that existed to manage oxidative S species [25]. Present day organisms still utilize S for diverse redox reactions. In particular the S amino acid cysteine (Cys) is ubiquitously used in the context

of oligo-peptides like glutathione (GSH; γ -glutamyl-Cys-glycine) or small proteins like thioredoxin (Trx) to generate [dithiol/disulfide] redox couples for transmission of intracellular reducing power. It should however be noted that Cys residues are used for a wide range of functions – catalytic, regulatory, structural, and others—whereas the related amino acid selenocysteine (Sec, further discussed below) seems to be uniquely dedicated to the catalysis of redox reactions [29–32].

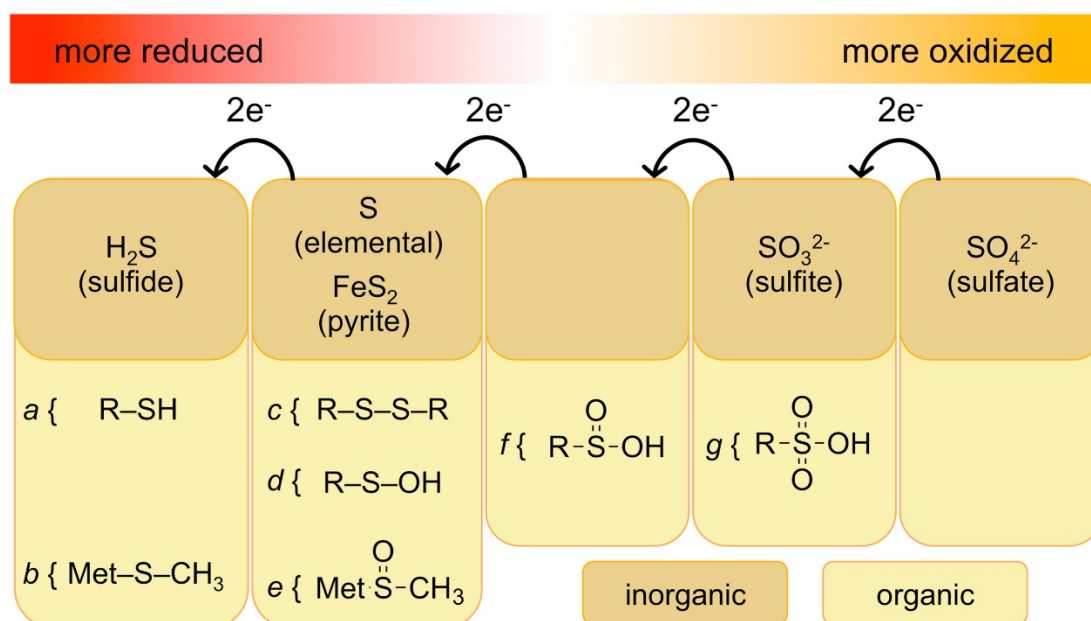


Fig. 1.2. Oxidation states of common inorganic and organic S-compounds. For simplicity, only species discussed in this review are included. Organic compounds are as follows: a, thiols, such as found in reduced proteins, Cys, and GSH; b, thioether within Met; c, disulfide, such as found in oxidized protein-disulfides or GSSG; d, sulfenic acid; e, sulfoxide within oxidized Met; f, sulfinic acid; g, sulfonic acid.

4. Disulfide reductase-driven reactions fuel DNA precursor biosynthesis

One important event in the early evolution of life was adoption of the DNA genome. Several factors are thought to have spurred the transition from an RNA- to a DNA-genome. DNA is more stable in an aqueous environment, is more resistant to damage by transition metals, and is less susceptible to damaging photoreactions than is RNA [33]. Also, cytosine (C) easily deaminates to uridine (U). In an RNA genome, this would lead to a uridine-guanosine (U-G) base-pair, which would be strand-ambiguous for repair, since either the U on one strand could be repaired to a C or the G on the other strand could be repaired to an adenosine (A). In a DNA genome, C-deamination also yields a U-G base pair, however since U is unnatural in DNA, and instead is replaced by thymidine (T), the U can be unambiguously recognized as a deaminated C and correctly repaired [34]. Thus, an RNA genome might be more prone to information degradation than is a DNA genome. For DNA synthesis, ribonucleotides have to be reduced to deoxyribonucleotides. This critical reduction reaction is catalyzed by the enzyme ribonucleotide reductase (RNR). Although three classes of RNR with various known permutations have been described, the enzyme likely arose only once, as all known RNR enzymes appear to share a common ancestor [35]. The complex chemistry of ribonucleotide reduction involves a transition state having an enzyme-associated free radical to drive the reaction. In most RNRs, this is a thiyl radical generated on a Cys residue within the active site of RNR [36–38]. With few exceptions (see below), generation of the radical intermediate, and therefore the reduction of ribonucleosides, is driven by electrons provided through a dithiol-disulfide exchange. This exchange, in turn, is fueled by an initial disulfide reduction reaction in which the electrons are derived from

NADPH and used to generate a dithiol. For more details about the enzymatic mechanisms and evolutionary relationships of RNRs, the reader is directed to excellent reviews specifically focused on these topics [35, 39–41].

5. NADPH-dependent disulfide reductases

Although there are hundreds of different NADPH-dependent enzymes in biology, each of which has the ability to use electrons extracted from NADPH to reduce specific organic or inorganic substrates, only two small groups of these enzymes, the Trx-disulfide reductases (TrxRs) and the glutathione-disulfide (GSSG)-reductases (Gsr), can reduce an intracellular disulfide [42]. In TrxRs, electrons from NADPH are used to reduce an active site disulfide in oxidized Trx (Trx-disulfide), yielding NADP^+ and a reduced Trx-dithiol [30, 36, 39]. In Gsr, electrons from NADPH are used to reduce GSSG, yielding NADP^+ and 2GSH [43]. The reduced Trx or GSH then distribute this NADPH-derived reducing power by their transmission of these electrons to other molecules, mainly via dithiol-disulfide exchange reactions. In all known living systems under nearly all conditions, reduction of RNR is fueled by either a TrxR system, a Gsr system, or both [44–47]. Beyond providing reducing power for production of DNA precursors by RNR, the NADPH-dependent TrxR- and Gsr-driven systems in most organisms fuel reductive “antioxidant” enzymes. These activities are used to sustain a moderately reducing status of most molecules in the cytosol, manage ROS, prevent oxidative stress, repair oxidative damage, and regulate signaling (see below). These activities occur both through direct protein-disulfide reductions and via the transfer of

this reducing power to Trx-dependent peroxiredoxins and methionine sulfoxide reductases [48, 49], or to GSH-dependent glutathione peroxidases (Gpx) [43].

Gsrs across phyla share a common ancestor, suggesting that this enzyme arose only once [50]. By contrast, two different non-homologous families of TrxRs are known. One family is found in archaea, eubacteria, protists, fungi, and plants, but is generally absent from metazoans (multicellular-animals). The second family of TrxRs is found in metazoans and, interestingly, is homologous to Gsr [48, 51, 52]. This indicates that the transition from single-celled heterotrophic protozoa to multicellular heterotrophic metazoans was associated with duplication and specialization of the Gsr gene to generate a new TrxR gene [53, 54], and in most cases, loss of the ancestral TrxR gene. In one exception, the single-celled green alga *Chlamydomonas* has both the “bacterial” and the “metazoan” form of TrxR [55]. The evolution of metazoan TrxRs from Gsr involved acquisition of a carboxy (C)-terminal extension to the enzyme that encodes the active site responsible for reducing Trx-disulfides [48, 51, 54]. Interestingly, in most metazoan TrxRs, this C-terminal active site contains a Cys-selenocysteine (Cys-Sec) pair rather than Cys-Cys pair [51]. Sec is typically considered a “hyperactive” version of Cys, as it is structurally the same as Cys, but has the S replaced by the chemically similar, yet more reactive, selenium (Se). Sec is a rare amino acid, being found in only 25 human proteins, and in nearly all situations it is found in the active site of a redox-active enzyme [56, 57]. However, the evolutionary history of Sec and the roles Se might have played in the early evolution of life are unclear. Sec is not used in all metazoan TrxRs or even in all metazoans. Selenoproteins are found in species from all three kingdoms, although this

distribution is scattered and varies widely between kingdoms [58–60]. Importantly, the entire Trx system in mammals is critically dependent on nutritional selenium status, since TrxRs are selenoproteins. In the GSH system several Gpx isoenzymes are selenoproteins. Among these, Gpx4 was recently shown to have critical cell survival functions when other selenoproteins were deleted [61]. The reader is directed to reviews on TrxR proteins and their evolution for more details on this [48, 49, 53, 62] and to other articles in this issue for more details on the roles of Se and Sec in living systems. Importantly for this review, Gsr and both families of TrxRs are NADPH-dependent; however the phylogenetic distribution of the metazoan TrxR appears to be similar to the predicted phylogenetic distribution of an NADPH-in-dependent disulfide reductase system (see below). In some organisms, the disulfide reductase-dependent redox systems have diverged away from the ancestral TrxR- and Gsr-driven systems. For instance, several microbes synthesize modified versions of GSH or have even replaced GSH with distinct but redox-similar analogs [63]. These include the small molecules bacillithiol, found in gram-positive bacteria of the genus *Bacillus* and comprised of L-cysteine-D-glucosamine and malic acid [64–66], or mycothiol, found in mycoplasma and other actinomycetes bacteria, and consisting of N-acetyl-L-cysteine linked to a glucosamine and inositol [67, 68]. Coenzyme A (CoA), a ubiquitous cellular cofactor associated most often with the tricarboxylic acid (TCA) cycle and fatty acid metabolism, serves as the primary low molecular weight thiol for transmission of disulfide reducing power in *Staphylococcus aureus* [69, 70]. Similarly, trypanothione, an important redox regulator in protozoan parasites, is a small molecule comprised of two GSH residues connected by a

spermidine linkage [71–73]. In each case, these thiol-containing molecules form redox couples that participate in dithiol-disulfide exchange reactions akin to those performed by the GSH or Trx systems in other organisms, and they are reduced by NADPH-dependent enzymes that are homologous to Gsr or TrxR, [64, 67, 69, 70]. Because many of these redox systems are diverged from GSH or Trx, yet are found in pathogens and essential to virulence, they are of interest as possible drug targets. For more detailed information on the specialized derivatives of the NADPH-dependent disulfide reductase systems, the reader is directed to other excellent reviews [74–79]

6. NADPH-independent disulfide reductase systems capable of sustaining DNA replication in microbes or plants

Trx was originally discovered as the electron-donor for RNR in *E. coli*, which subsequently led to the identification of TrxR as the NADPH-dependent reductase that recycled Trx-disulfide back to Trx-dithiol to support this reaction [47, 80]. However, *E. coli* mutants having disruptions in the TrxR system remained viable, which led to the discovery of glutaredoxins (Grxs; [45, 81]). All organisms use either the NADPH-dependent TrxR- or Gsr-driven systems, or both, to provide reducing power to RNR [35]. Moreover, *E. coli* coincidentally lacking both TrxR and Gsr (TrxR/Gsr-null) cannot support replication, although spontaneous TrxR/Gsr-null suppressor mutants are readily isolated (Fig. 3A), [82–84]. Notwithstanding, the requirement for additional genetic lesions to allow survival of TrxR/Gsr-null *E. coli* indicates that they can mutagenically acquire, but do not constitutively possess, an alternative disulfide reductase system. On the other hand, it also shows that the capacity for combinatorial TrxR- and Gsr-

independent disulfide reduction is easily programmed onto the existing machinery of bacteria, and is almost certainly used naturally in some microbes. TrxR/Gsr-null *E. coli* suppressor mutants were found to carry an expanded triplet repeat in the gene encoding alkyl hydroperoxide reductase C (AhpC), which encodes a Trx- and GSH-independent peroxidase (Fig. 3B;[82–84]). Wild type AhpC-dithiol reduces alkyl hydro-peroxides and related substrates, and in the process generates an active site disulfide. AhpC-disulfide is subsequently reduced by AhpF, a close homologue of bacterial TrxR that can use either NADPH or NADH as a source of reducing power. In the suppressor mutants, reduced Trx is not required for survival, but both GSH and Grx1 are. The mutant AhpC was found to have an altered substrate specificity, and can generate GSH through the disulfide reduction of glutathionylated-Grx1 (Grx1-SSG), an oxidized form of Grx1 [84]. This disulfide reductase system, while bypassing the need for either TrxR or Gsr, and while also being able to use NADH as a source of disulfide reducing power, does not likely generate substantial amounts of NADPH-independent disulfide reducing power. In living cells, the [NADH/NAD⁺] redox couple is typically maintained in an oxidized state whereas the [NADPH/NADP⁺] redox couple is kept in a reduced state (see above). Thus, even though AhpF can obtain electrons from NADH, based on the relatively low availability of NADH in cells, the suppressor mutant of AhpC is likely fueled predominantly by NADPH. After further complete disruption of the AhpC gene, generating TrxR/Gsr/AhpC-triple-null *E. coli*, additional suppressor mutants were isolated (Fig. 3C). These clones were found to carry a disrupting mutation in the gene encoding lipoic acid dehydrogenase-A (LpdA), an accessory protein of the TCA cycle

[85]. Lipoic acid (LA) is an important 2-S-containing cofactor for several enzymes in the TCA cycle. LA can exist in either a reduced dithiol (dihydrolipoamide) or an oxidized disulfide state. Normal bioenergetic oxidation of substrates in the TCA cycle generates reduced dihydrolipoamide cofactors, whose oxidation by LpdA is coupled to reduction of NAD^+ to NADH. The NADH is then used for production of ATP, thereby generating expendable energy and regenerating the oxidized NAD^+ . In the suppressor mutants, LpdA is compromised. As the TCA cycle oxidations drive the reduction of LA, the cycle stalls with a high reducing potential within the components of the TCA cycle and accumulation of dihydrolipoamide. At the same time, the absence of Gsr and TrxR in these cells results in an oxidized cytosol in which Grx1-disulfide or Grx-SSG accumulates. Under these conditions, the reduced LA can directly reduce the oxidized Grx, which in turn can support RNR activity [85]. This suppressor mutant therefore uses a bonafide NADPH-independent disulfide reductase system to drive RNR. To our knowledge, however, no naturally occurring organisms have been described as yet that use such a system constitutively. Moreover, since eukaryotes segregate the TCA cycle within the mitochondria while RNR is exclusively cytosolic [86], it is unlikely that the eukaryotic TCA cycle could be similarly modified to directly support DNA precursor production. *E. coli* have one other known TrxR- and Gsr-independent system of supporting RNR activity; however rather than using NADPH-independent disulfide reduction, this system might obviate the need for disulfide reduction. Under anaerobic conditions, *E. coli* express a type III RNR that can use either reducing power supplied by Trx or Grx, or can directly use the oxidation of formate, to generate a thiyl radical in the

RNR active site and thereby drive the reduction of ribonucleotides [87, 88]. Although the formate-driven reaction does not use Trx or Grx and is independent of NADPH, TrxR, and Gsr, it also might not involve reduction of a disulfide bond. Therefore, this might not constitute a bonafide NADPH-independent disulfide reductase system. Yeast also require either a functional TrxR1 or functional Gsr system for survival. Thus, TrxR1/Gsr-null yeast are non-viable [89, 90] as are colonies coincidentally lacking Gsr and both thioredoxins (Trx1 and Trx2) [91]. Indeed, even yeast only lacking Trx1 are severely compromised in their ability to synthesize DNA during S phase, resulting in growth arrest [92, 93]. Some algae and plants have an NADPH-independent thioredoxin reductase that, instead, obtains reducing power directly from the electron transport chain of the photosynthesis light-reaction. For this, a reduced ferredoxin within the electron transport chain can transfer reducing power directly to ferredoxin-thioredoxin reductase (FTR). FTR reduces Trx-disulfide; however it is unrelated to either TrxRs or Gsrs, and it uses a distinct mechanism involving an FeS-cluster [94–96]. This is, therefore, a bonafide NADPH-independent disulfide reductase; however its activity is restricted to photosystem activity and it is un-clear whether it supports DNA replication.

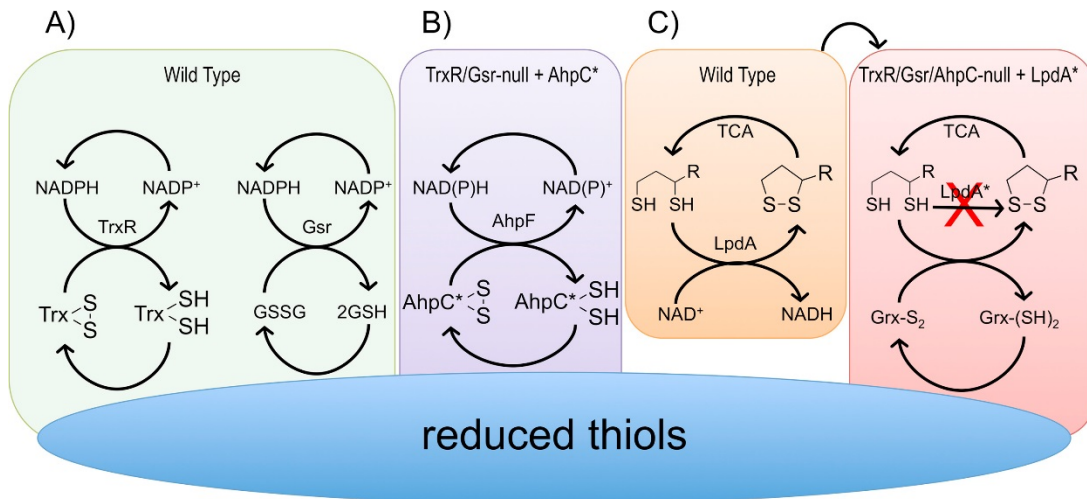


Fig. 1.3. Alternative pathways of *E. coli* disulfide reduction. A, constitutive disulfide reduction by NADPH-dependent TrxR- or Gsr-driven systems. B, in TrxR/Gsr-null *E. coli*, a suppressor mutation in the AhpC gene (AhpC*) results in the enzyme being able to use reducing power from AhpF to reduce Grx1-SSG. AhpF can use either NADPH or NADH as electron donors, so this provides a possible source of NADHP-independent disulfide reducing power. C, left, normal TCA cycle drives reduction of LA-disulfide cofactors to the dihydrolipoate state and LpdA uses this reducing power to drive reduction of $\text{NAD}^+ \rightarrow \text{NADH}$. The NADH is used for bioenergetics and does not contribute to disulfide reduction. Right, in triple-null TrxR/Gsr/AhpC mutants, suppressor mutations (LpdA*) compromise LpdA activity. Accumulated reducing power on the dihydrolipoate cofactors is then transferred to oxidized Grx-disulfide, generating Grx-dithiol. This regenerates the LA-disulfide, thereby restoring TCA cycle activity, and allows Grx to provide reducing power to RNR. This system is fully NADPH-independent.

7. Sources of disulfide reducing power in eukaryotic organelles

Eukaryotic evolution of organelles, including nuclei, mitochondria, endoplasmic reticula, and plastids, each with differing restrictions on molecular exchanges with the cytosol, required development of specialized subcellular disulfide reductase machineries. GSH is synthesized exclusively in the cytosol but is found in most organelles as a result of either diffusion or regulated transport [97, 98]. Mitochondria, which import GSH yet have no disulfide-dithiol exchange with the cytosol [99], have their own dedicated Trx

system (TrxR2, Trx2, and Prx3) and GSH system (Gsr, Grx2, 3, and 5, and Gpx1, 3, and 4). A similar situation is found for plastids in plants [100]. Cytosolic and mitochondrial Gsr are encoded by alternative mRNA splice-isoforms from the same gene [101], whereas the other mitochondrial components each have unique genes. The endoplasmic reticulum also has tight restriction on exchange with the cytosol and it, uniquely, maintains an oxidizing redox status to support protein folding and disulfide formation. The ER does not have either a TrxR or Gsr. Rather, it obtains the small amount of disulfide reducing power it needs from the free thiols that translocate into the ER in the context of Cys residues on nascent proteins and by import or perhaps exchange of GSH [97, 102]. Nuclei have freer ex-change of small molecules with the cytosol; however TrxR1 and Gsr do not localize to the nucleus. Nonetheless, the nucleus has at least one dedicated Trx-family member, nucleoredoxin [103], which can regulate the activity of some transcription factors. Also, during oxidative stress both Trx1 and Grx1 transit to the nucleus [104, 105]. It is unclear how redoxins within the nucleus obtain their reducing power from cytosolic TrxR1 or Gsr, but most likely this will be by a dithiol-disulfide shuttle involving GSH-GSSG and perhaps the redoxins, themselves. Organellar compartmentalization restricts the possibilities for having NADPH-independent disulfide reductase pathways in eukaryotes. For instance, the eukaryotic TCA cycle is confined to the mitochondria whereas RNR is strictly cytosolic [106]. As a result, the NADPH-independent pathway for generating reduced Grx that arose in TrxR/Gsr/AhpC-null/LpdA-mutant *E. coli* and supported RNR activity (see above, Fig. 3c) could not support RNR in eukaryotes.

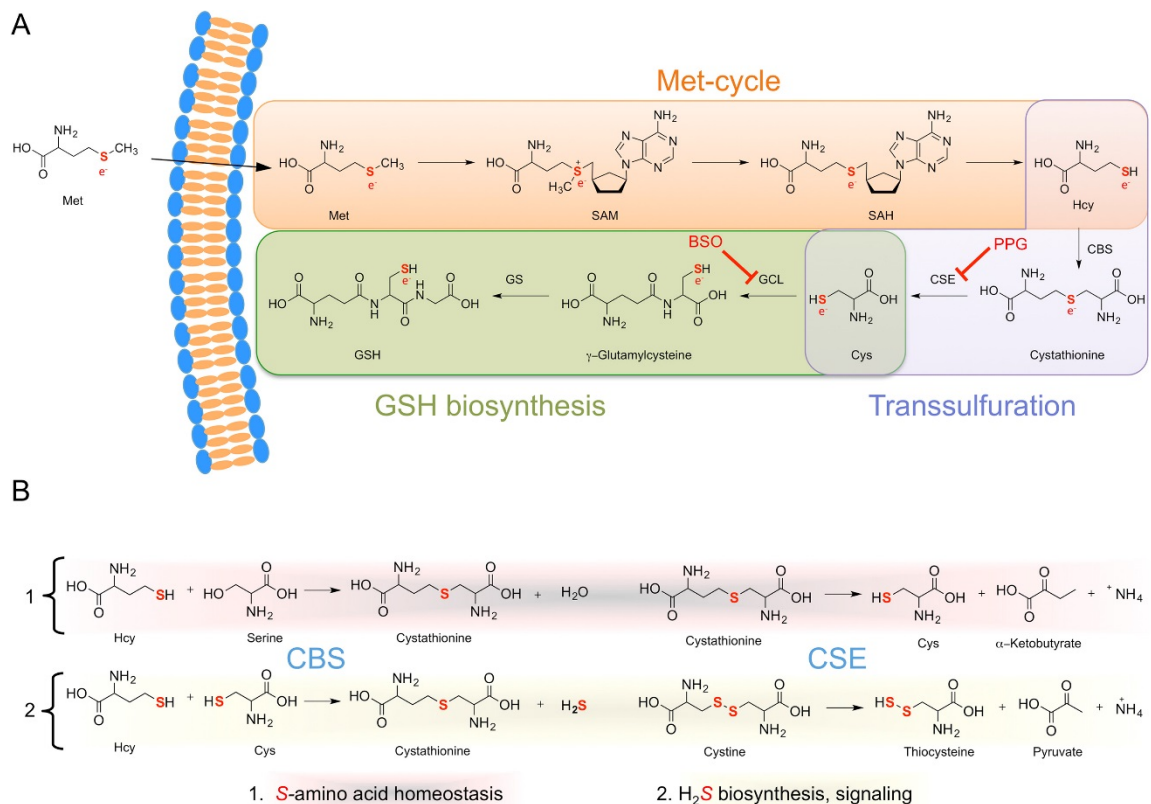


Fig. 1.4. Met metabolism and transsulfuration pathway activities. A, acquisition of disulfide reducing power from extracellular Met. The redox-active S is designated in red font as S. In extracellular fluids, the reduced S is protected from oxidation as a thioether. The Met-cycle generates Hcy bearing this reduced S, and transsulfuration moves this reduced S to Cys; no other portion of Met is transferred to the Cys. The Cys can be used in de novo GSH biosynthesis, which puts the reduced S into a context that can be used for disulfide reduction reactions. The points of pathway inhibition by PPG and BSO are shown. Ancillary reactants and products are not shown. B, Roles of CBS and CSE in S-amino acid homeostasis and in H₂S signaling. Row 1 shows the classical transsulfuration reactions for shunting S from the Met-cycle into Cys. Row 2 shows the reactions that lead to H₂S production for signaling. For the CBS reaction, Cys replaces Ser, so the reaction is Hcy + Cys → cystathionine + H₂S. For the Cse reaction, cystine replaces cystathionine, so the reaction is cystine → thiocysteine + pyruvate. In a subsequent step not shown, thiocysteine (Cys-persulfide) can be reduced by TRP14, Trx1, or Grx to generate H₂S + Cys.

8. The identification of a constitutive NADPH-independent cytosolic disulfide reductase system in mammals

Based on the well-established requirement for the NADPH-dependent disulfide reductase systems in microbial models, as discussed above, it seemed to be a foregone conclusion that mammalian cells would also require the presence of at least one of the two NADPH-dependent cytosolic disulfide reductases for survival. In 2015, however, we reported the surprising observation that mice in which all hepatocytes are constitutively TrxR1/Gsr-null are long-term viable and sustain relatively normal hepatic activities [107]. The TrxR1/Gsr-null hepatocytes exhibit robust proliferation during postnatal liver growth and during regeneration following surgical partial hepatectomy [107]. Mice with TrxR1/Gsr-null livers exhibit full recovery from sterile acute oxidative stress induced by surgical hepatic ischemia/reperfusion injury [108], indicating that antioxidant defenses in TrxR1/Gsr-null cells are at least not so deficient as to be at a critical lethal-threshold. These findings indicate that mammalian hepatocytes, unlike microbes, must have a constitutive alternative mechanism of supporting RNR activity and redox homeostasis that can sustain hepatocyte physiology in the complete combined absence of both TrxR1 and Gsr. Mice with TrxR1/Gsr-null livers are highly sensitive to pharmacological inhibition of GSH biosynthesis by buthionine sulfoximine (BSO; Fig. 4A) as well as to conjugation-mediated depletion of GSH by acetaminophen, either of which induces rapid necrotic death of all hepatocytes and fulminant acute liver failure [107, 108]. This indicates that survival is critically dependent upon GSH, and therefore is not being sustained by an alternative Trx-disulfide reductase, such as by re-purposing of either the mitochondrial TrxR2 or the testis-specific Tgr (TrxR3) [48, 109]. Curiously, like the livers of Gsr-null mice reported a decade earlier by Rogers and colleagues [110],

TrxR1/Gsr-null livers exhibit no detectable GSSG-reductase activity, yet they maintain normal or higher levels of total GSH, of which nearly all is in the reduced form [107, 108]. In combination, these results indicated that GSH was likely sustaining redox homeostasis in the TrxR1/Gsr-null livers, and that this was supported by de novo GSH synthesis rather than by reductive re-cycling of GSSG into 2GSH [107]. Although the above results indicated that essential cytosolic disulfide reducing power in TrxR1/Gsr-null livers was coming from newly synthesized GSH, it was unclear how the hepatocytes could acquire the Cys used to synthesize GSH. In blood plasma, Cys is rapidly oxidized to its disulfide form, cystine, both spontaneously and catalytically [111, 112]. Other chemical species of extracellular Cys are also largely or entirely in oxidized disulfide states (e.g., GSSG or protein-disulfides). Normally cells import extracellular disulfides and generate Cys by reduction of these in the cytosol using either the TrxR1- or the Gsr-system [112–114]. Based on known mechanisms, TrxR1/Gsr-null hepatocytes could therefore neither acquire reduced Cys from the blood plasma nor by disulfide reduction in the cytosol. However, another route of Cys biosynthesis is known that does not require either acquisition of an extracellular thiol or intracellular disulfide reduction. Thus, by using de novo biosynthesis of Cys via the methionine (Met) cycle and transsulfuration pathway to generate intracellular Cys, cells can “extract” the reduced S from Met (Fig. 4A), move it via Cys into GSH, and then utilize this in reduction reactions [107]. Although the thiol-S in Cys and the thioether-S in Met are both in the same redox state (Fig. 2), the thioether form is considerably more oxidation-resistant. Therefore Met generally retains a reduced state in the extracellular environment [107]. Work by Banerjee

and colleagues showed that in HepG2 human liver carcinoma cells, roughly half of the S in the intracellular glutathione pool could be derived from homocysteine (Hcy) added to the culture media [115, 116]. However, Hcy is not typically found at appreciable levels either in food or in extracellular fluids [112]. Rather, it is a key transient intermediate metabolite in the Met-cycle that also serves as a branchpoint-metabolite for fueling the transsulfuration pathway (Figs. 4A and 5). Therefore, the implication from this work is that a large portion of the S in normal cellular steady-state GSH pools is derived from Met via the Met-cycle and transsulfuration. These path-ways are exceptionally active in liver [117]. Because HepG2 cells are highly de-differentiated compared to normal hepatocytes in situ, and therefore are expected to have a less robust Met cycle and transsulfuration pathway than those found in normal liver, the GSH pool in normal liver could contain a substantially larger proportion of Met-derived S [115, 116, 118–120]. Based on the ability of the Met cycle and transsulfuration pathway to supply reduced S to hepatic GSH pools, we hypothesized that hepatocytes in the TrxR1/Gsr-null livers obtained all of their Cys and all of their cytosolic disulfide reducing power from consumption of Met [107]. Consistent with this, inoculation of propargyl glycine (PPG), which inhibits the last step of transsulfuration (Fig. 4A), caused fulminant acute liver failure in these mice, but had no effect in control livers having an active allele encoding TrxR1 [107]. Furthermore, whereas administration of either [³⁵S]-Met or [³⁵S]-cystine resulted in rapid [³⁵S]-labeling of hepatic GSH and protein pools in control mice, only administration of [³⁵S]-Met, not [³⁵S]-cystine, appreciably radiolabeled either hepatic GSH or protein pools in mice with TrxR1/Gsr-null livers [107]. These experiments

indicated that, in addition to requiring Cys-dependent de novo biosynthesis of GSH for survival (see above), TrxR1/Gsr-null livers require de novo biosynthesis of Cys via transsulfuration, and that they cannot effectively utilize cystine as a hepatic Cys source [107]. The radio-tracer studies also revealed the fate of the GSSG that was produced in TrxR1/Gsr-null livers. Following administration of [³⁵S]-Met, mice with TrxR1/Gsr-null livers but not wild type controls showed substantial accumulation of radiolabel in their blood serum GSSG pools [107]. This indicated that the TrxR1/Gsr-null livers excrete much more oxidized GSSG than do wild type livers, which likely is critical for sustaining the reduced state of the intracellular [GSH/GSSG] redox couple in the absence of GSSG-reductase activity [107]. To our knowledge, this Met-driven pathway is the only constitutive NADPH-independent disulfide reductase system that has been uncovered to date. It remains also the only identified source of cytosolic disulfide reducing power in TrxR1/Gsr-null livers (Fig. 4A).

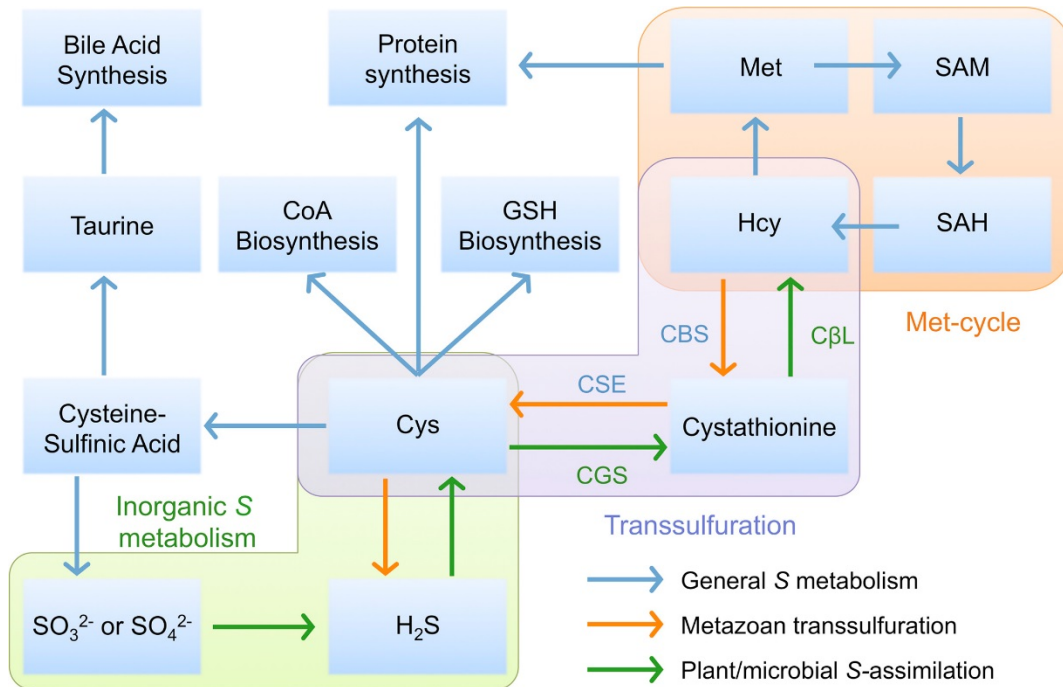


Fig. 1.5. Cellular metabolism of sulfur. Inorganic S metabolism, de novo production of Cys using sulfide, and de novo synthesis of Met from Cys are all restricted to plants and microbes. Metazoans have reversed the transsulfuration pathway, and thereby use Met for de novo synthesis of Cys. Scheme shows only routes and directions, not redox requirements at each step.

9. Collateral impacts of the mammalian NADPH-independent disulfide reductase system

Met is an essential amino acid in metazoans, meaning they are auxotrophic for Met and must acquire this amino acid nutritionally. In addition to using Met as a source of reducing power to sustain redox homeostasis, TrxR1/Gsr-null livers require Met and Met-derived Cys for protein translation and for synthesis of other S-containing metabolites [112], including S-adenosyl methionine (SAM; [118, 121]), CoA; [122], taurine [123], and H_2S [124–128]. Moreover, transit of each reduced S from Met to GSH is predicted to consume, in addition to one molecule of Met, one molecule each of serine,

glutamine, and glycine[107]. Thus, TrxR1/Gsr-null livers likely consume substantially more of these four amino acids than do normal livers. The surprising robustness of the TrxR1/Gsr-null livers suggests that the Met-driven system has the potential to provide a substantial alternative source of disulfide reducing power when needed. Nonetheless, the impacts of the dramatically altered amino acid utilization in these livers are unknown, and deserve further investigation. A better understanding of the metabolic realignments that allow survival under exclusive NADPH-independent cytosolic disulfide reduction conditions will likely provide important insights into how cells combat oxidative stress and recover from oxidative damage.

10. Is the Met-driven cytosolic disulfide reductase system truly NADPH-independent?

The universal role of NADPH as the primary electron donor for most biological reductions, including for supporting disulfide reduction systems, made the uncovering of an apparently NADPH-independent disulfide reduction system in mouse liver surprising. But is this system truly “NADPH-independent”? The answer to this question must certainly be “no”, as NADPH serves as the major first electron acceptor for both chemolithotrophic and photosynthetic reactions, and therefore as the predominant first electron donor for all biological reductions (discussed above). Thus, at some point NADPH must have been involved in generating the reduced S in Met that fuels this system. Where did that occur? As discussed above, utilization of Met as a source of disulfide reducing power in TrxR1/Gsr-null hepatocytes does not consume cellular NADPH. However since the TrxR1 mutation is liver-specific, the question arises: could

other cells in these mice, cells that do have TrxR1, supply reducing power to the TrxR1/Gsr-null livers? To date, the answer to this appears also to be “no”, but further investigation is needed. The experiments detailed above show that Met is the only detected source of cytosolic disulfide reducing power in these livers. Although the Met-cycle has the ability to produce Met from intracellular SAM, S-adenosylhomocysteine (SAH), or Hcy (Fig. 5), none of these are typically available in the diet or in extracellular fluids. As such, exogenous Met is the sole de facto source of intracellular Met [112, 129]. Since Met is an essential amino acid, the exogenous Met must come from either the diet or possibly from enteric biosynthesis by gut microbes. Possible contributions of the gut microbiota remain largely unexplored to date, but merit investigation. Tracing the Met farther back to its origins one finds that the generation of its reduced S is, indeed, NADPH-dependent. Microbes and plants produce Met from Cys using homologues of the same enzymes that mammals use to generate Cys from Met (Figs. 4 and 5; [130] see below). The Cys source in plants and microbes is typically not from disulfide reduction, but rather is generated by de novo synthesis using sulfide. With the nominal exception of the use of environmental sulfide for Cys biosynthesis by some chemolithotrophs (see above), this sulfide, in turn, is generated by NADPH-dependent reduction of inorganic sulfate or sulfite [131, 132]. Thus, the acquisition of disulfide reducing power in TrxR1/Gsr-null livers is NADPH-independent within the hepatocytes, themselves, and cannot likely be supplied by NADPH-dependent reductions within other cell types of these mice. Nonetheless, within the organisms from which the Met was originally

acquired, NADPH fuels the primary reduction of inorganic S to form sulfide and eventually Met.

11. Evolution of the Met-dependent disulfide reductase system

To date, the Met-dependent disulfide reductase system has been studied only in mice, so its evolutionary history can only be inferred. Interestingly, the enzymes involved in moving the reduced S from Met to GSH in mice are found across all phyla. The Met-cycle is ubiquitous and can generate Hcy from Met, or vice versa, in all species. The GSH biosynthetic pathway is also ubiquitous, and will assemble Cys into GSH across phyla [133]. However the two-step transsulfuration pathway that links Hcy to Cys, while also ubiquitous, changed directions during evolution. Specifically, most metazoans use the transsulfuration pathway to move the reduced S from Hcy to Cys, thereby using the Met cycle to feed the production of Cys and Cys-derived products, including GSH, taurine, and CoA. Most microbes, fungi, and plants instead use this pathway to move the reduced S from Cys onto Hcy, thereby feeding the Met-cycle (Fig. 5; [134]). This apparent “flux- reversal” prevents the biosynthesis of Met from Cys in metazoans, and thereby explains why Met is an essential amino acid. Interestingly, metazoans as a group exhibit a Met auxotrophy [135], suggesting the ability to generate Hcy from Cys was lost roughly coincident with the metazoan transition, when single-celled aquatic heterotrophic protozoans gave rise to the first primitive multicellular aquatic heterotrophic animals. However, some single celled organisms, like the brewer's yeast *Saccharomyces cerevisiae*, have been reported to transsulfurate in either direction, converting Cys → Hcy or Hcy →

Cys, as needed ([136]; see below). This suggests that transsulfuration did not simply “reverse”, but rather, it first became bidirectional, and later the ancestral Cys → Hcy direction was lost. Therefore, we predict that not only metazoans, but perhaps any organism that can transsulfurate Hcy → Cys and can effectively excrete the oxidized GSSG might, under appropriate conditions (*e.g.*, adequate Met, Ser, Gly, and Glu available; TrxR1 and Gsr compromised), be able to use the Met-dependent disulfide reductase system.

