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Mammalian Thioredoxin Reductase 1 in Antioxidant Defense, Regulation of Adipocyte Differentiation and as an Anticancer Drug Target

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family, friends and everyone who helped me

"I have no special talents, I am only passionately curious"

Albert Einstein

(*14th March 1879 - 18th April 1955*)

"The ones who are crazy enough to think that they can change the world, are the ones who do"

Steve Jobs

(*24th February 1955 - 5th October 2011*)

ABSTRACT

Reactive oxygen species (ROS) are oxygen containing reactive molecules generated as by-products of cellular metabolism. At physiological concentrations, ROS acts as secondary messengers in cellular signaling transduction, but excessive amounts of ROS result in oxidative stress and cellular damage. Several antioxidant enzyme systems include the thioredoxin (Trx)- and glutathione (GSH)-dependent systems together with superoxide dismutases and catalases may act in concert to protect cells and organisms from the toxic effects of excessive ROS. Mammalian thioredoxin reductase 1 (TrxR1), which is a cytosolic selenoprotein with a selenocysteine (Sec, U) residue in a conserved C-terminal GCUG motif, catalyzes the reduction of thioredoxin using NADPH and is known to be involved in antioxidant defense, redox regulation and cell proliferation. This thesis has focused on studying multiple aspects of cellular events and signaling pathways that are modulated by TrxR1.

Paper I. The Sec residue in the C-terminal motif of TrxR1 is highly nucleophilic and can be easily targeted by electrophiles. In this study, we found the mutant p53 activator and anticancer drug lead named APR-246 (PRIMA-1^{Met}) targeted and inhibited both recombinant and cellular TrxR activity. The inhibited TrxR1 still maintained its NADPH oxidase activity, which thus could contribute to the oxidative stress and cell death that are triggered by APR-246. Our findings provide insights into the p53 independent cytotoxicity mechanisms of APR-246 on tumor cells.

Paper II. In this study, we used thiophosphate (SPO_3) and selenite to modulate the Sec incorporation into TrxR1 in mammalian cells. We found that SPO_3 promoted expression of Sec-to-cysteine substituted forms of TrxR1 and, conversely, selenite increased Sec incorporation in TrxR1. SPO_3 treatment also attenuated cisplatin induced toxicity on A549 and HCT116 cells, while selenite supplementation sensitized NIH 3T3 cells to cisplatin but decreased the dependence of these cells on GSH. Taken together, these results show that the selenium status of cells can modulate the cytotoxicity of drugs that target TrxR1 and the glutathione dependence of the cells.

Paper III. Here we utilized *Txnrd1* depleted (*Txnrd1*$^{-/-}$) mouse embryonic fibroblasts (MEFs) and observed massive cell death upon cultured at low-density in high-glucose medium. The cell death was linked to excessive H_2O_2 production promoted by high-glucose metabolism. Reconstitution of the cells with Sec-containing TrxR1, but not with the Sec-to-Cys substituted variant, rescued the MEFs from this lethality. These results show that Sec-containing TrxR1 is essential to maintain self-sufficiency of MEFs under high-glucose conditions, due to an essential role in control of glucose-derived H_2O_2 production. This study is, to our knowledge, the first time identified an essential biological role of Sec-containing TrxR1 that cannot be sustained by the Cys-mutant of the enzyme.

Paper IV. *Txnrd1*$^{-/-}$ MEFs revealed a strong increase of spontaneous lipogenesis and hormonally induced adipocyte differentiation. The highly promoted adipocyte differentiation capacity was due to unlimited mitotic clonal expansion capacity and dramatically upregulated PPARγ expression. These effects were likely to be connected to increased oxidative signaling in *Txnrd1*$^{-/-}$ MEFs, because NAC treatment abolished the adipocyte differentiation by blocking mitotic clonal expansion. An increased Akt signaling in *Txnrd1*$^{-/-}$ MEFs induced by decreased cellular PTEN activity and increased ROS, also contributes to the enhanced adipogenesis. These results suggest that the selenoprotein TrxR1 suppress adipocyte differentiation through inhibition insulin signaling events, mitotic clonal expansion and PPARγ expression.

In summary, this study shows that TrxR1 plays an essential role in antioxidant defense, regulation of adipocyte differentiation and servers as an anticancer drug target.

LIST OF PUBLICATIONS

I. **Peng X**[§], Zhang MQZ[§], Conserva F[§], Hosny G, Selivanova G, Bykov VJN, Arnér ESJ, Wiman KG.
APR-246/PRIMA-1^{MET} inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase. *Cell Death Dis*. 2013; (4): e881.
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II. **Peng X**, Xu J, Arnér ESJ.
Thiophosphate and selenite conversely modulate cell death induced by glutathione depletion or cisplatin: effects related to activity and Sec contents of thioredoxin reductase. *Biochem J*. 2012; 447 (1): 167-74.

III. **Peng X**, Mandal PK, Kaminskyy VO, Lindqvist A, Conrad M, Arnér ESJ.
Sec-containing TrxR1 is essential for self-sufficiency of cells by control of glucose-derived H_2O_2. *Cell Death Dis*. 2014; (5): e1235.

IV. **Peng X**, Petrus P, Giménez-Cassina A, Conrad M, Rydén M, Arnér ESJ.
Thioredoxin reductase 1 suppresses adipocyte differentiation through inhibition of key regulatory signaling events. *Manuscript*.

Article not included in this thesis
Stafford WC, **Peng X**, Olofsson MH, Zhang X, Luci D, Lu L, Cheng Q, Dexheimer, TS, Tresaugues L, Molina DM, Coussens N, Augsten M, Ahlzén HSM, Nordlund P, Östman A, Stone-Elander S, Maloney D, Jadhav A, Simeonov A, Linder S and Arnér ESJ.
Drug Mediated Inhibition of Thioredoxin Reductase 1 is Sufficient for Anticancer Efficacy. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

$AONO_2$	Nitrooleic Acid
ASK1	Signal-regulating kinase 1
ATF1	Activating transcription factor 1
BSO	Buthionine sulfoximine
cDDP	Cisplatin
C/EBP	CCAAT-enhancer-binding protein
Cys	Cysteine
CM	Conditioned Medium
CREB	Cyclic AMP response element–binding protein
DNCB	Dinitrochlorobenzene
DTNB	5,5'-dithiobis-(2-nitrobenzoicacid)
DTT	Dithiothreitol
ERK	Extracellular signal-regulated kinase
FABP4/aP2	Fatty-acid-binding protein 4
FBS	Fetal bovine serum
GCS	γ-glutamyl-cystien synthase
GLUT	Glucose transport
Gpx	Glutathione peroxidase
GR	Glutathione reductase
Grxs	Glutaredoxins
GSH	Glutathione
GST	Glutathione transferase
IR	Insulin receptor
LAP	Liver-enriched activator protein
LDH	Lactate dehydrogenase
LIP	Liver-enriched inhibitory protein
LNO_2	Nitrolinoleate
MCE	Mitotic clonal expansion
MEFs	Mouse embryonic fibroblasts
MPTP	Mitochondrial permeability transition pore

MQ	Methylenequinuclidinone
Msr	Methionine sulfoxide reductase
mTORC	Mammalian target of rapamycin complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAC	N-acetyl cysteine
NADP	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
Nrf2	NF-E2-related nuclear factor 2
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3 kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKB	Akt/protein kinase B
PKC	Protein kinase-C
PPARγ	Peroxisome proliferator-activated receptor γ
Prxs	Peroxiredoxins
PTEN	Phosphatase and tensin homolog
PTP1B	Protein tyrosine phosphatase 1B
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
Sec	Selenocysteine
SecTRAP	Selenium compromised thioredoxin reductase-derived apoptotic protein
SOD	Superoxide dismutase
SPO_3	Thiophosphate
TCA	Tricarboxylic acid
Trx	Thioredoxin
TrxR	Thioredoxin reductase protein
Txnip	Thioredoxin-interacting protein
Txnrd	Mouse gene encodes thioredoxin reductase
TXNRD	Human gene encodes thioredoxin reductase
UCP1	Uncoupling protein-1