12. Flux-direction in the transsulfuration pathway

Transsulfuration flux-direction is not determined simply by substrate concentrations. In metazoans the catalytic direction is irreversible, thereby preventing the use of Cys for Met production [129, 135]. In plants, the transsulfuration enzymes are called cystathionine β -lyase (C β L) and cystathionine γ -synthase (CGS), whereas in metazoans the enzymes are called cystathionine β -synthase (CBS) and cystathionase (CSE, Cth), respectively, reflecting the reversal (Figs. 4 and 5; [136]). Interestingly, *S. cerevisiae*, which can run transsulfuration in either direction, has two sets of transsulfuration enzymes—one for the Hcy → Cys direction and one for the Cys → Hcy direction—thereby further emphasizing the dedicated directionality of the enzymes on the pathway[121,136]. Also, the non-metazoan *Dictyostelium* has a Met auxotrophy, suggesting that the Cys → Hcy pathway was lost from a common metazoan ancestor and from select other single-celled organisms (Fig. 6, see below). Many non-metazoan species use NADPH to reduce sulfate into sulfide, which is used for de novo biosynthesis of Cys. In these organisms, Cys is a “metabolic hub” that feeds protein synthesis as well

as the biosynthesis of all S-containing molecules, including Met, GSH, CoA, SAM, Hcy, LA, and others [112]. Non-sulfate-reducing organisms require an alternative source of Cys. This is provided heterotrophically by ingestion of organo-S-compounds, which were produced originally by the sulfate-reducing organisms. Metazoans still use Cys as the metabolic hub for the production of all S-containing molecules, with the notable exception of Met, which was blocked by reversal of the transsulfuration pathway, and Met-cycle intermediates such as SAM and Hcy. Acquisition of energetic nutrients by early free-living actively-feeding heterotrophs likely provided a collateral abundance of ingested amino acids, including Met and cystine. Indeed, most metazoans require a very similar set of essential amino acids in their diet [137]. This might suggest that the early metazoan lifestyle made many of the costly steps in the biosynthesis of certain amino acids, including sulfate reduction, superfluous. As a consequence, the genes encoding these processes, having become largely unused, could have been lost without substantial adverse consequences (“genetic atrophy”). In non-sulfate-reducing organisms, potential advantages of being able to convert Met $\rightarrow$ Cys include the following:

- (i) A more demanding role for Cys than Met. Non-metazoans require Met for protein synthesis and synthesis of Met-cycle intermediates. The latter are continuously recycled, so loss of protein is the only loss of Met. By contrast Cys is used for protein synthesis and is the pre-cursor for all S-containing metabolites. In sulfate-reducing organisms, Cys is the first organic product of S assimilation and is readily available as the precursor for GSH, CoA, and other S-metabolites. Non-sulfate-reducing organisms, by contrast, obtain S from ingestion of extracellular S-amino acids [129, 138]. Many cells

excrete S-containing metabolites, such as GSH (usually as a disulfide), taurine, and H₂S, all of which are derived from Cys. Hcy → Cys transsulfuration allows Met, not just cystine, to contribute to secretion of S-metabolites.

- (ii) Met is a “reductase-efficient” source of Cys; cystine is a more “reductase-demanding” source. In rodents and perhaps all metazoans, dietary Met is available in vast excess over what is needed for incorporation into protein and to support the Met-cycle [118]. Met enters cells ready for use in either protein-synthesis or the Met-cycle. Transsulfuration also allows the thioether-associated S in Met to be transferred to Cys and Cys-based metabolites without disulfide reduction or consumption of NADPH (discussed above). By contrast, cystine requires NADPH-dependent disulfide reduction into Cys before it can be used for protein synthesis or other metabolic pathways. Banerjee and colleagues [115, 116] showed that about half of the S in mammalian cellular GSH pools is Met-derived (discussed above). This supports the idea that the costs of utilizing Met to make Cys must be similar to those of reducing disulfide substrates to make Cys.

- (iii) Hcy → Cys transsulfuration allows Met to fuel an alternative NADPH-independent disulfide reductase system. The hepatic Met-driven cytosolic disulfide reductase system appears to be sufficiently robust to support liver functions and long-term survival of mice with TrxR1/Gsr-null livers [107, 108]. Having an additional robust NADPH-independent back-up system for the two NADPH-dependent cytosolic disulfide reductase systems could provide survival advantages in cases of severe oxidative or toxic stresses.

- (iv) Selenium metabolism. Selenoproteins including TrxRs, Gpxs, and others play critical roles in metazoans (see above). Plants do not have selenoproteins, yet they provide the major source of dietary Se for many metazoans in the form of Se-Met and other Se-metabolites, which plants produce as sequestration products [139]. In metazoans, Se-Met transits the Met-cycle and transsulfuration pathway, yielding Sec or selenide for use in the synthesis of selenoproteins [140, 141]. The metazoan transsulfuration direction thus supports selenoproteins.

- (v) Signaling. CBS and CSE have not only been flux-reversed, but also partially repurposed [24]. In mammals these two enzymes are the major sources of intracellular H₂S production (Figs. 4 and 5) [126]. H₂S is now recognized as an important signaling molecule in animals, akin to NO and H₂O₂ [142–144]. It can exert its effects through post- and pre-translational modifications of protein-Cys residues, resulting in protein- or metabolite-S-persulfides (R-SSH; [127, 145, 146]). The potential advantages of advanced signaling by regulated production of H₂S, for example to coordinate cellular activities for multicellular life, might have demanded both the reversal and repurposing of the transsulfuration pathway. Of the five explanations above for why the evolutionary transsulfuration pathway flux-reversal is advantageous for heterotrophs, the last option, Signaling, is the easiest to correlate to unique metazoan needs, and might be the most important attribute that offset potentially detrimental consequences of being Met-auxotrophic. Signaling is important in all cells and many single-celled organisms can communicate (e.g., quorum-sensing). However beyond even the needs in sessile multicellular plants, multicellularity in animals is critically dependent on advanced cell-

cell communication, and likely required a quantum leap in signaling. In metazoans, individual cells need to coordinate their differentiation and developmental activities, metabolic activities, reproductive activities, and physical activities. Rapid coordinated motions such as swimming and feeding in primitive metazoans, for example, would require that cellular contractions are synchronized. A classical paradigm is provided by the non-metazoan slime-mold, *Dictyostelium*, which is considered a “single-celled social amoeba”. *Dictyostelium* transits from a free-living single-cell amoeboid stage, to a coordinated motile pseudo-multicellular slug stage, to a pseudo-multi-cellular anchored stalk stage, in which individual cells differentiate into vegetative or sporulating cell types. Even though this is a more rudimentary coordination of cells than might have been needed in even the earliest metazoans, this life-cycle is dependent on a large number of advanced signaling mechanisms. Indeed, *Dictyostelium* continues to be an important model for studying signaling mechanisms [147]. In this context, it is intriguing that *Dictyostelium* is one of the few Met-auxotrophic non-metazoans (see above; Fig. 6). Thus, one might ask: was the loss of Cys $\rightarrow$ Hcy transsulfuration not simply “genetic atrophy” of an unused function, as suggested above, but rather a selected trait that enhanced signaling capacity and thereby empowered the metazoan transition?

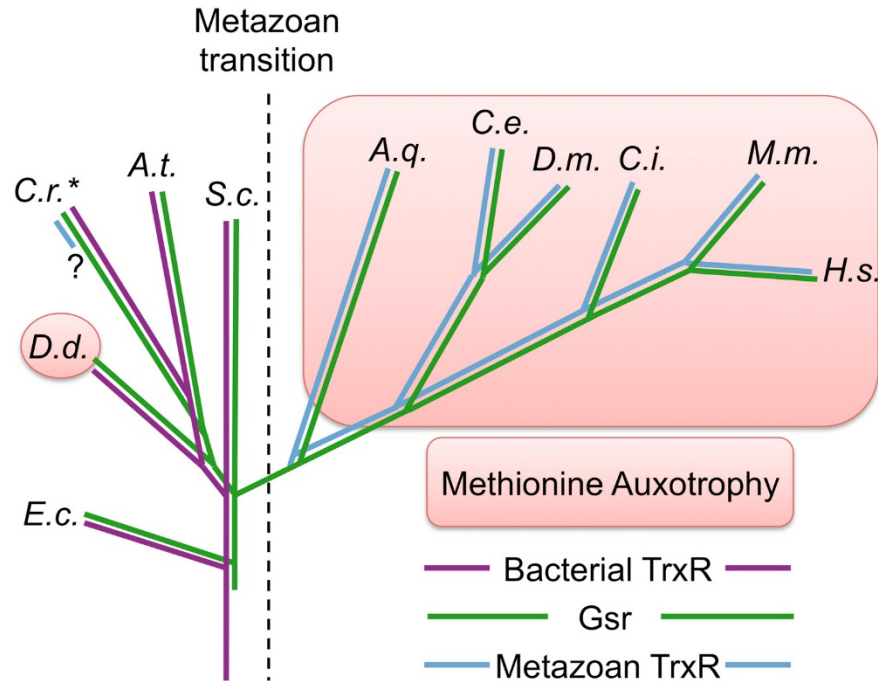


Fig. 1.6. Phylogeny of TrxR and Gsr enzyme families, and of Met auxotrophy. A.t.– *Arabidopsis thaliana*, A.q.– *Amphimedon queenslandica*, C.e.– *Caenorhabditis elegans*, C.i.– *Ciona intestinalis*, C.r.– *Chlamydomonas reinhardtii*, D.d.– *Dictyostelium discoideum*, D.m.– *Drosophila melanogaster*, E.c.– *Escherichia coli*, H.s.– *Homo sapiens*, M.m.– *Mus musculus*, S.c.– *Saccharomyces cerevisiae*. The asterisk denotes the single-celled green alga, *C. reinhardtii*, as representing a sparse but diverse set of non-metazoans that have the metazoan TrxR (blue line with “?”). Predominant others in this group are protozoan intracellular parasites, which might have acquired the gene laterally from a metazoan host. To our knowledge, no similar model yet explains the enigmatic presence of this gene in an alga. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

13. Similar phylogenetic distributions of TrxR-“type” and transsulfuration-“direction”

Intriguingly, metazoans acquired a novel TrxR enzyme via gene duplication and specialization of Gsr [53, 54] apparently coincident with loss of their Cys → Hcy transsulfuration capacity (Fig. 6). Other reviews have been published on the origins and enzymatic differences between the two classes of TrxRs, and the reader is referred to these for such details [48, 52, 54, 62]. Here we will consider why this transition might

have occurred. As the hallmark roles for TrxR are to provide reducing power for RNR and antioxidant enzymes, one might first ask whether the metazoan transition was associated with novel replication- or antioxidant-demands, which might have been better served by the new TrxR. However, there is no obvious reason to suggest that either replication or antioxidant requirements should have shifted at the metazoan transition. As emphasized in the previous section, perhaps the most critical improvement that would have been needed for the transition from protozoan to metazoan was advanced cell-cell communication and signaling.

14. Roles of the metazoan TrxR1 system in signaling

It is possible that the metazoan TrxR arose specifically for regulation of signaling pathways and not as an improved means of sustaining redox homeostasis. Investigations over the past 15 years have revealed the importance of the mammalian TrxR1 system in redox signaling. Trx1 modulates the oxidation status of protein-Cys residues, and thereby influences the properties or activities of those proteins or enzymes. Included in this, Trx1 plays regulatory roles on many transcription factors, including P53, HIF1, NFκB, AP1, and others [148]. In addition to reducing protein disulfides, however, Trx1 and some Trx-family members, in particular the Trx-related protein of 14 kDa (TRP14), also participate in signaling by regulating protein-S-nitrosylation in NO-signaling and protein-S-persulfidation in H₂S-signaling [114, 145, 149]. Trx1 and TRP14 have, in addition to their active site Cys residues, regulatory Cys residues that are subject to post-translational modification and can alter the activity of the proteins [150–152]. This provides another level of regulation on how Trx1 or TRP14 modulate signaling in cells. Intracellular signal

transduction from many membrane-associated receptors is redox-regulated by mechanisms involving the TrxR1 system. Seminal studies by Finkel's team and by Rhee's team showed that ligand binding by the PDGF receptor, EGF receptor, T cell receptor, and other transmembrane receptors was associated with, in addition to auto-phosphorylation of tyrosyl domains on the cytosolic portion of the receptor, activation of a membrane-associated NADPH-oxidase (NOX) complex [153–160]. NOX activation leads to extracellular H_2O_2 production, which enters the cell through aquaporins [161]. Inside the cell, the H_2O_2 inactivates ubiquitous protein phosphatases, thereby antagonizing dephosphorylation of the receptor. Trx1-dependent Prxs counteract this by eliminating the H_2O_2 . Trx1 also re-activates the protein phosphatases, after which the receptor is dephosphorylated and signaling stops [162–166]. This is now considered a general paradigm for signaling by many transmembrane receptors. More recently, the TrxR1 system has also become recognized as a critical component regulating the catalytic oxidation of some regulatory Cys residues. Winterbourn and colleagues demonstrated that most cellular thiols are poor substrates for oxidation by H_2O_2 . The reasons for this are, first, that the thiols are not reactive enough, so reaction rates with H_2O_2 are exceptionally slow ($\sim 1/\text{mol}/\text{sec}$; [167]). Second, peroxiredoxins (Prx), are highly abundant in the cytosol and have exceptionally nucleophilic active site Cys residues that react with H_2O_2 at rates $\sim 10^7$ -fold faster than most protein-Cys residues [168, 169]. Therefore, it would be nearly impossible for a molecule of H_2O_2 to evade reacting with a professional peroxidase long enough to oxidize another protein-Cys residue [170]. Dick and colleagues have shown that, for redox regulation of STAT3 protein, Prx2 serves as a

catalyst for STAT3 Cys-oxidation. For this, Prx2 rapidly reacts with H_2O_2 forming an active site sulfenic acid. It then transfers this oxidation-state to regulatory Cys residues on STAT3 [171]. Importantly, the enzymatic activity of the Prxs is regulated by Trx1, and so the TrxR1 system plays a critical role in this regulatory system. As another example, TrxR1 plays a role in regulating activity of the antioxidant and cytoprotective system driven by nuclear factor erythroid-2-related-2 (Nrf2). Nrf2 provides a robust response to oxidative stress and electrophilic toxins in cells [172]. Either genetic or pharmacologic inhibition of TrxR1 activity induces Nrf2 without inducing oxidative stress, indicating that a part of the stress-sensing regulatory mechanism of Nrf2 is controlled by TrxR1 [49, 173–176]. Finally it should be noted that homozygous disruption of Trx1 or TrxR1 in mouse zygotes results in severe defects in early embryonic patterning without having any detectable impacts on either the level of cellular oxidative stress or cell proliferation [49, 108, 177–181]. The findings above reveal that the mammalian TrxR1 system plays modulatory roles in redox signaling for a diverse spectrum of pathways and responses. Thus, one might ask: was acquisition of these signaling activities critically dependent on the evolution of a new TrxR enzyme, and could this advance in signaling have empowered the metazoan transition?

15. Closing perspectives: Balancing antioxidant activities and redox signaling

We now know that in hepatocytes and perhaps in many metazoan cell types, cytosolic disulfide reducing power can come from NADPH via TrxR1, from NADPH via Gsr, or from Met catabolism [107]. We also now know that the cytosolic disulfide

reducing power required for RNR activity and cell homeostasis can be delivered to its targets by either Trx1 in the absence of GSH or by GSH in the absence of Trx1 [108, 178]. By contrast, a similar TrxR1- Gsr-system redundancy is not evident in signaling. Disruptions of the TrxR1 system, although not substantially impacting either the cellular redox balance or GSH pools, disrupts embryonic patterning, Nrf2 regulation, and other signaling-dependent processes [49]. By contrast, adult animals including humans tolerate severe long-term whole-body pharmacologic depletion of GSH using BSO without evident defects in signaling [182–184]. Perhaps the redox-signaling roles of the TrxR1 system are so crucial in metazoans that its ancestral redox-homeostasis roles must be “excused”, when necessary, for the sake of sustained effective signaling. Gsr, on the other hand, might be more committed to sustaining cellular redox-homeostasis even in the absence of any contribution from TrxR1. Where would the NADPH-independent system fall in this hypothetical dichotomy? Met delivers its reducing power to the homeostatic machinery via GSH, which argues that it simply provides “back-up support” to the NADPH + Gsr-driven system. On the other hand, for this reducing power to arrive at GSH, the reduced S from Met must transit the metazoan Hcy → Cys transsulfuration pathway, the enzymes, substrates, and products of which supply H₂S for signaling activities. Continued studies on the disulfide reductase systems and in the rapidly advancing field of redox signaling will improve our understanding of both the evolution and the physiological roles of each of these systems. Lastly, these new insights on disulfide reductase systems should be considered in the context of human health and physiology. If the TrxR1-, Gsr-, and Met-driven disulfide reductase systems play distinct

roles in signaling and redox homeostasis, then it might be possible to target these pathways to selectively impact specific signaling activities versus homeostatic activities. The implications of this on modulating pathologies including inflammatory diseases, cancers, and neuropathologies could be immense. Almost certainly, there remain other important pathways to be uncovered and there will always be new untested clinical applications arising in the realm of Redox Biology.

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CHAPTER TWO

DISULFIDE REDUCTASE SYSTEMS

IN THE LIVER

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CHAPTER TWO - DISULFIDE REDUCTASE SYSTEMS IN THE LIVER

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Abstract

Intermediary metabolism and detoxification place high demands on the disulfide reductase systems in most hepatocyte subcellular compartments. Biosynthetic, metabolic, cytoprotective and signalling activities in the cytosol; regulation of transcription in nuclei; respiration in mitochondria; and protein folding in endoplasmic reticulum all require resident disulfide reductase activities. In the cytosol, two NADPH-dependent enzymes, glutathione reductase and thioredoxin reductase, as well as a recently identified NADPH-independent system that uses catabolism of methionine to maintain pools of reduced glutathione, supply disulfide reducing power. However the necessary discontinuity between the cytosol and the interior of organelles restricts the ability of the cytosolic systems to support needs in other compartments. Maintenance of molecular- and charge-gradients across the inner-mitochondrial membrane, which is needed for oxidative phosphorylation, mandates that the matrix maintain an autonomous set of NADPH-dependent disulfide reductase systems. Elsewhere, complex mechanisms mediate the transfer of cytosolic reducing power into specific compartments. The redox needs in each compartment also differ, with the lumen of the endoplasmic reticulum, the mitochondrial inter-membrane space and some signalling proteins in the cytosol each requiring different levels of protein oxidation. Here, we present an overview of the current understanding of the disulfide reductase systems in major subcellular compartments of hepatocytes, integrating knowledge obtained from direct analyses on liver with inferences from other model systems. Additionally, we discuss relevant advances in the expanding field of redox signalling.

The redox biology of sulfur and the need
for disulfide reductase systems

The reduction and oxidation of sulfur (S) in the context of either cysteine (Cys)-containing proteins or small Cys-derived-S-containing molecules mediates redox homeostasis. Biological S can exist in several different redox states and can undergo a variety of different modifications, although probably the most common of these is the [dithiol-disulfide] redox couple (Figure 1). Typically, a pair of thiols (dithiol) is oxidized to a disulfide with concomitant loss of two electrons ($2e^-$). Conversely, a disulfide bond can be reduced by the addition of $2e^-$, thereby yielding a dithiol. Dithiols provide the reducing power for a large number of reductases that support housekeeping, repair and antioxidant defence activities. Sustaining these activities requires a ready supply of thiol-based reducing power. One fundamental dithiol-driven reaction is the reduction of ribonucleoside diphosphates by ribonucleotide reductase (RNR) to generate DNA precursors (Thelander and Reichard, 1979). Most cells in adult animals are post-replicative and need only small amounts of RNR activity to support DNA re-pair and mitochondrial turnover. Other important dithiol-driven reactions are associated with redox homeostasis, that is the maintenance of a moderately reducing intracellular environment (Lopez-Mirabal and Winther, 2008). This is important for housekeeping, defence and repair activities. Free intracellular Cys must be maintained in a reduced state to contribute either to protein synthesis or as the metabolic precursor for the biosynthesis of S-containing metabolites, including coenzyme A (CoA) and GSH. Similarly, the function of many Cys-containing proteins, and most S-containing metabolites, requires maintenance of the reduced thiol status of the S. For defence functions, elimination of

ROS by peroxidases requires that these enzymes be supplied with reducing power via dithiol-based electron donors (Sies et al., 2017). Enzymatic repair of protein disulfides or methionine (Met)-sulfoxides also requires reducing power obtained from dithiol donors, which are thereby converted to disulfides (Arner and Holmgren, 2000; Boschi-Muller and Branlant, 2014).

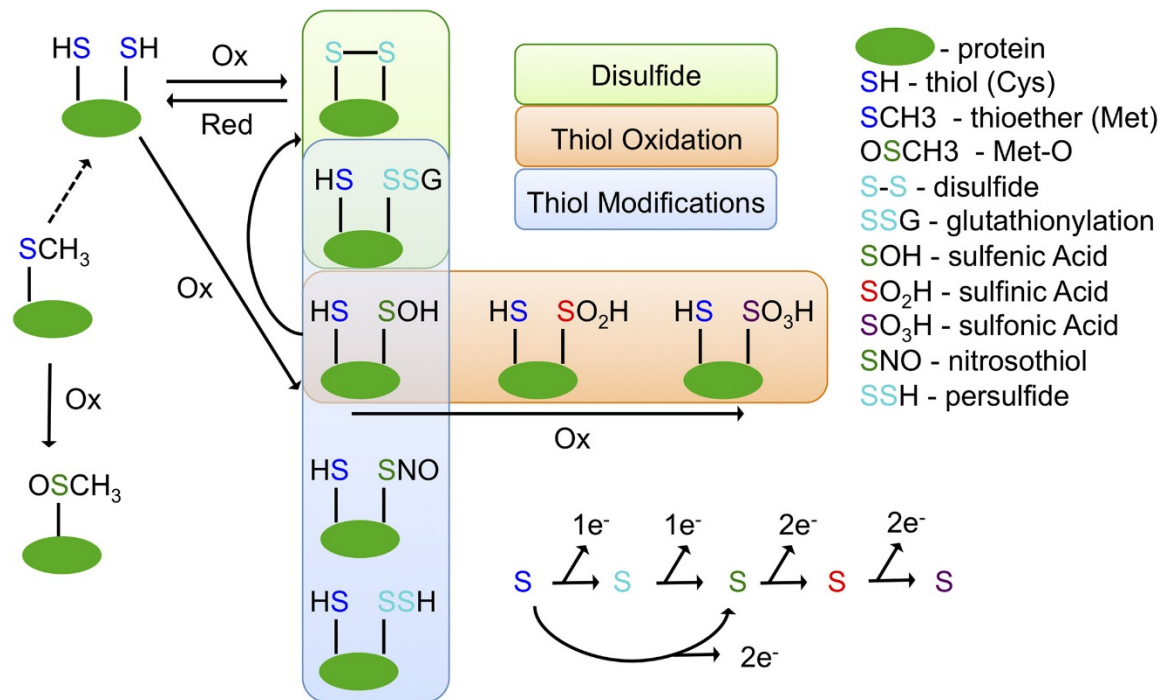


Figure 2.1 Oxidative modifications of S amino acids. For simplicity, only the species discussed in this review are included. Protein thiols (dark blue S) undergo two electron oxidation to a disulfide (light blue S) or are oxidized to the S-glutathionylated (light blue S), S-nitrosothiol (green S), S-persulfide (green S) or sulfenic acid (green S), which can be further oxidized to the sulfinic acid (red S) and sulfonic acid (purple S) by subsequent reactions with H₂O₂. Thioethers, like in Met, have the same S-redox state as thiols (dark blue S) and can be oxidized to the S-sulfoxide (Met-O)

The tripeptide GSH (γ -Glu-Cys-Gly) is the most abundant low molecular weight thiol in the liver and one of the major electron donors for disulfide reduction reactions.

The other major electron-donors for disulfide reductions are members of the thioredoxin

(Trx) and glutaredoxin (Grx) families of small proteins. Intracellular reduced Trxs and GSH provide reducing power to fuel diverse cellular reduction reactions. In the case of GSH, this can occur either by direct reduction of a substrate or by enzyme-facilitated reductions in concert with Grxs (Fernandes and Holmgren, 2004). In most compartments of living cells, both Trxs and GSH are thought to exist predominantly in a reduced state. Studies in yeast using sensitive fluorescent redox reporters show that ratios of oxidized GSH-disulfide : reduced GSH-thiol (GSSG : GSH) in the cytosol are ~1:1000–1:10 000 (Meyer and Dick, 2010; Morgan et al., 2011). This is achieved both by rapid reduction of GSSG once it forms and by active export of GSSG, coupled with de novo GSH biosynthesis, if reduction rates are insufficient (Morgan et al., 2013; Eriksson et al., 2015). One-step oxidation of a thiol by an H₂O₂-derived oxidant yields an unstable sulfenic acid (-SOH) intermediate, which will react spontaneously with another thiol to yield a disulfide and water. Over-oxidation of -SOH by another molecule of H₂O₂ yields a sulfinic acid, and a third consecutive oxidation with H₂O₂ yields a sulfonic acid (Figure 1). Disulfides can be reduced to dithiols by Trx or GSH + Grx. Uniquely in some peroxiredoxins (Prx), an active site sulfinic acid can be restored back into a thiol by sulfiredoxin-1 in a reaction that requires both Trx1 and ATP (Biteau et al., 2003; Woo et al., 2005). Other sulfinic and all sulfonic acids are considered irreversible, so that only protein turnover can eliminate these Cys adducts. Additional cellular modifications of Cys thiols include S-glutathionylation, S-nitrosylation and S-persulfidation (Figure 1). Each of these modifications is readily reversible, and these are thought to play defensive roles in preventing irreparable over-oxidations during periods of oxidative stress.

However, they each can also modify protein activities on their own and thereby are important contributors to redox signalling (see below).

The NADPH-dependent disulfide reductase systems

Disulfide reductase systems were originally identified in *Escherichia coli* as the essential electron donors for RNR (Thelander and Reichard, 1979). Trx and its reductase, TrxR, were discovered first. Later, when *E. coli* lacking Trx were found to be viable, Grxs were discovered as an alternative electron donor for RNR (Holmgren, 1976). *E. coli* or yeast coincidentally lacking both TrxR and GSSG-reductase (Gsr; in combination TrxR/Gsr-null) are not viable, indicating that, both in eubacteria and eukaryotes, no other constitutive systems could supplant their need to at least have either TrxR or Gsr (Nordlund and Reichard, 2006). Across phyla, all cells use reduced NADPH to generate intracellular disulfide reducing power (Miller et al., 2018). NADPH is generated intracellularly by reduction of NADP^+ ; in liver, this predominantly occurs during oxidation of glucose in the pentose phosphate pathway, oxidation of malate by malic enzyme, folate metabolism, or oxidation of isocitrate by isocitrate dehydrogenase. Mitochondrial NADPH is also generated by nicotinamide nucleotide transhydrogenase (NNT), which transfers reducing power from NADH to NADP^+ to generate NADPH. Like most mammalian cells, liver hepatocytes have only three enzymes that can use NADPH to reduce a disulfide. These are (i) Gsr, (ii) cytosolic TrxR1 and (iii) mitochondrial TrxR2 (Arnér, 2009). All three of these are homologous flavoenzymes that extract electrons from NADPH and pass these via a flavin cofactor to an amino-terminal active site, wherein it reduces a disulfide to a dithiol. In Gsr, this active site dithiol then

reduces GSSG. Unlike Gsr, the mammalian TrxRs are selenoproteins, meaning they contain the rare 21st translationally inserted amino acid selenocysteine (Sec). The Sec occurs in a Cys-Sec containing carboxy (C)-terminal active site domain. In mammalian TrxRs, the reduced N-terminal active site dithiol reduces the C-terminal seleno-sulfide bond on the complementary sub-unit of the homodimeric enzyme. This selenol-thiol pair can then reduce the active site in Trx1-disulfide (Arnér, 2009). Although only Gsr, TrxR1 and TrxR2 can use NADPH to reduce a disulfide bond, and these enzymes each typically reduce only very specific substrates, a broad range of disulfide reduction reactions are required for homeostatic, biosynthetic, repair and defence activities. Without known exceptions, these reductions are driven by reducing power delivered via GSH + Grx-family proteins or by Trx-family proteins (Miller et al., 2018). Thus, the NADPH-dependent Gsr and TrxRs are considered the primary disulfide reductases, which each fuels a downstream disulfide reductase chain. Indeed, until recently, it was thought that all cellular disulfide reducing power was derived from intracellular NADPH via Gsr, TrxR1 or TrxR2. We now know that mammalian cells also have an NADPH-independent source of cytosolic disulfide reducing power (see below). Trxs and Grxs are related families of small proteins (~11–15 kDa) with overlapping functions. Grxs are typically reduced by GSH whereas Trxs are typically reduced by TrxRs. The major substrate of TrxR1 is Trx1, yet some other Trx-related proteins also have active-site dithiols that can donate reducing power to select substrates, thereby becoming disulfides, and then can be reduced back to dithiols by TrxR1 (Arnér, 2009). GSH + Grx traffics reducing power to RNR, GSH peroxidases (Gpx), some Prxs and other protein disulfides, for example, those

arising from oxidative stress. Trx1 traffics reducing power to disulfide-containing enzymes including RNR, Met-sulfoxide reductases (Msr), most Prxs and to other protein disulfides (Lillig and Holmgren, 2007). The precise functional overlap between the Grxs and the Trxs, as well as the specific roles of other related redoxin family members, remains incompletely characterized (see below)

Disulfide/dithiol redox in the cytosol and nucleus

Segregation of cellular activities into discrete organelles mandates allocated disulfide reductase activities. In hepatocytes like in most cells, the cytosolic compartment is the largest compartment, it maintains a reduced status with a ratio of GSSG : GSH of <1:1000, and its disulfide reductase systems are used for fuelling biosynthetic and repair activities. It also supports many antioxidant defences and redox signalling activities. Nonetheless, each of the other subcellular compartments has its own critical needs that must be met to maintain cell homeostasis. Many antioxidant functions of the disulfide reductase systems are carried out by Gpx- and Prx-family proteins, which catalyse both the reductive detoxification of peroxides and, in some situations, the catalytic transfer of oxidation-equivalents to another protein (see below). Both peroxidase families function via similar mechanisms, although Gpxs are predominantly selenoproteins fuelled by GSH, whereas the Prxs have, instead, highly activated Cys residues and are fuelled by Trxs or, in some cases, by GSH. In Prxs, the initial reaction with peroxide will form an active site Cys-SOH (Rhee et al., 2001). For peroxidase activity, the 2-Cys subclass of Prxs, including human Prx 1, 2, 3, 4 and 5, resolve the SOH by formation of a disulfide with a second active site Cys residue and the release of water. The disulfide is

subsequently reduced by Trx1. 1-Cys Prxs, such as human Prx6, typically use GSH or ascorbate to restore the active site thiol (Kang et al., 1998; Monteiro et al., 2007). Not all ROS are removed by the antioxidant systems. Respiration, metabolism, extracellular exposures or redox signalling oxidize some cytosolic proteins, which are subsequently repaired. Oxidized adducts include Cys-disulfides and Met-sulfoxides (Figure 1). Protein-disulfides are reduced by either Trx1 or GSH + Grx, whereas the reduction of Met-sulfoxides requires a dedicated repair system. MsrA and MsrB are enantiomer-specific enzymes that preferentially use reducing power from Trx1 to reduce oxidized Met-(S)-sulfoxide or Met-(R)-sulfoxide, respectively, to Met (Tarrago and Gladyshev, 2012; Boschi-Muller and Branlant, 2014). The cytosol and nucleus share disulfide reductase systems; however, the systems-components are unequally partitioned between the two domains (Figure 2). The nuclear membrane is generally permissive for transit of small molecules but restrictive for macromolecules. Neither TrxR1 nor Gsr normally localize to the nucleus and, as such, the nucleus does not have independent primary disulfide reductase activity. Nuclear contents are spatially isolated away from most ROS sources (extracellular, metabolic and mitochondrial), so basal oxidative damage is likely to be low in nuclei. However, nuclei still depend on disulfide reductase activity for other activities. Genomic DNA synthesis, either for replication or repair, requires DNA precursors that universally are synthesized by the disulfide reductase-dependent enzyme RNR (see above). RNR, however, is strictly cytosolic, so disulfide reductase requirements for DNA precursor synthesis are cytosolic. Deoxyribonucleotides are synthesized in the cytosol and must be imported into the nucleus or mitochondria to sup-

port genomic or mitochondrial DNA synthesis (Nordlund and Reichard, 2006). During oxidative stress, some Trx1 and Grx1 transit to the nucleus, where they function in gene regulation rather than fuelling antioxidant systems (Karimpour et al., 2002). At least one Trx-family protein, nucleoredoxin (Nxn), has dedicated nuclear activities (Kurooka et al., 1997). Nxn can regulate the activity of transcription factors including NF- κ B, AP-1 and others, typically by reducing disulfide bonds on the transcription factors that impede DNA-binding. Indeed, evidence from diverse mammalian cell models suggests that nuclear Trx1, Grx1 and Nxn all target distinct transcription factors for redox regulation of gene expression (Hirota et al., 2000; Go and Jones, 2010). In the absence of a primary disulfide reductase within the nucleus, however, reductive recycling of all nuclear redoxins needs to be fuelled by primary reductase activities in the cytosol (Arnér, 2009). Intuitively, this requires redox-shuttling, likely via transit of Trx1, GSH or GSH + Grx1 between the nucleus and the cytosol. Although the kinetics of shuttling is likely slow, the demand will be correspondingly low.

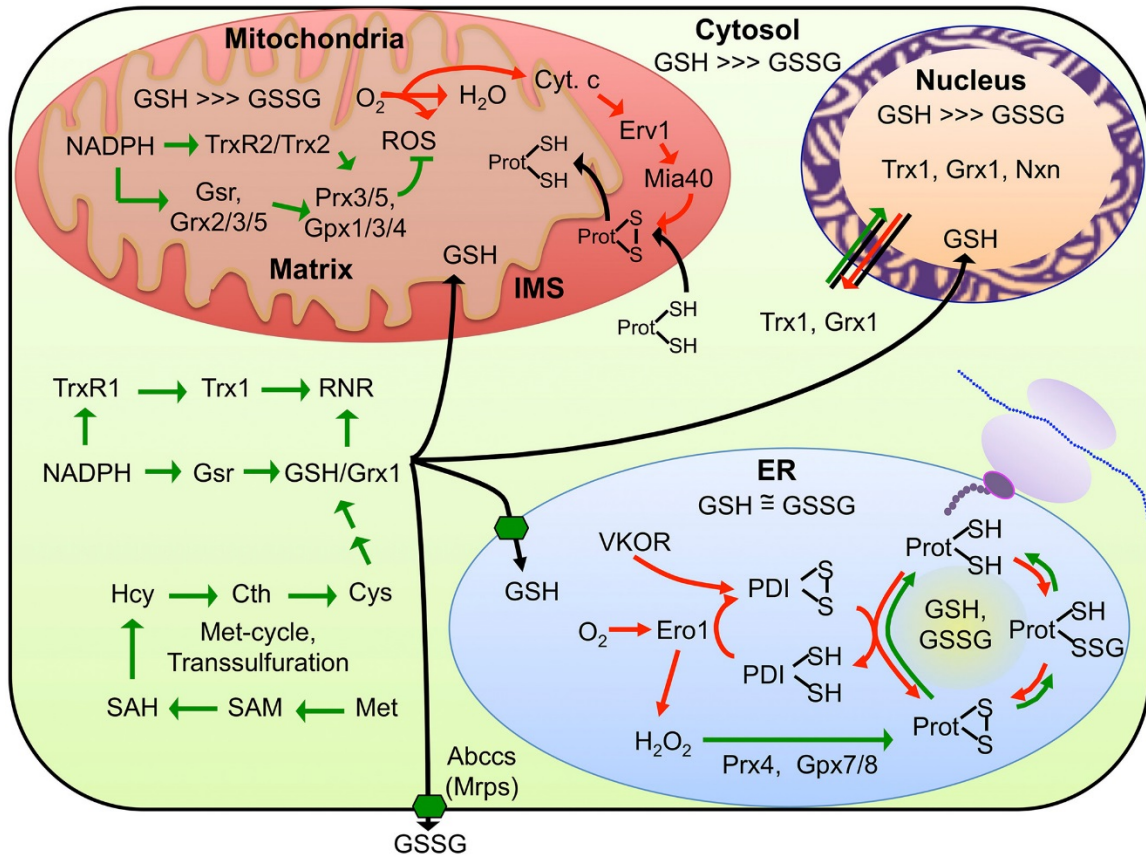


Figure 2.2 Disulfide reductases of the eukaryotic cell. The various disulfide reductase systems are shown in each major organelle. Also represented is the trafficking of cytosolic GSH to all compartments and the GSH : GSSG ratios for each compartment. The NADPH-independent disulfide reductase pathway is shown in the lower left of the panel, demonstrating the conversion of Met to Cys and subsequently GSH. Green and red arrows represent reductive and oxidative processes, respectively; black arrows depict molecular trafficking. See text for more details

Disulfide/dithiol redox in mitochondria

Respiratory activities in mitochondria generate collateral ROS. However, the restrictions on molecular transport between the cytosol and either the mitochondrial intermembrane space (IMS) or matrix severely limit the routes by which cytosolic reducing power can enter the mitochondria (Figure 2). GSH, for example, is only

synthesized in the cytosol, yet it is imported into mitochondria by a unidirectional high-affinity transporter. Notwithstanding, no dithiol-disulfide redox shuttle is detected between the cytosol and the mitochondria (Albrecht et al., 2011). Instead, mitochondria have independent disulfide reductase systems. TrxR2, Trx2, Prx3 and Prx5 constitute a mitochondrial NADPH-dependent Trx-based antioxidant system. Also, the nuclear gene encoding Gsr issues an mRNA splice-isoform encoding a protein variant with a mitochondrial signal peptide. Mitochondrial reduction of GSSG by Gsr, along with mitochondrial Grx2, 3 and 5, and mitochondrial Gpx1, 3 and 4, provide an NADPH-dependent GSH-based antioxidant system. Similar to the cytosol, mitochondria produce NADPH from malic enzyme and isocitrate dehydrogenase, yet, uniquely, mitochondria also utilize NNT to shuttle reducing power from NADH, found abundantly in the mitochondria for respiratory and metabolic activities, to NADPH. Normally, NNT supplies half of the mitochondrial NADPH; knockdown of NNT severely impacts the mitochondrial redox state (Yin et al., 2012). Curiously, in mouse heart under severe metabolic restriction, the direction of NNT reverses. NADPH is then consumed to make NADH and support ATP production, which coincidentally increases ROS levels (Nickel et al., 2015). To date, it remains unclear whether similar starvation responses also act in hepatocyte mitochondria. The IMS is a distinct compartment between the cytosol and the matrix that contains only ~60 different proteins, as compared to the more than 500 proteins found within the matrix and hundreds more inner mitochondrial membrane proteins (Figure 2). The mitochondrial genome encodes few proteins, instead relying on import from the cytosol for the majority of its essential proteins. Studies in yeast models

show that importing from the cytosol to the matrix involves, in part, the oxidation of protein thiols to disulfides in the IMS, referred to as the disulfide relay (Erdogan and Riemer, 2017). Like the lumen of the endoplasmic reticulum (ER; discussed below), the IMS facilitates oxidative protein folding, yet unlike in the ER, this is achieved in a reducing environment. Protein dithiols in the yeast IMS are oxidized to disulfides by Mia40, which is re-oxidized by the sulfhydryl oxidase Erv1 (Kojer et al., 2015). Reduced Erv1 transfers electrons to cytochrome c, thereby linking the disulfide relay to the respiratory electron transport chain. Details of the mechanisms used in mammalian cells remain unclear. The known mammalian Erv1 homologue, growth factor-Erv1-like (Gfer, also called Alr, for augmenter of liver regeneration), is reported to be both cytosolic and within the IMS, with tissue-restricted expression (Fischer et al., 2013). Consistent with a role in mitochondrial function, Gfer is essential for fetal survival and liver-specific Gfer-disruption severely impacts hepatic mitochondrial activities (Gandhi et al., 2015).

Disulfide/dithiol redox in the endoplasmic reticulum

Unlike the subcellular compartments discussed above, the ER maintains an oxidizing environment to accommodate protein folding-associated disulfide bond formation (Figure 2). Also unlike other compartments, wherein GSH is at least 1000-fold more abundant than GSSG, the ER maintains near equivalent levels of GSSG and GSH (Birk et al., 2013). The ER contains a unique set of Trx-related proteins, the protein-disulfide isomerases (PDI), of which ~20 are found in mammals. Like Trx1, PDIs can transfer reducing-equivalents from their dithiol to a target protein-disulfide; however, in the disulfide form, whereas Trx1-disulfide accepts electrons almost exclusively from

TrxR1, most PDIs readily accept electrons from reduced thiols of diverse proteins, thereby installing a disulfide on the target protein. In this process, the PDI becomes reduced to a dithiol. Members of the PDI family exhibit active site Cys-pairs of differing reactivities, with most acting preferentially as dithiol oxidases, but some behaving preferentially as disulfide reductases. The PDI-dithiol is regenerated to the disulfide form by ER oxidase-1 (Ero1) using molecular oxygen as the electron acceptor; by Prx4, Gpx7, Gpx8 using H₂O₂; or by vitamin K epoxide reductase (Figure 2) (Tavender et al., 2010; Zito et al., 2010; Rutkevich and Williams, 2012). The ER lumen does not contain a primary disulfide reductase; however, it still must maintain a balanced redox status and not become overly oxidizing. Molecular O₂ provides oxidizing equivalents, but in the absence of TrxR1 and Gsr, what is the source of reducing power to balance this? The most obvious source is the influx of free thiols associated with the Cys-residues of nascent proteins. Also, the ER does not synthesize GSH but rather imports it. In yeast and perhaps all eukaryotic cells, import occurs via facilitated diffusion through the Sec61 transporter (Ponsero et al., 2017). Transport by Sec61 is regulated by the redox status of the ER lumen, and it has been suggested that regulated shuttling of GSH into the ER via Sec61 and GSSG out of the ER, either also by diffusion through Sec61 or another route, might provide a redox-responsive gate to sustain an appropriate balance (Ponsero et al., 2017). GSH has complex interactions with PDI proteins, effectively distributing electrons by reducing some PDI family members and being reduced by others (Lappi and Ruddock, 2011; Rutkevich and Williams, 2012). Interestingly, depletion of ER GSH does not disrupt protein folding (Tsunoda et al., 2014), suggesting that, although GSH likely

facilitates redox-dependent protein folding, Ero1 and PDIs are sufficient without GSH. The reader is directed to a recent review on redox activities in ER protein folding for more details on these interactions (Ellgaard et al., 2018).

Genetic disruptions of the cytosolic NADPH-dependent disulfide reductase systems in mice

In humans, familial disruptions of glucose-6-phosphate dehydrogenase (G6pd), a key enzyme in the pentose phosphate pathway, diminish cellular NADPH levels but are generally not pathological, presumably due to residual NADPH production by other pathways. However, under some toxic oxidative challenges, most famously from fava beans, the consumption of which in these patients causes a potentially fatal crisis termed ‘favism’, erythrocytes fail to maintain a sufficiently reduced GSH : GSSG balance and haemolytic anaemia ensues (Salvador and Savageau, 2003). Although the most obvious candidate cause for this was that the diminished NADPH levels resulted in insufficient Gsr activity, it was later shown that full constitutive disruption of Gsr in mice is benign and that these mice tolerate many toxic oxidative stresses (Rogers et al., 2006). Indeed, Gsr-disruption across phyla has surprisingly little effect on either total GSH levels or GSH : GSSG ratios (Rogers et al., 2006; Toledano et al., 2007). In yeast, GSSG export plays a critical role in maintaining a reduced GSSG : GSH balance in a Gsr-null background, and when export is also disrupted, Grx2 and Trx2 contribute to GSSG reduction by mediating cross-trafficking of reducing power from the TrxR systems to GSH (Morgan et al., 2013). Based on these models, it is unlikely that favism results solely from unabated oxidative stress. Rather, it might also involve secondary activities

of the toxic challenge. Based on the diversity of critical homeostatic and defence functions supported by the Trx1 system, it was anticipated that genetic disruption of TrxR1, unlike disruption of Gsr, might be lethal in mice. However, it is known that long-term treatments with pharmacological compounds that systemically inhibit TrxRs, such as auranofin, are well tolerated (Cai et al., 2012). Early reports of knockouts in the Trx1 system showed that mouse embryos lacking either TrxR1 or Trx1 died around embryonic days 6–8. Interestingly, this length of survival suggested that basal cell homeostasis and proliferation was sustained. Deeper analyses on the TrxR1-null embryos indicated that failure was due to defective embryonic patterning. Patterning requires that cells respond appropriately to extracellular stimuli, such as cell–cell, paracrine or endocrine factors, the response to which, in turn, is dependent on intercellular signal transduction. This suggested that the mutants have a defect in how cells communicate with each other or their environment and not a basal defect in cell physiology. Subsequently, tissue-specific knockouts of TrxR1 were shown to be compatible with survival of diverse cell types. In liver, TrxR1 elimination results in increased glycogen storage and induction of the cytoprotective Nrf2 (nuclear factor erythroid-derived 2-like 2) response (Suvorova et al., 2009; Iverson et al., 2013; Cebula et al., 2015). Liver-specific disruption of Trx1 is also non-lethal, with only subtle phenotypic effects (Prigge et al., 2017). The mouse models revealed that either the TrxR1- or the Gsr-system can adequately support all essential hepatic cytosolic disulfide reduction requirements in the absence of the other. More unexpectedly, however, combined mouse models, in which all hepatocytes concurrently lack both TrxR1 and Gsr, or even ones concurrently lacking TrxR1, Gsr and Trx1, are

also viable and support long-term mouse survival (Eriksson et al., 2015; Prigge et al., 2017). This remarkable finding is contrary to the situation in both bacteria and yeast, which cannot survive without these enzymes (Nordlund and Reichard, 2006), and led to the discovery of the only known constitutive NADPH-independent disulfide reductase system that can support cell homeostasis and proliferation (see below).

NADPH-independent disulfide reduction in the liver

Until recently, disulfide reduction in all cells was thought to be NADPH-dependent (Nordlund and Reichard, 2006; Miller et al., 2018). However, mice with liver-specific deletion of both TrxR1 and Gsr were found to be long-term viable (see above). TrxR1/Gsr-null livers exhibit robust developmental and regenerative hepatocyte proliferation, and they tolerate acute oxidative stress from ischaemia/reperfusion injury. However, the TrxR1/Gsr-null livers are extremely susceptible to treatments that either block GSH synthesis (buthionine sulfoximine) or deplete hepatic GSH stores (acetaminophen) (Eriksson et al., 2015; Prigge et al., 2017). These observations indicated that, although WT livers tolerate GSH depletion (Martensson et al., 1990), TrxR1/Gsr-null livers are critically GSH-dependent. TrxR1/Gsr-null livers are incapable of reducing GSSG to GSH, indicating that they do not have an alternative GSSG-reductase activity. They are also incapable of effectively using extracellular cystine, the oxidized disulfide of Cys. Instead, Met, an essential S-amino acid, supplies the only detected source of disulfide reducing power and of hepatic S in these livers (Eriksson et al., 2015). The thioether-S within Met is in the same redox state as a thiol (Figure 1), yet is resistant to oxidization in the extracellular milieu. Met is imported into cells, and therein, the S is

extracted from Met and inserted into Cys via the combined activities of the Met-cycle and the transsulfuration pathway (Mosharov et al., 2000; Eriksson et al., 2015). This provides Cys for translation and for S-metabolite synthesis, including GSH synthesis. Remarkably, synthesis of GSH using the Met-derived S is the only source of cytosolic disulfide reducing power detected in these livers. The newly synthesized GSH is used in single-turnover reactions, and the oxidized product, GSSG, which cannot be reductively recycled in these livers, is excreted (Eriksson et al., 2015). Thus, just like yeast have been shown to use GSSG export to sustain reduced GSSG : GSH pools during Gsr insufficiency (Morgan et al., 2013), it is likely that GSSG export is also maintaining the reduced status of the TrxR1/Gsr-null hepatocyte cytosol. The major GSSG exporters in mammalian cells are members of the ATP-binding cassette (also called multiple drug resistance) family of transporters (Keppler, 2011), yet their specific roles in TrxR1/Gsr-null hepatocytes remain untested. Although transit of S from Met to GSH had previously been shown to account for roughly half of the S in hepatocellular GSH pools (Mosharov et al., 2000), it was not predicted that flux through this pathway in the absence of Gsr activity could be sufficient to provide all S and concurrently support all cytosolic disulfide reductase requirements in cells.

Cross-trafficking of disulfide reducing power between cytosolic systems

It has remained unclear how effectively reducing power can transit between the mammalian GSH and Trx1 systems. Biochemical studies showed that GSH + Grx2c, a cytosolic isoform of the normally mitochondrial Grx2 protein, could reduce Trx1-

disulfide, and TrxR1 could reduce Grx2c-SSG, an oxidized form of Grx2c (Du et al., 2012; Sengupta and Holmgren, 2014). The mouse models described above have provided a useful system to investigate cross-trafficking. For instance, yeast cultures cannot support S phase RNR activity without Trx1 (Nordlund and Reichard, 2006). This led to the speculation that in TrxR1-null mammalian models, reducing power likely cross-traffics from GSH to Trx1 to support RNR. To test this, our team generated a floxed allele of Trx1, which revealed that liver development, growth, function and regeneration are retained in Trx1⁻, Trx1/TrxR1⁻ and even Trx1/TrxR1/Gsr-null livers (Prigge et al., 2017). Interestingly, using genetically modified mouse-derived primary fibroblast models, we also showed that Trx1 can provide an oxidative stress survival advantage in the absence of both TrxR1 and Gsr, suggesting that reducing power very likely does cross-traffic from GSH to Trx1 in cells (Prigge et al., 2017). To date, it remains unclear how Met-sulfoxide is repaired in cells lacking Trx1. One possibility is that another as yet unidentified redoxin, perhaps a Trx- or Grx-family member, can support Msr activity. Importantly, however, Trx1/TrxR1-null livers are also viable (Prigge et al., 2017), so if these cells are able to support Msr activity, the underlying redoxin would need to be able to obtain its disulfide reducing power from GSH. Alternatively, it is possible that Met-sulfoxide is simply not repaired in these livers. In this case, proteolytic turnover of the oxidized proteins might be the only route of replacing damaged Met-sulfoxide residues in critical sites. Further investigations will be required to understand how Trx1/TrxR1-null livers tolerate Met-sulfoxide and whether Msrs participate in this process.

Redox perspective on liver physiology

The liver is a major contributor to intermediary metabolism, glucose homeostasis, lipid and sterol metabolism and turn-over, energy storage and detoxification (Rui, 2014). It produces most of the proteins in blood plasma and the bileacids that aid digestion (Miller et al., 1951; Russell, 2003). Many of these activities impinge on the disulfide reductase systems. Proliferative regeneration requires RNR activity; detoxification often consumes GSH; protein synthesis and excretion require S-amino acids and ER-mediated protein maturation; and in mice, bile acids are usually conjugated to the S-containing molecule taurine, which is synthesized from Cys. Moreover, the high metabolic and respiratory rates of liver will challenge the cytosolic and mitochondrial antioxidant systems. Experimentally, the liver's large size (~5% of body mass) and the fact that it is predominantly composed of hepatocytes, which constitute ~95% of the organ's mass, supports biochemical and enzymatic analyses as well as surgical procedures. Powerful transgenic, transduction and transfection tools facilitate hepatocyte genetic manipulations and cell lineage tracing in liver, and reliable protocols exist for inducing proliferative regeneration, sterile oxidative stress, metabolic diseases, inflammatory conditions, cholestatic injuries and cancers in liver (Michalopoulos, 2017). Above, we covered roles of the disulfide reductase systems in basal biosynthetic, homeostatic, antioxidant or repair activities. However, disulfide reductase systems also modulate hepatic responses and adaptations to extracellular signals through redox signalling, as discussed below. One major function of liver is the regulated storage and release of glucose, which is under the control of the pancreatic hormone insulin. Hepatic insulin signalling provides a paradigm for H₂O₂-mediated redox signalling. On hepatocytes, the insulin receptor associates with

anNADPH-oxidase4 (Nox4) complex on the cell membrane. Insulinbinding induces both autophosphorylation of the receptorand Nox4 activity, the latter of which causes transient localized intracellular H₂O₂ accumulation (Mahadev et al., 2004). H₂O₂ inactivates local protein tyrosine phosphatases (PTP) by Cys-oxidation in the enzyme active site.

However, both the levels of H₂O₂ and the longevity of PTP inactivation are antagonized by the Trx1 system (Dagnell et al., 2013; Dagnell et al., 2017). Thus, Trx1-fuelled Prxs reduce the H₂O₂ and Trx1, itself, reduces the PTP active site. Reduction of the oxidized Prx also oxidizes Trx1, which may hinder reactivation of PTPs and further contribute to extending the signalling duration (Brown et al., 2013). The balance between H₂O₂ production versus Trx1-driven remediation determines the longevity of receptor phosphorylation and, therefore, of signalling by the activated receptor (Mahadev et al., 2004). These activities occur downstream of hormone-receptor binding and can influence, or perhaps even supersede, the normal outputs of receptor binding. ROS, in particular H₂O₂ but likely in other species in some situations, for example, HOCl from inflammatory responses, influence many cellular signalling networks, so conditions that increase levels of intrinsic oxidative stress can result in impaired signal transduction (Sies et al., 2017). The combination of compromised antioxidant defences and increased oxidative stress are hallmarks of insulin resistance, which is the predominant symptom of type 2 diabetes. Similar mechanisms of signal modulation are expected to occur in all situations wherein a balance between ROS and Trx1 activity might influence the activity of a PTP that, in turn, affects receptor phosphorylation. Other examples include modulation of growth factor signalling, liver regeneration and bile acid production

(Winterbourn and Hampton, 2015; Michalopoulos, 2017; Dagnell et al., 2018). Another means by which ROS influence signalling is through the formation of disulfide bonds, thereby causing either direct or indirect modulation of target protein activities.

Importantly, however, this seemingly simple redox reaction is irrelevant in cells and, therefore, must be the product of more complex mechanisms. Oxidation of a Cys-thiol by H_2O_2 often leads to the formation of a disulfide; however, except in the case of exceptionally reactive Cys residues, for example, in the active site of some enzymes, this reaction is typically too slow ($\sim 10^0 \text{ M}^{-1}\text{s}^{-1}$) to have general importance. More-over, the relatively high reactivity ($\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$) and concentrations ($\sim \text{mM}$) of Prxs in the cytosol will effectively eliminate H_2O_2 before it can react with less reactive or less abundant thiols (Nagy et al., 2011; Winterbourn and Hampton, 2015). Thus, Cys oxidation as a regulatory mechanism within the context of redox signalling likely requires facilitated thiol oxidation, which can occur through several mechanisms. These are (i) ROS bursts in ‘privileged’ situations; (ii) primary oxidation by peroxidase-resistant species; and (iii) catalytically mediated oxidation.

(i) ROS bursts in ‘privileged’ situations. In the example of H_2O_2 -mediated control of insulin signalling above, two factors contribute to redox signalling. First, the H_2O_2 is generated in a concentrated burst, stimulated by receptor binding, that generates a localized pocket of very high $[\text{H}_2\text{O}_2]$. This increases the burden on the local Prxs, possibly to the point of inactivation via sulfinic acid formation (Wood et al., 2003), and it increases the probability of oxidizing other less reactive thiols. Second, the Cys of the PTP active site is already more reactive than most structural Cys thiols, thereby

increasing the reactivity rate with H_2O_2 (Winterbourn and Hampton, 2015; Dagnell et al., 2018).

(ii) Primary oxidation by peroxidase-resistant species. NO was first recognized as a gaseous signalling molecule for its activities in vasodilation (Ignarro et al., 1981). Cellular NO is produced via the NADPH-dependent consumption of L-arginine by the three NOS: endothelial NOS, inducible NOS and neuronal NOS. Activated NO species (e.g. NO^+ , ONOO and Fe^{3+}NO) react with reduced thiols to form S-nitrosothiols (SNO). The stability of –SNO moieties is regulated by the TrxR1 system (Nikitovic and Holmgren, 1996; Benharet al., 2008). Both Trx1 and the Trx-related protein of 14 kDa (TRP14) denitrosylate S-nitrosothiols (Pader et al., 2014). Importantly, however, Cys-SNO modifications can also readily resolve spontaneously with a vicinal thiol to generate a disulfide and HNO (Gupta and Carroll, 2014). Thereby, in some situations NO-mediated processes might be more effective than H_2O_2 -mediated processes in generating protein disulfides for redox signalling.

(iii) Catalytically mediated oxidation. Recent studies have uncovered catalytic thiol-oxidation by H_2O_2 , in which a predominating deterrent to oxidation, the Prxs, are proposed to play the role of ‘turncoat’ and, instead, mediate targeted oxidation (Figure 3). The high reactivity of the Prx thiol causes it to react with H_2O_2 in preference to nearly any other cellular thiol (see above), resulting in a Prx-SOH. Pioneering studies in yeast showed that peroxidases can transduce redox equivalents to target proteins for H_2O_2 -responsive transcription factor activation (Ross et al., 2000; Delaunay et al., 2002). In mammalian cells, H_2O_2 -Prx-transcription factor relays underlie Trx1-dependent redox

regulation of several gene regulatory pathways (Sobotta et al., 2015; Stocker et al., 2018).

It is anticipated that the paradigm set by these mechanisms will help explain, at least conceptually, many of these other regulatory processes as well.

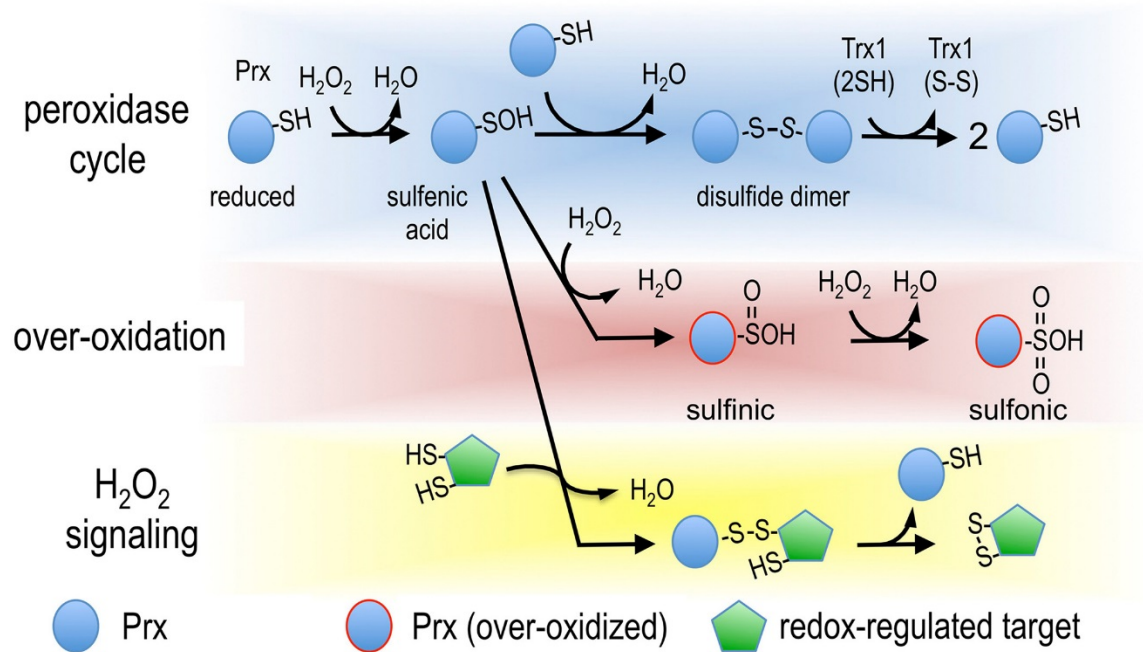


Figure 2.3 Prxs in catalysis, over-oxidation and signalling. Peroxidase activity, over-oxidation (loss of peroxidase activity) and signalling are each likely to be initiated by the rapid reaction between H_2O_2 and a reduced Prx (blue circle) to form a sulfenic acid. In the catalytic cycle (top, blue panel), the reaction of the SOH with a ‘resolving’ Cys on a second reduced Prx yields an intermolecular disulfide and H_2O , and the disulfide is subsequently reduced by Trx or Grx. In severe oxidative stress (middle, red panel), it is proposed that the sulfenic acid can be over-oxidized by H_2O_2 , yielding sulfinic or sulfonic forms respectively. The sulfinic form can be repaired by sulfiredoxin to a thiol, whereas the sulfonic form is considered irreparable (Biteau et al., 2003). The over-oxidized forms, however, might have other functions in cells (Chae et al., 2012). In signalling (bottom, yellow panel), Prx-SOH is proposed to induce a disulfide on a redox-regulated target protein (green pentagon), such as STAT3.

Other protein S-modifications can influence signaling and participate in redox signalling. Similar to S-nitrosylation, hydrogen sulfide (H_2S) and its thiol adduct, S-persulfidation (S-sulfhydration, -SSH), participate in redox signalling (Nagy, 2015). Like

NO, H₂S is produced in cells. H₂S and Cys thiols are in the same oxidation state and will not react (Figure 1). Instead, protein-SSH is formed through the reaction of a thiol with sulfane-S or the reaction of H₂S with an oxidized S (disulfide, SOH, SNO or SSG) (Greiner et al., 2013; Mishanina et al., 2015). Additionally, H₂S has been shown to react with both NO and H₂O₂ to form highly reactive intermediates capable of either persulfidation or nitrosylation/oxidation (Cortese-Krott et al., 2014; Garai et al., 2017). Cys-SSH easily transfers the persulfide to other thiols and can be reduced by GSH or the TrxR1 system (Doka et al., 2016). Intriguingly, reduced Trx1 is required to activate 3-mercaptopyruvate sulfurtransferase (MPST), a major producer of H₂S, indicating that the TrxR1 system is responsible for both production and reduction of protein-SSH species (Nagahara et al., 2007). Cellular H₂S is produced primarily by the transsulfuration enzymes cystathionine- β -synthase (CBS) and cystathionine γ -lyase (CSE) (Figure 4) and during Cys metabolism by MPST (Nagy et al., 2014). A recent study from Akaike's team also showed the formation of Cys-SSH by the mitochondrial Cys tRNA synthetase, CARS2 and the subsequent co-translational incorporation of CysSSH and CysSSSH into proteins (Akaike et al., 2017). To date, the source of the Cys-SSH for this mechanism remains uncertain, as Cys-SH and H₂S will not react directly (see above). Moreover, it is presumed that most protein-SSH groups would then need to be reduced by GSH or the TrxR1 system to achieve a thiol-status. These compelling findings expand the possible sources of cellular protein persulfidation but also raise more questions regarding the oxidation state of newly synthesized proteins. The reader is directed to other reviews in this issue for further information on protein persulfidation. Another protein-S-

modification that can participate in redox regulation is glutathionylation. Whereas, like H_2S , GSH will not react directly with a thiol (see above), it can exchange with disulfide, -SOH, -SNO or -SSH modifications. Moreover, these reactions can occur spontaneously or can be catalyzed by GSH-S-transferases (Gst). The high concentration of GSH (mM-range) and GSTs in cells makes detection of specific regulated protein-SSG events difficult (Ghezzi, 2013). Under oxidative stress, for example, following loss of the NADPH-dependent disulfide reductase systems, a high accumulation of protein-SSG adducts is observed (Prigge et al., 2017), which might have both signalling and protective roles (Ghezzi, 2013). The reader is directed to other reviews in this issue for more detailed presentations of glutathionylation and redox signalling.

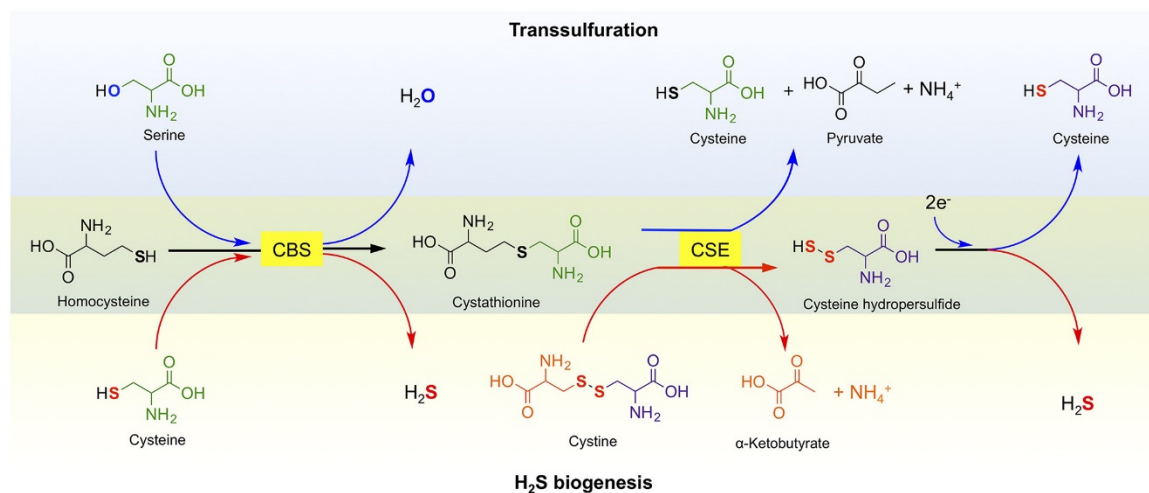


Figure 2.4 Hepatic transsulfuration and H_2S generation. Upper panel (blue) depicts transsulfuration in which homocysteine and serine are converted into Cys and pyruvate. In the lower panel (yellow), transsulfuration of Cys or cystine yields cystathionine and H_2S or α -ketobutyrate and Cys persulfide, respectively, which is converted to Cys and H_2S . Each carbon backbone and reactive S is colour coded, so the fate of each amino acid and each S can be followed.

Conclusions and perspectives

Although the basal cytosolic disulfide reductase systems were characterized in bacteria many decades ago, new functions of these systems are regularly being discovered. Yeast models have revealed important eukaryotic/organelle-specific modifications. Mammalian cell cultures have provided advancements because mammalian disulfide reductase systems evolved new components, activities and responsibilities beyond those in bacteria or lower eukaryotes. However, cell culture models can be problematic for genetic analyses, and they lack systemic interactions. Beginning roughly a decade ago, the development of mouse models that have genetically modified disulfide reductase systems has brought new discoveries to the field. These models have revealed roles in systemic homeostasis, system redundancy, cross-trafficking of reducing power and redox signaling and have allowed these functions to be studied in the context of important diseases. The genetically modified mouse liver, in particular, has attributes that have made it instrumental in these advances, not the least of which is its accessibility for tissue-specific gene conversion. Noteworthy among the discoveries made possible by these mouse models was the recent recognition of the only known NADPH-independent disulfide reductase system that can support cell homeostasis and proliferation. In summarizing what is known about the disulfide redox systems in the liver here, it is our hope that this information will also help enable future discoveries.

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CHAPTER THREE

HEPATOCTE HYPERPROLIFERATION UPON LIVER-SPECIFIC CO-
DISRUPTION OF THIOREDOXIN-1, THIOREDOXIN REDUCTASE-1,
AND GLUTATHIONE REDUCTASE

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Summary

Energetic nutrients are oxidized to sustain high intracellular NADPH/NADP⁺ ratios. NADPH-dependent reduction of thioredoxin-1 (Trx1) disulfide and glutathione disulfide by thioredoxin reductase-1 (TrxR1) and glutathione reductase (Gsr), respectively, fuels antioxidant systems and deoxyribonucleotide synthesis. Mouse livers lacking both TrxR1 and Gsr sustain these essential activities using an NADPH-independent methionine-consuming pathway; however, it remains unclear how this reducing power is distributed. Here, we show that liver-specific co-disruption of the genes encoding Trx1, TrxR1, and Gsr (triple-null) causes dramatic hepatocyte hyperproliferation. Thus, even in the absence of Trx1, methionine-fueled glutathione production supports hepatocyte S phase deoxyribonucleotide production. Also, Trx1 in the absence of TrxR1 provides a survival advantage to cells under hyperglycemic stress, suggesting that glutathione, likely via glutaredoxins, can reduce Trx1 disulfide in vivo. In triple-null livers like in many cancers, deoxyribonucleotide synthesis places a critical yet relatively low-volume demand on these reductase systems, thereby favoring high hepatocyte turnover over sustained hepatocyte integrity.

Introduction

Cell homeostasis requires cytosolic disulfide reduction reactions. For example, the activities of most cytosolic proteins require maintenance of the thiol state of their cysteine (Cys) residues. When these oxidize into disulfides or other modifications, they must be reduced back into thiols. Also, reduction of disulfide bonds drives the catalytic

cycle of ribonucleotide reductase (RNR) in generating deoxyribonucleotide triphosphates (dNTPs) for DNA synthesis (Holmgren and Sengupta, 2010, Thelander and Reichard, 1979).

NADPH generated from NADP⁺ via intracellular oxidation of energetic nutrients provides the reducing power for most anabolic, homeostatic, and cytoprotective reduction reactions. However, only two cytosolic enzymes can channel reducing power from NADPH into disulfide reduction reactions: thioredoxin reductase-1 (TrxR1) and glutathione reductase (Gsr) (Arnér and Holmgren, 2000, Couto et al., 2016). TrxR1 reduces the active site disulfide in oxidized thioredoxin-1 (Trx1) into a dithiol. Gsr reduces glutathione disulfide (GSSG) into 2GSH, which can reduce oxidized glutaredoxins (Grxs, also called Glrxs). Trx1 and reduced glutathione (GSH)/Grx distribute this reducing power to substrates, including RNR, peroxidases, protein disulfides, and low-molecular-weight disulfides (Arnér, 2009, Holmgren, 1985, Laurent et al., 1964).

Highlighting the fundamental activities of NADPH-dependent disulfide reductase systems, *E. coli* coincidentally lacking both thioredoxin reductase and glutathione reductase are non-viable (Feeney et al., 2011). By contrast, we recently showed that TrxR1/Gsr-null mouse livers, which have the equivalent deficiency, sustain redox homeostasis using what is, to date, the only known constitutive NADPH-independent cytosolic disulfide reductase system (Eriksson et al., 2015). In this system, the reduced sulfur in methionine (Met) is transferred via the Met cycle and transsulfuration (in combination, MTS) onto Cys without changing its redox state. Besides providing an

essential source of Cys for protein and coenzyme A synthesis, this Cys source is used for de novo GSH synthesis, thus putting the Met-derived reduced sulfur into a molecule that can support cytosolic disulfide reductions (Eriksson et al., 2015). Despite the predicted inefficiency of this pathway, lifespans of mice with TrxR1/Gsr-null livers can exceed 20 months.

The extent to which reducing power can be exchanged between GSH and Trx1 systems remains incompletely defined (Arnér, 2009). Mammalian TrxR1 is homologous to Gsr (Novoselov and Gladyshev, 2003), yet TrxR1 cannot reduce GSSG and Gsr cannot reduce Trx1 disulfide (Arnér, 2009). Nonetheless, TrxR1 can directly reduce cytosolic Grx2c disulfide in vitro (Johansson et al., 2004), Grxs can reduce Trx1 disulfide (Zhang et al., 2014), and in some conditions reduced Trx1 can reduce GSSG (Kanzok et al., 2001). In yeast, Trx1 is essential for maintaining S phase dNTP pools (Camier et al., 2007, Koc et al., 2006), suggesting that GSH/Grx in the absence of Trx1 cannot fully support RNR. In mammals the need for Trx1 is less clear. Trx1-knockout mice are embryonic lethal (Matsui et al., 1996); however, TrxR1-null embryos are also non-viable (Jakupoglu et al., 2005), yet this is associated with defects in embryonic patterning, not in cell proliferation or other basal cellular processes (Bondareva et al., 2007). Indeed, since both TrxR1-null and TrxR1/Gsr-null livers exhibit robust physiology and normal replication (Eriksson et al., 2015, Prigge et al., 2012), it is clear that enzymes including RNR can obtain adequate reducing power without the hepatocytes being able to use NADPH/TrxR1 to reduce Trx1. Either this reducing power must be coming directly from

GSH/Grx or it must be cross-trafficked from the GSH system to Trx1, thereby maintaining the reduced state of Trx1 in the absence of TrxR1.

To distinguish between these possibilities, we here developed a floxed allele encoding Trx1 (Txn1fl). Mice having homozygous liver-specific disruption of this allele, as well as all combinatorial disruptions of hepatic Trx, TrxR1, and Gsr, proved to be viable. Pathway analyses revealed that, much like the strategy utility companies use to maintain a robust electrical power grid, mammalian cells have a robust disulfide-reducing power grid that diversifies re-routing possibilities to favor redox homeostasis. Most surprisingly, the predominant response to major disruptions in the hepatocyte cytosolic disulfide systems is adoption of a seemingly cancer-like phenotype that includes dramatic hyperproliferation and rapid cell turnover. This is discussed in the context of cellular reducing power requirements and cell survival strategies.

Results

Development and Survival of Mice with Trx1-, Trx1/TrxR1-, Trx1/Gsr-, and Trx1/TrxR1/Gsr-Null Livers

No previous studies have shown in vivo evidence of TrxR1-independent Trx1 activity, suggesting that TrxR1-null cells should be functionally Trx1 null. Since TrxR1-null cells and livers are generally robust (Mandal et al., 2010, Peng et al., 2014, Suvorova et al., 2009), we investigated whether Trx1-null livers were also viable. A floxed allele of Txn1 (Txn1fl), encoding Trx1, that becomes protein null following Cre-mediated recombination was developed (Figures 1A–1C). Germline disruption, like for the original Txn1-knockout allele (Matsui et al., 1996), was recessive lethal in pre-implantation

embryos (data not shown). Using *Txn1^{fl}*, we generated homozygous liver-specific *Trx1*-null mice (Figure 1D, lanes 5 and 6), revealing that *Trx1* is dispensable for overtly normal liver development and function. We then generated mice with all possible combinations of liver-specific disruptions of *Trx1*, *TrxR1*, and *Gsr*. Remarkably, mice survived through adulthood with each of these combinations, including even the liver-specific triple-homozygous *Trx1/TrxR1/Gsr*-null combination (see below).

Liver histology in each of the single-homozygous mutations was similar to that of wild-type (WT) controls (Figure 1E, top). By contrast, double- and triple-null livers showed a range of histopathologies, including mild inflammation, ductular reactions, cell death (including both feathery and ballooning hepatocytes, individual necrotic hepatocytes, and intermittent multi-hepatocyte necrotic foci), intermittent presence of giant Langhan's macrophages, and heteromorphic or hypertrophic hepatocytes (Figure 1E, bottom, and see below). Livers of double- and triple-null combinations were 2- to 3-fold larger per body mass than WT livers (Figure 1F), and necrotic foci were often visible from the surface (e.g., Figure 1G, green arrows). Immunoblots verified the hepatic protein-null status for each allele (Figure 1H). Residual *TrxR1* or *Trx1* protein in *TrxR1*- or *Trx1*-null condition, respectively, was from non-hepatocyte liver cell types, as shown previously for *TrxR1*-null livers (Eriksson et al., 2015, Suvorova et al., 2009). Levels of several other key redox proteins were also assessed. Mitochondrial *Trx2* was modestly lower in livers lacking either *Gsr* or *Trx1*; *Grx1* was modestly elevated in *TrxR1*- or *TrxR1/Gsr*-null livers; and the *Trx*-related protein of 14 kDa (TRP14) was subtly increased in *TrxR1/Gsr*- and *Trx1/TrxR1*-null livers. Curiously, levels of peroxiredoxin-2

(Prdx2 and Prx2), which requires reducing power from Trx1 for activity, were elevated in all genetic combinations lacking Trx1 (Figure 1H). Trx1/TrxR1-null livers also showed a small increase in mitochondrial TrxR2 (black asterisk in Figure 1H).

Because both the NADPH + Gsr system and the Met + MTS system traffic reducing power via GSH (Eriksson et al., 2015), we measured GSH system enzyme activities in representative liver lysates. Most genotypes had similar total glutathione levels, although these were 2-fold elevated in lysates from livers lacking Trx1, and Grx activity was subtly elevated in TrxR1- and TrxR1/Gsr-null livers, but not in livers lacking Trx1 (Figure 1I). Genotypes lacking Gsr showed no GSSG reductase activity using either NADPH or NADH (Figures 1I and 1J), verifying they did not contain a cryptic GSSG reductase activity.

The histopathologies associated with the double- and triple-null combinations suggested mice with these combinations might exhibit liver disease or dysfunction. As compared to sera from WT controls, mice with TrxR1/Gsr-null livers showed significantly elevated serum markers of liver damage, including alanine aminotransferase, aspartate aminotransferase, γ -glutamyltransferase, alkaline phosphatase, and total bile acids (Table 1). They also showed significantly elevated levels of cholesterol; however, other markers of hepatic function, including serum glucose, protein, albumin, and bilirubin contents, did not differ significantly from those in WT controls, and serum immunoglobulin and urea contents were also normal (Table 1). This suggests that, despite having substantial chronic liver damage, maintenance of

intermediary hepatic functions in mice with TrxR1/Gsr-null livers is remarkably unaffected.