1 INTRODUCTION

1.1 THE ORIGINS OF OXYGEN AND EVOLUTION OF AEROBIC LIFE

About four and a half billion years ago, the earth was formed by accretion from the solar nebula [1]. The meteorites we have, which share the highest similarity to the material from the very beginning of earth, are highly reduced. The original atmosphere under this reducing environment would contain a mixture of hydrogen (H_2), methane (CH_4), nitrogen (N_2) and ammonia (NH_3), with small amounts of carbon dioxide (CO_2), carbon monoxide (CO), and hydrogen sulfide (H_2S) etc. Under these circumstances, oxygen (O_2) could not be present in more than a few parts per million [2]. However, how does the organic life evolve from this environment and what is the energy source used for the synthesis of original organic compounds?

Sunlight is the main energy source, but only a small fraction with short wavelength can be absorbed by CH_4, NH_3, H_2O, CO_2, and so on. UV light is probable, however, most of the photochemical reactions at these low wavelengths would have happened in upper atmosphere, so that the compounds formed might have been decomposed by this UV light before reaching the oceans. Therefore, lightning is believed to be an important source of energy that occurs closer to the oceans [2]. During the 1950s, Stanley Miller and Harold Urey passed electric sparks that simulating lightning through a gas mixture of (H_2), methane (CH_4), and ammonia (NH_3). After collecting the end-produces, they found a complex mixture of organic compounds, including different types of amino acids, which are the components of protein and building blocks of life. These reactions are believed to turn the early ocean into a soup containing all organic precursors of live. The only ingredients needed for life creation out of this soup are chance and time, both of which are limitless. It seems that it took billions of years to form the life since the first fossils of large animals is from half a billion years ago [3].

The first organisms are presumably evolved in O_2 free atmosphere. For these organisms, O_2 would have severe toxic effects to them since they have few, if any, antioxidants, to protect against O_2. These O_2-hating organisms are called anaerobic. Yeasts and many other single cell organisms live on anaerobic respiration (fermentation) for energy production with simple structure, which may present the ancient life. Subsequently, the evolution of the first oxygen-evolving photosynthetic bacteria (cyanobacteria) starts changing the components of atmosphere. Cyanobacteria evolved to utilize the sunlight energy to break down water. They used hydrogen equivalents to drive their metabolism, and released the byproducts O_2 to the

atmosphere. The accumulation of O_2 has two advantages: it formed the ozone (O_3) layer in the stratosphere that protected the organisms from UV radiation damage, which may have helped the life to move from the sea to the land; in addition, ferrous iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) to produce super toxic hydroxyl radical ($OH^{\cdot}$) (Fenton reaction, see details in section 1.2.2). So life that use O_2 for energy production (aerobic) would be difficult to be evolved with abundant Fe^{2+} around. High amount of O_2 in the atmosphere precipitated Fe^{2+} from water by forming insoluble Fe^{3+} complexes, leaving the sea and river suitable for life with trace amounts of soluble iron today [4].

"Excessive amount of O_2 was by far the greatest crisis on earth", wrote by professor Lynn Margulis at University of Massachusetts in 1986. Microorganisms that had no defense system against this toxic gas died out quickly. The resistant organisms propagated and quickly replaced the sensitive strains on the surface of the earth. The survivors began to develop antioxidant defense systems to protect against the toxicity of O_2 [5]. The adaptation to O_2 further evolved the organisms to have metabolic transformations, utilized different oxygenases for biosynthesis. Further, they started using O_2 for energy production (aerobic respiration), which increased the ATP production efficiency dramatically. After the development of aerobic respiration, multicellular organisms were subsequently evolved with highly efficient energy production. Evolving such systems could ensure controlled distribution of O_2 to the body: for example, most parts in human body are never exposed to the high amount of O_2 in atmosphere (approx. 21%) due to a much lower O_2 concentration in blood [4].

The 21% O_2 content in atmosphere is believed to be formed already half billion years ago by the outcome of a sustainable natural balance. When the O_2 levels are too high, it will suppress the plant growth, which then results in a lower amount of O_2 production by the decrease of photosynthesis. On the contrary, if the O_2 levels decreases, the animals would suffocate, which leads to less consumption of O_2. Thereby, the nature regulates the atmospheric oxygen content through a dynamic equilibrium [3].

1.2 REACTIVE OXYGEN SPECIES (ROS)

Evolution of aerobic respiration in organisms inevitably causes production of reactive oxygen species (ROS), referring to oxygen containing reactive molecules including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitric oxide (NO) and peroxynitrite ($OONO^-$) [6, 7]. Some of these molecules are highly reactive, for example, hydroxyl radical can easily cause oxidative damage to proteins,

DNA, and lipids; however, some of them are relatively inert, such as superoxide and hydrogen peroxide [7]. Physiological concentrations of ROS act as secondary messengers in cellular signaling by modulating the activities of protein kinases, phosphatases and transcription factors. However, excessive amount of ROS causes cell damage and cell death [8].

1.2.1 Sources of ROS

Various organelles and enzyme systems are sources of ROS generation in mammalian cells, including mitochondrion, endoplasmic reticulum (ER), peroxisome, NADPH oxidase, xanthine oxidase (XO), nitric oxide synthase (NOS), lipoxygenase, cyclooxygenase (COX), and cytochrome P450 monooxygenase (P450) [9], some of which are further discussed below:

1.2.1.1 Mitochondria

Mitochondria have been known to be an important generator of intracellular ROS for decades [5]. During mitochondrial respiration, electron donors in tricarboxylic acid (TCA) cycle donate electrons to mitochondrial electron transport chain, which contains five protein complexes named complex I-V respectively. Many studies show that mitochondrial ROS are formed predominantly at complex I, coenzyme Q or complex III, which can donate electrons one at a time to oxygen molecules and thereby generate superoxide (Fig. 1). The mitochondrial isoform of superoxide dismutase (MnSOD) converts superoxide to hydrogen peroxide, which is less reactive and can be degraded to H_2O and O_2 by antioxidant enzymes glutathione peroxidases (GPxs), peroxiredoxins (Prxs) or catalases [8, 10] (see section 1.3 for more details).