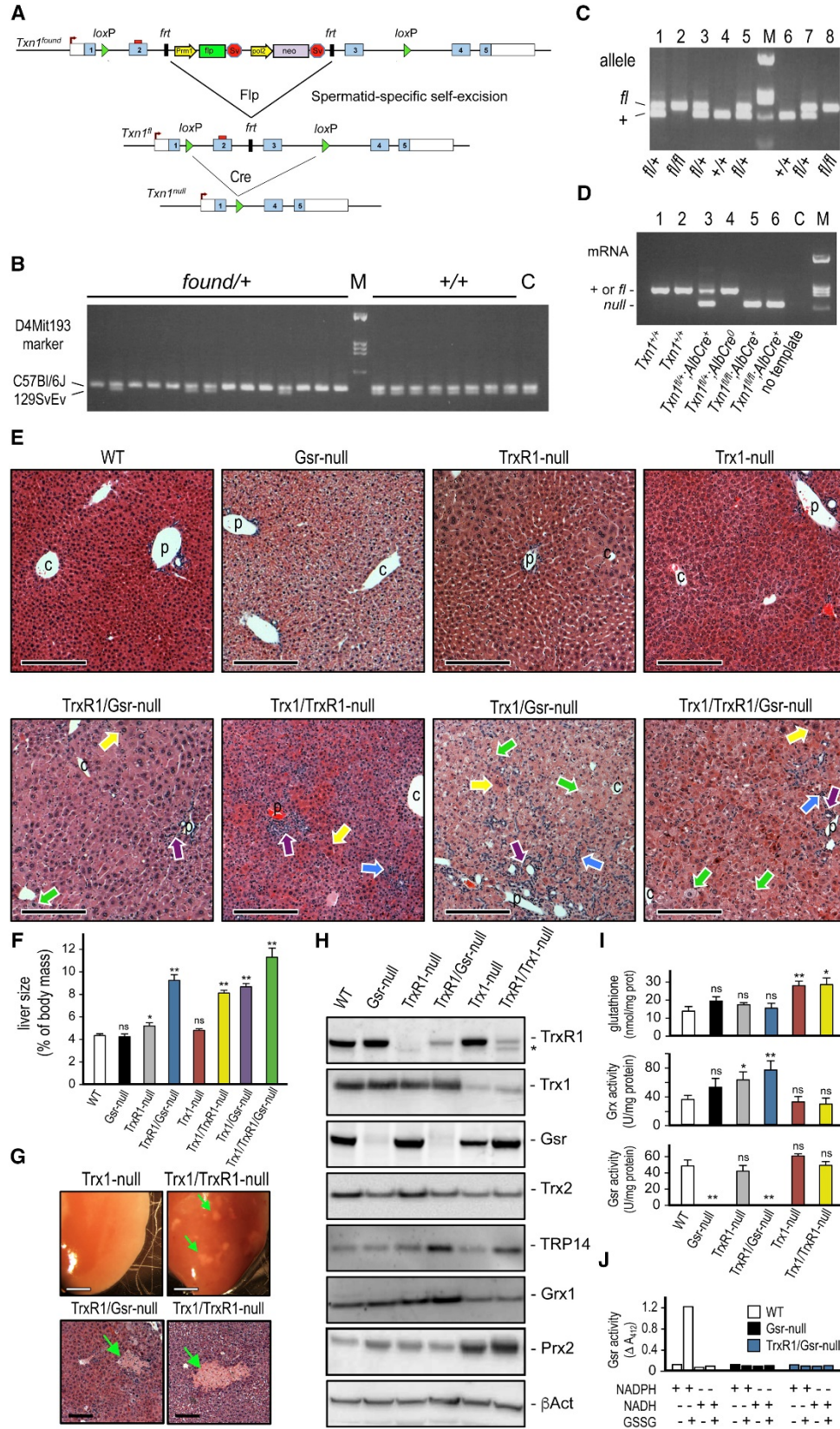


Figure 3.1 Development of Mice with Liver-Specific Co-disruptions of Trx1, TrxR1, and Gsr

(A)Txn1 fl allele. The targeting vector generating the founder allele (Txn1 found) contained a self-excising Flp/neo cassette that matures into the floxed allele (Txn1 fl) in male germ cells. Cre recombination generates Txn1 null. Details are in the Supplemental Experimental Procedures.

(B) Chromosomal targeting. C57BL/63129 Sv Ev hybrid embryonic stem cells (ESCs) were used. Founder pups (chimera3 C57BL/6) that either did (left) or did not (right) contain the Txn1 found allele were analyzed for D4Mit193 (17.9 cM from Txn1), distinguishing C57BL/6 and 129SvEv chromosome 4. Most pups with Txn1 found were homozygous for the C57BL/6 D4Mit193 marker with ratios reflecting meiotic recombination frequencies, indicating the allele had targeted the C57BL/6 chromosome. M, marker; C, WT ESC control.

(C) Mendelian representation of WT (+) and Txn1 fl alleles in genomic DNA from F1 sib-matings.

(D)Conversion of Txn1 fl to Txn1 null in liver by AlbCre and survival of mice with Trx1-null livers. Total RNA from adult livers was analyzed for the presence of mRNA from the WT or Txn1 fl alleles (molecularly identical) versus from the Txn1 null allele by RT-PCR. Lanes 5 and 6 show adult mice with Trx1-null livers. C, no template control.

(E) Histology of adult livers of each homozygous combination. Arrows show inflammation (blue), ductular reactions (purple), or hypertrophic (yellow) or degenerating (green) hepatocytes. Scale bars, 400mm. C or P, representative central veins or portal triads, respectively.

(F) Liver size at harvest. Bars depict mean and SEM (*p < 0.05 and **p < 0.01; ns, not significant; versus WT, Student's t test).

(G) Representative macroscopic and microscopic necrotic foci (green arrows) in double-null, but not in single-null, livers. Upper panels, liver lobes; scale bars, 1 mm. Lower panels, H&E-stained histology; scale bars, 200mm.

(H) Immunoblots on liver lysates of representative genotypes. Asterisk denotes mitochondrial TrxR2, which cross-reacts with the TrxR1 antibody.

(I) Levels of GSH system component activities. Graphs depict means and SEM (* $p < 0.05$ and ** $p < 0.01$; ns, not significant; versus WT, Student's t test).

(J) Thiol generation in lysates detected by reaction with DTNB, measured at 421 nm. Reactions contained liver lysates, GSSG, NADPH, or NADH as indicated

Redistribution of Cytosolic Disulfide-Reducing Power

Survival of mice having Trx1/TrxR1/Gsr-null livers indicates mammalian cells have unexpectedly robust means of redistributing cytosolic disulfide-reducing power under diverse conditions. This was further investigated using a short-term synthetic lethality assay in primary fibroblasts (Figure S1) (Suvorova et al., 2009). Cell survival was assessed over a 48-hr period following disruption of MTS with D,L-propargylglycine (PPG), of GSH synthesis with L-buthionine-(S,R)-sulfoximine (BSO) (Eriksson et al., 2015), or under hyperglycemia-induced oxidative stress (Peng et al., 2014) (Figure 2A). WT and Gsr-null cells tolerated each of the challenges. TrxR1- and Trx1/TrxR1-null cells tolerated PPG, but not BSO. TrxR1/Gsr-, Trx1/Gsr-, and Trx1/TrxR1/Gsr-null cells were killed by PPG as well as by BSO, and only Trx1/TrxR1/Gsr-null cells were susceptible to hyperglycemic stress in these conditions (Figures 2A and 2B). These results were mapped onto the cytosolic disulfide reductase pathways (Figure 2C).

The ability of Gsr-null cells to tolerate BSO or PPG (e.g., Figure 2, condition 1) indicated that the NADPH + TrxR1-driven system alone, in the absence of both the NADPH + Gsr- and the Met-driven systems, supported survival. In the Trx1/TrxR1-null fibroblasts, tolerance of PPG (condition 5) indicated that the NADPH + Gsr-driven system, in the absence of both the NADPH + TrxR1-driven and the Met-driven systems,

supported survival. Survival of Trx1/Gsr-, TrxR1/Gsr-, and Trx1/TrxR1/Gsr-null cultures (e.g., conditions 3 and 7) confirmed that the Met + MTS-driven pathway in the absence of both NADPH-dependent systems was sufficient (Eriksson et al., 2015). Thus, survival was supported under all tested conditions in which reducing power could flow to Trx1 from TrxR1 or to GSH from either Gsr or MTS. Cross-trafficking from TrxR1 to GSH/Grx (Johansson et al., 2004) (gray arrow i in Figure 2C) did not support survival (condition 8). Also, the susceptibility of Trx1/TrxR1/Gsr-null, but not TrxR1/Gsr-null, fibroblasts to hyperglycemic stress (condition 10) suggests that, in the TrxR1/Gsr-null condition, Trx1 in the absence of TrxR1 provides protection against oxidative stress. One explanation for this could be that cross-trafficking of reducing power from GSH/Grx to Trx1 (gray arrow ii) supports Trx1 in this role (see the Discussion).

	ALT U/L	AST U/L	GGT U/L	AlkP U/L	BA μm/L	Chol mg/dL	Gluc mg/dL	Prot g/dL	Alb g/dL	Glob g/dL	A:G ratio	Bili mg/dL	Urea mg/dL
Control n = 13 (SEM)	23.23 (2.02)	34.61 (1.59)	0 (0)	194.9 (19.27)	6.62 (3.08)	103.8 (6.69)	290.4 (13.72)	4.48 (0.14)	2.62 (0.08)	1.88 (0.08)	1.42 (0.05)	0.115 (0.018)	21.92 (1.79)
TrxR1/Gsr null n = 13 (SEM)	206.7 (67.60)	198.7 (54.96)	50.31 (18.77)	351.8 (69.49)	112.7 (33.9)	204.9 (28.6)	237.8 (23.63)	4.65 (0.13)	2.55 (0.08)	1.80 (0.07)	1.44 (0.06)	0.109 (0.016)	22.69 (0.91)
Student's t test	0.012 ^a	0.006 ^a	0.013 ^a	0.040 ^a	0.014 ^a	0.002 ^a	0.069	0.452	0.331	0.499	0.781	0.843	0.714

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; AlkP, alkaline phosphatase; BA, total bile acids; Chol, cholesterol; Gluc, glucose; Prot, total protein; Alb, serum albumin; Glob, immunoglobulin; A:G, Alb:Glob; Bili, total bilirubin.
^aStudent's t test $p \leq 0.05$.

Table 3.1. Serum Markers of Hepatic Health and Function

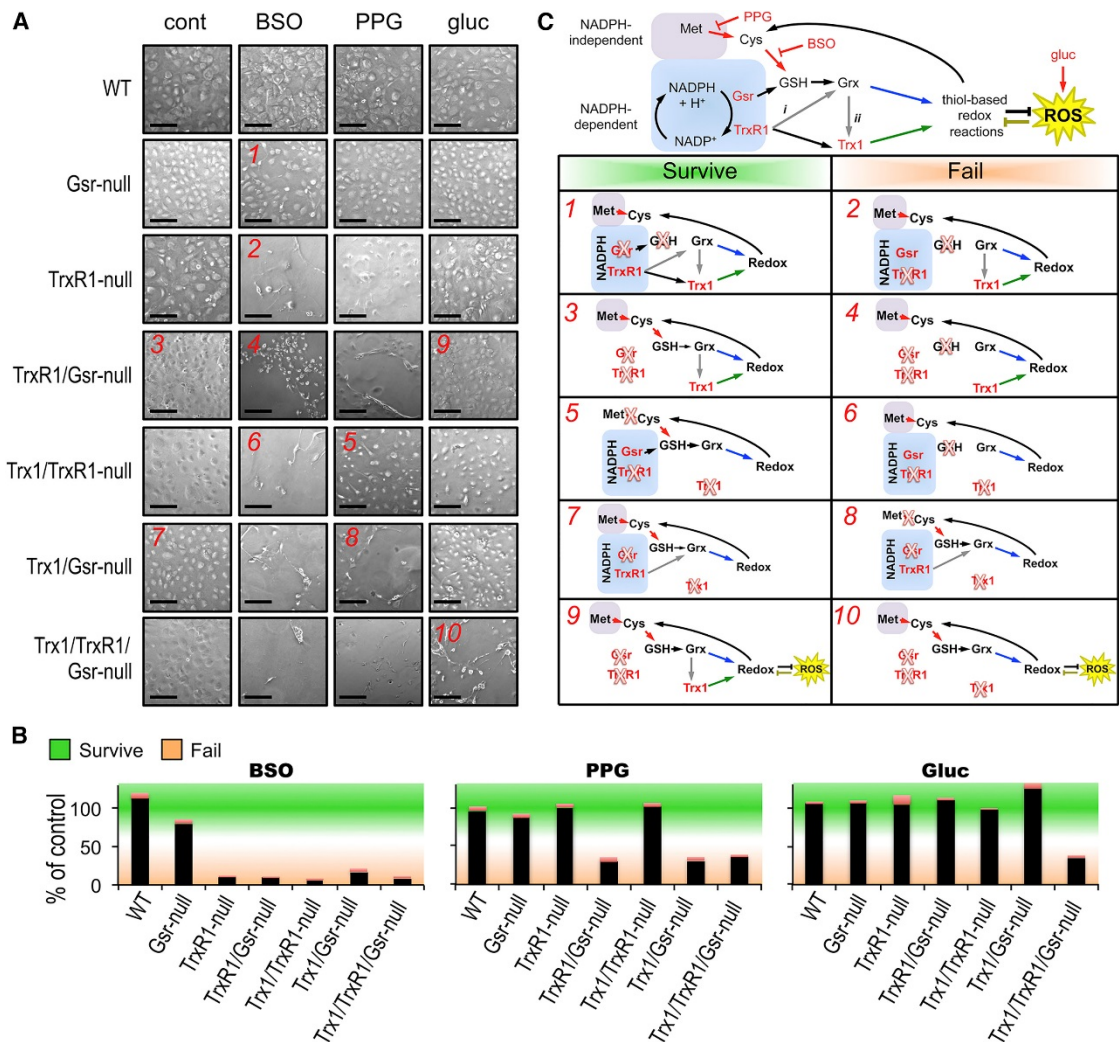


Figure 3.2 Redistribution of Reducing Power among Cytosolic Disulfide-Reducing Systems in Primary Fibroblasts

(A) Synthetic lethality at 48 hr. Stably WT or Gsr-null fibroblast cultures or cultures that had been converted to the indicated genotypes 4 days earlier (see Figure S1) were untreated (cont, standard medium, 5 mM glucose) or treated for 48 hr with 3 mM BSO, 3 mM PPG, or 33 mM glucose, as indicated at top, and photographed. Red numerals in select panels refer to conditions modeled in (C). Scale bars, 200 μ m.

(B) Quantification of cell survival. Adherent live cells were counted on replicate plates as in (A), presented as the percentage of live cells present compared to the untreated control value for that cell line. Bars represent mean and SEM of surviving cells of the indicated

line under the conditions indicated. Green shading and orange shading correspond to values considered to represent cell survival or cell failure under these conditions.

(C) Pathway interpretations. Top diagram depicts reducing power trafficking from NADPH-independent (rose) or -dependent sources (blue). Enzymes or pathways that are disrupted genetically or pharmacologically in (A) are indicated by red font or red arrows, respectively. Yellow star, hyperglycemia-induced oxidative stress; gray arrows, putative cross-trafficking of reducing power from TrxR1 to Grx (arrow i) or from Grx to Trx1 (arrow ii); blue and green arrows, the outputs of the GSH or Trx1 pathways, respectively. Below, the modeling pathway activities and outcomes under key conditions tested in (A) are shown. Assignments of survive or fail (green or orange, respectively) were based on values depicted with the same colors in (B). Red numerals refer to correspondingly labeled conditions in (A). Red Xs denote enzymes or activities that were disrupted in each condition.

Oxidative Stress and Xenobiotic Tolerance in Disulfide Reductase-Deficient Livers

Prxs and glutathione peroxidases require reducing power from Trx1 or GSH, respectively, to support their functions as peroxidases or redox sensors (Brigelius-Flohé and Maiorino, 2013, Sobotta et al., 2015). It was, therefore, expected that livers having deficiencies in their Trx1, GSH, or both systems might exhibit increased steady-state levels of oxidatively damaged macromolecules and would exhibit increased susceptibility to oxidative challenges. To assess basal accumulation of oxidative damage, sections from WT, TrxR1-, or TrxR1/Gsr-null livers harvested from resting adult mice were stained for the posttranslational protein modifications 4-hydroxynonenal (4-HNE), acrolein, or glutathionylation (Brand et al., 2004, Forman, 2016, Shearn et al., 2016, Uchida et al., 1998). Whereas neither WT nor TrxR1-null livers showed substantial staining for any of these antigens (Figures 3A–3H), TrxR1/Gsr-null livers exhibited a checkerboard-pattern of abundant scattered individual hepatocytes with strong staining for protein modification by 4-HNE (Figure 3J) and uniformly strong staining for protein acrolein adducts and

protein glutathionylation (protein-SSG) in most hepatocytes (Figures 3K and 3L, respectively). Curiously, hepatocytes undergoing ballooning cell death (BHs in Figures 3I–3L) showed little staining for either 4-HNE or acrolein, yet they showed dramatic levels of glutathione conjugates. Intense protein-SSG staining was also observed as inclusions within giant Langhan's macrophages (LMs), but not in their surrounding cytosol or nuclei (Figure 3L). These observations suggest that, although TrxR1/Gsr-null hepatocytes accumulate increased macromolecular oxidative damage as compared to WT or TrxR1-null hepatocytes and have a high frequency of BHs undergoing cell death, these terminal cells appear to have cleared 4-HNE and acrolein modifications and instead accumulate protein-SSG adducts. Following cell death, BH cells are likely engulfed by the macrophage precursors to LMs, which then become LMs and exhibit the protein-SSG staining in their phagolysosomes.

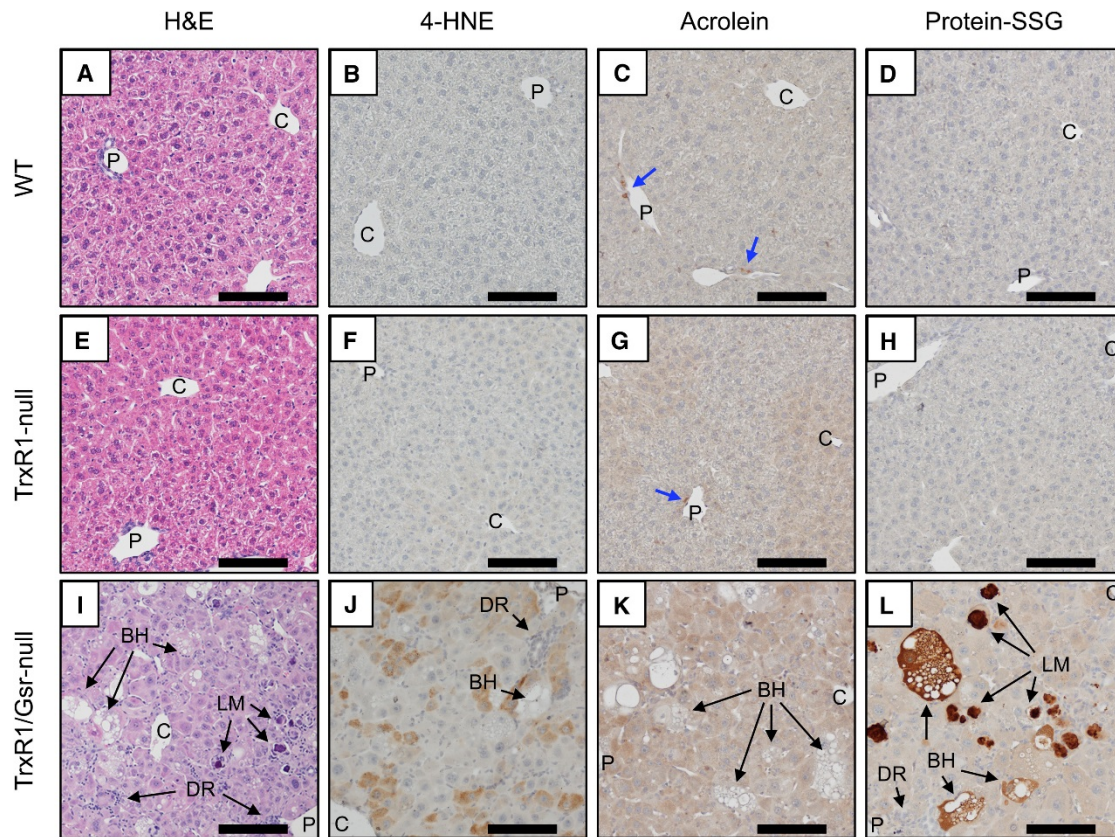


Figure 3.3 Accumulation of Oxidative Damage in Resting Adult Liver

(A–L) Panels show representative images of H&E-stained histology (A, E, and I) or immunostaining for 4-HNE (B, F, and J), acrolein adducts (C, G, and K), or glutathionylated proteins (protein-SSG; D, H, and L) in WT (A–D), TrxR1-null (E–H), or TrxR1/Gsr-null (I–L) livers, as indicated. Blue arrows denote small rare acrolein-positive cells of unknown type, which are present in all genotypes. Abbreviations as in Figure 1; BH, ballooning hepatocyte; DR, ductal reaction; LM, giant Langhans' type macrophage. Scale bars, 200 μ m.

In the absence of normal NADPH-dependent routes of supplying reducing power to the cytosolic antioxidant systems, it was anticipated that endogenous ROS generated as a byproduct of mitochondrial oxidative phosphorylation would be the predominant cause of the oxidative damage and cell death observed in the double- and triple-null livers.

Transmission electron microscopy of livers revealed that all of the mutant liver genotypes

analyzed, including Trx1 and TrxR1 null, which showed no overt pathology, have significantly reduced mitochondrial cross-sectional areas, indicating their mitochondrial networks are composed of finer branches (Figures 4A and 4B, top panel). However, morphometric analyses indicated that total mitochondrial area per cytoplasmic area was similar across genotypes (Figure 4B, middle panel). Also, the levels of mitochondrial citrate synthase (Cs) activity per liver protein, as well as the levels of activity for all five mitochondrial electron transport systems, were similar among all genotypes (Table S1). This further indicated that the total hepatic mitochondrial content and total mitochondrial activity were not substantially affected by these disruptions in the cytosolic disulfide reductase systems. Curiously, compared to WT hepatocytes, the TrxR1/Gsr-, Trx1/Gsr-, and Trx1/TrxR1/Gsr-null hepatocytes also showed a significantly increased abundance of coarse electron-dense bodies (DBs) surrounded by one or more distinct layers of membrane (yellow arrows in Figure 4A and quantified in Figure 4B), as well as coarse electron-dense formations within some mitochondria (yellow asterisks in Figure 4A). The identities of these structures and their relationship to each other are uncertain, although they might reflect mitochondrial fission and mitophagic activities (Khraiwesh et al., 2013, Quist et al., 2015).

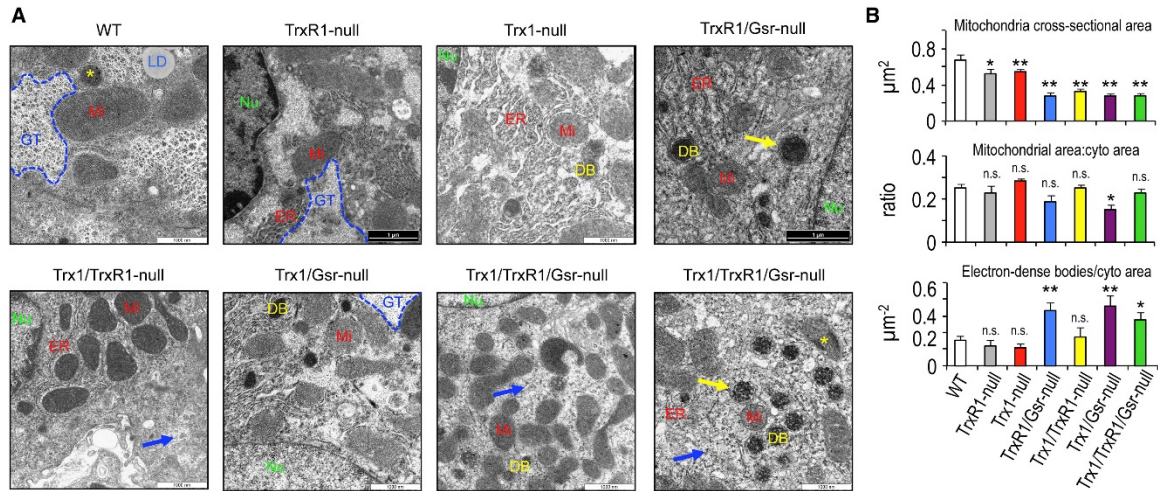


Figure 3.4 Hepatocytic Mitochondrial Ultrastructure

(A) Representative transmission electron micrographs, captured at 5,000 $\times$ magnification. Yellow arrows denote regions where DBs have distinct multilamellar surrounding membranes; yellow asterisks denote Mi containing distinct electron-dense condensations; blue arrows denote glycogen rosettes dispersed outside of GTs. DB, coarsely electron dense bodies; ER, endoplasmic reticulum; GT, glycogen tracts; LD, lipid droplet; Mi, mitochondrion; Nu, nucleus. Scale bars, 1,000 nm.

(B) Quantification of average mitochondrial cross-sectional area (top panel), average total mitochondrial area per cytoplasmic area (middle), or average number of DBs per cytoplasmic area (bottom).

To test whether the reductase-deficient livers, which exhibited already dramatically elevated oxidative damage and hepatocyte death (see above), had increased susceptibility to oxidative challenges, we used a model of hepatic ischemia/reperfusion (I/R) injury-induced oxidative stress (Jaeschke, 2003) on mice with WT, Trx1-, Trx1/TrxR1-, Trx1/Gsr-, TrxR1/Gsr-, or Trx1/TrxR1/Gsr-null livers. Unexpectedly, nearly all animals of all genotypes survived until the 2-week harvest point (Figure 5A, top). The double- and triple-null livers harvested at 2 weeks post-I/R surgery showed

increased necrotic cells and acellular debris (Figure 5A, bottom), but this did not lead to liver failure.

To investigate how reductase system disruptions affected susceptibility to hepatotoxins, we challenged adult male TrxR1/Gsr-null or WT mice with the pro-hepatotoxin acetaminophen (paracetamol, APAP) at doses from zero to 165 mg/kg (LD50 = 338 mg/kg for WT mice). Previous studies showed that disruption of only TrxR1 in mouse liver results in high APAP tolerance, which is associated with increased activities of the pathways for detoxifying APAP, itself, and its cytotoxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Iverson et al., 2013). WT mice showed no response to any of the APAP doses, and, even at the highest dose, their livers revealed no overt histopathology at 24 hr (Figure 5B, top panel). By contrast, at the highest dose (165 mg/kg; $\sim 0.5 \times$ LD50), one of the three mice with TrxR1/Gsr-null livers died in < 20 hr. At the 24-hr harvest, the other two mice with TrxR1/Gsr-null livers challenged at this dose were lethargic and their livers appeared uniformly pale (data not shown). Histology revealed that nearly all hepatocytes in these livers were necrotic (Figure 5B, second panel). Although all TrxR1/Gsr-null mice challenged with 83, 26, or 16.5 mg/kg APAP survived until the 24-hr harvest, following even the lowest dose of APAP ($\sim 0.05 \times$ LD50), histology revealed extensive centrilobular hepatocyte death and sinusoidal congestion (Figure 5B, lower panels). Thus, although TrxR1-null livers are highly resistant to APAP (Iverson et al., 2013) and TrxR1/Gsr-null livers show surprising tolerance to hepatic oxidative stress (Figure 4), the TrxR1/Gsr-null livers are highly sensitive to APAP.

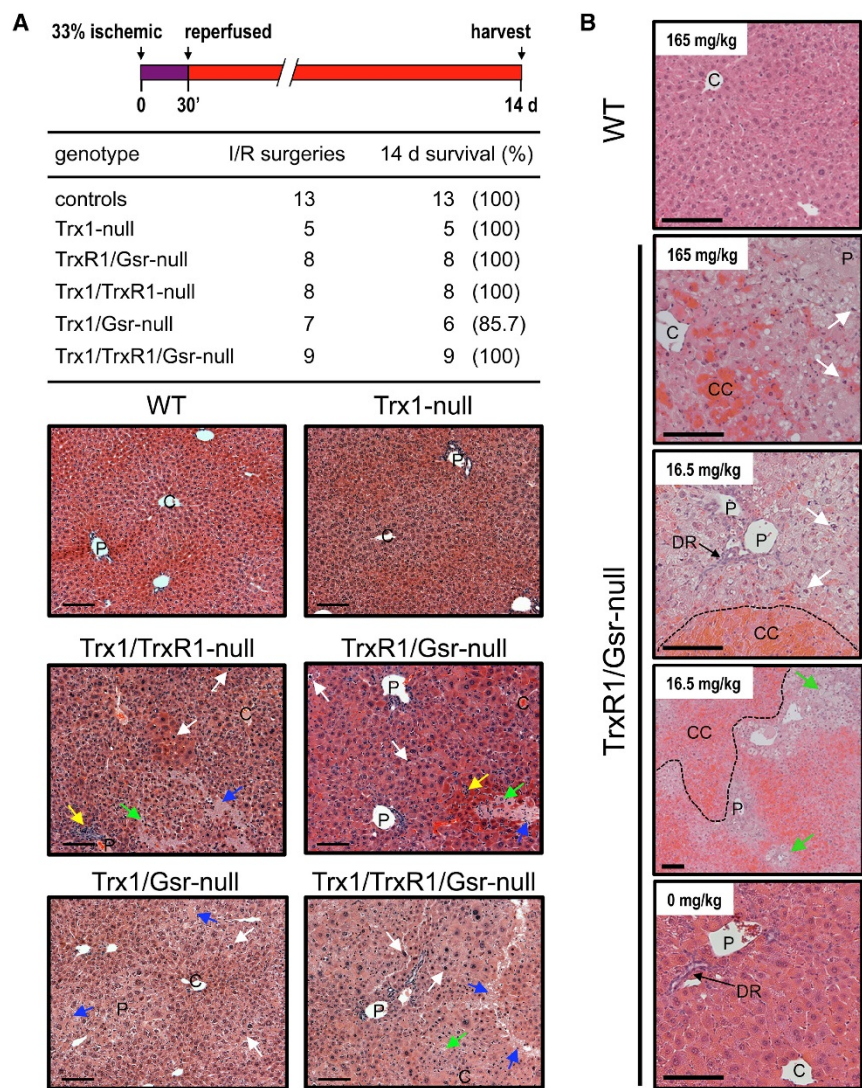


Figure 3.5. Impacts of Acute Oxidative or Hepatotoxic Stress

(A) Acute oxidative stress. Top: experimental strategy for I/R injury model is shown. The left lateral lobe (one-third of liver mass) was occluded for 30 min to induce ischemia; 14-day survival is tabulated below. Bottom: representative liver micrographs at 14 days post-I/R injury are shown (see also Figures S3 and S4). Inflammatory foci, pyknotic cells, necrotic hepatocytes, or post-necrotic acellular debris are denoted by yellow, white, green, or blue arrows, respectively. Scale bars, 200 μ m.

(B) Hepatic acetaminophen toxicity. Adult male mice were challenged with the indicated doses of APAP and harvested 24 hr later for histopathological evaluation. Representative tracts of centilobular congestion (CC) are denoted with dashed lines; other designations are as above. Scale bars, 200 μ m.

Hyperproliferation in Hepatocytes Having Disulfide Reductase System Deficiencies

Because RNR requires disulfide-reducing power and because RNR inhibition arrests proliferative growth (Holmgren and Sengupta, 2010), it was expected that the double- and triple-null livers in this study would have compromised proliferative capacities. However, morphometry revealed that the average hepatocyte volumes in TrxR1/Gsr- and Trx1/TrxR1/Gsr-null livers were ~16-fold greater than those in WT livers (Figure 6A). The volume of hepatocyte nuclei in these livers was similarly increased (Figures 6B and 6D), so there were no substantial differences in the nuclear:cytoplasmic ratios between genotypes (Figure 6C). Most surprisingly, resting adult double- and triple-null livers showed frequencies of proliferatively active hepatocytes, as distinguished by the expression of proliferative cell nuclear antigen (PCNA) and Ki67 antigen, 20- to 300-fold above those in WT livers (Figures 6E, 6F, and S3), and frequencies of hepatocytes in M phase of the cell cycle, as visualized by staining for phospho-histone H3 (PHH3), 7- to 30-fold higher than those in WT livers (Figure 6G). Many hepatocytes in the double- and triple-null livers exhibited ballooning or feathery degeneration (Figure 1E, green arrows), which are forms of programmed parenchymal cell death generally associated with steatohepatitis or cholestasis, respectively (Li and Crawford, 2004, Yip and Burt, 2006). The elevated rates of proliferation and cell death indicate that these livers have exceptionally high hepatocyte turnover rates and survive in a chronic regeneration-like state.

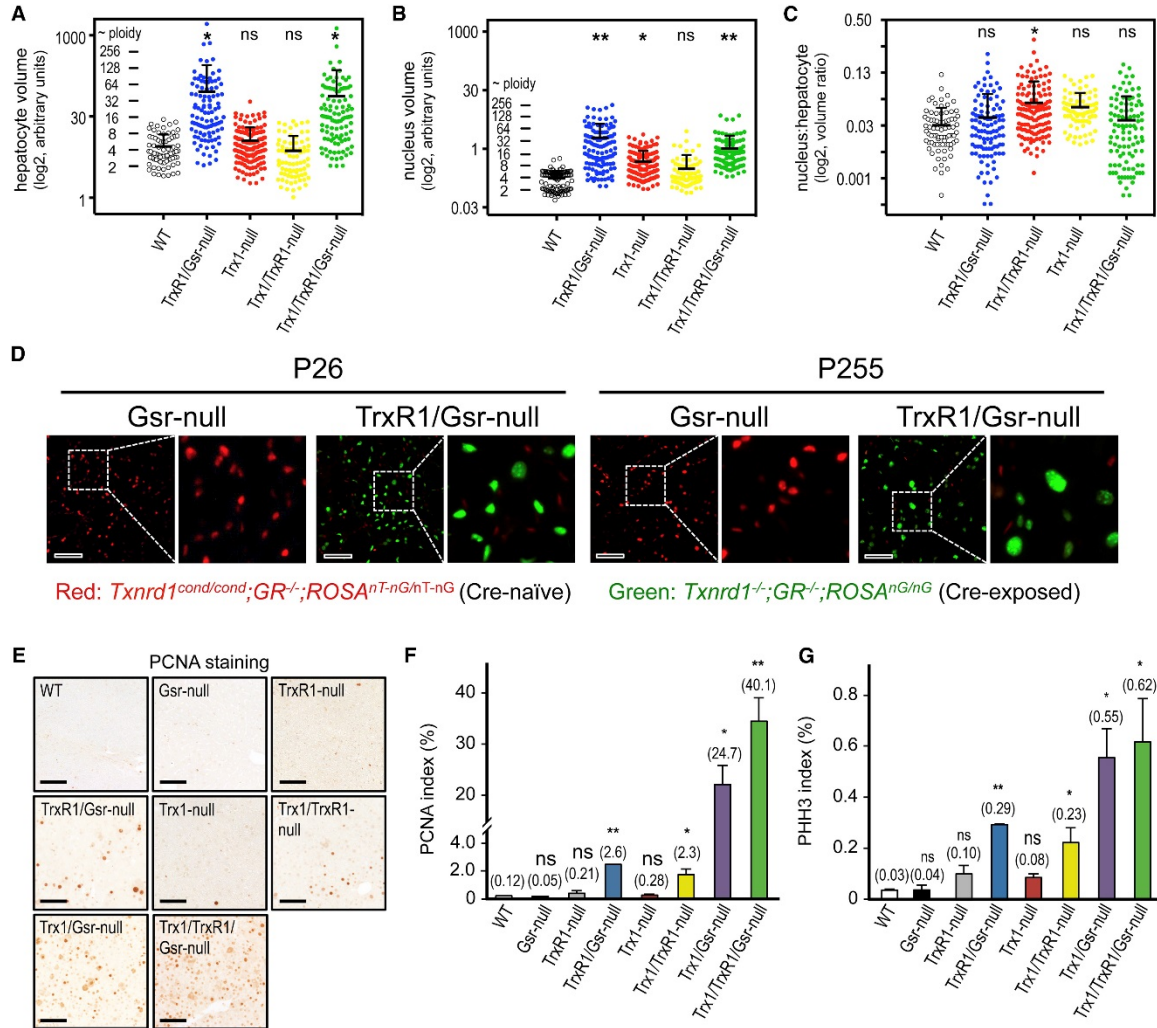


Figure 3.6. Hepatocyte Morphometry and Proliferation

(A–C) Genotype-specific hepatocyte morphometry. Volumetric values are plotted on a log₂ scale. Each point represents a single hepatocyte or nuclei (A and B, respectively). The nuclear:total hepatocyte volumetric ratio for each hepatocyte is plotted in (C). WT livers are predominated by diploid and tetraploid nuclei, as seen by clustering of values for WT livers in (B), and these landmarks were used to assign approximate ploidy values. See the Supplemental Experimental Procedures for details.

(D) Hepatocyte nuclear sizes in juvenile or aged mice. Cryosections of livers from post-natal day 26 (P26) or P255 mice of the indicated genotypes were prepared and imaged by fluorescence microscopy. In TrxR1/Gsr-null mice, hepatocyte nuclei are green and nuclei of all other cell types are red; in Gsr-null mice all nuclei are red. For each condition, a

low-magnification (left; scale bar, 100 μm) and embedded zoomed image pair is shown for nuclei size comparisons. Results show that hyperploidy is already pronounced by P26 in TrxR1/Gsr-null livers (e.g., compare P26 TrxR1/Gsr-null nuclei sizes to P255 Gsr-null nuclei sizes) yet increases further with age.

(E–G) Proliferation in resting adult livers. Representative immunostaining or compiled data for PCNA (E and F, respectively) or PHH3 (G) staining are shown. Graphs depict means and SEM (* $p < 0.05$ and ** $p < 0.01$; ns, not significant; versus WT, Student's t test). Scale bars, 200 μm . See also Figure S4.

Discussion

Survival and proliferation of mouse hepatocytes lacking Trx1 contrasts with the situation in lower eukaryotes in which Trx1 is required to support S phase replication (Camier et al., 2007, Koc et al., 2006). Remarkably, when hepatic Trx1 disruption is combined with the elimination of TrxR1 and Gsr, the predominant phenotype is hyperproliferation rather than a replicative deficiency.

To better understand the paradox of how TrxR1/Gsr- or Trx1/TrxR1/Gsr-null hepatocytes could support increased DNA replication without the use of canonical NADPH-dependent pathways to drive RNR, we estimated the burden that S phase places on the cytosolic disulfide reductase systems (Figures S2A and S2B). This revealed that hepatocytes contain within their steady-state GSH pools roughly three times the reducing power required for RNR to support a replication cycle. Moreover, normal hepatocyte S phase is ~ 7 hr whereas GSH normally has a < 2 hr half-life, so during the S phase the GSH pools chemically turn over several times (Figure S2C). Because half of the sulfur in WT liver GSH pools comes from Met via MTS (Mosharov et al., 2000), the molar consumption rate of Met for GSH synthesis in normal resting hepatocytes is therefore

already several times higher than the molar consumption rate of reducing power by RNR during the S phase (Figure S2C). In TrxR1/Gsr-null hepatocytes using only Met to fuel cytosolic disulfide reduction (Eriksson et al., 2015), an increase of <10% in the rate of GSH synthesis over basal would be sufficient to support S phase RNR activity (Figure S2C). Actual demand could be lower yet if deoxyribonucleoside salvage also contributes (Arnér and Eriksson, 1995). Thus, the burden of S phase RNR activity is quite small compared to the steady-state rate of GSH turnover.

The highly enlarged average cell and nuclear volume in Trx1/TrxR1/Gsr-null hepatocytes (Figures 6A–6D) suggests proliferative hepatocytes in these livers frequently bypass mitosis (endoreplication) to become super-polyploid. More modest super-polyploidy phenotypes occur in hepatocytes of patients or mice with non-alcoholic fatty liver disease (NAFLD) and in normal rodent livers following partial hepatectomy-induced liver regeneration (Gentric et al., 2015, Melchiorri et al., 1993). In the NAFLD models, inefficient progression through S and G2 phases activates a G2/M checkpoint that blocks entry into M phase but can be bypassed, thereby allowing G2-arrested hepatocytes to directly enter S phase (Gentric et al., 2015). This reveals that replication-dependent hepatocyte super-polyploidy can be caused, counterintuitively, by replication inefficiency coupled with G2/M checkpoint activation and mitosis bypass. In the current study, we show that Trx1/TrxR1/Gsr-null hepatocytes can support and sustain dramatically elevated proliferation rates, yet it remains possible that RNR output and replication efficiency in these livers is nevertheless compromised and actually underpins the super-polyploidy phenotype. Further studies will be required to resolve the

mechanisms underlying the cell cycle phenotypes of these hepatocytes and how this program might contribute to long-term survival of mice with Trx1/TrxR1/Gsr-null livers.

The current study shows that liver homeostasis, and therefore survival of animals with hepatocyte defects, follows classic principles of steady-state kinetics and population biology. Thus, because hepatocytes can have variable rates of both production (cell proliferation, equivalent to a synthesis rate) and loss (cell death, a decay rate), organ stability is maintained as long as hepatocyte proliferation and death rates are balanced. The liver genotypes with the highest proliferation indexes in this study also show the most cell death, indicating these livers have rapid hepatocyte turnover despite also maintaining a stable steady-state hepatocyte population. These dynamics are reminiscent of an *r* strategy in population biology's classic *r/K* selection theory, wherein high production of low-fitness progeny allows persistence of the population despite the high individual mortality rates (Wilson and MacArthur, 1967). Intriguingly, cancer cells also frequently have high rates of both proliferation and cell death. Following I/R injury, evidence of hepatocyte death further increased, yet animals of all genotypes recovered and exhibited long-term survival (Figure 5A). This suggests that rates of proliferation must also increase following I/R injury and, indeed, TrxR1/Gsr-null livers are able to proliferatively regenerate following two-third hepatectomy (Eriksson et al., 2015). In the case of post-I/R injury, however, animal-to-animal variance in both resting and post-I/R injury proliferative values precluded detection of an increase in proliferation rates (Figure S3).

Cytosolic disulfide-reducing power can be disseminated via diverse routes in mammalian cells. We previously found that the Met + MTS pathway alone was able to support homeostasis in the absence of the NADPH-dependent systems (Eriksson et al., 2015). In the current study, we show that either NADPH + TrxR1 alone or NADPH + Gsr alone can also support cell survival and that Trx1 in the absence of both TrxR1 and Gsr can provide a survival advantage under some conditions. In the latter case, we infer that the previously described cross-trafficking of reducing power from GSH/Grx to Trx1 (Zhang et al., 2014) underpins this advantage, possibly by allowing Trx1 to support some Prx activity. Interestingly, Trx1-null livers exhibit increased levels of Prx2 protein despite lacking Trx1, the obligatory reducing power donor for Prx activity. This might suggest that a feedback circuit regulates Prx2 protein levels.

In conclusion, mammalian cells are here shown to have a surprisingly robust cytosolic disulfide reductase grid. This grid can be fueled either by reducing power generated intracellularly via the oxidation of energetic nutrients yielding NADPH to power TrxR1 and Gsr, or by reducing power assimilated from extracellular Met to drive de novo NADPH-independent GSH biosynthesis. From any of these sources, the disulfide-reducing power can be redistributed via diverse routes, such that cytosolic disulfide reductase system functions are maintained despite disruption of key components within individual pathways. When the outcomes of these compensatory pathways in liver become insufficient, macromolecular oxidative damage accumulates and cell death increases. However, our results indicate that this increased cell death is compensated for

by increased hepatocyte proliferation rates, which match the increased cell turnover rates and maintain cell population homeostasis.

Experimental Procedures

Mice, Surgeries, and Harvests

All animal procedures presented in this study had been reviewed and approved in advance by the Montana State University Institutional Animal Care and Use Committee (IACUC). Detailed methods are provided in the online Supplemental Experimental Procedures. A floxed allele of the mouse *Txn1* gene, in which exons 2 and 3 are flanked by codirectional loxP sites (*Txn1^{fl}*), was generated on a C57BL/6J background. These mice, as well as mice with the *Txnrd1^{fl}* allele, are publicly available through Jackson ImmunoResearch Laboratories (stocks JAX 030221 and JAX 028283, respectively). Mice with *TrxR1⁻*, *Gsr⁻*, *Trx1⁻*, *TrxR1/Gsr⁻*, *Trx1/TrxR1⁻*, *Trx1/Gsr⁻*, and *Trx1/TrxR1/Gsr*-null livers are all overtly normal as adults and fertile in both genders. Hepatic I/R injury involved 30 min of occlusion of the left lateral lobe (~33% of liver) followed by reperfusion. All analyses shown were done on adult mice (60–90 days of age) of both genders except as specified in the text or figure legends. Animals were maintained on a 14:10-hr light:dark cycle with unrestricted access to feed and water. Except where indicated otherwise, all harvests were performed between 9:00 and 11:30 a.m.

Immunoblotting and Enzymatic Assays

For immunoblotting and enzymatic analyses, snap-frozen liver samples were pulverized with a mortar and pestle, homogenized in ice-cold buffer, and clarified by centrifugation. Immunoblots used 20 µg total protein of the clarified lysate per lane. Antibody sources are specified in the Supplemental Experimental Procedures. Glutathione levels were determined as described previously (Eriksson et al., 2015). Gsr activity was measured using 30 µg protein lysate with the Gsr activity analysis kit (Cayman Chemical 703202; Figure 1I) or the DTNB reduction assay (Sigma GRSA; Figure 1J). Grx activity was measured on 20 µg protein lysates by a fluorescent assay (IMCO FkGRX-01). Measurements of mitochondrial enzymatic activities were performed on liver samples that had been saline-perfused at harvest and snap-frozen in liquid nitrogen prior to shipment on dry ice to the NIH/NIDDK Diabetes Research Center Bio-Analytical Redox Biology Core at the University of Alabama at Birmingham.

Primary Fibroblast Systems

Cultured cells were grown on plastic cell culture wells at 37°C in ambient air using standard medium, consisting of DMEM (Gibco 31885, which includes 5.6 mM D-glucose, 4.0 mM L-glutamine, and 1.0 mM sodium pyruvate) supplemented with 10% fetal calf serum. Procedures for establishing and Cre-converting mouse primary fibroblast cultures were described previously (Suvorova et al., 2009) and are validated in Figure S1. As indicated, cultures were treated for 48 hr with 3 mM BSO (Acros Organics 235520050), 3 mM PPG (Sigma P7888), or with an additional 28 mM D-glucose.

Statistical Analyses and Resource Sharing

Statistical analyses were performed on Excel or GraphPad Prism 7 software.

Graphs show means and SEM. Significance was determined by a Student's t tests versus WT samples. All renewable resources developed in this study are available for unrestricted non-profit use on request unless otherwise restricted by an outside party.

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CHAPTER FOUR

TRXR1, GSR, AND OXIDATIVE STRESS DETERMINE HEPATOCELLULAR
CARCINOMA MALIGNANCY

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Manuscript in Chapter 4

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Contributions: Performed research

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TRXR1, GSR, AND OXIDATIVE STRESS DETERMINE HEPATOCELLULAR
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Significance

Oxidative DNA damage is considered carcinogenic, yet the increased oxidative stress in cancer cells could make these cells critically dependent on endogenous antioxidant systems for survival. Accordingly, activation of the major cellular antioxidant network correlates with increased malignancy in many cancers. This report shows that genetic co-disruption of the two major reductase systems within this network in mouse liver increases oxidative stress yet is apparently neither carcinogenic nor tumoricidal. Instead, chronic lifetime oxidative stress in the liver strongly augments the malignant progression of chemically induced cancers. Thus, the balance between oxidative stress and the endogenous antioxidant network is shown to have a much greater impact on determining malignant progression of cancers than on determining rates of cancer initiation.

Abstract

Thioredoxin reductase-1 (TrxR1)-, glutathione reductase (Gsr)-, and Nrf2 transcription factor-driven antioxidant systems form an integrated network that combats potentially carcinogenic oxidative damage yet also protects cancer cells from oxidative death. Here we show that although unchallenged wild-type (WT), TrxR1-null, or Gsr-null mouse livers exhibited similarly low DNA damage indices, these were 100-fold higher in unchallenged TrxR1/Gsr-double-null livers. Notwithstanding, spontaneous cancer rates remained surprisingly low in TrxR1/Gsr-null livers. All genotypes, including TrxR1/Gsr-null, were susceptible to N-diethylnitrosamine (DEN)-induced liver cancer, indicating

that loss of these antioxidant systems did not prevent cancer cell survival. Interestingly, however, following DEN treatment, TrxR1-null livers developed threefold fewer tumors compared with WT livers. Disruption of TrxR1 in a marked subset of DEN-initiated cancer cells had no effect on their subsequent contributions to tumors, suggesting that TrxR1-disruption does not affect cancer progression under normal care, but does decrease the frequency of DEN-induced cancer initiation. Consistent with this idea, TrxR1-null livers showed altered basal and DEN-exposed metabolomic profiles compared with WT livers. To examine how oxidative stress influenced cancer progression, we compared DEN-induced cancer malignancy under chronically low oxidative stress (TrxR1-null, standard care) vs. elevated oxidative stress (TrxR1/Gsr-null livers, standard care or phenobarbital-exposed TrxR1-null livers). In both cases, elevated oxidative stress was correlated with significantly increased malignancy. Finally, although TrxR1-null and TrxR1/Gsr-null livers showed strong Nrf2 activity in noncancerous hepatocytes, there was no correlation between malignancy and Nrf2 expression within tumors across genotypes. We conclude that TrxR1, Gsr, Nrf2, and oxidative stress are major determinants of liver cancer but in a complex, context-dependent manner.

Introduction

Liver cancer is the third most lethal cancer worldwide. There is a major need to better understand the mechanisms underlying progression of the disease and to identify novel molecular targets for therapy (1). Oxidative stress in hepatocytes has been implicated in liver cancer, but the mechanisms and overall impacts remain uncertain (2, 3). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), which arise

primarily from respiration, metabolism, or inflammation, can cause point mutations or larger genomic lesions (4).

Since mutation underlies carcinogenesis, oxidative stress is considered carcinogenic (5–8); however, H_2O_2 is now also recognized as a signaling molecule that modulates inflammation, proliferation, differentiation, cytoprotection, autophagy, metastasis, and metabolic pathways (9–11). The activities of these pathways can either increase or decrease malignancy in a context-dependent fashion (12). Therefore, H_2O_2 likely can be either procarcinogenic or anticarcinogenic depending on context.

Excess cytosolic ROS is eliminated by peroxiredoxins using reducing power from thioredoxin reductase-1 (TrxR1) or by glutathione peroxidases using reducing power from glutathione reductase (Gsr) (11). In response to oxidative stress, nuclear factor erythroid-derived 2-like 2 (Nrf2), which is normally posttranslationally repressed by Kelch-like ECH-associated protein-1 (Keap1), becomes derepressed and drives expression of components within the TrxR1- and Gsr-driven systems above their basal levels (13). Other Nrf2 target genes encode detoxification, energy metabolism, or other cytoprotective functions (14, 15). Homozygous single disruption of TrxR1, Gsr, or Nrf2 in mouse livers is benign (16–18), yet each of these conditions influences hepatic responses to oxidative stress (18–20). TrxR1 disruption activates Nrf2 (16), and mounting evidence suggests that TrxR1 is a critical participant in the redox signaling mechanism that makes Nrf2 H_2O_2 -responsive (21). Together, the TrxR1-, Gsr-, and Nrf2-driven systems constitute an integrated antioxidant network (21).

Besides safeguarding normal cells from carcinogenesis, the Nrf2 pathway protects cancer cells from oxidative stress, cytotoxic immune responses, radiation therapy, and chemotherapy, any of which might otherwise kill the cancer (22–26). Indeed, many aggressive cancers have an activated Nrf2 pathway (27–30). Thus, the Nrf2 pathway itself can be either procarcinogenic or anticarcinogenic, depending on the context of its activation (21, 31). Similarly, based on its roles in eliminating cytosolic H₂O₂ but also in redox signaling and Nrf2 activation, we hypothesized that TrxR1, like H₂O₂ and Nrf2, might also have context-dependent procarcinogenic or anticarcinogenic activities.

N-diethylnitrosamine (DEN) is a procarcinogen that is activated to yield an ethyl diazonium ion by hepatocyte-specific cytochrome P450s. The reactive ethyl diazonium ion alkylates diverse substrates in the hepatocytes, including DNA (32). DEN treatment of proliferative liver (juvenile or regenerating) causes a high incidence of subsequent hepatocellular carcinoma (HCC) (33–35). Like clinical HCC, the DEN-induced rodent model is initiated by arbitrary mutations in the host's own cells. This provides carcinogenic diversity, and it models cancer progression in an isogenic immune-competent host. Male mice are more susceptible than females to DEN-induced HCC; the sex-specific roles of signaling, cell adhesion, metabolism, and inflammation are still being resolved (34, 36–39). Glutathionylation and glucuronidation, both of which are induced by hepatic Nrf2 activation, participate in DEN detoxification (32, 40, 41).

Here we investigated the formation of either spontaneous or DEN-induced cancers in WT vs. TrxR1-, Gsr-, and TrxR1/Gsr-null livers, to characterize the impacts of the antioxidant systems on tumor initiation or progression. Although TrxR1/Gsr-double-

null livers showed high levels of basal DNA damage, this was associated with a surprisingly low incidence of spontaneous liver cancer. However, our results also show that reductase-deficient livers remained susceptible to DEN-induced carcinogenesis, and that in the postinitiation phase, TrxR1 and Gsr deterred oxidative stress-driven malignant progression.

Results

TrxR1/Gsr-Null Livers Have Chronically Elevated DNA Damage Markers Yet Rarely Develop Spontaneous Cancer.

Although neither TrxR1- nor Gsr-null livers show oxidative stress, mice with double-null (TrxR1/Gsr-null) livers, while still able to sustain long-term homeostasis via a backup system that uses the catabolism of methionine (Met) to support essential GSH-dependent activities (42), accumulate extensive oxidative damage (42, 43). TrxR1/Gsr-null livers also exhibit hepatomegaly, ploidy anomalies, hyperproliferation, hepatocyte death, and inflammation (42, 43). To investigate whether chronic oxidative stress in TrxR1/Gsr-null livers causes DNA damage, we immunostained liver sections for the modified histone γ -H2A.X (44). Our results show low frequencies of γ -H2A.X-positive nuclei in WT, Gsr-null, and TrxR1-null livers (~ 0.1 – 1% of hepatocytes positive), whereas $\sim 40\%$ of all nuclei in resting adult TrxR1/Gsr-double-null livers were γ -H2A.X-positive (Fig. 1). Since genome damage underlies cancer initiation, we expected TrxR1/Gsr-null livers to have a high rate of spontaneous cancers. However, of 32 aged mice (9–14 mo old, standard care) with TrxR1/Gsr-null livers, only 2 (6.3%) had liver

tumors at harvest (Table 1). Whereas no spontaneous liver tumors were found in similar groups of mice with WT, Gsr-null, or TrxR1-null livers, in considering the high chronic level of damage in TrxR1/Gsr-null livers, their incidence of spontaneous tumors was unexpectedly low.

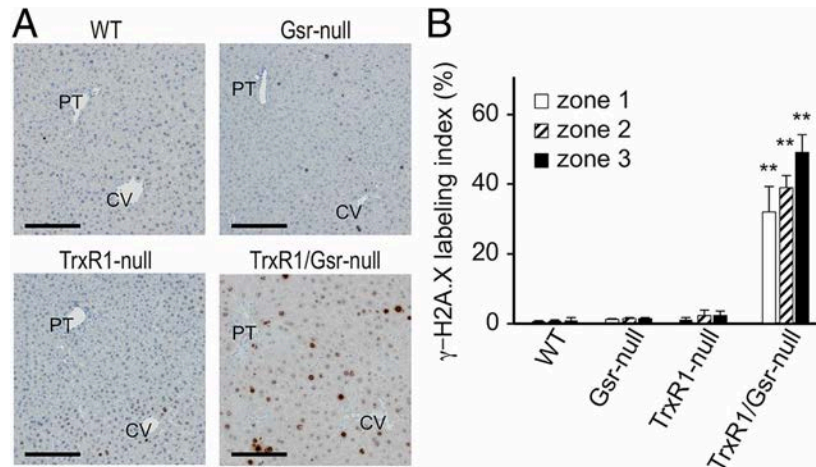


Figure 4.1 TrxR1/Gsr-null livers have high basal levels of DNA damage. (A) Liver sections of the indicated genotypes from resting adult mice immunostained for γ -H2A.X. CV, central vein; PT, portal triad. (Scale bars: 100 μ m.) (B) Zone- and genotype-specific staining quantification. Bars show mean and SEM. ** $P < 0.01$, pairwise vs. all other genotypes, Student's *t* test.

TrxR1/Gsr-Null Livers Are Susceptible to Chemically Induced Cancer.

It has been hypothesized that cancer cells are more dependent on endogenous antioxidant systems compared with normal cells (45). Therefore, we tested whether the low incidence of spontaneous liver cancer in TrxR1/Gsr-null livers might reflect an intrinsic inability of liver cancer cells to thrive without these antioxidant systems, unlike noncancerous hepatocytes (42). Groups of juvenile [postnatal day (P) 14] male mice with WT, Gsr-null, TrxR1-null, or TrxR1/Gsr-null livers each received DEN, were maintained

under standard care thereafter, and were harvested 8 mo later. TrxR1/Gsr-null livers readily developed severe liver cancer (Fig. 2A). WT and Gsr-null livers showed indistinguishable cancer burdens, TrxR1-null livers developed less cancer than WT livers, and TrxR1/Gsr-null livers developed more cancer than WT livers (Fig. 2B). Histology showed that the lesions in TrxR1/Gsr-null livers included large adenomas and malignant HCCs (Fig. 2C and SI Appendix, Fig. S3).

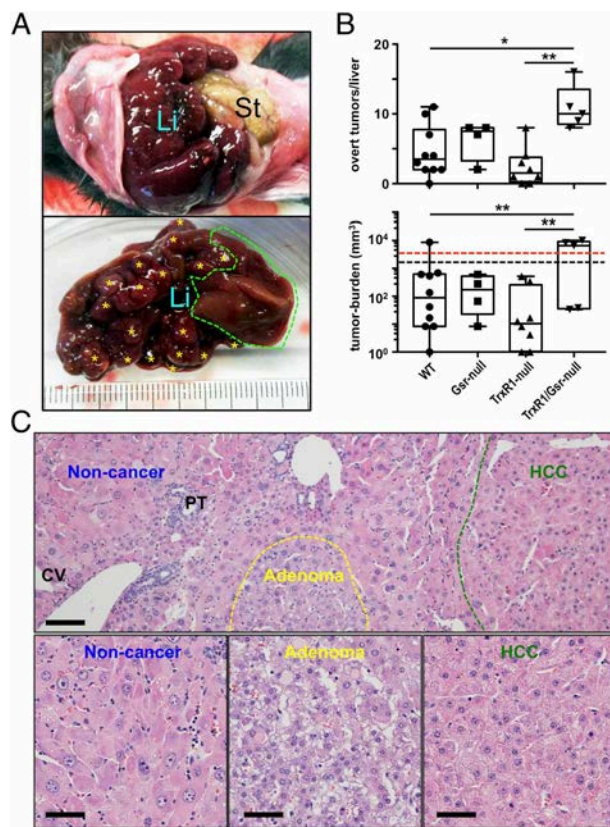


Figure 4.2 DEN induces cancer in TrxR1/Gsr-null livers. Male mice were challenged with 25 mg/kg DEN at P14, maintained under standard care, and harvested at P260–P265. (A) Cancerous TrxR1/Gsr-null liver. The “grapeshot” appearance is from the many tumors (e.g., yellow asterisks); relatively healthy tissue is seen in the right third of the organ (green dashed line). The small ticks on the scale at the bottom are 1 mm. Li, liver; St, stomach. (B) Overt tumor counts (Top) and tumor burden (Bottom) of individual mice. Tumor burden represents the sum of all tumor volumes, calculated from their measured diameters. Black and red dashed lines represent the volume of normal adult WT (~1,700 mm³) and TrxR1/Gsr-null (~3,200 mm³) livers, respectively. *P < 0.05;

****P ≤ 0.01, Student's t test. (C) Representative histology of cancerous TrxR1/Gsr-null liver. (Top) Region containing noncancerous tissue, an adenoma, and an HCC. (Bottom) Higher-magnification images, as indicated. (Scale bars: Top, 200 μm; Bottom, 50 μm.)**

Cancer Resistance in TrxR1-Null Livers Correlates with Altered Metabolite Profiles.

Following DEN challenge, mice with TrxR1-null livers developed threefold fewer tumors ($P = 0.015$) compared with their WT littermates (SI Appendix, Table S1). This indicates that hepatocellular TrxR1 disruption provided partial protection against DEN-induced cancer. Because TrxR1-null livers exhibit chronic activation of Nrf2 (16, 19), and the GSH system is a predominant target of Nrf2 activation, we measured basal and DEN-induced levels of Gsr and glutathione-S-transferase (GST) activity. Gsr activity was constitutively higher in TrxR1-null livers compared with WT livers and did not respond to the DEN challenge, whereas GST activity was constitutively higher in TrxR1-null livers compared with WT livers and was induced in both genotypes on DEN challenge (SI Appendix, Fig. S1 A and B). Metabolomics analyses revealed dramatic differences in metabolite profiles between WT and TrxR1-null livers in both unchallenged and DEN-challenged conditions (SI Appendix, Fig. S1 C–J). Affected metabolites included nucleosides, amino acids, and oxidative stress/repair products, and affected diverse pathways (SI Appendix, Fig. S1 E–J). This analysis verified that the basal enzymatic and metabolic profiles in mouse liver are substantially different between WT and TrxR1-null livers, and that DEN challenge differently impacts metabolic activities in each genotype.

Genotype (n)	Tumors (tumors/liver)				
	Total	0.5–2 mm	2–5 mm	5–10 mm	>10 mm
WT (31)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gsr-null (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TrxR1-null (22)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TrxR1/Gsr-null (32)	3 (0.094)	0 (0)	0 (0)	2 (0.03)	1 (0.031)

Table 4.1 TrxR1/Gsr-null livers have high basal levels of DNA damage. (A) Liver sections of the indicated genotypes from resting adult mice immunostained for γ -H2A.X. CV, central vein; PT, portal triad. (Scale bars: 100 μ m.) (B) Zone- and genotype-specific staining quantification. Bars show mean and SEM. ***P < 0.01, pairwise vs. all other genotypes, Student's t test.

WT and TrxR1-Null Liver Tumors Progress
Similarly Under Normal Care, but TrxR1-Null
Tumors Are More Aggressive
Under Chronic Stress.

One of the most robust ways to evaluate the impacts of mutations on in-context cell fate is clonal analysis (46), wherein a marked subset of mutated cells develops alongside, and potentially in competition with, WT cells. To test whether persistence or progression of DEN-initiated liver cancers differs between WT and TrxR1-null cells, we used a cell lineage-tracing strategy based on clonal analysis of postinitiation marked-mosaic gene conversion (Fig. 3A). By this approach, the entire liver showed WT TrxR1 activity at the time of DEN exposure (P14). One month later (P44), allowing time for clonal expansion of the cancer-initiated cells, an arbitrary subset of cells was converted to TrxR1-null using limited Cre-activation (Materials and Methods).

At harvest, the relative contributions of functionally WT (red) and TrxR1-null (green) cells in tumors were monitored by fluorescence imaging. This analysis revealed extreme

variation in the composition of TrxR1-null cells vs. WT cells to tumors (Fig. 3 B–D). For example, Fig. 3B shows a large (>1 cm diameter) tumor containing clones of red (WT) or green (TrxR1-null) cells. Fig. 3C shows a liver lobe containing a major tumor also composed of clones of WT or TrxR1-null cells, as well as several smaller lesions with various ratios of WT to TrxR1-null cells (quantified in Fig. 3D). Region 1 shows the relative representation of WT vs. TrxR1-null hepatocytes in noncancerous parenchyma of this liver. Compared with this region, cancerous regions either showed similar red:green ratios (e.g., regions 2 and 3), substantially more WT cells (regions 4 and 7), or substantially more TrxR1-null cells (regions 5 and 6). In addition, many apparently equivalent tumors were either fully WT or fully TrxR1-null (Fig. 3E, T1 and T2, respectively). Of 112 tumors analyzed in 25 marked mosaic animals, 55 had mixed cell composition, and 28 had predominantly WT cells, and 29 had predominantly TrxR1-null cells. Thus, even under conditions in which WT and TrxR1-null cancer cells competed with each other developmentally for contribution to tumors, neither cell type had an overarching advantage over the other within DEN-initiated liver tumors.

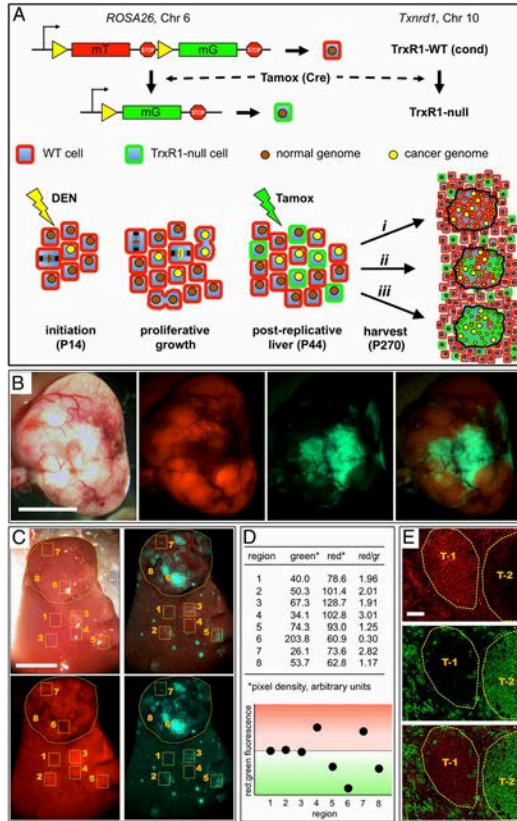


Figure 4.3 Marked-mosaic analysis of cancer progression by WT vs. TrxR1-null hepatocytes. (A) Strategy. The dual-fluorescent Cre-responsive ROSAmT-mG allele was used to distinguish Cre-naïve (red fluorescent) from Cre-exposed (green fluorescent) cell lineages. Cre activity was supplied by activation of tamoxifen-inducible CreER. DEN (25 mg/kg) was administered i.p. to tamoxifen-naïve (functionally WT) P14 males. At P44, tamoxifen was used to induce mosaic activation of Cre in an arbitrary subset of cells. Mice were incubated under standard care and harvested at P270. If cancer cells required TrxR1, tumors were red fluorescent (condition i). If the absence of TrxR1 favored cancer progression, tumors were green fluorescent (iii). If TrxR1 was superfluous for cancer, both red and green fluorescent cells contributed (ii). (B) Contributions of WT and TrxR1 null cells within a single tumor. Bright field and red, green, and merged red + green fluorescence images are shown. (Scale bar: 1 cm.) (C) Diversity of WT and TrxR1-null cell contributions to tumors. Shown are bright field and red, green, and merged red + green fluorescence images of the surface of a liver lobe containing a large tumor (region 8, yellow dashed line) and several smaller lesions. (Scale bar: 1 cm.) (D) Relative contributions of red (WT) and green (TrxR1-null) cells were determined in the eight numbered regions circumscribed in yellow lines in C. Region 1 is apparently normal parenchyma; the red:green ratio in region 1 represents the baseline hepatocyte mosaicism in noncancerous liver of this animal. All other demarcated regions were cancerous. (E) Representative fluoromicrographs of a cryosectioned cancerous marked-mosaic liver: red (Top), green (Middle), and merged red + green (Bottom) fluorescence. The region

exhibits two comparable tumors, one composed of WT cells (T-1, red fluorescent) and the other composed of TrxR1-null cells (T-2, green fluorescent). (Scale bar: 165 μm .)

We next investigated whether DEN-initiated cancers in TrxR1-null vs. WT livers developed differently under conditions of lifetime chronic hepatic oxidative stress. For this, P14 male mice with WT or TrxR1-null livers were treated with 25 mg/kg DEN, and at P44, one-half of the mice were switched permanently onto drinking water containing 0.07% phenobarbital (PB) (47, 48). Metabolism of PB by hepatocyte-specific cytochrome P450s results in chronic NADPH-dependent ROS generation (49–51). At harvest (P240), most livers exhibited only few and small tumors, with the exception of TrxR1-null livers that had been under chronic PB stress, which exhibited dramatic cancer (Fig. 4A), including increased numbers of tumors and an increased total tumor burden (Fig. 4B and C). Because the animals were not started on PB until 1 mo after the DEN challenge, this dramatic difference in liver cancer likely arose from differences in cancer persistence or progression rather than initiation.

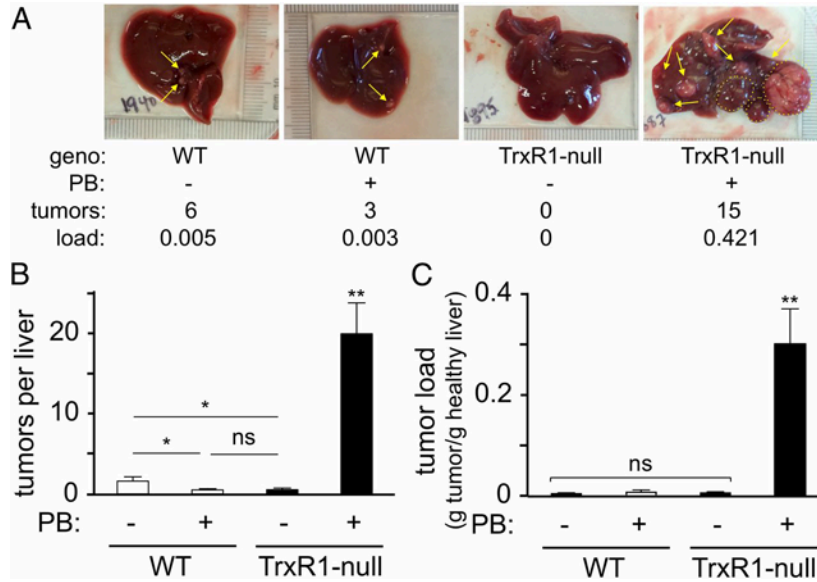


Figure 4.4 Chronic post-DEN initiation PB stress promotes increased liver cancer in TrxR1-null livers. P14 male mice with constitutively WT or TrxR1-null livers were challenged with 25 mg/kg DEN. One month later, groups of mice were either maintained under standard care or switched to drinking water containing 0.07% PB. All mice were harvested at P240 (n = 5 mice per condition). (A) Gross morphology and quantification on representative whole livers of each condition at harvest. Yellow arrows and yellow dotted lines indicate visible small and large tumors, respectively. Tumor counts and loads are for the liver shown above; tumor load refers to the measured volume of tumor tissue per total liver weight for the liver shown, presented as a ratio. (B and C) Average number of tumors per liver (B) and tumor load (C) for all five animals in each condition, presented as mean and SEM. *P < 0.05; **P < 0.01; ns, P > 0.05, Student's t test.

Nrf2 Activity in TrxR1-, Gsr-, and TrxR1/Gsr-Null Livers.

TrxR1-null livers have been shown to chronically activate Nrf2 (16, 19), whereas the status of Nrf2 activity in Gsr- and TrxR1/Gsr-null livers has not been reported.

Transcriptomes of adult Gsr-null livers were similar to those of WT livers and did not have a marked Nrf2 response (SI Appendix, Fig. S2), whereas TrxR1/Gsr-null livers showed dramatically altered expression of hallmark Nrf2-response genes (Fig. 5A) (52); in the figure, red asterisks at right denote Nrf2-response genes identified in previous

studies (15, 53, 54). Immunoblots confirmed that TrxR1/Gsr-null hepatocytes had increased levels of nuclear Nrf2 protein and the Nrf2-response proteins Cbr3 (carbonyl reductase-3) and HO-1 (heme-oxygenase-1) (Fig. 5B). TrxR1/Gsr-null livers expressed higher levels of many Nrf2-response mRNAs than have been reported for either TrxR1-null (16, 19) or Keap1-null (chronic derepression of Nrf2) livers (15) (Fig. 5C). Nonetheless, these comparisons also show that each genotype had a large number of responses that were either unique or were shared with only one of the other two genotypes (Fig. 5D), suggesting that differences in WT, TrxR1-null, TrxR1/Gsr-null, and Keap1-null liver transcriptomes extend beyond having different degrees of Nrf2 activation. qRT-PCR analyses for sentinel Nrf2-response mRNAs verified that most were not induced in Gsr-null livers yet were significantly stronger in TrxR1/Gsr-null livers compared with TrxR1-null livers (Fig. 5E).

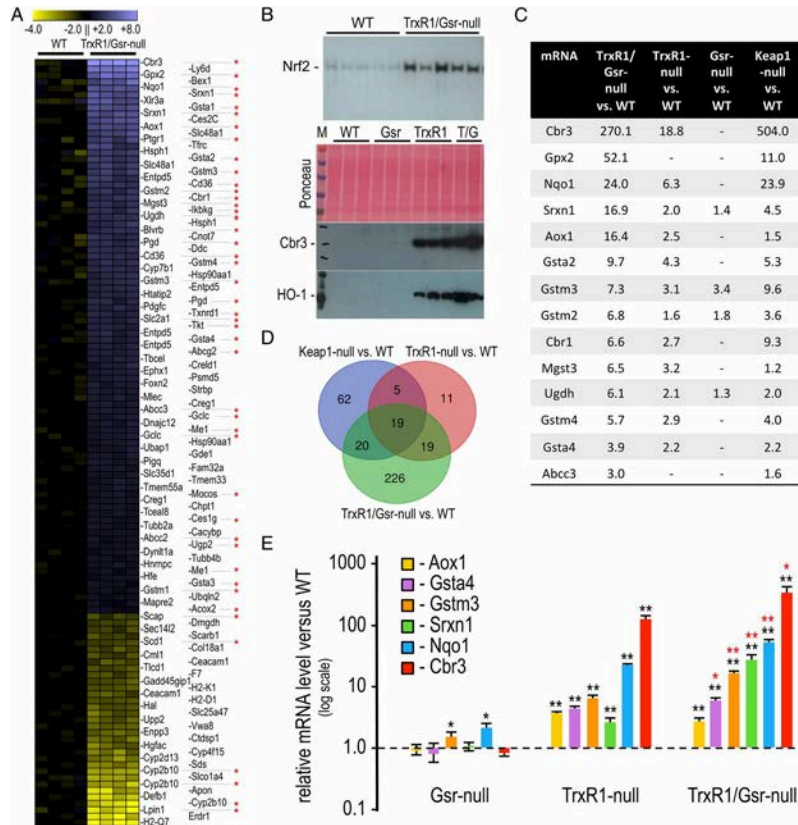


Figure 4.5 The Nrf2 response predominates in the transcriptome of TrxR1/Gsr-null livers compared with WT livers. (A) Comparative heat map of resting adult WT and TrxR1/Gsr-null liver transcriptomes. Mean values for each gene in WT livers were assigned the heat designation of black (“normal”). Red asterisks at right indicate reported Nrf2 targets. (B) Nrf2 protein levels in purified liver nuclei from WT and TrxR1/Gsr-null livers (Top) and of Cbr3 or HO-1 protein from total lysates of WT, Gsr-null, TrxR1-null, and TrxR1/Gsr-null (T/G) livers (Bottom). (C) Relative expression of 14 Nrf2 target genes compared with WT liver controls in TrxR1/Gsr-null (this study), TrxR1-null (16, 19), Gsr-null (this study), and Keap1-null (65) livers, analyzed using uniform criteria. (D) Gene list comparisons for Keap1-, TrxR1-, and TrxR1/Gsr-null livers from the uniform datasets used in C. (E) Relative mRNA levels assessed by qRT-PCR for six Nrf2 target genes in Gsr-, TrxR1-, and TrxR1/Gsr-null livers compared with basal levels in WT livers (log scale). Bars show mean and SEM; $n = 5$ for all genotypes. Black asterisks indicate values that differed significantly from WT; red asterisks represent values that differed significantly from TrxR1-null liver. * $P < 0.05$; ** $P < 0.01$, Student’s t test.

Immunohistochemistry was used to identify the cell types that participated in the Nrf2 response in TrxR1- and TrxR1/Gsr-null livers (Fig. 6). Across genotypes, Nrf2 was largely restricted to hepatocyte nuclei. Within the liver lobule, however, the distribution was nonuniform. In WT livers, staining for Nrf2 was weak and centrilobular (zone 3, Fig. 6). Matching the transcriptome data, proteins from Nrf2-response genes NADPH-quinone oxidase-1 (Nqo1) and Cbr3 were undetectable in WT liver (Fig. 5). Another Nrf2-response protein, HO-1, was absent from hepatocytes but was strongly expressed in Kupffer cells of WT livers (purple arrows). Since Nrf2 was not detected in Kupffer cells, this HO-1 expression was likely Nrf2-independent. In TrxR1-null livers, nuclear staining for Nrf2 protein in centrilobular hepatocytes was increased, and, correspondingly, Nqo1, Cbr3, and HO-1 were detected in centrilobular TrxR1-null hepatocytes. In TrxR1/Gsr-null livers, Nrf2 protein was enriched in nuclei across all zones, although this remained strongest in zone 3. Cbr3, Nqo1, and HO-1 were strongly increased in TrxR1/Gsr-null livers. There remained large hepatocyte-to-hepatocyte differences in expression levels of each of these proteins in TrxR1/Gsr-null livers, but the distribution was “checkerboard” rather than zonal. Nrf2 and Nrf2-response gene proteins were expressed both in apparently healthy and in degenerating hepatocytes (“ballooning” hepatocytes, blue asterisks, Fig. 6). HO-1 expression in Kupffer cells was similar in all genotypes (purple arrows), indicating that this expression was independent of the status of surrounding hepatocytes.