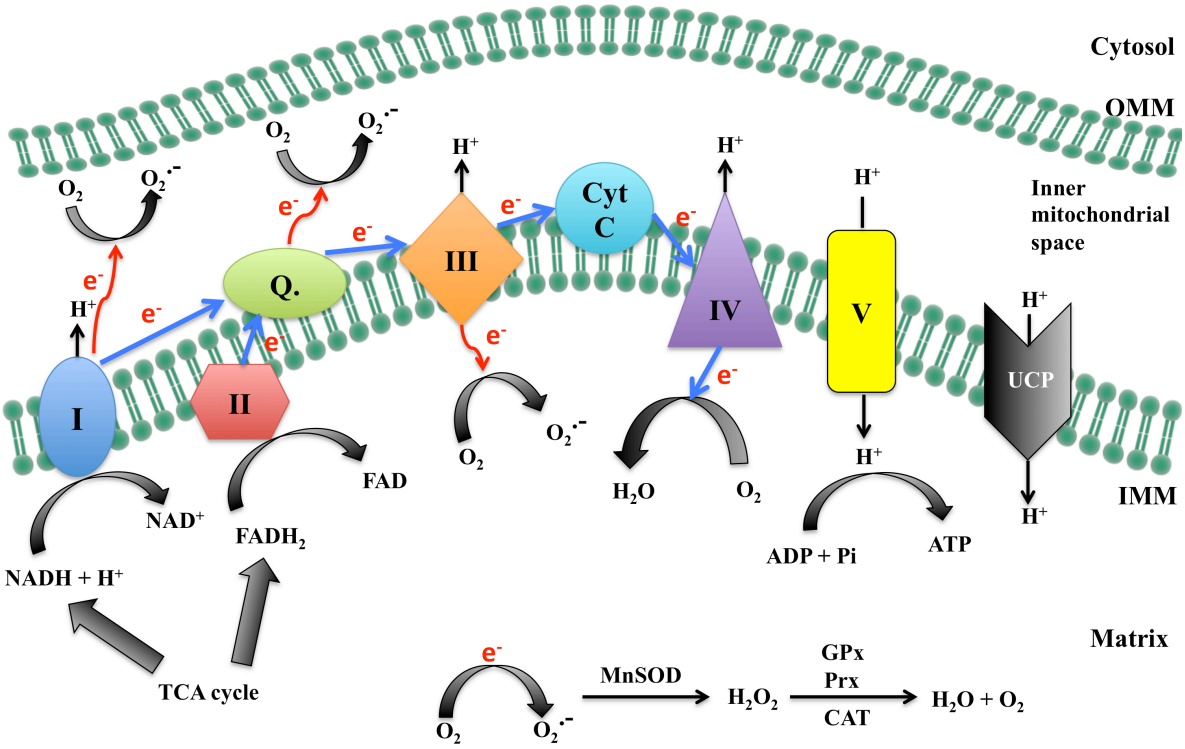

Figure 1. **ROS generation through mitochondrial electron transport chain**. Mitochondria consist of outer mitochondrial membrane (OMM), inner mitochondrial space, inner mitochondrial membrane (IMM) and matrix. Mitochondrial electron transport chain is located at IMM and consists of complex I to V. NADH and $FADH_2$ are the electron donors generated by TCA cycle and give electrons to complex I or II respectively. Electrons are then passed to coenzyme Q, complex III and finally complex IV for the reduction of O_2 to H_2O. This process generates a H^+ voltage gradient across the mitochondrial membrane. The energy from this voltage gradient drives the synthesis of ATP by complex V (ATP synthase). Alternatively, uncoupling proteins (UCPs) can diminish the voltage gradient to generate heat to control the rate of ATP generation. Single electron leakage from complex I, coenzyme Q or complex III reduces O_2 to superoxide intermediate. Mitochondrial superoxide dismutase (MnSOD) converts this reactive species to hydrogen peroxide, which will further be degraded to H_2O and O_2 by GPxs, Prxs and catalases. The figure is modified from Finkel [8].

1.2.1.2 Peroxisome

Another oxygen consumption organelle in the cells is the peroxisome. It was first described that in the peroxisome respiratory pathway, electrons are transferred from various metabolites to reduce O_2 to H_2O_2 with release of heat instead of production of ATP [11]. The main metabolic processes contributing to the generation of ROS in peroxisomes are catalyzed by

oxidases such as acyl-coA oxidases, urate oxidase and xanthine oxidase, which may contribute to 35% of all hydrogen peroxide generated in rat liver [12]. In addition, iron and copper ions are abundant in peroxisomes and can catalyze the formation of hydroxyl radical in the Fenton reaction (see below for more details) [13].

1.2.1.3 Endoplasmic reticulum

ER is the cellular compartment in which proper protein folding and disulfide formation of proteins take place. These processes are dependent on the oxidizing environment in the lumen of ER with a high ratio of oxidized to reduced glutathione (GSSG/GSH). It estimates that 25% of the cellular ROS is generated from disulfide bonds formation in the ER during protein folding. The plausible mechanism is that during disulfide bonds formation, ER oxidoreductin 1 (ERO1) and protein disulfide isomerase (PDI) transfer electrons from free thiol groups to oxygen and thereafter form ROS [14].

1.2.1.4 NADPH oxidase

Superoxide generation by NADPH oxidase (NOX) was thought to only be present in "professional" phagocytes that promote microbial killing for a long time. Over the past years, six homologs of the cytochrome subunit of the phagocyte NADPH oxidase have been found (NOX1 to NOX5, DUOX1 and DUOX2) and they are now referred to as the NOX family of NADPH oxidases. These NOX enzymes are in fact found virtually in every tissue with the primary function of ROS generation [15]. NOX activation can be stimulated by a large group of stimuli, including toxic compounds, irradiation exposure, microorganism infection, growth factors and hormone activation. Upon activation, NOX transports one electron at a time from NADPH to an oxygen molecule to generate superoxide and other downstream ROS, like hydrogen peroxide [16].

1.2.1.5 Nitric oxide synthase

There are three nitric oxide synthase (NOS) isoforms found in mammalian cells: neuronal NOS (NOS I) that is mainly expressed in neurons and are involved in synaptic functions in the central nervous system (CNS); inducible NOS (NOS II) that is expressed in many different cell types and is responsive to stimulation of cytokines, LPS (lipopolysaccharide) and other agents; endothelial NOS (NOS III) that is mainly expressed in endothelial cells and controls blood pressure. Monomeric forms of NOS have limited capacity to transfer electrons to molecular oxygen to generate superoxide. However, in presence of heme, NOS can form

functional dimers and transfer electrons from NADPH to O_2 and $_L$-arginine to form NO and $_L$-citrulline [17] (see more details below).

1.2.2 ROS formation and conversion

When molecular oxygen is reduced by a single electron, it forms superoxide ($O2^{\cdot-}$), which is highly reactive and plays an important role in many signaling pathways such as activation of protein kinases and inactivation of phosphatases [18]. Superoxide is also quite toxic and used by phagocytes to kill invading microorganism [16].

$$O_2 + e^{\cdot-} \rightarrow O_2^{\cdot-}$$

Because superoxide is toxic, nearly all organisms that depend on aerobic respiration contain analogues of the superoxide-scavenging enzyme, superoxide dismutase (SOD). SOD catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2) [18]. Hydrogen peroxide is less reactive and involves in regulation of various physiological responses such as cell proliferation, differentiation, and migration. Hydrogen peroxide oxidizes and activates protein tyrosine kinases and at the same time, oxidizes and inactivates protein tyrosine phosphatases, which results in exaggerated phosphorylation signaling cascades. This has led to implications of cellular "redox" signaling in regulating normal processes and disease progression [19].

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Hydrogen peroxide reacts with superoxide to generate the highly reactive and toxic hydroxyl radical, which is the Haber-Weiss reaction. This reaction is very slow and can be catalyzed by iron ions. The first step involves oxidation of superoxide to oxygen molecule by a ferric ion:

$$(1) \quad Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

The second step is the Fenton reaction that involves the reduction of hydrogen peroxide to hydroxyl radical:

$$(2) \quad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^-$$

Net reaction:

$$O_2^{\cdot -} + H_2O_2 \rightarrow HO^{\cdot} + OH^- + O_2$$

Hydroxyl radical ($OH^{\cdot}$) is by far the most reactive oxygen species with a half-life of about a nanosecond. It causes bases oxidation, thereby results in DNA mutation and carcinogenesis.

Hydroxyl radical also readily targets the double bonds in polyunsaturated fatty acids to form lipid peroxides, which may induce apoptosis [20]. Further, tyrosine and tryptophan residues on proteins are additional targets to hydroxyl radicals, the oxidation of which may cause function alteration in protein and remodeling of cellular events [21].

Nitric oxide (NO) is synthesized by converting $_L$-arginine to $_L$-citrulline, which is catalyzed by NOS. Most of the physiological effects of NO come from binding to guanylyl cyclase (GC)-coupled receptors, which results in conformational changes and generation of cGMP. This process affects multiple downstream targets including protein kinases, phosphodiesterases and ion channels. Nitric oxide can also modify proteins through post-translation modification, such as the attachment of Nitric oxide to free a thiol group of the cysteine residue, named *S*-nitrosylation. This modification regulates the function of many proteins in cell signaling and can be reversed by several redox regulators [22].

$$Arg + 2O_2 + NADPH + H^+ \xrightarrow{NOS} Cit + NO + 2H_2O + NADP^+$$

Nitric oxide can also react with superoxide to form peroxynitrite ($OONO^-$). Peroxynitrite is highly reactive and can directly react with DNA, proteins and lipids. However, these reactions happen at a relatively slow rate, which allows it to react more selectively in the cell. In addition, peroxynitrite can react with other molecules to form additional types of reactive nitro species, including nitrogen dioxide ($^{\cdot}NO_2$) and dinitrogen trioxide (N_2O_3).

$$O_2^{\cdot -} + NO \rightarrow ONOO^-$$

1.3 ANTIOXIDANTS

Since high amounts of ROS result in oxidative stress and cellular damage, the mammalian cell have developed different antioxidant enzymes and utilizes low molecular weight compounds to prevent ROS toxicity and to keep redox homeostasis for the cellular events [7]. This section will give a brief introduction to these antioxidant enzymes with the focus on mammalian thioredoxin (Trx) system.

1.3.1 Selenium

Jöns Jacob Berzelius (1779 - 1848), one of the first professors of Karolinska Institutet, is considered to be one of the founders of modern chemistry [23]. He had a great interest in mineralogy, which fostered his passion, knowledge and analytic skills for discovery new elements. In 1817, Berzelius found the pyrite created a red precipitate when working in the Falun mine, Sweden. It was first believed to be a tellurium compound. However, Berzelius doubted that since tellurium had never been found in the Falun mine. After scrutinizing and reanalyzing, Berzelius finally identified it as a newly found element with similar properties to sulfur and tellurium. Since tellurium is named after the latin name of earth, Tellus, Berzelius decided to name the new element "Selenium" for moon from the Greek word "selene" [24]. The naming was described in Swedish:. . . "skola beskrifvas vara en egen förut okänd brännbar mineralkropp, hvilken jag har kallat Selenium af ∑ελην η, måna, för at dermed utmerka dess nära slägtskap med Tellurium" [24].

Selenium is an essential trace element of fundamental importance to human biology. Humans take up selenium from diet. Meat, milk, eggs, seafood, cereal and nuts are selenium rich food [25, 26]. The antioxidant properties of nutrient selenium have received great attention. Most of its antioxidant activity is correlated with selenocysteine synthesis and incorporation into selenoproteins (see details below). However, another selenium-containing amino acid, selenomethionine, can scavenge ROS directly. Both free selenomethionine and selenomethionine residues in protein can rapidly react with peroxynitrite to form methionine selenoxide [27, 28], which can be reduced back to selenomethionine by glutathione in a non-enzymatic reaction [29].

Selenium deficiency has been linked to cardiovascular disease, reproductive impairment, inflammation and obesity [30, 31]. However, overdose of selenium becomes highly harmful to human health. It was reported that a commercial company provided a liquid dietary selenium supplement, which actually contained 200 times concentration as it labeled. This accident caused severe toxic effects to the users, such as diarrhea, fatigue, hair loss, joint pain, nausea and headache [32].

1.3.2 Selenocysteine and Selenoproteins

1.3.2.1 Selenocysteine in general

A main biological role of selenium is to be used for selenocysteine (Sec, U) synthesis. Sec is the 21st naturally synthesized amino acid and an analogue to cysteine (Fig. 2) with a

selenium atom that replaces the sulfur in cysteine. This alteration gives Sec unique biochemical properties, such as lower p*K*a and higher nucleophilicity [33].

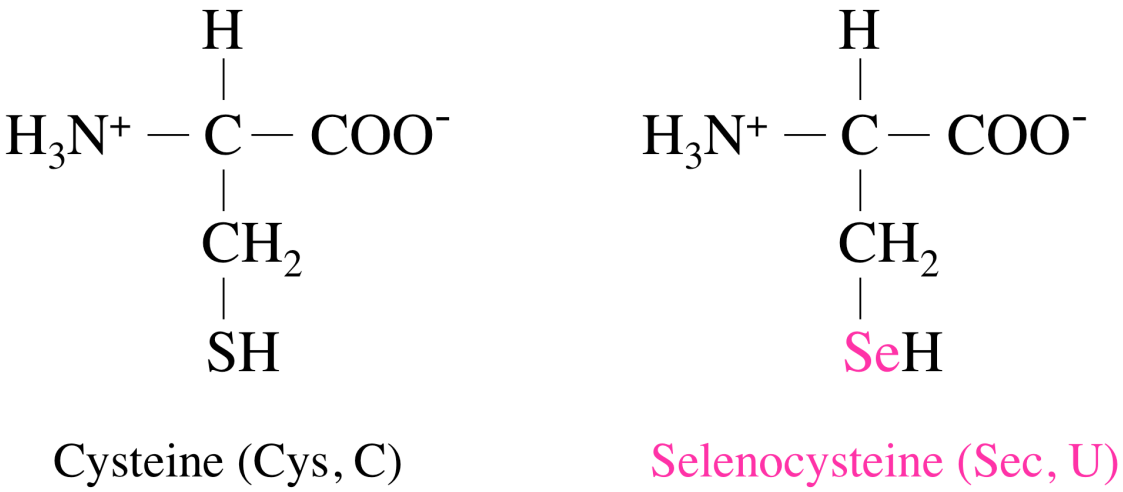

Figure 2. **Chemical structure comparison of Cys and Sec.**

1.3.2.2 Selenoprotein distribution

Selenoproteins refers to proteins that contain at least one Sec, and they are present in all three major forms of life, archaea, bacteria and eukaryotes. Among bacteria and archaea, selenoproteins are not common and only found in certain phylogenetic groups [34]. In eukaryotes, selenoproteins are present in most animals with only a few identified in green alga *Chlamydomonas*, one in *C. elegans*, but completely missing from other plants or fungi [35, 36]. In recent years, through the development of bioinformatics and genomic analysis methods, the number of discovered selenoproteins has been rapidly expanded. So far, 25 and 24 selenoproteins are identified in human and mouse, respectively [37], together with a few more found in bacteria and archaea [38]. Further, 310 different selenoprotein genes belonging to 25 families are found in samples collected from the Sargasso Sea [39] and more than 3600 distinct selenoprotein genes derived from 58 selenoprotein families are identified in samples from the Global Ocean Sampling (GOS) expedition [40]. Nonetheless, selenoproteins seem to be dispensable for many organisms, such as plants, fungal and certain insects, which only use Cys-dependent homologue of the selenoproteins found in other species. It is apparent that Cys-dependent oxidoreductases are more common than their selenoprotein counterparts in nature [33]. All of these raise the question that what are the reasons for the existence of selenoproteins, and what unique properties can arise with selenocysteine in place of cysteine?

1.3.2.3 Selenocysteine versus Cysteine

It is still an ongoing discussion why proteins use Sec instead of Cys. When comparing the chemical properties of selenium and sulfur, the two elements are very similar in terms of ion radii, redox potentials and electronegativity. However, selenium has lower p*K*a, higher polarizability and thus also higher nucleophilicity.