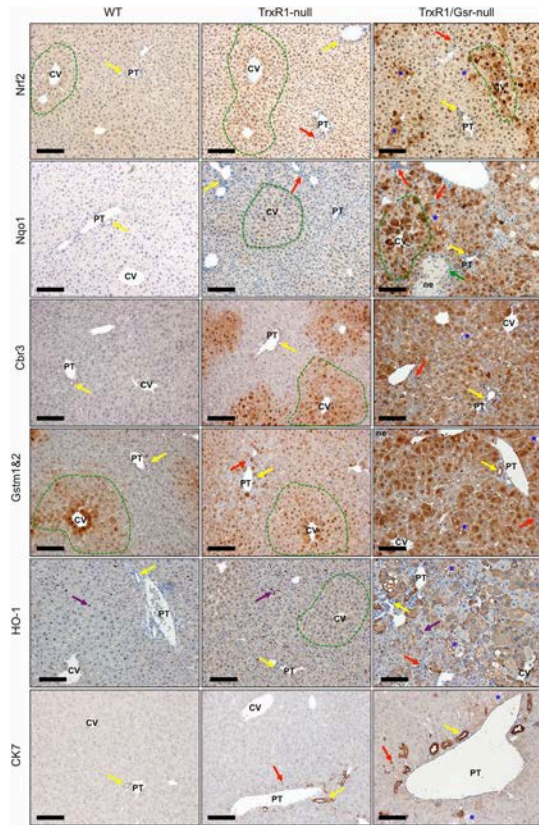


Figure 4.6 Nrf2 activity in WT, TrxR1-null, and TrxR1/Gsr-null livers. Sections of livers were immunostained for the markers indicated at left (brownish-red) and counterstained with hematoxylin (bluish nuclei). Representative centrilobular (zone 3) expression circumscribed by green dashed lines. ne, necrosis. Yellow arrows, bile ducts; red arrows, ectopic ductular responses; green arrow, inflammatory cells; purple arrows, HO-1+ Kupffer cells. Blue asterisks denote representative ballooning (degenerating) hepatocytes. (Scale bars: 100 μ m.)

Phenotypic Heterogeneity in DEN-Induced Liver Tumors.

DEN-induced liver tumors in each genotype were pathologically diverse, with WT, Gsr-null, TrxR1-null, and TrxR1/Gsr-null livers each exhibiting all grades of lesions, including benign hyperplasias, adenomas, and malignant HCCs (Fig. 7 and SI Appendix, Table S1). Quantitatively however, TrxR1/Gsr-null livers contained predominantly larger and more advanced lesions, whereas Gsr-null littermates had more

benign lesions (SI Appendix, Fig. S3). This prompted us to ask whether there was a correlation between Nrf2 activation and tumor grade, particularly since noncancerous TrxR1- and TrxR1/Gsr-null livers showed high basal Nrf2 activity compared with WT and Gsr-null livers (Figs. 5 and 6). Sections of cancerous livers of each genotype were stained for Cbr3 and Nqo1, and protein levels were compared between normal parenchyma and cancerous lesions in the livers (Fig. 7). Within tumors of all genotypes, expression of Cbr3 and Nqo1 varied from no detectable expression in some tumors to uniformly strong expression in others (Fig. 7).

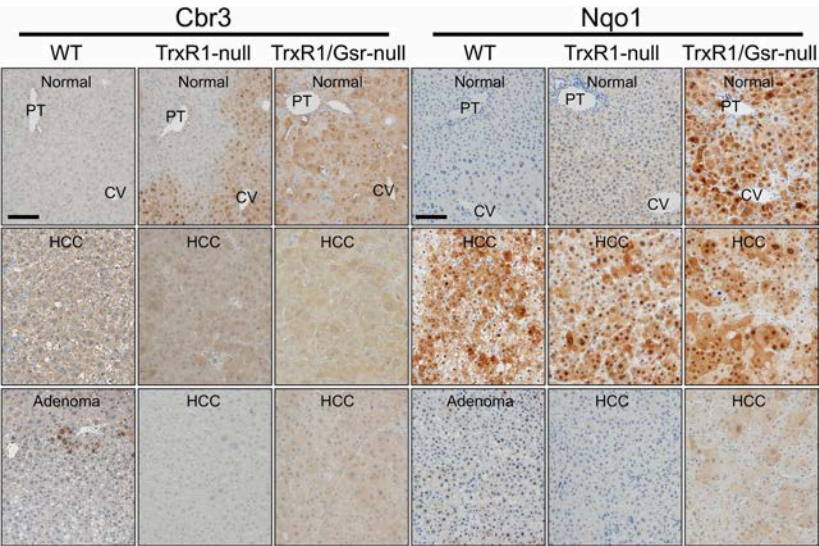


Figure 4.7 Nrf2 activity in tumors. Sections of DEN-induced cancerous livers stained for expression of Cbr3 or Nqo1. The top panels represent noncancerous regions; below are representative tumors. (Scale bars: 100 μ m.)

Discussion

ROS and Cytosolic Disulfide Reductase
Systems in Liver Carcinogenesis.

The ability of ROS to damage DNA makes these likely carcinogens. Endogenous ROS arise as collateral products of respiration, β -oxidation of fatty acids, and other activities in hepatocytes. Extracellular ROS can also impact hepatocytes. Many liver cancers are associated with inflammation (55) and are likely modulated by oxidative stress (2, 3). The cytosolic NADPH-dependent disulfide reductase systems, driven by TrxR1 and Gsr, support potent peroxidases and repair activities. Therefore, these pathways are expected to provide a critical barrier against liver carcinogenesis.

Neither TrxR1- nor Gsr-null livers show evidence of basal oxidative stress (16, 17, 20), indicating that the TrxR1- and Gsr-driven pathways robustly complement each other to combat ROS. However, TrxR1/Gsr-double-null livers accumulate extensive chronic oxidative damage. This indicates that Met-fueled reductase activity (42) by itself is insufficient to prevent pervasive oxidative stress. Contrary to our initial expectations, we found that the number of spontaneous cancers in TrxR1/Gsr-null livers remained low, yet DEN exposure increased their cancer frequency by 115-fold over the rate for unmitigated endogenous oxidants (cf. Table 1 and Fig. 2B). Mutation sites are likely arbitrary for both ROS and DEN. Although we are unable to quantitatively compare the one-time lesion incidence from single-dose DEN-exposure with lifetime lesion incidence from chronic oxidative stress in TrxR1/Gsr-null livers, our data might suggest that compared with alkylation damage, oxidative DNA damage is dramatically less carcinogenic. Interestingly, living organisms have been adapting to endogenous ROS since the evolution of respiration (56). In contrast, exposure to chemicals that would be metabolized into intracellular alkylating agents, as DEN is, might instead have been

largely restricted to occasional environmental exposures and thus perhaps have had less impact as a driver of natural selection over evolutionary time. If lesions induced by oxidative stress are indeed less carcinogenic than lesions induced by DEN, this would suggest that postmutation defenses (i.e., repair, tumor suppression, and immune responses) are more effective against oxidative damage than alkylation damage. Further investigations are needed to test this.

The significantly diminished tumor incidence under normal care seen in DEN-treated animals with TrxR1-null livers compared with those with WT livers (Table 1) is intriguing. Based on the strong chronic Nrf2 response (16, 19) and the altered basal- and DEN-responsive metabolome in TrxR1-null livers (SI Appendix, Fig. S1), it is likely that Nrf2-driven expression of the phase-2 and -3 xenobiotic metabolism pathways, including both the glucuronyl and GSH conjugation systems that detoxify DEN and its products (32, 40, 41), effectively diminished the alkylation damage in these livers at the time of DEN treatment. Interestingly, the TrxR1/Gsr-null livers, in which Nrf2 target genes are induced to yet-higher levels (Fig. 5), developed more severe cancer under standard care. Although it is tempting to speculate that this reflects the balance between enhanced detoxification and increased oxidative stress, other possibilities should also be considered. For example, TrxR1-null livers overaccumulate glycogen, providing the hexose source for glucuronidation, and have an increased ability to generate excess GSH for GSH conjugation. In combination, this makes them exceptionally resistant to acetaminophen-induced hepatotoxicity (19). In contrast, TrxR1/Gsr-null livers have low glycogen levels and are critically dependent on their GSH for survival, and thus are

exceptionally susceptible to acetaminophen (43). As such, the Nrf2-induced phase 2 enzymes in TrxR1/Gsr-null livers might also plausibly be substrate-limited for detoxification of DEN.

Cytosolic Disulfide Reductase Systems in Liver Cancer Persistence or Progression.

Many cancers appear to be critically addicted to cytosolic disulfide reductase system activities. Metabolic realignments in cancer cells increase intracellular levels of endogenous ROS and thus make the cells more susceptible to oxidative stress-induced cell death compared with noncancerous cells (45, 57). This is consistent with our own studies on inhibition of TrxR1 in cultured cancer cells, head and neck cancers, and breast cancer tumors (58), and also with a recent report showing that knockdown of TrxR1 in mouse models of xenografted human HCCs and transgene-driven mouse HCCs led to diminished tumor size (59).

In line with this, we expected HCCs to be critically dependent on the endogenous antioxidant systems, and when the oxidative damage in TrxR1/Gsr-null livers did not result in abundant fulminant HCC, we originally suspected that TrxR1/Gsr-null cancers formed but became self-limiting via a “cell suicide mechanism” that prevented transformed cells from tolerating their own cancer-associated ROS. Conversely, however, the highly abundant malignant tumors that developed in DEN-exposed TrxR1/Gsr-null livers in the present study indicate that the absence of effective endogenous cytosolic antioxidant systems is not necessarily self-limiting for HCC.

It remains unclear what roles the diversity of initiating mutations in the DEN model, followed by long-term natural selection and evolution of the tumors in the absence of the disulfide reductase systems, play in their ability to tolerate increased oxidative stress. Moreover, hepatocytic metabolic eccentricities, such as their exceptionally strong Met cycle and transsulfuration pathway, will make the Met-driven reductase pathway more robust in hepatocytic cancers (60, 61). Importantly, clinical HCC, like DEN-induced HCC, presents with diverse genetic lesions that are not well represented in some preclinical models. Moreover, long-term therapeutic efficacy will require that the cancers do not readily evolve a therapy-resistant physiology. The present study emphasizes that HCC can readily adapt and persist without TrxR1 and Gsr activities. In considering the strong propensity of cancers to acquire resistance mechanisms, therapeutic approaches targeting reductase pathways in cancers should likely be restricted to combinatorial regimes that discourage evolution of “hepatocyte-like” oxidative stress-insensitivity survival strategies.

The results presented in this study show that postinitiation oxidative stress, as modeled either by coincidental loss of both TrxR1 and Gsr (Fig. 2) or by chronic hepatic PB-induced oxidative stress in livers only lacking TrxR1 (Fig. 4), can increase liver cancer malignancy. Indeed, malignant HCCs predominated in DEN-induced TrxR1/Gsr-null livers, whereas Gsr-null livers exhibited more benign hyperplasias (SI Appendix, Fig. S3). Currently we do not have a mechanistic explanation for this, but three possibilities might contribute. First, it is possible that accumulated oxidative DNA damage during cancer progression favors malignant progression through a genetic

mechanism, as predicted in the “multihit” hypothesis (5, 6) and suggested in at least one mouse HCC study (62). Second, it is possible that alterations in redox signaling resulting from these disruptions favor malignant progression through an epigenetic mechanism, for example, by favoring proliferation or morphogenic transitions. Third, disruption of TrxR1 and Gsr may trigger secondary enzymatic and metabolic realignments through Nrf2 (Fig. 5 and SI Appendix, Fig. S1) or other systems that contribute to the altered cancer phenotypes. Further investigations are needed to test each of these possibilities. The mouse liver cancer models reported here should support such investigations.

Interplay of the Cytosolic Disulfide Reductase Systems and Nrf2 in Liver Cancer.

A previous study showed that in HeLa cells, pharmacologic depletion of GSH did not induce Nrf2, knockdown of TrxR1 did induce Nrf2, and combination treatments further increased Nrf2 activation (63). We now extend these observations with full liver transcriptome analyses (Fig. 5 and SI Appendix, Fig. S2). The distinct impacts of TrxR1-, Gsr-, and TrxR1/Gsr-null conditions on Nrf2 activation show that these three antioxidant systems are integrated into a cross-regulated network and might provide further clues to understanding Nrf2 regulation. The activities of this network optimize the protection of cells from oxidative or toxic exposures. Whereas this will effectively antagonize cancer initiation, in some cases the network could favor the malignant progression of existing tumors by preventing cell death-based defenses (Fig. 8).

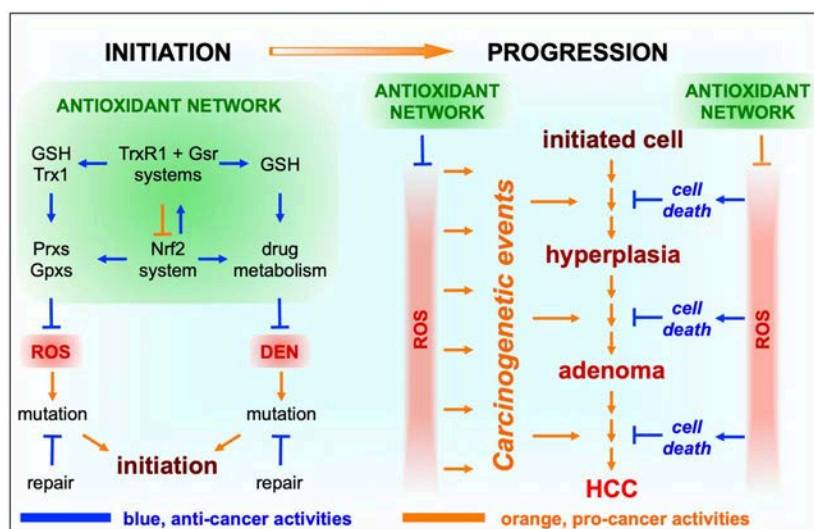


Figure 4.8 Roles of the endogenous antioxidant network in cancer initiation and progression. The antioxidant systems form an integrated cross-regulated network that will effectively protect cells from carcinogens, but in existing cancers, the network likely plays both anticancer and procancer roles.

A genome-wide association study of human squamous cell lung cancers revealed a strong correlation between malignancy and mutations that activate Nrf2 (29). Several mouse and human studies have also shown that malignant progression in diverse cancers is favored by chronic activation of Nrf2 (22, 27, 28), and a recent study indicated that Nrf2-deficient mice are resistant to HCC (64). Therefore, in the present study, we expected more severe liver cancers to correlate with Nrf2 activation. Conversely, we found that individual tumors within a liver can have diverse Nrf2 activity levels that show no obvious correlation with tumor grade (Fig. 7). This unexpected finding might be peculiar to liver cancers. Indeed, the diversity of molecular, genetic, redox, and metabolic phenotypes of the HCCs reported here likely presages the diversity of “programs” that individual cancers might adopt for survival. This should be considered a general caveat

for studies seeking an “Achilles’ heel” that makes cancer cells more susceptible than normal cells to a simple therapy.

Materials and Methods

Mice.

All procedures were reviewed and approved by the Montana State University Animal Care and Use Committee. DEN was administered to male pups at P14 $\pm$ 1 d at 25 mg/kg i.p. in sterile saline (33). PB at 0.07% (wt/vol) was added to drinking water continuously starting at P44 $\pm$ 2 d. For marked-mosaic analyses, male pups at P14 $\pm$ 1 d of age, genotype Txnrd1^{cond/cond}; ROSA^{mT-mG/CreER}, were inoculated i.p. with 25 mg/kg DEN in sterile saline. At P44 $\pm$ 2 d, 0.2 mL of 1 mg/mL tamoxifen-citrate was administered i.p.

Transcriptome Analysis, qRT-PCR, Histology, and Immunohistochemistry.

Transcriptomes from WT, Gsr-null, and TrxR1/Gsr-null livers were analyzed using Affymetrix 430A 2.0 arrays. Probes were synthesized from total liver RNA using the MessageAmp II-Biotin Enhanced system and hybridized for 16 h at 45 °C. Transcriptome data generated in this study have been deposited in the Gene Expression Omnibus (accession nos. GSE124444, GSE124445, and GSE124446). qRT-PCR was performed on cDNA generated from total mouse liver RNA using a mixture of oligo-dT and random primers and GoTaq PCR Master Mix. cT values were calculated using RotorGene 6000 series software v. 1.7, and differences between Nrf2-regulated

transcripts compared with endogenous β -actin control were calculated. Histology, immunohistochemistry, and immunoblotting were performed following standard protocols and the antibodies listed in SI Appendix.

Statistical Analyses.

Except where indicated in figure legends, statistical significance was determined by pairwise Student's *t* tests. Transcriptomes were analyzed using FlexArray software v. 1.6.2 and National Center for Biotechnology Information Mouse Genome Annotation 35. Array comparison output datasets were normalized by GeneChip robust multiarray averaging, and variance was calculated by one-way ANOVA. Datasets were restricted to fold change twofold or more up- or down-regulated, false discovery rate ≤ 0.05 , and raw value expression level >200 units for comparisons between sets.

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CHAPTER FIVE

SUPPLEMENTAL ASCORBATE COMPROMISES HEPATOCYTE SURVIVAL AND
INCREASES RISK OF ACUTE LIVER FAILURE DURING
SEVERE OXIDATIVE STRESS

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SUPPLEMENTAL ASCORBATE COMPROMISES HEPATOCYTE SURVIVAL AND
INCREASES RISK OF ACUTE LIVER FAILURE DURING
SEVERE OXIDATIVE STRESS

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Abstract

Cellular oxidants are primarily managed by the thioredoxin reductase-1 (TrxR1)- and glutathione reductase (Gsr)-driven antioxidant systems. The highly nucleophilic active sites of TrxR1 and Gsr are dependent on NADPH availability and are disrupted by electrophilic toxins; loss of hepatic TrxR1 and Gsr is modeled in mice having hepatocyte-specific co-disruption of TrxR1 and Gsr (TrxR1/Gsr-null livers). In these livers, methionine catabolism sustains levels of reduced glutathione (GSH) and liver homeostasis. Although most mice with TrxR1/Gsr-null livers exhibit long-term survival, ~30% undergo spontaneous liver failure between 4- and 7-weeks of age. Here we tested whether liver failure was ameliorated by dietary ascorbate supplementation. Unexpectedly, rather than protecting the animals, ascorbate (5 mg/ml in acidified drinking water) nearly doubled the frequency of liver failure (57%). In adults, ascorbate (4 mg/g/day x 3 days intraperitoneal) caused extensive cell death in TrxR1/Gsr-null but not in Gsr-null livers. Intraperitoneal administration of ascorbate (4 mg/g) or dehydroascorbate (0.3 mg/g) to adult mice with TrxR1/Gsr-null livers depleted hepatic GSH levels, whereas only dehydroascorbate significantly depleted GSH in Gsr-null control livers. Our results reveal that ascorbate supplementation can be a liability for livers under severe oxidative stress, such as might occur following exposure to an electrophilic toxin.

Introduction

Oxidative stress is a substantial oxidative deviation from normal cellular redox steady state [1]. Cells generate reactive oxygen species (ROS) as a byproduct of cellular respiration and metabolism or in response to external factors such as xenobiotic exposure, radiation, or environmental pollution [2]. Inflammation also exposes surrounding cells and tissues to high levels of ROS. Hydrogen peroxide (H_2O_2), the major cellular oxidant, is generated from superoxide ($\text{O}_2^{\cdot-}$) both spontaneously and catalytically by superoxide dismutases [3]. Superoxide is produced primarily by the mitochondria as a byproduct of cellular respiration and other metabolic activities [4]. Some membrane or cytosolic enzymes, including NADPH-oxidases and cytochrome p450's, also generate superoxide under in certain conditions [5-7]. Less commonly, oxidants including hypochlorous acid (HOCl), nitrous oxide ($\cdot\text{NO}$), hydroxyl radical ($\cdot\text{OH}$), and singlet oxygen are generated in cells [8]. All of these ROS have the ability to damage proteins, lipids and nucleotides. Oxidative damage has been implicated as contributors to numerous disease states including neurodegeneration, heart disease, and cancer [2].

All cells have potent endogenous antioxidant systems, which play critical roles in both defense against ROS and repair of oxidative damage. Predominant among these are the two cytosolic disulfide reductase systems: one driven by thioredoxin reductase-1 (TrxR1) and one driven by glutathione reductase (Gsr) [9]. In the former system, reducing power (electron pairs) extracted from reduced nicotinamide adenine dinucleotide phosphate (NADPH) by the flavin-containing TrxR1 are used to reduce a disulfide bond in oxidized Trx1 , generating reduced (dithiol) Trx1 . In the later system, electron-pairs from NADPH are extracted by the flavin-containing Gsr and used to

reduce glutathione-disulfide (GSSG) into two molecules of the thiol-containing tripeptide glutathione (GSH) [10]. Fueled by reducing power from the Trx1 and GSH systems, abundant cytosolic peroxiredoxins (Prx) and glutathione peroxidases (Gpx) rapidly reduce H_2O_2 and other hydroperoxides [11, 12]. Protein disulfides arising from ROS exposure are repaired back to thiols by cytosolic thioredoxin-1 (Trx1), GSH, and the GSH-dependent glutaredoxins (Grx). Similarly, methionine (Met)-sulfoxides arising from ROS exposure are repaired by Trx1- or GSH/Grx-dependent Met-sulfoxide reductases (Msr)[13-15].

Severe oxidative stress conditions demand considerable consumption of NADPH. To sustain an adequate supply, several regulatory systems, including direct oxidant-inhibition by a key glycolytic enzyme, glyceraldehyde phosphate dehydrogenase (GAPDH) and metabolic realignments by oxidant-responsive transcription factor (Nrf2) and signaling (AMPK) pathways coordinately (1) re-prioritize glucose metabolism to favor NADPH production from NADP^+ over glycolysis; (2) suppress competing anabolic consumption of NADPH; and (3) induce NADPH-generating pathways. In addition, since ROS generation is frequently a secondary consequence of exposure to electrophilic toxins, the oxidant-response pathways also activate drug metabolism phase-2, which glutathionylate, glucuronidate, or sulfate the compounds, as well as phase-3 transporters, which then export the conjugated toxins out of the cell.

In yeast or bacteria, genetic co-disruption of TrxR1 and Gsr is lethal [16, 17]. Mice with hepatic deletion of both TrxR1 and Gsr (TrxR1/Gsr-null), however, are long-term viable and exhibit relatively normal liver function. TrxR1/Gsr-null livers sustain

redox homeostasis by an NADPH-independent system that uses catabolism of dietary methionine (Met), via the Met cycle and transsulfuration pathway, to generate Cys, which is then used for de novo GSH synthesis [9, 18]. This GSH supports critical reduction reactions, generating GSSG, which is subsequently excreted from the cells [18].

Although this pathway is able to support long-term survival, export of the oxidized GSSG makes this highly inefficient, and resting TrxR1/Gsr-null livers accumulate extensive oxidative protein and DNA damage [18, 19]. Moreover, mice with TrxR1/Gsr-null livers and experience a “crisis period” between 4- and 7-weeks of age, during which a subset of the animals of both sexes die from acute liver failure [18]. The high levels of oxidative damage to liver macromolecules and the high frequencies of acute liver failure in these mice suggest that the balance between redox homeostasis and cell death is at the threshold at this critical period, and is easily tipped toward failure.

A diverse group of dietary supplements are categorized antioxidants. These nutrients have the potential to support or synergize with endogenous antioxidant systems, thereby bolstering maintenance of cellular redox homeostasis. Mechanisms of antioxidant action are similarly broad. Some, like GSH, are true reductants that can provide the electrons to support elimination of H_2O_2 or otherwise reduce oxidants or oxidized cellular molecules. Conversely others, such as sulforaphane, are themselves oxidative electrophiles that induce cells to elicit robust cytoprotective gene expression responses [20]. Vitamin E (α -tocopherol) stably traps free radicals, thereby quenching free radical chain propagation [21, 22]. N-acetylcysteine (NAC) is a potent antioxidant that can function in thiol-disulfide exchanges to liberate other thiols [23], provide a

source of the limiting sulfur amino acid cysteine (Cys) for GSH biosynthesis during severe glutathionylation-induced GSH-depletion, or provide a source of intracellular hydrogen sulfide (H_2S) which, in turn, can protect cellular thiols from overoxidation [24] or directly quench H_2O_2 [23]. Still others, including many complex natural products, might provide antioxidant support through mechanisms that remain unresolved [25]. One of the most potent antioxidants is ascorbate (Vitamin C), which is a true reductant, a potent free radical trap, and an essential cofactor for the generation of hydroxyproline by hydroxyl prolase [26-28]. Although nearly all plants and animals can synthesize ascorbate, humans cannot, making Vitamin C an essential nutrient for humans [29].

Importantly, all redox-active antioxidants are also pro-oxidants. Whereas their reduced state will have antioxidant activity, the utilization of this reducing power will generate the oxidized form of the compound, which will be a pro-oxidant. Examples include most thiol-containing antioxidants, such as GSH, Cys, or NAC, which readily form disulfides during oxidation. Upon take-up into cells, these disulfides must, in turn, be reduced to dithiols by the cellular disulfide reductase systems, thereby consuming NADPH and disulfide reducing power. Another example is ascorbate, which oxidizes to dehydroascorbate (DHA). DHA, itself, is not active as an enzymatic cofactor, a free radical trap, or an antioxidant, although also unlike ascorbate, it has the ability to pass the blood-brain barrier and provide a precursor of ascorbate to the central nervous system [30]. DHA is also actively taken-up by hepatocytes and is thereafter reduced to ascorbate by GSH, Grxs, GSH-S-transferases (Gst), and TrxR1 [29]. In this context, however, DHA is a pro-oxidant that requires consumption of cellular NADPH for the subsequent

reduction of GSSG to GSH or recycling of oxidized TrxR1 [31]. Importantly, since most extracellular fluids are oxidizing, many dietary antioxidants, such as GSH, Cys, NAC, or ascorbate, will spontaneously oxidize into their pro-oxidant forms before they can enter cells, and therefore will require consumption of NADPH and cytosolic disulfide reducing power before antioxidant benefits can be realized.

It remains unclear how well supplemental ascorbate can substitute for insufficiencies in the endogenous disulfide reductase systems. In one study, ascorbate supplementation in newborn rats was shown to compensate for GSH depletion caused by buthionine sulfoximine (BSO), which inhibits glutamate-cysteine ligase (Gcl), the first committed step in GSH biosynthesis. In that study, ascorbate supplementation resulted in both an increase in GSH levels and a decrease in mortality [32]. Also, in a 4-year old girl with a deficiency in glutathione synthase (GS, the last step in GSH biosynthesis), administration of ascorbate resulted in increased plasma GSH levels [33]. The mechanisms underlying the ability of ascorbate to increase GSH levels in either of these reports, however, remain uncertain. GSH, itself, cannot be synthesized without having both Gcl and GS activity [34]. This suggests that ascorbate could not increase synthesis of GSH in these studies, but rather might have been able to re-prioritize residual biosynthetic activities. In the current study, we tested whether ascorbate supplementation could decrease levels of hepatic oxidative stress and rates of acute liver failure in mice with TrxR1/Gsr-null livers. Results showed that, rather than either protecting these livers or favorably re-prioritizing hepatic GSH, ascorbate treatment caused hepatic GSH depletion and increased the frequency of spontaneous acute liver failure. These results

emphasize the ability of ascorbate supplements to have pro-oxidant activities in some situations. In the absence of robust NADPH-dependent disulfide reductase systems, such as might occur during severe oxidative stress or exposure to electrophilic toxins or drugs, supplemental ascorbate might be a liability for liver health.

Materials and Methods

Mice, Supplementations, and Harvests

Animal procedures presented were approved by the Montana State University and McLaughlin Research Institute Institutional Animal Care and Use Committees (IACUC). The Gsr-null allele used in this study is a radiation-induced deletion that disrupts all protein-coding function of the allele. Mice homozygous for this mutation (Gsr-null) are phenotypically normal and have been reported previously [35]. The Txnrd1cond allele is a targeted Cre-dependent conditional-null allele that encodes normal TrxR1 in the Cre-naïve state but is null in the Cre-recombined state, which has been described previously [36] and is available through Jackson Labs (JAX Stock #028283). Mice with TrxR1/Gsr-double-null livers in this liver are also whole body Gsr-null, but TrxR1/normal in all cell types except hepatocytes. These mice are all overtly healthy as adults and are fertile in both genders, although their livers show extensive chronic cell death, accumulation of oxidative damage, hepatomegaly, and hyperproliferation [18, 19]. Liver transcriptomes in Gsr-null mice reveal almost no significant gene expression differences from wild-type (WT) livers [37], and therefore the current study used Gsr-null mice as littermate-controls for the TrxR1/Gsr-null mice. All analyses shown used adult mice (60–90 days of age) of both genders except as specified in the text or figure legends. Animals were maintained

on a 14:10-hr light:dark cycle with unrestricted access to feed and sterilized acidified tap water (pH 2.9-3.1, adjusted with HCl by a Hawkins automated doser. Except as indicated otherwise, all harvests were performed between 9:00 and 11:30 a.m. For dietary supplementation, ascorbate was added to the acidified drinking water to a final concentration of 0.5% and this was replaced weekly. Importantly, at the low pH of the acidified drinking water, spontaneous oxidation of ascorbate to DHA is negligible [38].

Total Glutathione

To measure total glutathione (GSH and GSSG levels), snap-frozen liver pieces (~0.3 g) were homogenized in 0.8 ml of 10 mM HCl and proteins were removed by adding 5-sulfosalicylic acid to 1% (w/v) followed by centrifugation. Reaction mixes contained 120 mM NaH₂PO₄, pH 7.4, 5.3 mM EDTA, 0.75 mM 5,50-dithiobis-(2-nitrobenzoic acid (Sigma D8130), 0.24 mM NADPH and 1.2 IU/ml GR (Sigma-Aldrich G9297), and 5 ml clarified lysate, and were followed at room temperature by absorbance at 412 nm in a Versamax plate reader. Standard curves contained dilutions ranging from 0 to 1600 pmole GSH. Liver GSH concentrations were calculated by comparison with standard curves assuming 100% recovery at all steps. Protein content was determined by the Bradford method.

Statistical Analyses

Statistical analyses were performed on Microsoft Excel 14.7 or Graphpad Prism 8.1 software. Bar graphs show means and SEM. Significance was determined by a Student's-T test versus control samples. Significance was assigned at $P < 0.05$.

Results

Ascorbate Supplementation Exacerbates Acute Liver Failure Frequencies in Mice With TrxR1/Gsr-null Livers

Mice with TrxR1/Gsr-null livers are born at expected Mendelian frequencies; a portion of the animals of both sexes die of spontaneous acute liver failure between postnatal days 28-49 (P28-49); and animals surviving to P50 can live past P200 with survival curves not differing substantially from those of matched WT mice [18]. At all ages, TrxR1/Gsr-null livers exhibit pronounced accumulation of oxidatively damaged protein and DNA, high hepatocyte death indexes, and hyperproliferation [18, 19]. Although it is not yet clear why liver failure is restricted to only the three-week window of age from P28-49, the association with oxidative damage led us to hypothesize that liver failure results when individuals exceed a threshold of hepatocyte oxidative stress-induced cell death that becomes incompatible with liver survival and function. Therefore, we tested whether supplemental ascorbate could decrease the frequency of acute liver failure by ameliorating hepatic oxidative stress. Cages of pups having either TrxR1/Gsr-null (experimental mice) or Gsr-null (littermate controls) were placed on drinking water containing either 0% or 0.5% ascorbate at weaning (~P19) and maintained under these conditions until P63. Whereas the survival rate of animals with Gsr-null livers was nearly 100% for mice on water with or without ascorbate, mice with TrxR1/Gsr-null livers exhibited ~30% lethality on water without ascorbate and, contrary to our expectations, this increased to 57% lethality with ascorbate supplementation (Fig. 1). Pathological analyses of representative animals revealed that lethality was associated with acute liver

failure, with severe congestion and most if not all hepatocytes appearing necrotic (not shown)[18].

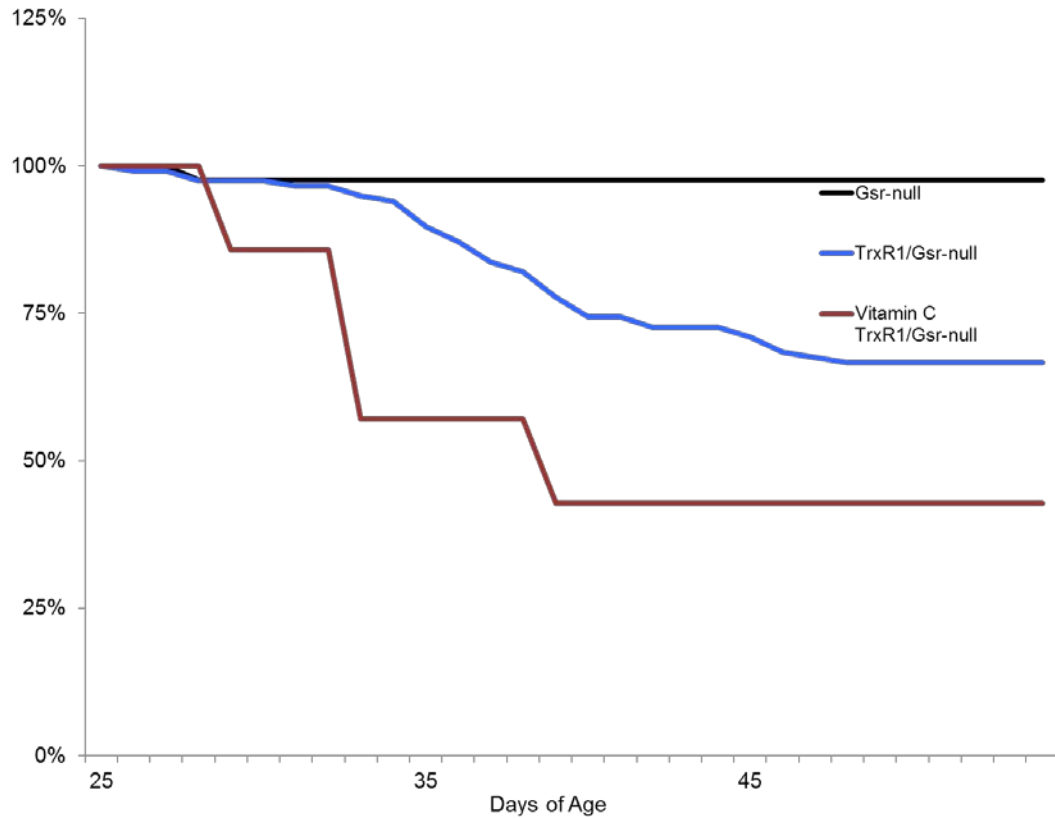


Fig. 5.1. Survival rates of mice given supplemental ascorbate. Mice with livers lacking Gsr (black line, n = 85) or TrxR1 and Gsr (blue line, n = 117) were given standard acidified water, while mice with livers lacking TrxR1 and Gsr (red line, n = 7) were Asc supplementation in their water.

Ascorbate Causes Severe Histopathology and Hepatocyte Necrosis in TrxR1/Gsr-null Livers

The increased frequency of spontaneous death in mice with TrxR1/Gsr-null livers was unexpected, as we had anticipated that ascorbate should alleviate hepatic oxidative stress in these mice. To further investigate whether ascorbate, itself, was compromising the TrxR1/Gsr-null livers, we administered ascorbate to resting adult mice (P60-90) via

daily intraperitoneal (i.p.) injections with 0 (control) or 4 mg/g body weight in sterile saline, which is half the daily dose that had previously been shown under chronic administration to inhibit growth of oncogene-driven tumors in mice without adversely affecting the mice, themselves [39]. After less than three days the mice with TrxR1/Gsr-null livers exhibited overt signs of adverse effects, including lethargy and weight-loss (10-20% of starting body weight lost). Gsr-null controls did not exhibit adverse effects. Mice were sacrificed 3 h after the third daily inoculation and liver histology was examined. The histology of Gsr-null livers from the ascorbate-treated mice was indistinguishable from that of saline-treated Gsr-null control livers. By contrast, the livers of ascorbate-treated TrxR1/Gsr-null mice all showed dramatic pathology which was far more severe than the basal pathology in TrxR1/Gsr-null livers under standard care [18, 19]. In many histological sections, nearly all hepatocytes were necrotic and had pyknotic nuclei, and large regions showed loss of cellularity with or without congestion (e.g., Fig. 2D). Nonetheless, sections could be found from most liver that, while still having extensive hepatocyte necrosis, lacked congestions and still had some apparently surviving hepatocytes (not shown).

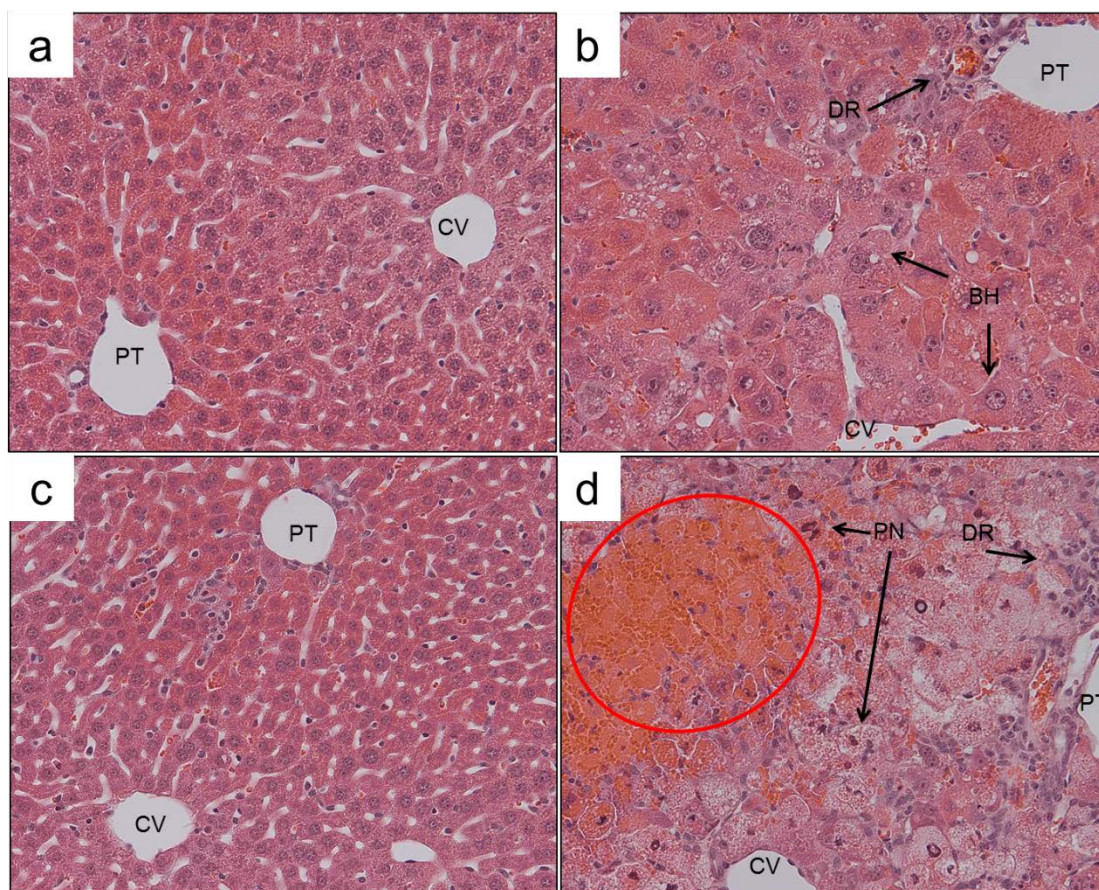


Fig. 5.2. Haematoxylin and eosin-stained histology of Gsr-null and TrxR1/Gsr-null livers treated daily with Asc. Gsr-null (a,c) or TrxR1/Gsr-null (b,d) animals received no treatment (a,b) or 3 daily IP injections of 4mg / g Asc (c,d). Congestion (red circle), ballooning hepatocytes (BH) ductular reaction (DR) pyknotic nuclei (PN) portal triad (PT) and central vein (CV)

Ascorbate Treatment Modestly Depletes GSH in TrxR1/Gsr- But Not In Gsr-null Livers

The severe hepatic necrosis induced by treatment of mice having TrxR1/Gsr-null livers with ascorbate strongly implicated ascorbate, itself, as being hepatotoxic in these severely reductase-compromised livers. Since these livers are highly sensitive to treatments that either block GSH biosynthesis or deplete GSH stores, we investigated whether the ascorbate was impacting hepatic GSH levels. Mice were given a single dose

of ascorbate in sterile saline (4mg/g body weight) and were sacrificed 60 or 180 minutes later. Livers were harvested and total GSH+GSSG levels were measured in the liver lysates. As reported previously, non-stressed TrxR1/Gsr-null livers had similar GSH levels as did the Gsr-null littermate controls (Fig. 3, black bars). Moreover, 60 min after treatment with ascorbate, no significant differences in GSH was measured (gray bars). However, at 180 min after treatment, the TrxR1/Gsr-null livers had half as much total GSH as untreated controls, whereas the Gsr-null control livers were not significantly affected (white bars). Although this indicated that administration of ascorbate led to hepatic GSH depletion, the modest magnitude (50% of initial levels) and slow kinetics (not apparent at 1h) was dramatically different than the kinetics and magnitude of the loss of hepatic GSH we had previously measured upon administration of acetaminophen to mice [40], in which nearly all GSH was lost within 1 hour of treatment.