The p*K*a difference between Sec and Cys (5.2 for the selenolate and 8.5 for the thiolate) is significant and often cited. Under physiological pH, most Cys molecules are likely to be in their protonated forms, which are less active. Sec, on the other hand, would mainly be deprotonated and more readily to be involved in chemical reactions. The low p*K*a also renders the selenolate become a superior leaving group relative to thiolate, which may accelerate the bond exchange between selenosulfide and disulfide in selenoproteins, such as mammalian thioredoxin reductase (TrxR) [41]. However, it is notable that these p*K*a values are only referred to free status of the amino acids, and the situation of Sec or Cys residues are highly dependently on the specific microenviroment of the protein structure [33]. For example, *D. melanogaster* has a-SCCS carboxyterminal active site motif in thioredoxin reductase (TrxR), instead of -GCUG as found in the mammalian orthologue. It is found that the activity of this non-selenorpotein TrxR is surprisingly high, due to the two flanking Ser residues that activate the two redox active Cys residues [42].

Nucleophilicity refers to the capability to donate electrons to another atom or to supply a pair of electrons for the formation of a new bond with a foreign atom. Nucleophilicity consists of a combination of multiple factors such as atomic radius, p*K*a, polarizability and electronegativity. Significantly higher nucleophilicity of Sec as compared to Cys is believed to be the truly unique aspect of selenoproteins [33, 43]. A comparative study of the rate between selenol/diselenide and thiol/disulfide exchange reactions showed that at physiological pH, the higher nucleophilicity of selenium reacted more than 10^7 times faster than the corresponding sulfur compounds [44]. However, since the p*K*a of Sec is much lower than Cys as mentioned above, others argue that comparing activities under physiological conditions is in fact comparing the charged selenolate to a thiol (RSH). Therefore, the large difference in reaction rate betwen selenolate and thiolate can be mainly attributed to the concentration of nucleophilic species in the reaction [41]. Interestingly, an early study concluded that at very acidic pH, upon which Sec should be mainly in its protonated form still reacted readily with electrophiles due to the high nucleophilicity [45]. This inherent high nucleophilicity of Sec to efficiently react with electrophilic compounds may significantly contribute to its biological relevance. For example, it is attributing the high reactivity and broad substrate specificity of mammalian thioredoxin reductase (TrxR) to the high

nucleophilicity of the Sec residue in this enzyme [41]. The highly accessible Sec residue, which is easily targeted by a large number of electrophiles, may serve as an anticancer target [46]. It is also possible that in redox signaling pathways, the selenoproteins may catalyze the formation of intermediates using nucleophilic substitution reactions, where the Cys-containing orthologues are unlikely to mimic such reactions efficiently even if having lowered p*K*a values [33].

There is a new rationale for the requirement of Sec in proteins. It was noted that selenoprotein P, the only mammalian selenoprotein carries multiple Sec residues [47], evolved to appear when the amount of oxygen was increasing dramatically in the atmosphere [48]. It is paradoxical since it is well known that Se is much more susceptible to be oxidized to form selenenic (RSeOH) than S to form sulfenic (RSOH). However, further oxidation can occur on selenenic and sulfenic to form seleninic ($RSeO_2^-$) and sulfinic (RSO_2^-). The formation of Cys-SO_2^- in the protein is named "over-oxidation" [49], because it is extremely difficult be reduced back to a thiol, whereas Sec-SeO_2^- has the superior ability to be recycled back to selenol. One example in cellular context is that when Prxs are oxidized to form Cys-SO_2^-, they become inactivated and needs sulfiredoxin to restore their functions [50]. Further, it is showed that it is very difficult to further oxidize $RSeO_2^-$ to $RSeO_3^-$, while RSO_2^- can be readily oxidized to RSO_3^-. In biochemical context, it means that Sec-SeO_2^- cannot be further oxidized to the selenonic acid (Sec-SeO_3^-), while Cys-SO_2^- can be oxidized to the sulfonic acid (Cys-SO_3^-), which is referred to as "hyper-oxidation". There is no known pathways or enzymes of reducing Cys-SO_3^- to thiol in biological systems. Thus, Sec will be favored by those proteins, which are needed to be highly resistant to irreversible oxidation and inactivation [48].

1.3.2.4 Selenocysteine synthesis and incorporation in eukaryotes

The biosynthesis of Sec in eukaryotes is initiated by aminoacylation of the tRNA that carrying Sec ($tRNA^{[Ser]Sec}$) with serine (Ser). This reaction is catalyzed by seryl-$tRNA^{[Ser]Sec}$ synthetase (SerS), and then the $tRNA^{[Ser]Sec}$ with serine moiety is converted to phosphoseryl-$tRNA^{[Ser]Sec}$ by phosphoseryl-$tRNA^{[Ser]Sec}$ kinase (PSTK). Under selenium sufficient condition, selenophosphate ($SePO_3$) will be synthesized by selenophosphate synthesase 2 (SPS2), which will be used as the selenium donor for converting phosphoseryl-$tRNA^{[Ser]Sec}$ to Sec-$tRNA^{[Ser]Sec}$ by selenocysteine synthase (SecS) [51]. However, it shows that upon selenium deficient condition, SPS2 would provide thiophosphate (SPO_3) instead of $SePO_3$ to SecS for synthesis of Cys instead of Sec onto the $tRNA^{[Ser]Sec}$ (Fig. 3). As a result, selenium deficient

condition promotes Cys incoporation into the site of Sec residue in selenoproteins in mice [52, 53].

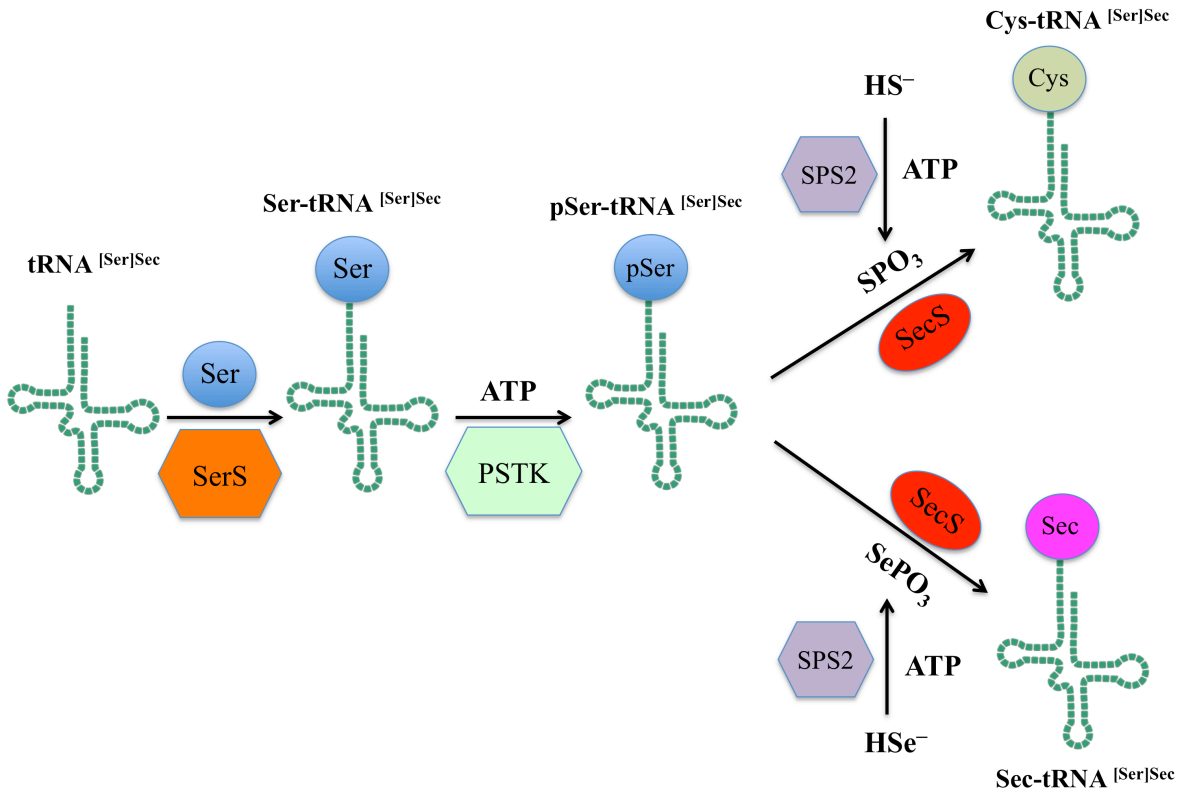

Figure 3. **Biosynthesis of Sec and de novo synthesis of Cys**. SerS and PSTK catalyzes successively to convert $tRNA^{[Ser]Sec}$ to phosphoseryl-$tRNA^{[Ser]Sec}$. SPSP2 utilizes selenide or sulfide as the substrates to produce $SePO_3$ or SPO_3, which is thereafter utilized by SecS to convert the phosphoseryl-$tRNA^{[Ser]Sec}$ intermediate to either Sec-$tRNA^{[Ser]Sec}$ or Cys-$tRNA^{[Ser]Sec}$. The figure is modified from Turanov et al. [51].

Selenoprotein synthesis utilizes a unique and complex machinery to incorporate Sec into in-frame UGA codon, which is normally a stop codon that terminates the translation [54]. Sec incorporation sequence (SECIS) is a secondary structure of mRNA that forms stem loop sharp, which plays an essential role for Sec incorporation in UGA codon [37]. When the ribosome encounters the in-frame UGA codon in mRNA of a selenorprotein, Sec-$tRNA^{[Ser]Sec}$ with a anticodon UCA translates UGA as Sec under the coordination of SECIS element [55]. SECIS binding protein 2 (SBP2) is well known to bind SECIS element with high specificity and affinity to recruit Sec-$tRNA^{[Ser]Sec}$ and facilitates the incorporation of Sec into the synthesized peptide [56]. Sec-specific translation elongation factor (EFSec) is another important player in eukaryotic Sec incorporation machinery, which forms complex with SBP2 and thereafter facilitates the interaction with SECLIS element and Sec encoding [57].

Ribosomal protein L30 is a component of large ribosomal subunit in eukaryotes, which has also been shown to bind SECIS element. Studies have revealed that L30 binds equally well to open and kinked SECIS elements, whereas SBP2 preferentially binds to open form of SECIS structures [58]. However, the exact role of L30 in Sec incorporation is yet unclear and needs further investigation. Nucleolin is found to be another SECIS element binding protein that influences the translation efficiency of UGA into Sec, whereas there is no consensus on the role of nucleolin in regulation of Sec incorporation. One study shows that nucleolin preferencially bind SECIS elements in the high ranked selenoproteins in the hierarchy of selenoprotein expression, but can hardly bind the SECIS elements from nonessential selenoproteins [59]. Another study with conflicting data shows that necleolin bind SECIS elements in variety of selenoproteins without major differences [60]. A eukaryotic translation initiation factor eIF4a3 has been shown to be an important regulator of Sec incorporation, which contributes to the preferential translation of the high ranked selenoproteins in the hierarchy of selenoprotein expression [61]. It has been shown that eIF4a3 is dramatically induced under Se deficient condition, which is proposed to selectively bind SECIS elements in the nonessential selenoproteins mRNA to compete with the binding of SBP2 and results in prevention of Sec incorporation into the nonessential selenoproteins [62]. Take into consideration that multiple proteins can bind SECIS elements, including SBP2, L30, nucleolin, eIF4a3, and possibly more, it is plausible that these regulations act in concert to regulate the expression of selenoproteins (Fig. 4). Additional studies are needed to investigate the exact mechanism of different proteins that bind SECIS element in regulation of selenoprotein synthesis and dictation of the hierarchy of selenoprotein expression.

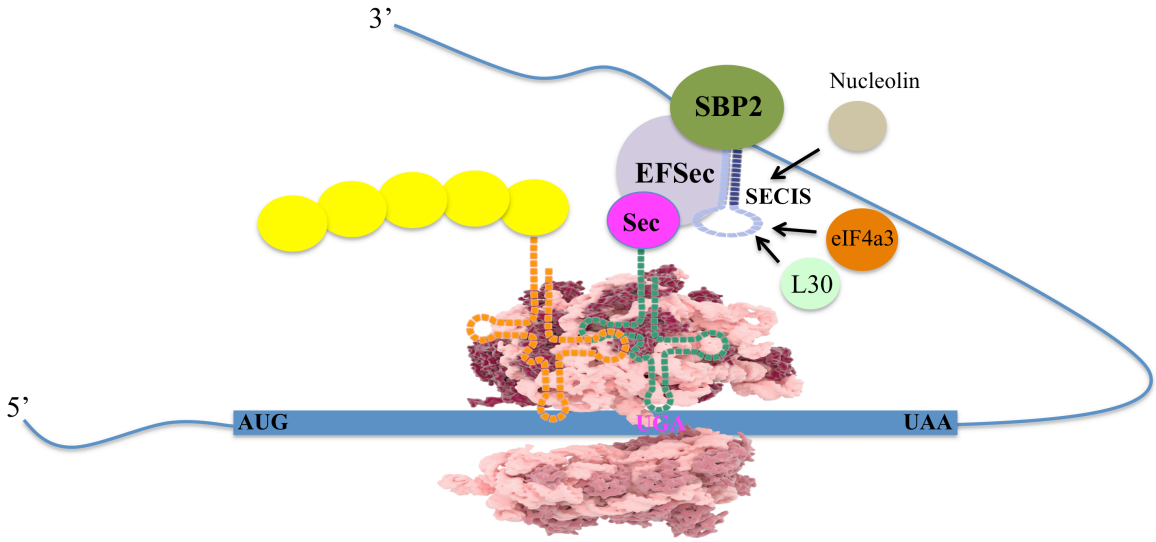

Figure 4. **Sec incorporation into selenoproteins in eukaryotes**. SBP2 and EFSec formed complex binds the stem loop like SCEIS element and facilitate the Sec incorporation at in-frame UGA codon decoded by $tRNA^{[Ser]Sec}$. L30, nucleolin and eIF4a4 are involved in the regulation of Sec incorporation. The figure is modified from Vyacheslav et al. [54].

1.3.3 Trx system

1.3.3.1 Trx system in general

The thioredoxin (Trx) system consists of Trx, thioredoxin reductase (TrxR) and the cofactor NADPH. Mammalian TrxR are selenoproteins having a Sec in the C-terminal -GCUG motif. TrxR uses reducing equivalents from NADPH to reduce oxidized Trx and thus supports a range of Trx-dependent cellular functions, including cell proliferation, redox regulation and antioxidant defense [46, 63].

1.3.3.2 Trx isoforms

Trx is a small (approx. 12 kDa) disulfide reductase universally expressed in all living cells [64]. There are two major isoforms of Trx in mammals. Trx1 is predominately located in cytosol and nucleus. However, Trx1 and its truncated form Trx80 have also been found in the membrane and extracellular space. Trx2 is specifically located in the mitochondria. The

active site of Trx contains two cysteine residues with the sequence of -Cys-Gly-Pro-Cys- [65]. Active site oxidized Trx is reduced to dithiol by NADPH and TrxR, and GSH together with glutaredoxin may serve as a backup system for Trx reduction [66]. Genetic depletion of both *Txn* and *Txn2* that encodes Trx1 and Trx2 respectively, gave early embryonic lethality in mice [67, 68].