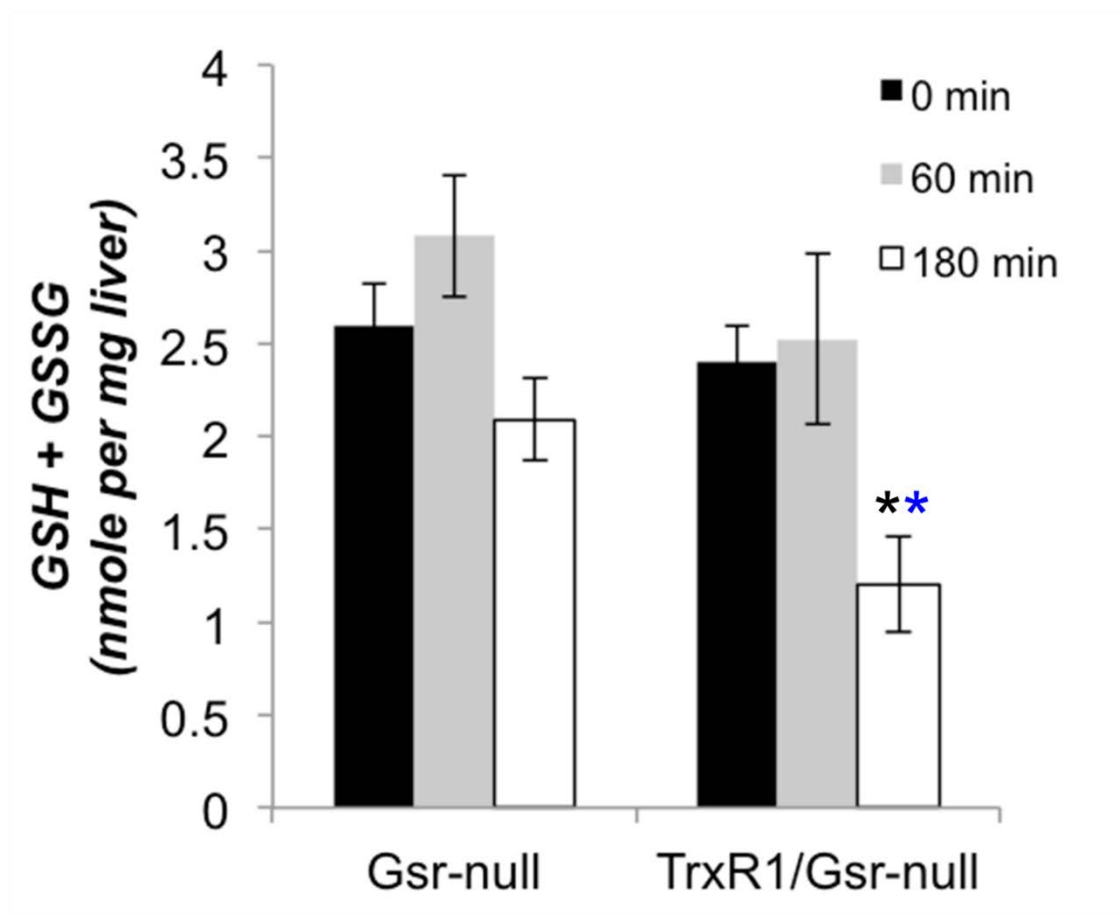


Fig. 5.3. Hepatic levels of total glutathione (GSH+GSSG) in animals given IP Asc (4mg / g) and harvested at indicated times are shown, $n > 3$ animals for each condition; bars represent mean $\pm$ s.e.m. * (black) indicates significance ($p < 0.05$) compared to 0 min control, * (blue) indicates significance between genotypes at indicated timepoint using Student's t-test

DHA Treatment Strongly Depletes GSH in Both Gsr- and TrxR1/Gsr-null Livers

Although the oxidized form of ascorbate, DHA, is reduced by GSH, ascorbate is not expected to consume GSH. This knowledge, in combination with the relatively long time and modest impact of ascorbate on hepatic GSH levels (Fig. 3) suggested that the observed GSH depletion might have been caused by hepatic GSH-dependent reduction of

DHA that was generated by spontaneous in vivo oxidation of the administered ascorbate in the relatively oxidizing environment of the extracellular fluids. Mice were treated with DHA at 1/12th the dose (0.3 mg/g body weight) that we had used for ascorbate and were harvested at 0, 20, 60, and 180 min after inoculation. Results showed that DHA caused significant depletion of hepatic GSH in TrxR1/Gsr-null livers as early as 20 min post inoculation, and at 60 and 180 min, hepatic GSH levels were only 20% of the initial level (Fig. 4). In the Gsr-null controls, significant GSH depletion was also observed, but this was delayed (no depletion detected until 60 min) and more modest (67% of the initial level; Fig. 4). The relative resistance of the Gsr-null livers to GSH depletion from DHA treatment is likely attributed, at least in part, to the ability of TrxR1 in these livers being able to directly reduce DHA (see Discussion).

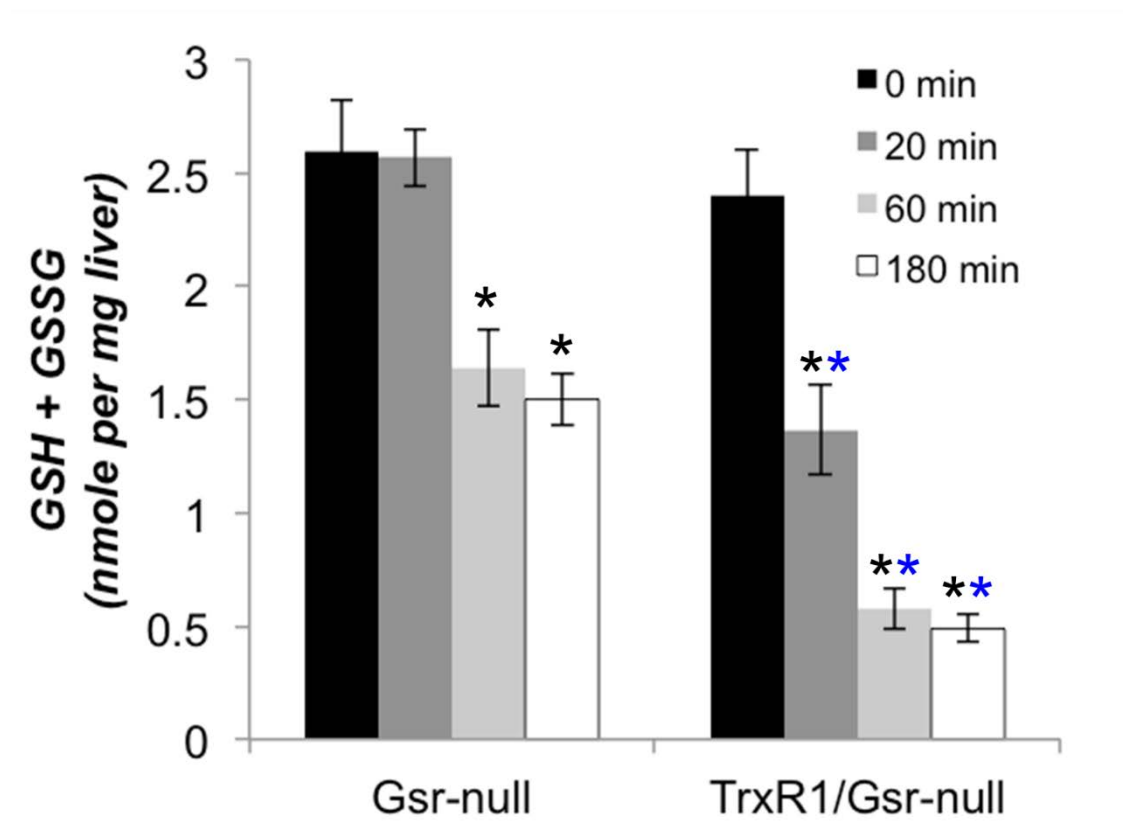


Fig. 5.4. Hepatic levels of total glutathione (GSH+GSSG) in animals given IP DHA (0.3mg / g) and harvested at indicated times are shown, n>4 animals for each condition; bars represent mean $\pm$ s.e.m. * (black) indicates significance ($p<0.05$) compared to 0 min control, * (blue) indicates significance between genotypes at indicated timepoint using Student's t-test

Discussion:

The rationale for dietary antioxidant supplements is to help support or bolster the endogenous antioxidant systems during oxidative stress. The efficacy of antioxidants has been demonstrated in diverse situations in cell culture models, animal models, and in the clinic. Perhaps most dramatically, treatment of patients following exposure to hepatotoxic levels of acetaminophen with the antioxidant NAC prevents acute liver failure in ~70% of affected patients [41, 42]. However, even in this well-studied situation, the exact mechanisms by which NAC promotes survival remain unclear. NAC, a simple N-acetylated version of Cys, undergoes complex and in many ways still mysterious extracellular and intracellular metabolism following administration. The relative importance of its roles as a true reductant, a Cys-donor, a driver of thiol-disulfide exchange, or an H₂S donor are not yet fully elucidated [23].

Vitamin C is perhaps the most common dietary antioxidant. Its requirement as an essential micronutrient, its potency as a free radical trap, and its natural occurrence in many foods indicates it is a safe and likely efficacious dietary supplement. Because the non-enzymatic reaction of ascorbate with H₂O₂ is slow and mammals lack ascorbate peroxidase, ascorbate supplementation will not directly eliminate H₂O₂ [43, 44]. Nonetheless, through its other activities, ascorbate is expected to support redox homeostasis in mammalian cells. The original goal of this project was to determine if

dietary ascorbate supplementation could ameliorate the oxidative damage and frequency of acute liver failure in severely oxidatively stressed livers, as modeled by mice with TrxR1/Gsr-null livers. Importantly, these livers are thought to model the effects of coincidental inhibition of TrxR1 and Gsr by strong electrophilic toxins [45], and as such might have value for testing therapeutic interventions for such conditions. As might be the case for many people considering ascorbate supplementation, we anticipated that this supplement likely would help, and we did not anticipate that it could possibly cause harm. Thus, the dramatically increased frequency of acute liver failure experienced with ascorbate was not expected.

We used intraperitoneal administration of ascorbate to test whether the increased frequency of acute liver failure was, indeed, due to the ascorbate. The dramatic hepatopathology that we found (Fig. 2D) supported this conclusion; however the modest and sluggish impacts that ascorbate inoculation had on TrxR1/Gsr-null livers (Fig. 3) suggested the effect might be indirect, and involve *in vivo* oxidation of the ascorbate to DHA. This prediction is supported by the rapid and precipitous loss of GSH that occurred in livers of mice inoculated with a much lower dose of DHA (Fig. 4).

At high physiological concentrations (> 0.1 mM), DHA is rapidly reduced to ascorbate by GSH, Grxs, Gsts, and TrxR1 [29, 46]. TrxR1 is reported to account for at least 75% of the DHA reduction in rat liver cytosol [47]; however in TrxR1/Gsr-null livers, all DHA reduction must utilize GSH. Since these livers lack Gsr, the resultant GSSG cannot be reduced. GSH/GSSG ratios are tightly regulated in the absence of Gsr by GSSG export [48]. Because TrxR1/Gsr-null livers are critically dependent on *de novo*

synthesized GSH, reduction of substantial amounts of DHA could lead to direct depletion of GSH, as seen in this study.

Like NAC, discussed above, ascorbate undergoes complex and in many ways mysterious metabolism both extra- and intra-cellularly. In the case of our initial survival study (Fig. 1), the ascorbate was added to acidified drinking water (see methods), in which its oxidation is slow, and this was changed every week. However, even if oxidation was negligible in the water bottle, it could have been substantial after ingestion, in both the gut and the blood plasma. Similarly, when we administered ascorbate by intraperitoneal inoculation (Figs 2,3), we minimized the likelihood of pre-administration oxidation to DHA, but not of *in vivo* oxidation. Indeed, we suspect that the slow and modest GSH depletion caused by intraperitoneal ascorbate (Fig. 3) likely reflects the rate of oxidation of the ascorbate to DHA in these animals.

The question then arises: when is administration of ascorbate safe and effective as an antioxidant, and when might it be dangerous? Clearly ascorbate, likely through *in vivo* oxidation to DHA, can deplete GSH and consume disulfide reducing power. However, survival of mice with TrxR1/Gsr-null livers emphasizes that, in normal hepatocytes, the NADPH-fueled disulfide reductase systems can generate far more reducing power than is needed for survival [18]. Thus, we anticipate that cells in which these systems are functional will not be adversely affected by ascorbate supplementation and, for combatting exposures that do not inhibit TrxR1 and Gsr, ascorbate supplementation could be beneficial. By contrast however, the current study shows that, when TrxR1 and Gsr are disrupted, as might occur with many strong electrophilic toxins or drugs, ascorbate

supplementation could be detrimental. Consistent with this, the electrophilic metalloid arsenic is known to be a potent inhibitor of TrxR1 and likely also of Gsr [45, 49, 50]. Ascorbate supplementation was shown to increase oxidation of lipids in human leukemia cells challenged with the electrophilic metalloid arsenic [51]. Similarly, the reactive hepatocytic metabolite of acetaminophen, N-acetyl-p benzoquinone imine (NAPQI), is known to directly and potently inhibit TrxR1 and to disrupt the GSH pathway [40]. Although acetaminophen overdose is associated with physiological responses in the liver that include severe oxidative stress [42], it might be prudent to caution against including ascorbate supplementation in therapeutic regimes to treat this condition. As in the mouse models presented here, it might not be effective as an antioxidant in these livers and, by contributing to GSH depletion, it could instead do harm.

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CHAPTER SIX: L-METHIONINE AND L-CYSTINE WITH
STABLE S ISOTOPE, ^{34}S , USED TO DETECT
AND FOLLOW S-METABOLITES

Abstract

Reduction of endogenous oxidants by S amino acids is thought to be driven most frequently through the formation and subsequent reduction of a disulfide on the active reducing entity, be it a protein or small molecule. However, in scenarios of overwhelming oxidation, such as in TrxR1/Gsr-null livers, disulfide formation may not be the most efficient mechanism. We hypothesize that, instead, a thiol can potentially react with multiple equivalents of oxidants to form the irreversible sulfinic ($-\text{SO}_2\text{H}$) or sulfonic ($-\text{SO}_3\text{H}$) acids, form a persulfide ($-\text{SSH}$) which can also be over to the persulfonic ($-\text{SSO}_3\text{H}$) acid or react to the most completely to yield the most oxidized form of S, SO_4^{2-} . S-metabolites are difficult to detect and follow by standard MS, so a stable isotope of S, ^{34}S , was incorporated into Met to yield L-(^{34}S)Met. The L-(^{34}S)Met was confirmed by MS and the amino acid was biovalidated in HEK-293 cells where ^{34}S peaks of S-adenosylmethionine, S-adenosylhomocysteine, homocysteine, cystathionine, and Cys were detected. A ^{34}S version of Cys was also synthesized using the enzyme cysteine synthase. L-(^{34}S)Met and L-(^{34}S)Cys are potentially useful synthetic molecules to track the metabolism of S in cells under stress.

Introduction

Co-disruption of both of the NADPH-dependent cytosolic disulfide reductase systems in liver via hepatocyte-specific genetic ablation of the genes encoding thioredoxin reductase-1 (TrxR1) and glutathione reductase (Gsr; in combination the double-homozygous state entitled “TrxR1/Gsr-null”) substantially impacts metabolic pathways in the liver, in particular relating to sulfur (S) amino acid metabolism. For example, elimination of a single molecule of hydrogen peroxide (H_2O_2) via the abundant disulfide reductase-fueled peroxiredoxin (Prx) or glutathione peroxidase (Gpx) enzymes (Nagy *et al.*, 2011, Arteel & Sies, 2001) in TrxR1/Gsr-null cells will require consumption of two molecules each of methionine, glutamic acid, glycine, and serine (Met, Glu, Gly, and Ser, respectively) and hydrolysis of ten phosphodiester bonds from adenosine triphosphate (ATP). The pathway will collaterally generate one molecule of glutathione-disulfide (GSSG), two molecules each of α -ketobutyrate (α KB), ammonia, and water, and will generate two methyl groups that must be utilized in S-adenosylmethionine (SAM)-dependent methyltransferase reactions (Eriksson *et al.*, 2015, Prigge *et al.*, 2017, Miller & Schmidt, 2019, Miller *et al.*, 2018, Fig. 6.1). Although the specific fate of some of the products of this pathway remain to be determined in the context of these cells, the TrxR1/Gsr-null cells are known to be incapable of reducing the disulfide bond in GSSG, and therefore this product must be excreted from the cells (Eriksson, *et al.*, 2015).

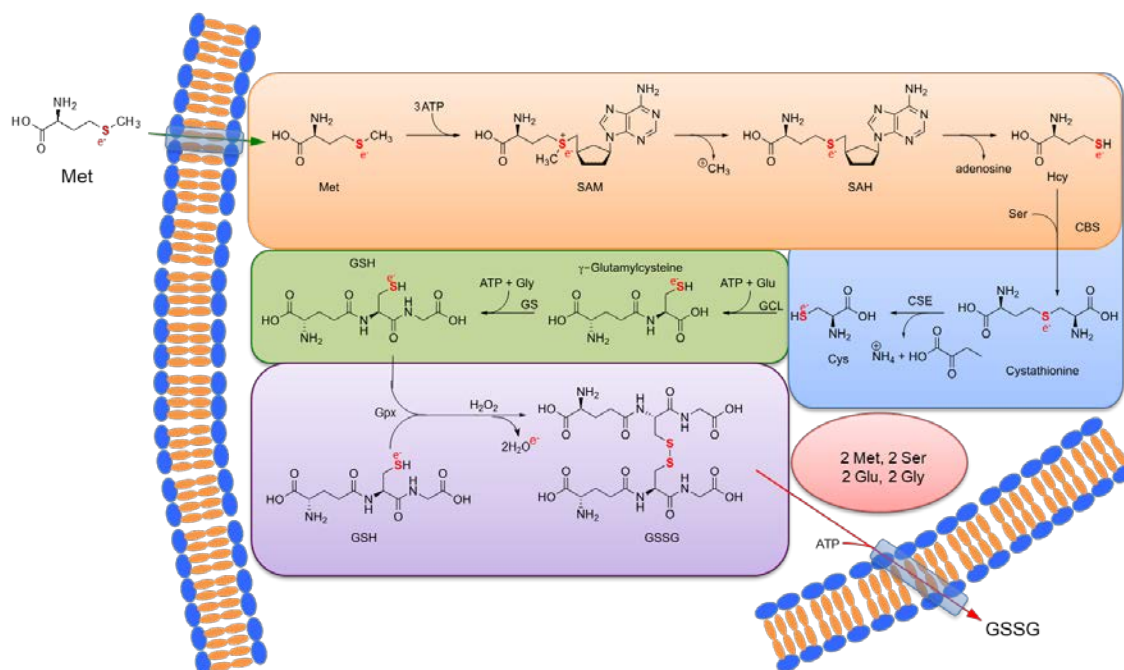


Figure 6.1: Energy and amino acid requirements of Met-driven H_2O_2 reduction. The energy and amino acid requirements of the Met-cycle (orange), transsulfuration (blue), and glutathione synthesis (green) resulting in one molecule of GSH which is utilized with another equivalent of GSH to reduce one H_2O_2 to $2\text{H}_2\text{O}$ (purple). The 8 amino acids (2 Met, 2 Ser, 2 Glu and 2 Gly) are excreted from the cell (red circle).

Although it remains unclear how the byproducts of this reaction will be subsequently used by the TrxR1/Gsr-null hepatocytes, or how much energy might be recovered in the process, the consumption of amino acids and energy in this pathway is surprising. However, there are several other known reactions in cells that could, potentially, provide mechanisms by which the Met-derived S moiety might more efficiently contribute to detoxification of H_2O_2 and thereby decrease the resource-cost for maintaining redox homeostasis in TrxR1/Gsr-null hepatocytes. For example, cystathionase (CSE), which normally converts cystathionine (Cth) into cysteine and α -ketobutyrate will, under certain conditions, use cysteine instead of cystathionine as a substrate, and yield as products cysteine-persulfide (Cys-SSH) and pyruvate and NH_3^+

(Fig. 2.4). Importantly, due to the penultimate S, the terminal S of Cys-SSH is highly electrophilic ($pK_a \sim 4.5$), and therefore, unlike Cys ($pK_a \sim 8.2$), will exist as a reactive perthiolate anion ($\text{Cys-SS}^- + \text{H}^+$) at physiological pH. Whereas the reaction of Cys and H_2O_2 at physiological pH is slow ($2.9 \text{ M}^{-1}\text{S}^{-1}$; Winterbourn & Metodiewa, 1999) compared to the reaction of H_2O_2 with a Prx ($1\text{-}4 \times 10^7 \text{ M}^{-1}\text{S}^{-1}$; Winterbourn & Hampton, 2008) and therefore not biologically relevant at neutral pH, Cys-SS⁻ is highly reactive with H_2O_2 , forming Cys-SSOH and possibly Cys-SSO₂H (Heppner *et al.*, 2018, Benchoam *et al.*, 2019, Fig. 6.2, orange square). Unlike Cys over-oxidation, which yields irreversible sulfinic (-SO₂H) and sulfonic (-SO₃H) acids, oxidation of Cys-perthiolate anion to Cys-SSO₂H and Cys-SSO₃H is reversible, as Cys-SSO₃H can be reduced with reductants like Trx to yield Cys and HSO_3^- (Filipovic *et al.*, 2018). The reduction product, Cys, can re-enter this cycle while HSO_3^- can neutralize yet another molecule of H_2O_2 to yield sulfate (discussed below). Cys-SSH is also readily reduced by Trx, with reaction rates more than twice as fast as the Trx-driven reduction of cystine (Wedmann *et al.*, 2016), yielding Cys and H_2S . Under normal conditions, H_2S is in much lower concentrations than other thiols such as GSH and is unlikely to act as a direct antioxidant, however when H_2S levels are increased, or in the case of an enzyme-mediated reaction as seen between H_2S and the oxidants produced by myeloperoxidase (Garai *et al.*, 2017), H_2S can be a potential sink for H_2O_2 . The reaction of H_2S and H_2O_2 , which yields sulfenic acid (HSOH), proceeds at a rate that is likely not biologically relevant ($k = 0.48 \text{ M}^{-1}\text{S}^{-1}$) (Rabai *et al.*, 1992), but may become relevant when the reaction is enzymatically driven (Garai *et al.*, 2017, Pálinkás *et al.*, 2015). HSOH can either react with additional

equivalents of H_2S , producing sulfane sulfur (HS_nH) species or with two additional H_2O_2 at a substantially slower rate ($k = 0.04 \text{ M}^{-1}\text{S}^{-1}$) to produce sulfite (HSO_3^-). HSO_3^- reacts slowly with another H_2O_2 to produce HSO_4^- ($k = 0.7 \text{ M}^{-1}\text{S}^{-1}$), but this rate may be increased dramatically by the mitochondrial-localized sulfite oxidase ($k = 85 \text{ M}^{-1}\text{S}^{-1}$; Ritzmann & Bosshard, 1988, Kessler & Rajagopalan, 1974), yielding HSO_4^- as the terminal oxidation of S (Rabai *et al.*, 1992, Fig. 6.2, purple square). This pathway is potentially the most “sulfur efficient” in that two Cys can reduce 1 H_2O_2 (classic disulfide mechanism), the resulting cystine can be converted to Cys-SSH by CSE and upon the addition of 2 e^- , yield H_2S , capable of reducing 4 more H_2O_2 , as well as another Cys. Utilizing the Cys-SSH or H_2S pathways instead of the disulfide formation/reduction to reduce oxidants might limit catabolism of vital nutrients (Met, Ser, Glu, Gly, ATP) for these hepatocytes; GSH, which powers Prx’s and Gpx’s, appears to be excreted from hepatocytes as GSSG after consuming only 1 equivalent of H_2O_2 . Furthermore, Met must go through 7 reactions to yield 1 molecule of GSH, which is not only energy and resource intensive but also potentially very slow, making TrxR1/Gsr-null hepatocytes more susceptible to waves of oxidation. However a biochemical method to track the metabolism of Met in TrxR1/Gsr null livers was not readily available. Importantly, although these reactions are all known to occur individually, it is unknown if these pathways might exist in oxidatively stressed cells and contribute to reductive consumption of H_2O_2 .

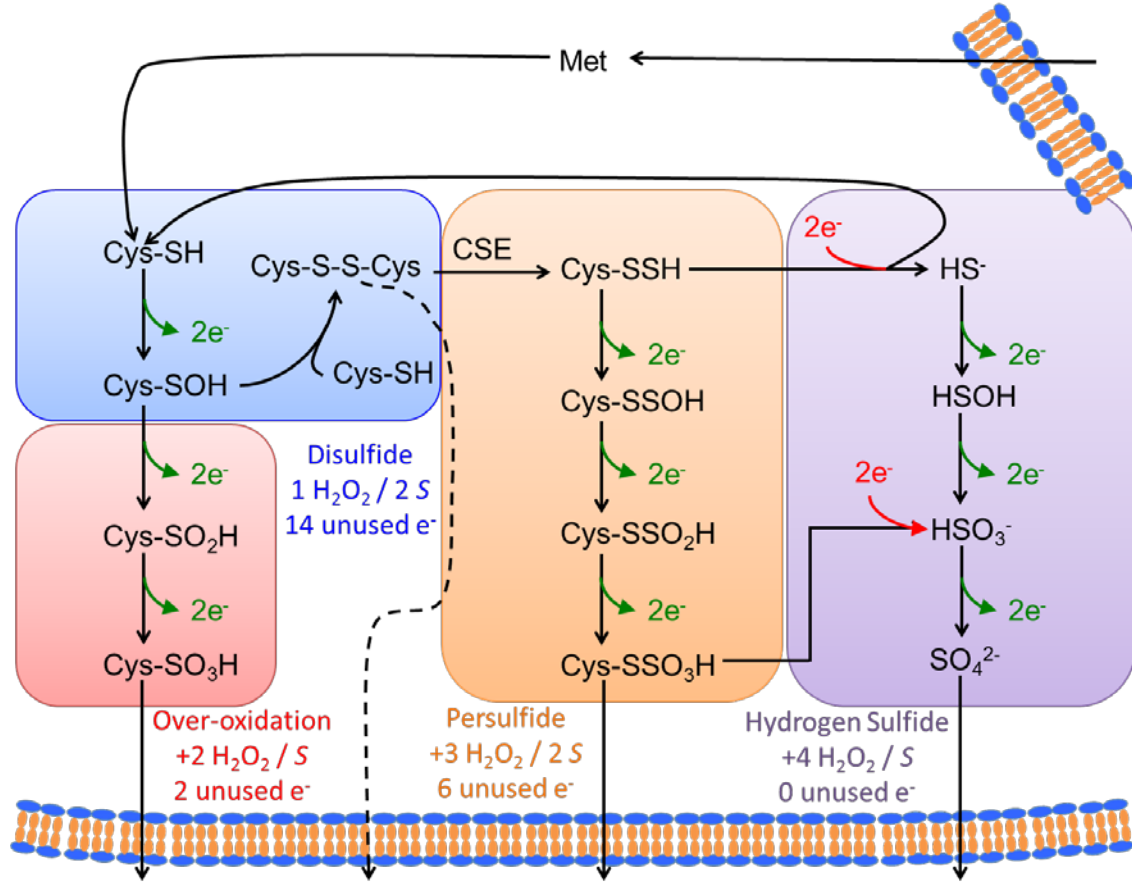


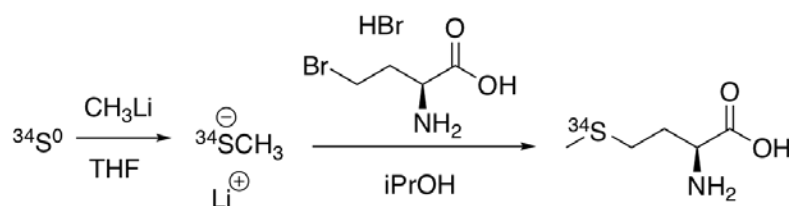
Figure 6.2: S-efficient oxidation pathways of Cys. In the canonical disulfide formation, 2 Cys each donate an electron to form a disulfide (blue square) which must be excreted in TrxR1/Gsr-null hepatocytes (dotted line). Cys may also donate 4 additional electrons via over-oxidize to Cys-SO₃H (red square), which is exported. Alternatively, Cys-SSH could provide 3 additional reducing equivalents (orange square) or HS⁻ could provide 4 additional reducing equivalents (purple square), both of which provide more reducing electrons per S-atom. S from the persulfide pathway may cross into the sulfate from either Cys-SSH → HS⁻ or Cys-SSO₃H → HSO₃⁻ by the addition of 2 electrons (red arrows).

Regardless of how it is used, in TrxR1/Gsr-null livers the critical and, to date only, detected source of S available is the thioether-S from dietary Met (Eriksson *et al.*, 2015). To investigate how this S is being used in oxidatively stressed conditions, as modeled by the TrxR1/Gsr-null liver system, we plan to track the Met-derived S using

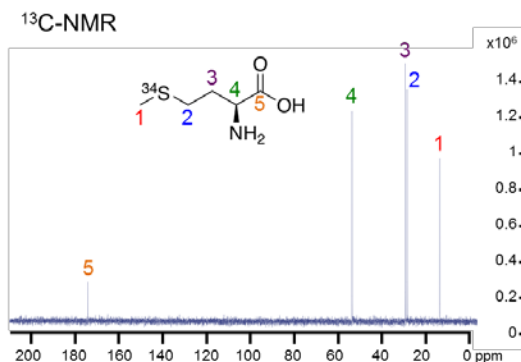
stable isotope-labeled Met (L-(^{34}S)Met). In this way, we intend to quantitatively trace flux of the sulfur through all possible pathways in these cells.

To accurately determine metabolic flux of S metabolites, the stable isotope L-(^{34}S)Met would need to be administered at a constant rate over the course of several hours. The most effective means to achieve this administration is through jugular venous catheterization, which allows a constant and adjustable introduction of L-(^{34}S)Met directly into the bloodstream. L-(^{34}S)Met will be administered at a rate of 82 mg/kg/hr, proportional to the [2,3,3- ^2H]-L-Serine infusion utilized by Ducker *et al.* (2016). To monitor the S metabolism of a 30g mouse over 4h would require about 10mg of L-(^{34}S)Met. Purchasing from the only commercial supplier of L-(^{34}S)Met (Toronto Research Chemicals) would cost \$1,800 / 10mg, making this experiment financially infeasible.

A



B



C

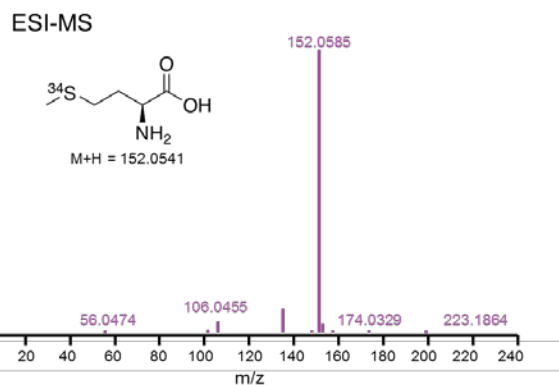


Figure 6.3: Synthesis and characterization of L-(^{34}S)Met. (A) – Synthetic scheme for the preparation of L-(^{34}S)Met from ^{34}S metal and (S)-(+)-2-Amino-4-bromobutyric acid hydrobromide. (B) ^{13}C -NMR spectrum of isolated L-(^{34}S)Met. (C) ESI-MS chromatogram of L-(^{34}S)Met.

Results

In order to obtain an economically feasible source of isotopically labeled Met, L-(^{34}S)Met was synthesized to incorporate the stable S isotope ^{34}S (yielding pure L-(^{34}S)Met) following the method of Ogra *et al.* (2008) (reaction scheme 6.1). $^{34}\text{S}^0$ metal, purchased from Cambridge Isotope Labs, was reduced with CH_3Li to yield the $\text{CH}_3^{34}\text{S}^-$ methyl thiolate, which was used *in situ* with the brominated 4-carbon amino acid backbone, (2S)-2-Amino-4-bromo-butyric acid hydrobromide (Fig. 6.3 A). The compound was purified by reverse phase HPLC analysis on a Phenomenex Synergi 4u Polar RP 80A HPLC column (250 x 30mm) monitoring absorbance at 235nm. After the sample was confirmed to be pure by ^{13}C -NMR (Fig. 6.3 B) and isotopically pure by mass spec ($\text{M}+\text{H} = 152.0468$, L-(^{34}S)Met = 149.0510 g/mol) (Fig. 6.3 C), HEK-293 cells were incubated in DMEM lacking cysteine and methionine but supplemented with 100 μM L-(^{34}S)Met. After 1 or 3 hours incubation, the cells were collected, washed and metabolites extracted, either in a 80:20 methanol:water buffer or a thiol blocking buffer, 80:20 methanol: 10mM ammonium formate, 25mM N-ethylmaleimide (NEM), pH 7.0. Mass spectroscopy of the cellular metabolites showed a substantial enrichment in the amount of L-(^{32}S)Met versus L-(^{34}S)Met between the control, which received normal DMEM and no isotopic label, and the L-(^{34}S)Met supplemented cells (Fig. 6.4 A). The ^{34}S could be tracked through the Met cycle, showing ^{34}S -enriched peaks for S-adenosylmethionine

(SAM), S-adenosylhomocysteine (SAH), and the NEM thiol adduct of Hcy (NEM-Hcy). Because HEK-293 cells maintain both NADPH-dependent disulfide reductase systems, they are not reliant on Met as their sole source of reduced sulfur, like in the TrxR1/Gsr null livers, so as expected, very little of the ^{34}S was trafficked through transsulfuration, with only a slight enrichment in [^{34}S]-cystathionine and [^{34}S]-Cys visible. ^{34}S isotopes of g-Glu-Cys, GSH/GSSG, hypotaurine and taurine were not detected. Interestingly, very substantial enrichment of ^{34}S -5'-methylthioadenosine (MTA), produced by the removal of a 3-amino-3-carboxyl-propyl group from SAM, was detected. While MTA is known to contribute to polyamine synthesis, the reason for its enrichment in this circumstance is not clear.

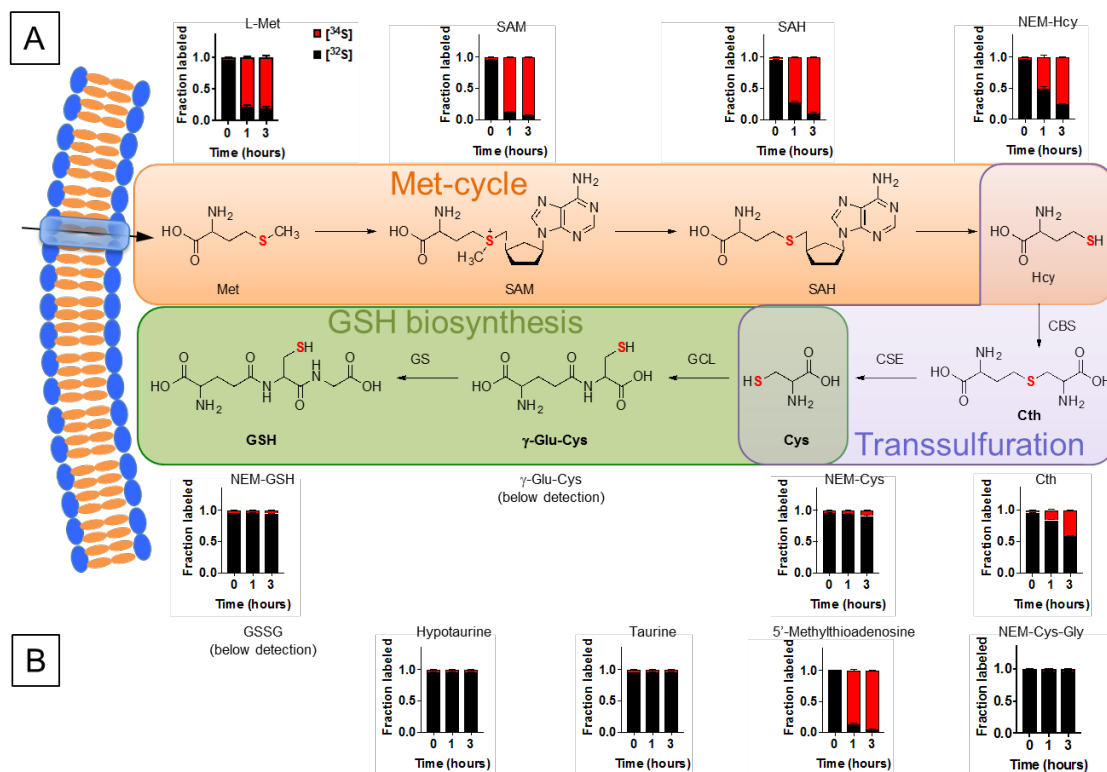


Figure 6.4: Identifying metabolites of [L-³⁴S]-Met-treated HEK-293 cells. (A) S-metabolites of HEK-293 cells grown for 1 or 3 hours with [L-³⁴S]-Met as the only source of S were identified by ESI-MS. Isotopic abundance of ³²S (black bars) and ³⁴S (red bars) shown as fraction of total metabolite concentration. (B) – Isotopic enrichment of downstream S-metabolites.