1.3.3.3 TrxR isoforms

There are three isoforms of TrxR in both human and mouse. TrxR1 is the major form of TrxR that locates mainly in cytosol and expresses in most tissues (See more details below). TrxR2 is predominantly located in mitochondria with much lower levels than TrxR1 in most tissues. Together with the mitochondrial version of thioredoxin (Trx2), TrxR2 is involved in antioxidant defense against ROS generated from mitochondrial electron transport chain [69]. TrxR2 has also been shown to be essential for hematopoiesis and heart development [70]. The transcription of the genes encoding TrxR1 and TrxR2 involve different splicing variants at the N-terminal end, resulting in different protein variants [46]. Another isoform of TrxR is thioredoxin glutathione reductase (TGR), which consists of a TrxR module with a N-terminal elongation containing an atypical monothiol -CPHS- motif in a glutaredoxin domain [71]. TGR is found to be present at very low levels in various tissues but the levels of TGR increases in testis after puberty. This enzyme is absent from mature sperm but is highly enriched in elongating spermatids at the site of mitochondrial sheath formation, which indicates unique functions of TGR in relation to spermatogenesis [71].

1.3.3.4 Catalytic mechanism of Mammalian TrxR1

Mammalian TrxR1 is a homodimer arranged head-to-tail with a Sec in its C-terminal -GCUG motif in each subunit (Fig. 5). Both subunits are required for electron transfer in the reduction reaction from NADPH to Trx. In the initiation of the reaction, the FAD bond with each subunit obtains the electrons from NADPH and thereafter transfers them to the -CVNVGC- motif of the same subunit in N-terminal and thus forming a dithiol motif. The dithiol-containing motif in one subunit of TrxR1 reduces the selenenylsufide motif in the other subunit of the dimer. The reduced C-terminal active site with a selenothiol motif subsequently reduce a broad number of substrates in cells including the active site disulfide of Trx (Fig. 5). However, some substrates such as certain quinones and 5,5'-dithiobis-(2-nitrobenzoicacid) (DTNB), may be reduced directly by N-terminal -CVNVGC- active site [72].

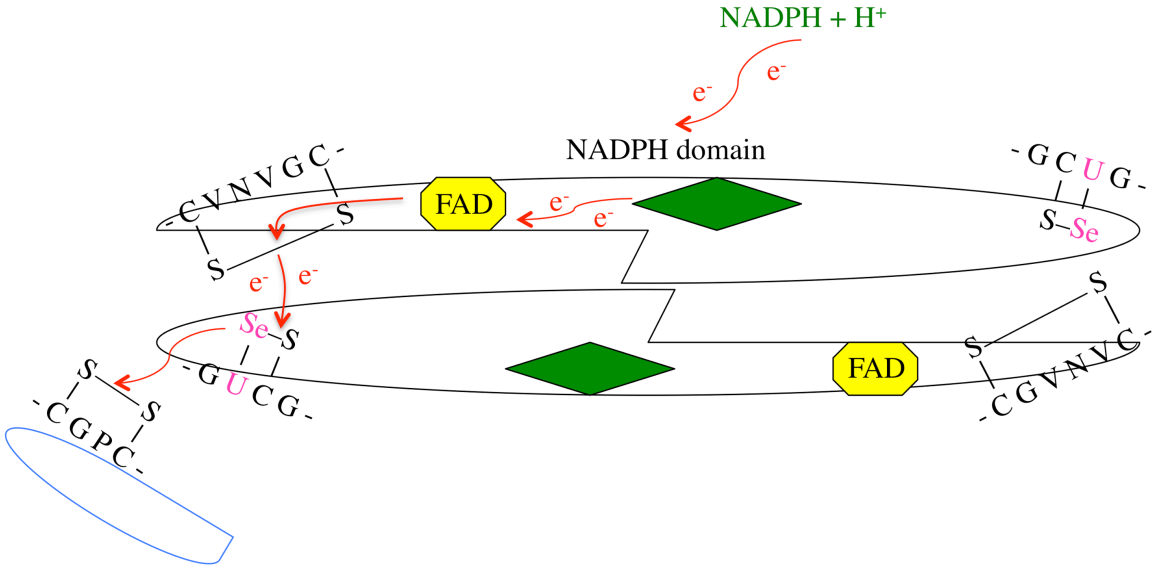

Figure 5. **The catalytic mechanism of Mammalian TrxR1**. During catalytic reaction, NADPH transfers electrons to enyme-bound FAD, which thereafter reduces the disulfide bond in the N-terminal -CVNVGC- motif. The reduced N-terminal sebsequently reduce the selenenylsufide-containing motif in the C-terminal of the other subunit. The formed selenothiol motif can reduce a broad range of substrates, including active site disulfide of Trx, other protein disulfide substrates, as well as non-protein substrates such as selenium compounds, peroxide, DTNB, lipoic acid and etc.

1.3.3.5 Cellular functions and substrates of mammalian TrxR1 and Trx1

The main physiological substrate of TrxR1 is Trx1. Reduced Trx1 is the reducing equivalent donor for many enzymes and proteins. Through reduction of Trx1, TrxR1 exert many important cellular functions, such as the reduction of ribonucleotide reductase (RNR), which is essential for DNA synthesis and cell proliferations [63]. TrxR1 also supports a range of antioxidant enzymes through Trx1, including peroxiredoxins (Prxs) and methionine sulfoxide reductases (Msrs). Prxs catalyze the scavenging of H_2O_2 with a high affinity to prevent oxidative stress and modulate signaling cascades [49]. Msrs recycle methionine sulfoxide back to methionine to repair oxidative damage on methionine residues of proteins [73].

In addition to supporting different enzymes, TrxR1 is also involved in regulation of cellular signaling transduction through altering activities of transcription factors, kinases and phosphatases. Via Trx1 reduction, TrxR1 either directly supports NF-κB through reducing its Cys residues [74, 75] or indirectly sustains HIF1a and AP1 via redox factor-1 APE/Ref-1 [76, 77]. Reduced Trx1 can non-covalently interact with apoptosis signaling-regulating kinase 1 (ASK1) and inhibit apoptosis signaling [78]. Recently, Trx system is shown to reduce and

reactivate oxidized protein-tyrosine phosphatase 1B (PTP1B), thereafter negatively regulate Platelet-derived growth factor β (PDGF-β) receptor tyrosine kinase signaling and potentially also suppress insulin signaling [79]. Likewise, reduced Trx1 may reduce oxidized phosphatase and tensin homolog (PTEN) and inhibit PI3K/Akt pathway [80]. However, conflicting data shows that Trx1 in reduced form can covalently interact with PTEN and inhibit PTEN activity, thereby augmenting PI3K/Akt signaling [81].

Further, through reduction of Trx1 or thioredoxin-related protein of 14 kDa (TRP14), TrxR1 catalyzes efficient cystine reduction reactions, which might be an essential source of cysteine that is required for protein synthesis and cellular redox homeostasis [82, 83]. TrxR1 also plays a role in denitrosylation of S-nitrosylated glutathione, caspase 3 or cathepsin B through either Trx1- or TRP14-catalyzed denitrosylation reactions [82]. Additional protein substrates to TrxR1 are glutaredoxin 2 which is predominant in mitochondrial and protein disulfide isomerase (PDI) in ER, more studies are required to understand how these reaction occurs [84, 85].

p53 is a redox sensitive transcription factor that plays an essential role in DNA repair, cell cycle arrest and apoptosis [86]. TrxR1 is reported to be required for p53 functions. Depletion of gene *trr1* that encodes TrxR1 in yeast dramatically decreases p53 activity but not p53 protein level [87, 88]. Electrophilic prostaglandins are shown to covalently modify and inhibit TrxR, which results in impairment of conformation and function of p53 [89, 90]. TrxR1 also indirectly facilitates DNA binding activity of p53 through redox regulation of Trx and APE/Ref-1 [91, 92].