TrxR1/Gsr-null livers cannot reduce cystine and instead must utilize Met as their sole source of reduced S, so providing a labeled source of S via Met should label all downstream S metabolites. This is because Met must be converted to Cys, a metabolic hub for S metabolism, in TrxR1/Gsr-null livers, however, as shown in the metabolic labeling of HEK-293 cells, this strategy is not as effective in cells that possess an NADPH-dependent disulfide reductase system. After successful synthesis of L-(³⁴S)Met, it was clear there was a need for another ³⁴S-derived metabolic marker to label S entering the metabolic pathways via uptake and intracellular reduction of cystine. Therefore, we initiated a project to synthesize L-(³⁴S)cystine.

Similar to the situation for obtaining L-(³⁴S)Met for mouse-labeling studies, L-(³⁴S)cysteine/cystine is not commercially available, so we needed to find a synthetic strategy to generate the labeled amino acid for our studies. As discussed above, the synthesis of L-(³⁴S)Met proceeded by the simple displacement of the (2S)-2-Amino-4-bromo-butyric acid with methyl thiolate; however an alternative strategy was required to avoid the possibility of a double displacement of the terminal thiol, Cys, with another equivalent of a bromo-backbone to form L-lanthionine. Similar strategies have been employed using benzyl mercaptan or thioacetic acid instead of HS⁻; however these approaches suffered from low yields and were inefficient in regards to amount of the expensive ³⁴S used (Crack *et al.*, 2019, Siebum *et al.*, 2004). Instead, the method of our

collaborators in Japan, Ono *et al.* (2017) was employed with minor variations. Briefly, ^{34}S metal was reduced to Na_2^{34}S using liquid NH_3 , Na^0 (quantitative yield), and *O*-acetylserine was produced by addition of L-serine to acetyl chloride in acetic acid (81% yield) (Fig. 6.5 A). The final step, displacement of the acetate of OAS by HS^- , was achieved enzymatically by the enzyme CysK, from the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium LT2 expressed in BL21(DE3) *E. coli* (expression clone kindly provided by our collaborators, T. Akakaie, Sendai, Japan). The protein was purified by Ni-NTA agarose. Reaction yields of the CysK enzymatic step varied from 20-40%, using the method of Gaitonde (1967) to measure Cys formed in the reaction (thiol agents such as 4-DPS or DTNB would give a positive result with the HS^- present.) The sample was confirmed to be pure by ^{13}C -NMR (Fig. 6.5 B) and was reduced by NaBH_4 (Brown, 1960). The reduced product gave a yellow color when mixed with DTNB and the reaction yield was calculated at 20%.

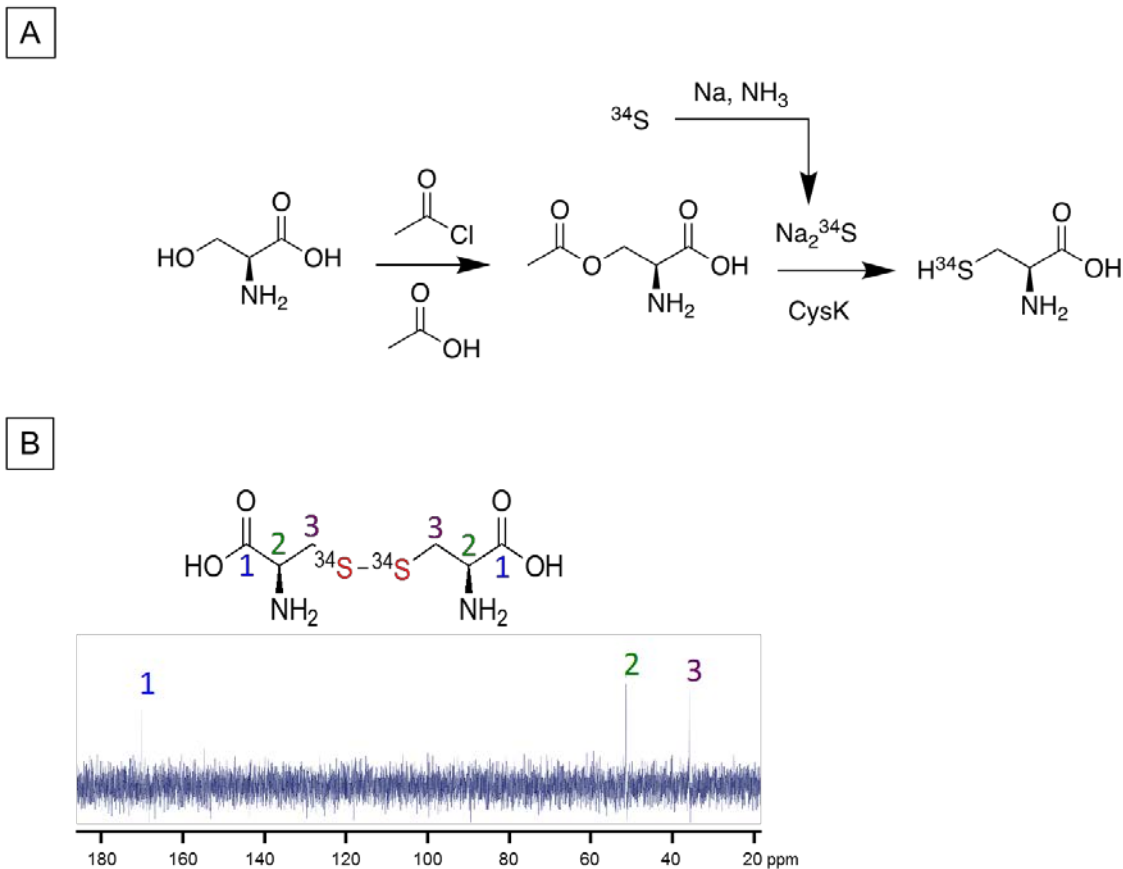


Figure 6.5: Synthesis and characterization of [L-³⁴S]-cystine. (A) – Synthetic scheme for the preparation of [L-³⁴S]-Cys from ³⁴S metal, O-acetylserine and the enzyme, CysK. (B) ¹³C-NMR spectrum of isolated [L-³⁴S]-Cystine.

Materials and Methods

Materials.

³⁴S was purchased from Cambridge Isotope labs as a dry powder. (S)-(+)-2-Amino-4-bromobutyric acid hydrobromide, L-serine, and all other reagents were purchased from Sigma Aldrich. THF was distilled over Na⁰ and was stored on molecular sieves. Reversed phase HPLC purification was performed with a Waters 2487 Dual 1 Absorbance detector, 600 controller and pump, and a Phenomenex Synergi 4μ Polar RP

80A HPLC column (250 x 21.2 mm,) using Waters Empower 3 software. NMR spectroscopy was performed on a Bruker Avance DPX 300 NMR spectrometer. The expression clone for CysK was generously provided by T. Akakaie, Sendai, Japan.

CysK expression

The pQE-80L-CysK (Ampr/His6) expression plasmid was heat shock transformed into DH5 α cells and grown overnight on TBG + Amp₁₀₀ plates at 37 °C. A single clone was picked and plasmid was purified by midiprep. The plasmid was transformed into BL21(DE3) competent *E. coli* cells and induced with IPTG. CysK was purified on Ni-NTA agarose. Purified CysK solutions were diluted 1:1 with glycerol and stored at -80 °C.

Synthetic Procedures.

L-(34S)Met. The method of Ogra *et al.* (2008) was followed. Briefly, 0.0351g ³⁴S metal (1.03 mmol, 1 equiv) was dissolved in 10 mL dry THF in a flame dried, 3-neck, 250mL round bottom flask and fitted with reflux condenser in center neck and all holes closed with stoppers. The flask was evacuated and refilled with N₂ three times, then submerged in dry ice/ acetone for 5 min and 4.0 mL 2.2 M methyl lithium (8.8 mmol, 8.5 equiv) added dropwise. A deep yellow color was noted initially but after the cooling bath removed and the solution brought up to room temp for 20 min, the solution was gray. The flask was returned to -78°C bath and 0.2701 g (S)-(+)-2-Amino-4-bromobutyric acid hydrobromide (1.03 mmol, 1 equiv), was dissolved in 30 mL IPA and added slowly to side of flask to chill before entering the CH₃³⁴S⁻ solution. After addition was complete, the solution was stirred for 10 min, then removed from dry ice bath. After

it reached room temperature (rt), the solution was refluxed for 1.5 hours and was a red color and cloudy after refluxing. The solution was neutralized with 5 mL 1 N HCl rotovaped until only the water later remained. The pH of this layer was about 4 (pH paper). The solution was extracted twice with 30mL diethyl ether and the red color was removed into the ether layer. The water layer was frozen and lyophilized to dryness. The sample was purified by RP HPLC using an isocratic method of 25% A (0.2% AcOH in H₂O) and 75% B (50% MeOH, 50% H₂O, 20 mM ammonium formate). Drying by lyophilization provided 27.1 mg of L-(³⁴S)Met as a white solid (40% yield). ¹³C-NMR (125 MHz, D₂O): δ 13.835, 28.716, 29.553, 53.781, 174.133; HRMS-ESI (*m/z*): calcd for C₅H₁₂NO₂³⁴S [M + H]⁺ 152.0541, found 152.0585

Na₂³⁴S. 0.0247g ³⁴S (0.727 mmol, 1 equiv) metal was added to a 2-neck, 25 mL round bottom flask. The center neck was fitted with a dry ice / acetone condenser and the other port was sealed with a septum. Both the reaction flask and the condenser were chilled with dry ice / acetone and about 10 mL of NH₃(l) was condensed to the reaction flask. 0.478 g Na⁰ (20.8 mmol, 29 equiv) was added and the solution turned a deep blue color. The mixture was stirred for 1.5 hours at -78 °C. The flask was removed from the cooling bath and allowed to come up to rt overnight, yielding a white precipitate. The flask was chilled on ice and the white precipitate was dissolved in 4 mL 0.3 N NaOH added dropwise. S²⁻ of the solution was quantified by DTNB, using Na₂S·9H₂O as the standard. The reaction yielded 4.0 mL of 181 mmol Na₂S (0.726 mmol, quantitative yield).

O-acetyl-L-serine. The synthesis of O-acetyl-L-serine was performed following the literature procedure (Wilcheck and Patchornik, 1964). Briefly, 1.115 g L-serine (10.6 mmol) was dissolved in 5.5 mL AcOH and 2 mL 6N HCl. The flask was purged with N₂ and submerged in an ice bath. 22 mL of acetyl chloride was added dropwise over 15 min and a white precipitate formed. The mixture was stirred for 3 hours, rt and the precipitate filtered. The filtrate was washed three times with dry Et₂O and lyophilized to dryness, yielding 1.7141 g of OAS·HCL as a white solid (88% yield). ¹H NMR (300 MHz, D₂O): δ 1.996 (s, 3H, COCH₃), 4.278 (t, 1H, CHCH₂), 4.401-4.475 (m, 2H, CHCH₂); ¹³C-NMR (125 MHz, D₂O): δ 19.902, 52.086, 61.815, 169.136, 173.057; HRMS-ESI (*m/z*): calcd for C₅H₁₀NO₄ [M + H]⁺ 148.0604, found 148.0441.

L-(³⁴S)cystine. The synthesis of L-(³⁴S)cystine was performed following the literature procedure (Ono *et al.*, 2017) using a modified procedure (Crack *et al.*, 2019). 0.1335g OAS·HCl (0.73 mmol, 1 equiv) was added to a 50mL Falcon tube with 25 mL 100 mM Tris pH 8.0 and 230 µL 1.5 mg/mL CysK (345 µg CysK protein). 4 mL of 180 mM Na₂³⁴S (0.73 mmol, 1 equiv) was added to the tube and the pH was adjusted to 8.0 using using 1 N HCl. The reaction was left at rt for 3h, dark, and was then transferred to 4 °C for 72 hours. The solution was yellow and still had a distinct smell of sulfur after 72 hours, so another 0.1341 g OAS·HCl was added and the reaction proceeded at 4 °C for another 72 hours, after which the solution was white and cloudy. CysK protein was precipitated by acidifying the solution to pH 1.0 with 12.1 N HCl and storing overnight at 4 °C. The precipitated protein was filtered away and any Cys was converted to cystine by

the addition of H₂O₂ to a final concentration of 0.1%. The solution was returned to pH 8.0 with 6 N NaOH and diluted 1:1 with acetone. L-(³⁴S)cystine was precipitated at -4 °C overnight, yielding 16.5 mg L-(³⁴S)cystine as a gray powder (18.3% yield). ¹H NMR (300 MHz, D₂O): δ 2.960-2.999 (m, 2H, CHCH₂), 4.093 (t, 1H, CHCH₂); ¹³C-NMR (125 MHz, D₂O): δ 35.875, 51.378, 170.023

Cell Culture Experiments

HEK-293 cells were grown to 80% confluency in DMEM. At 1 or 3h prior to harvest, media was removed, cells were washed twice with sterile PBS, and given DMEM lacking L-Met, L-cystine and L-glutamine, supplemented with L-(³⁴S)Met to 100 μM. At harvest, media was removed and saved and cells were washed twice with sterile PBS, then removed from the plate with trypsin. Cells were spun at 2,000XG for 5min, rt and the supernatant removed. Cell pellets were suspended in either 80:20 MeOH:H₂O or 80:20 MeOH:10 mM ammonium formate, 25 mM NEM, pH 7.0, briefly vortex and gently sonicated. Cell lysates sat on ice for 30 min, dark. Metabolite extracts were cleared by centrifugation at 13,000XG for 15 min, 4 °C.

Discussion

S-reducing power in normal and potentially harmful cells. The reducing power carried by S-metabolites like Cys and GSH are critical to drive cellular functions and maintain cell viability and redox homeostasis in healthy cells (Miller *et al.*, 2018). However, maintenance of redox homeostasis and reduction of oxidants is also critically important to potentially harmful cells. These might be our own cells, as in the case of

cancer cells, virally infected cells, or autoimmune effector cells, or they might be other cells in our bodies, such as those of pathogenic bacteria, fungi, or parasites like *Plasmodium falciparum*. As an example, cancer cells produce large amounts of oxidants through increased metabolism and the infiltration of inflammatory neutrophils and are critically dependent on the endogenous disulfide reductase systems to maintain viability and drive proliferation (Güngör *et al.*, 2009). In fact, recent work in the lab of Martin Bergo has shown that administration of classic antioxidants like NAC and vitamin E, once thought to prevent, slow or even cure cancer, actually increases rates of metastasis (Le Gal *et al.*, 2015, Wiel *et al.*, 2019, Lignitto *et al.*, 2019). Using a molecular tool like L-(³⁴S)Cys would allow the direct comparison of normal versus cancer cell S-metabolism and would hopefully identify pathways of vital importance to tumor that could be targeted. Similarly, parasites like *P. falciparum* must manage oxidants produced by their own metabolism as well as those of the host's immune response. The World Health Organization estimated that, in 2017, 435,000 people died of malaria, the majority of cases caused by *P. falciparum*. Like almost all other species, *P. falciparum* maintains the NADPH-dependent glutathione reductase and thioredoxin reductase pathways, but unlike metazoans, *P. falciparum* lacks the two enzymes of transsulfuration, CBS and CSE, and thus cannot utilize Met as a source of reducing power (Beri *et al.*, 2017). Similarly, the causative agent of anthrax, *Bacillus anthracis*, lacks the glutathione system and is thus critically dependent on the thioredoxin system to reduce ribonucleotide reductase and drive proliferation (Gustafsson *et al.*, 2012). The missing pieces of each species' sulfur redox metabolism represents critical vulnerabilities that can be exploited as possible

treatment options. The TrxR1 / Gsr-null live represents an important experimental model to continue to evaluate the contributions of both the NADPH-dependent and -independent disulfide reductase systems to help target the vulnerabilities of other, potentially harmful organisms *S* metabolism.

L-(³⁴S)Met and L-(³⁴S)Cys are precursors to other important *S*-metabolites. There are several other important *S*-metabolites that can be easily accessed through the ³⁴S compounds synthesized herein. For example, Met may also serve as a potential sink for oxidants through the formation of methionine sulfoxide (Met-SO). Reduction of Met-SO is accomplished in metazoans by two thioredoxin-dependent methionine sulfoxide reductases, MsrA and MsrB (Weissbach *et al.*, 2002, Kim & Gladyshev, 2003), but it remains unclear whether: 1) Met oxidation will protect cells from oxidative events; or 2) cells with impaired NADPH-dependent disulfide reductase systems will power the Msrs to reduce Met-SO. L-(³⁴S)Met-SO can easily be accessed by oxidation of L-(³⁴S)Met with *N*-chlorosuccinimide (Kikuchi *et al.*, 1988) and could serve as a critical tool to help answer these questions. As noted above, Cys represents a central metabolic hub for *S*-metabolites. This is also true synthetically, where L-(³⁴S)Cys could easily be converted to L-(³⁴S)-*N*-acetylcysteine (³⁴S-NAC) (Akeike *et al.*, 2017 Jones & Young, 1968), L-(³⁴S)cysteine sulfinic or L-(³⁴S)cysteine sulfonic acid (L-(³⁴S)Cys-SO₂H and L-(³⁴S)Cys-SO₃H, respectively) (Corpuz *et al.*, 2017, Bahrami *et al.*, 2012), or L-(³⁴S)Cysteine persulfide (L-(³⁴S)Cys-SSH)(Akaike *et al.*, 2017). None of these products are available commercially and could be used to better understand how cells utilize critically important *S* reducing power during oxidative events. All of the ³⁴S molecules presented could be

potentially useful to determine how cells metabolized oxidized *S* species or if partially oxidized *S* amino acids can still serve as reducing equivalents for cells experiencing oxidative crises.

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Concluding Remarks

The importance of thiol reducing power has been known perhaps since 1964 when Laurent, Moore and Reichard showed the need for a thiol-driven reduction reaction to generate DNA precursors. The balance of cellular oxidants and reductants is controlled by complex mechanisms and loss of this balance has been implicated in different disease states such as cancers, aging, neurodegenerative, cardiovascular, and skeletomuscular diseases. Thiol redox biology, however, remains a field of study with large knowledge gaps, and as shown by the surprise discovery of an NADPH-independent disulfide reductase system, many misconceptions. Clearly, there is a need for further investigation. Cellular disulfide reduction is accomplished by the two NADPH-dependent disulfide reductase systems as well as the NADPH-independent, Met-driven system, however possible overlap or redundancy between these pathways is largely unknown. Similarly, the reduction of H_2O_2 via a disulfide formation/reduction mechanism is generally accepted as the most common method, however intriguing alternate routes, such as the oxidation of persulfides or H_2S , have been proposed. To investigate the contributions of these mechanisms to the maintenance of a reduced cellular milieu may not be simple. The transient and labile nature of many thiol modifications, especially their susceptibility to spontaneous oxidation, makes this an especially difficult task and will require advances in the models used to generate oxidative stress, such as the TrxR1/Gsr-null mouse liver, the reagents used to label these modifications, like L- (^{34}S) Met and L- (^{34}S) cystine, but also in the methods used to isolate and detect them.

This thesis includes two reviews that cover the background of cellular disulfide reduction, from its earliest form in hydrothermal vents and its evolution to the current, multifaceted systems that maintain cellular redox homeostasis, to the roles of the disulfide reductase systems in different subcellular compartments, as well as provide a current status for many of the unknown roles disulfide reductase enzymes play. Furthermore, this thesis includes two published research articles, both relating changes in the NADPH-dependent disulfide reductase systems to altered physiology and the possible impacts of these changes to human health (ie cancer, acetaminophen overdose or toxic arsenic exposure.) A third research paper is also included in this thesis, which demonstrates the pro-oxidant effects of administration of the antioxidant ascorbate to TrxR1/Gsr-null livers. This paper is potentially valuable both in a clinical aspect, where ascorbate might be prescribed to counter the effects of excess oxidants, but also to the general public, as ascorbate is one of the most commonly taken over-the-counter supplements. The final chapter of this thesis is fundamental groundwork for future projects aimed at identifying how cells manage accumulation of oxidants/compromised disulfide reductase systems. The two isotopically labeled amino acids, L- (^{34}S) Met and L- (^{34}S) cystine, are valuable tools to monitor S-metabolism, both in the TrxR1/Gsr-null livers but also in other disease states, such as those mentioned above. L- (^{34}S) cystine is of particular interest to one of our collaborators, Dr. Gina DeNicola, who plans to use L- (^{34}S) cystine to monitor S-metabolism in pancreatic organoids to study pancreatic adenocarcinoma.

Building off the groundbreaking and fundamental work of Eriksson et al (2015), and contributing further biochemical characterization and new organic synthesis products, the work presented in this thesis has contributed to the understanding of disulfide reduction and thiol redox biology and has opened further avenues for investigation into the effects of altered disulfide reductase systems.

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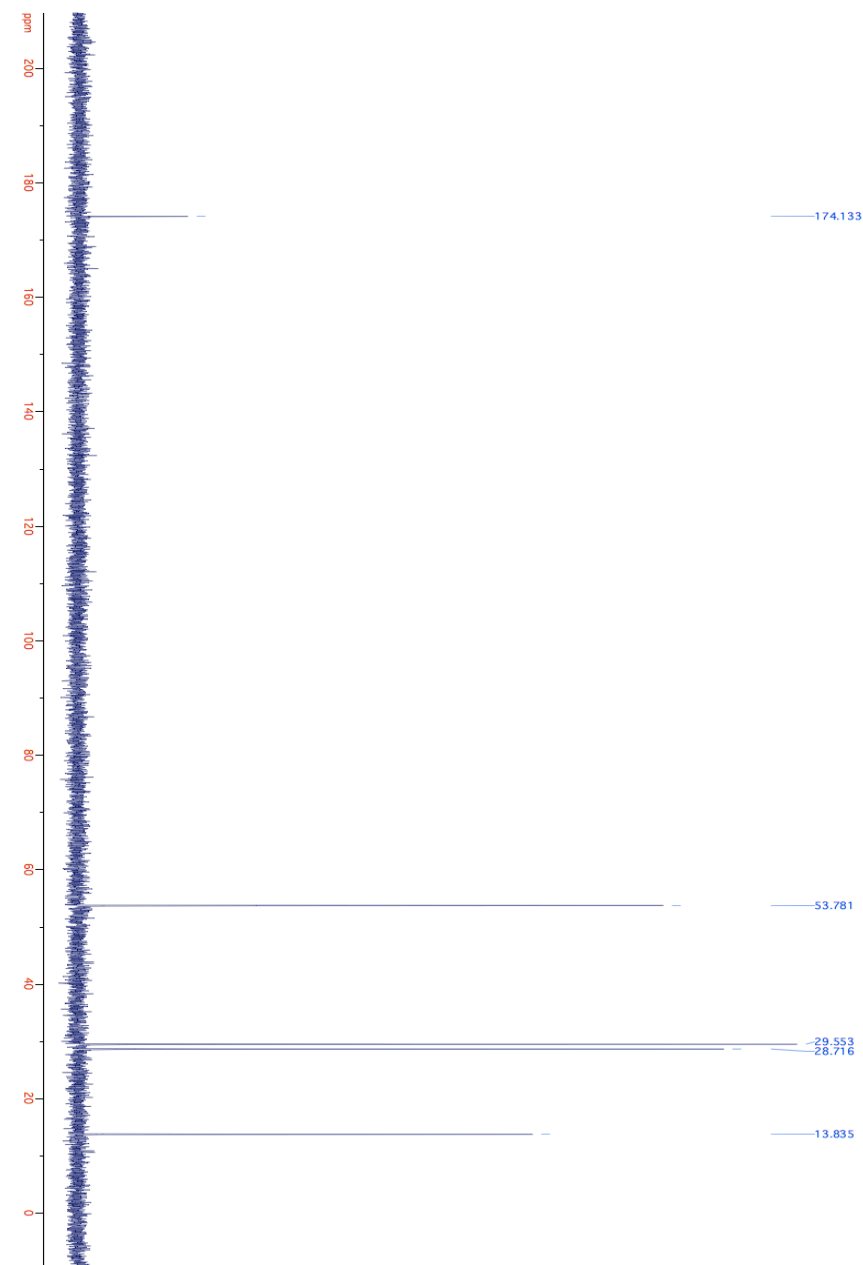
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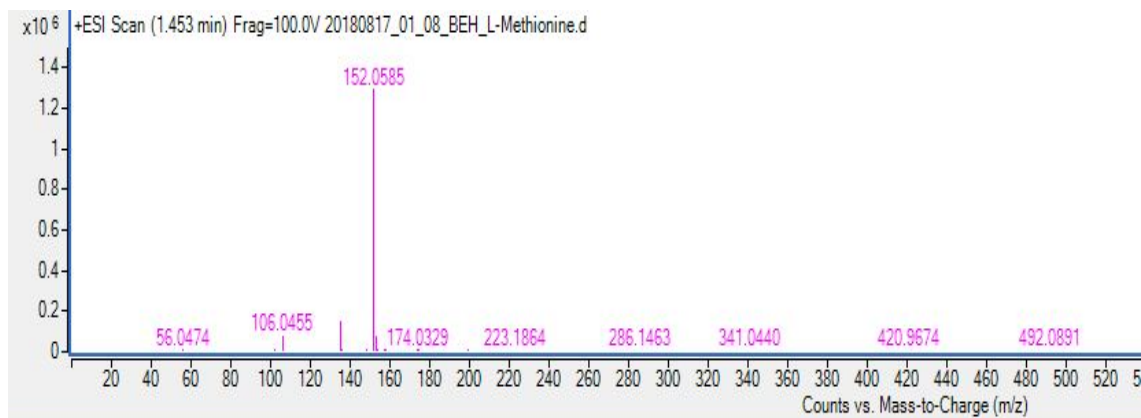
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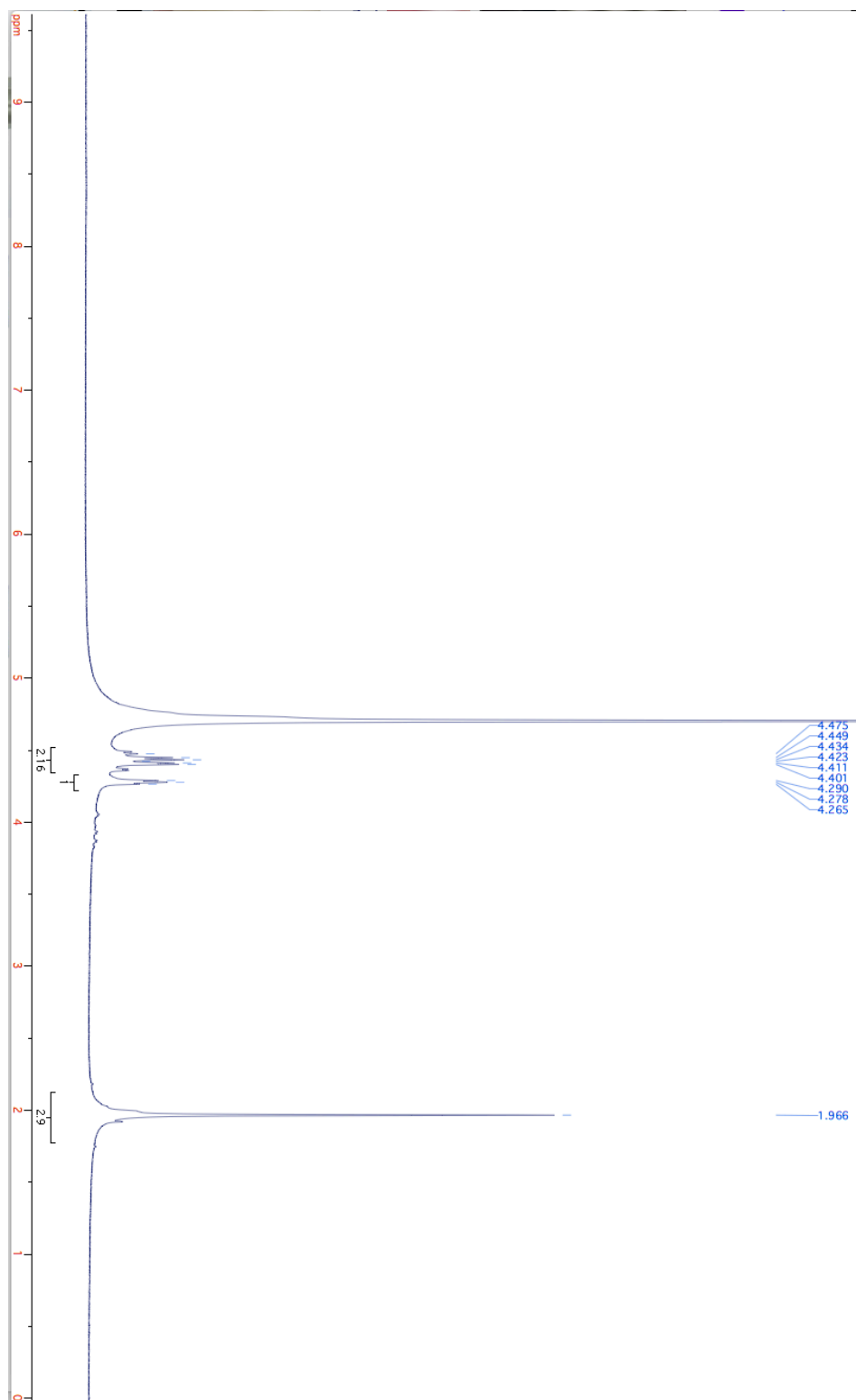
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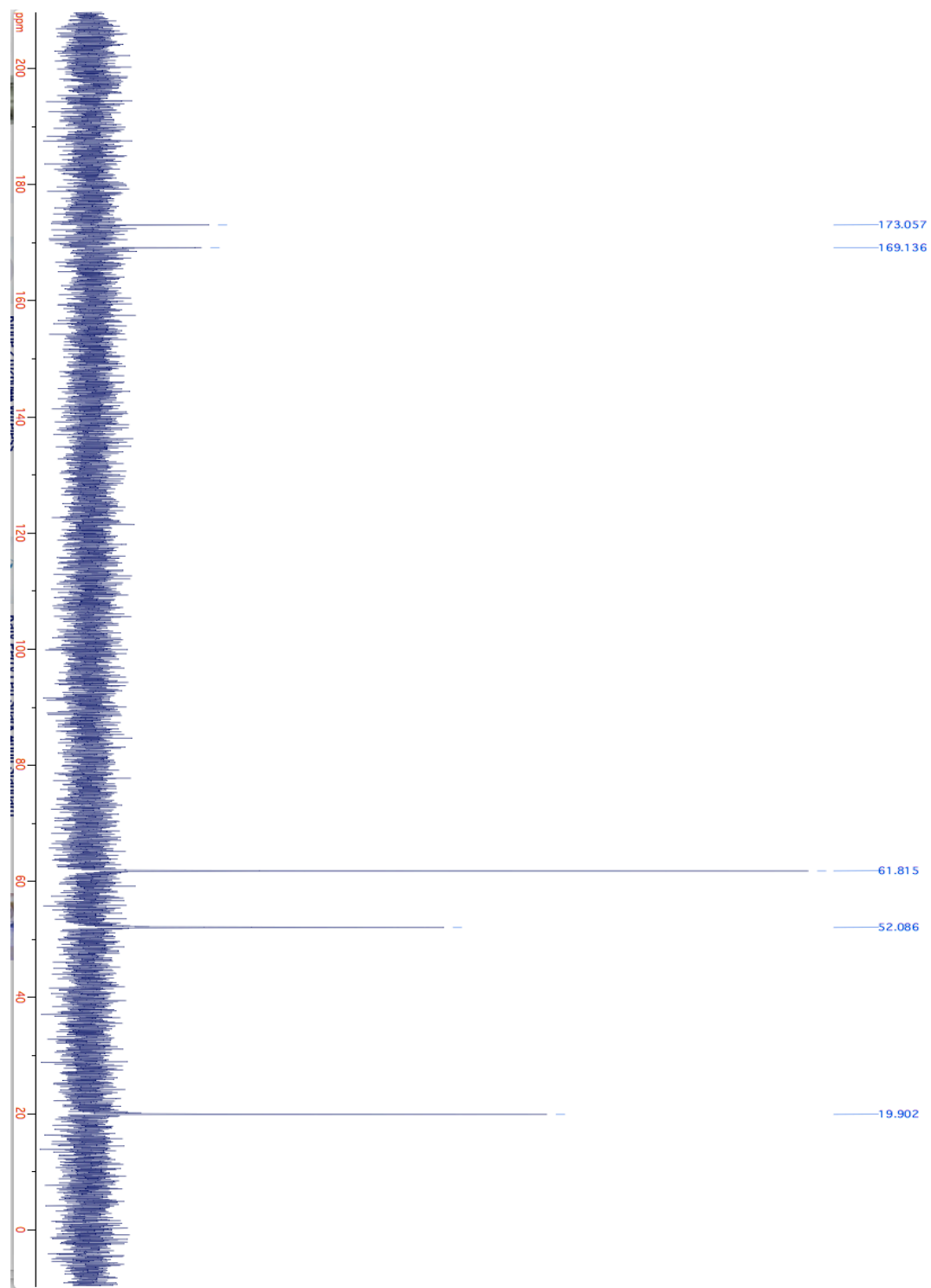
APPENDIX A

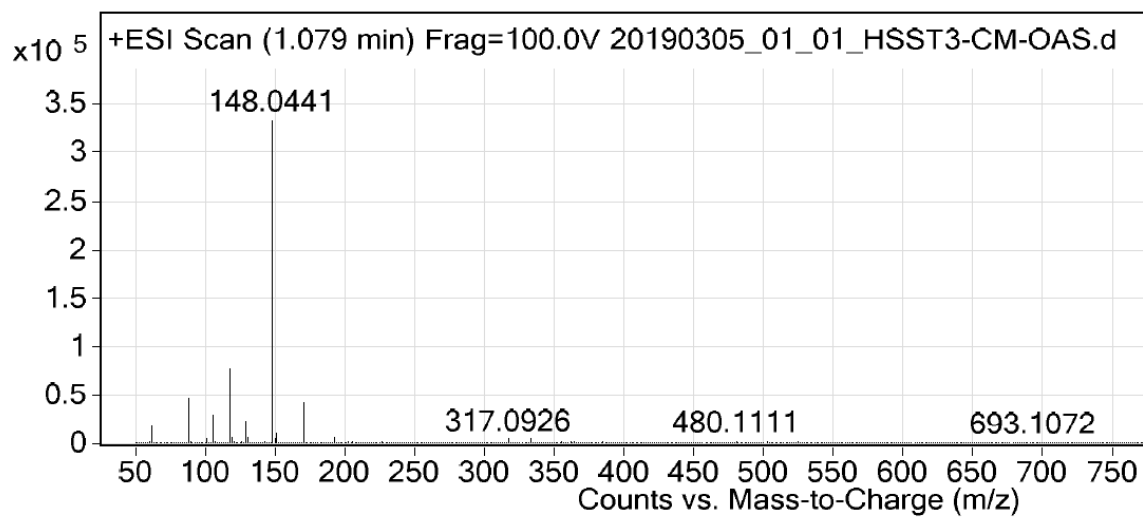
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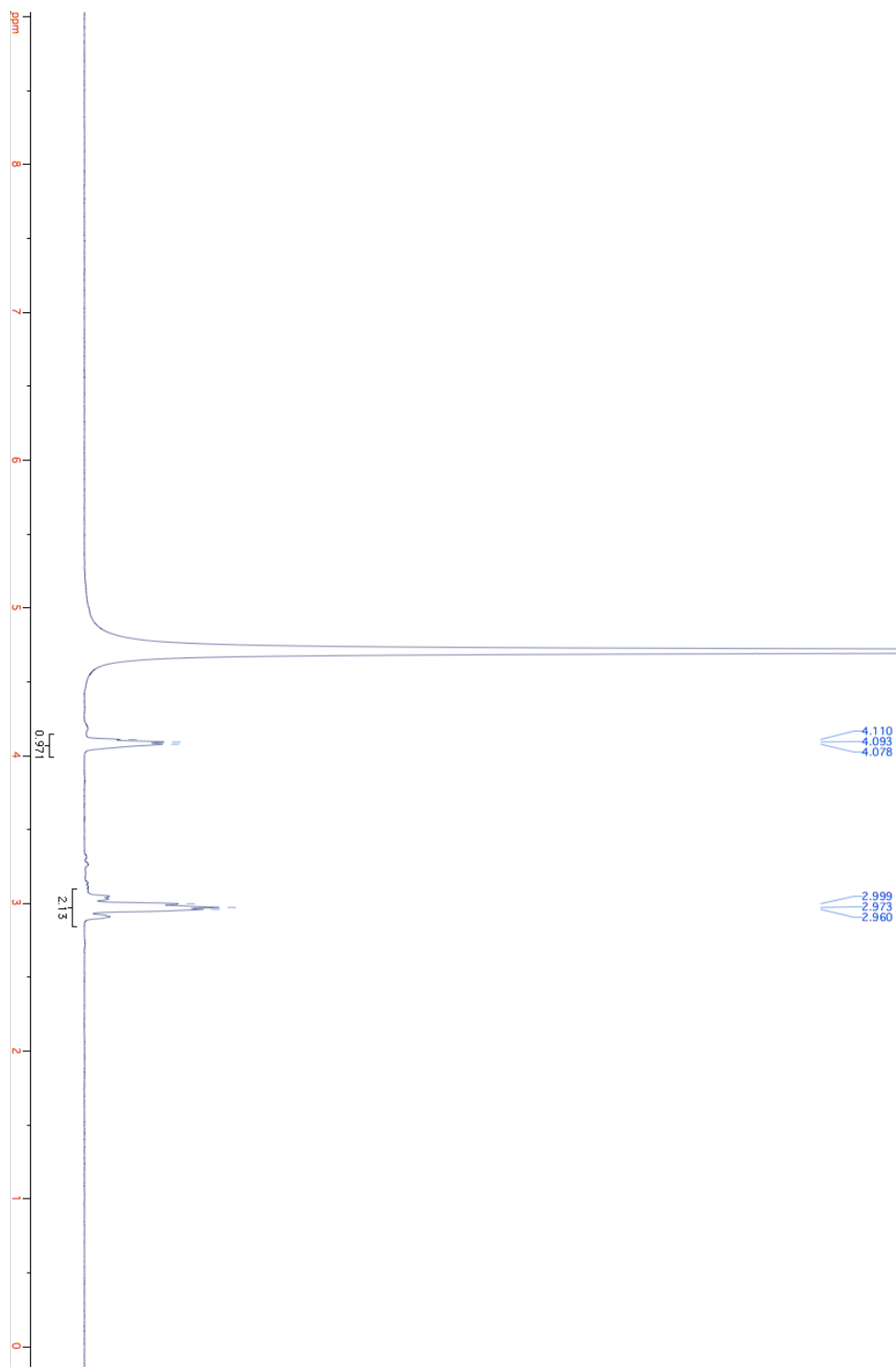
A1. ^{13}C -NMR spectra of L-(^{34}S)Met

A2. HRMS-ESI of L-(³⁴S)Met

A3. ^1H -NMR spectra of *O*-acetyl-L-serine

A4. ^{13}C -NMR spectra of *O*-acetyl-L-serine

A5. HRMS-ESI of O-acetyl-L-serine

A6. ^1H -NMR spectra of L-(^{34}S)cystine

A7. ^{13}C -NMR spectra of L-(^{34}S)cystine