TrxR1 also has additional non-proteins substrates in cells. It can reduce different forms of selenium compounds. Reduction of selenite to selenide consumes NADPH and triggers a redox cycling process in the cells, which might contribute to the prooxidant and lethal effects of high selenite concentrations [93]. TrxR1 is also known to be able to regenerate Vitamin C through reduction of dehydroascorbate to asorbate [94], which may be part of the total antioxidant activity of TrxR1. Ubiquinone is shown to be another endogenous substrate for both purified and cellular TrxR1. It is not yet known the biological importance of this reduction, but it indicates that selenium and ubiquinone may protect the cells from oxidative damage in a combinatory manner [95].

1.3.3.6 TrxR1 knockdown or knockout

Two independent studies observed that deletion of *Txnrd1* (gene encodes TrxR1 in mouse) results in early embryonic lethality [96, 97]. The earlier study shows that embryonic death occurs between embryonic day E9.5 and E10.5 with normal gastrulation and mesoderm

formation [96]. However, the latter study reports that *Txnrd1* null embryos are viable at embryonic day E8.5 but not at E9.5 with failure of gastrulation and mesoderm formation [97]. Even though the time points for the death of the *Txnrd1* null embryos are slightly different between the studies, it actually imposes a major difference in terms of the functional impact of depletion of *Txnrd1*. The molecular mechanism for this difference is still unknown. Notably, although these knockouts are embryonically lethal, the lethality occurs after several rounds of cell division and forms thousands of cells, showing that TxrR1 is not essential for cell proliferation but vital for certain signaling events that are required for embryonic development. Nervous system (NS)-specific deletion *Txnrd1* results in development of massive cerebellar hypoplasia, which indicates that TrxR1 is important for postnatal brain development [98]. Under serum starvation, attenuation of TrxR1 in a *k-ras* driven mouse cancer cell line causes a loss of self-sufficiency of growth [99]. TrxR1 knockdown in a mouse lung cancer cell line also leads to a reversal in morphology and anchorage-independent growth features, completely abrogating the ability to form tumors in a xenograft model system [100]. Recently, depletion of *Txnrd1* is shown to affect cellular signaling and nutrient metabolism. Upon serum starvation condition, protein tyrosine phosphatase 1B (PTP1B) is more oxidized in TrxR1-defiecent mouse embryonic fibroblasts (MEFs), which results in augmented platelet-derived growth factor (PDGF) stimulated response [79]. Hepatocyte-specific disruption of *Txnrd1* is shown in one study to repress lipogenesis and increase glycogen storage dramatically in liver [101], while another study reports hepatic lipidosis with liver-specific TrxR1 knockout [102].

Interesting, tissue- or cell- specific suppression of TrxR1 does not necessarily give apparent phenotype. Conditional heart-specific *Txnrd1* deletion does not affect Cardiac development and cardiomyocytes derived from knockout embryos with *Txnrd1*-deficiency survive and grow *in vitro* [96]. Neuron-specific removal of *Txnrd1* does not cause any disorder in brain development [98]. Likewise, hepatocytes lacking TrxR1 proliferate normally during development and regeneration in mice without exhibiting apparent phenotype [103]. In cellular systems, downregulation of TrxR1 neither induces oxidative stress nor causes Trx1 oxidation in HeLa cells [104]. Knocking down TrxR1 with approx. 90% efficiency gives no effect on A549 cells [105]. It also shows that depletion of *Txnrd1* lacks apparent phenotype on c-*myc* and *H-ras* driven transformed cells with similar proliferative, clonogenic and tumorigenic capability [106]. *Txnrd1* is further found to be dispensable for *c-myc* driven B-cell lymphomagenesis, however, the survival and growth of this tumor is strictly dependent on the present of sufficient GSH, suggesting that the antioxidant defense and DNA synthesis in *Txnrd1* deficiency cells is compensated by upregulation of GSH-dependent system [106].

1.3.3.7 TrxR1 as an anticancer target

As discussed above, the Sec-containing C-terminal of mammalian TrxR1 is highly nucleophilic and serves as an easily accessible target for electrophiles [33]. Intriguingly, many studies observe that targeting TrxR1 gives more potent cytotoxicity and cell death than that caused by TrxR1 knockdown or knockout. It is plausible that the electrophiles have many other targets in the cells addition to TrxR1, but genetic depletion or transcription suppression specifically affects the expression of TrxR1. However, it shows that TrxR1 with Sec truncation or compromised by reaction with electrophiles such as cisplatin, results in gain of a pro-oxidant function that induces ROS production and extensive cell death [107, 108]. The pro-oxidant forms of TrxR1 are termed SecTRAPs, referring to selenium-compromised thioredoxin reductase-derived apoptotic proteins). Further studies show that an intact FAD/N-terminal motif in the SecTRAPs is required for the pro-oxidant and cell-killing activity, suggesting that N-terminal dependent NADPH consumption and redox cycling is responsible for the ROS production of SecTRAPs [108].

Cisplatin was approved for clinical use of cancer therapy in 1970s [109]. It has been known for long time that the success of cisplain in chemotherapy derives from its DNA crosslinking ability, which causes DNA damage and cell death [110, 111]. However, targeting TrxR1 and inducing SecTRAPs formation have also been indicated to contribute significantly to the reaction mechanism of cisplatin [105, 112]. Downregulation of TrxR1 to approx. 10% causes no apparent phenotype in A549 cells, but increases the resistance to cisplatin dramatically [105]. A p53 activator anticancer drug lead RITA have also been shown to target TrxR1 and cause a p53 dependent ROS generation, which may synergize with p53-dependent induction of apoptosis thus resulting in a robust cancer cell death [113]. Arsenic trioxide (ATO) has been used as a drug for thousands of years in traditional medicine [114] and has been used recently to treat acute promyelocytic leukemia (APL) [115, 116]. It shows that ATO targets Sec on TrxR1 and irreversibly inhibits the enzyme activity, which subsequently leads to oxidative stress and Trx oxidation. This indicates that targeting TrxR1 might contribute to the anticancer acticity of ATO [117]. The Gold compound auranofin, a clinically approved drug to treat rheumatoid arthritis, is also found to be potent inhibitor of TrxR1. Targeting TrxR1 by auranofin induces apoptosis in cisplain-resistant human ovarian cancer cells, adramycin-resistant human leukemia cells and human lung adenocarcinoma epithelial cells [112, 118, 119]. Clinical studies evaluating the possibility to use auranofin as an anticancer drug against different cancer types are in progress [120, 121].

Targeting TrxR1 seems to be much more toxic to cancer cells than healthy tissues, which make it become an intriguing anticancer strategy. Many studies show that cancer cells have increased ROS levels compared to their normal counterparts [122, 123]. In order to adapt to the increased oxidative stress, the cancer cells utilize enhanced antioxidant systems to counteract ROS generation and balance redox hemostasis. The activation of antioxidant responsive transcription factor Nrf2 (nuclear erythroid 2-related factor 2), which regulates the expression of multiple enzymes involved in antioxidant defense, is an important mechanism for this adaptation [124-127]. However, the accumulated ROS levels in cancer cells are close to reach the threshold that triggers cell death. Cancer cells are thus highly dependent on cellular antioxidant systems. Compounds that disrupt the antioxidant capacity in cells might elevate the ROS further to reach beyond the threshold and causes extensive cell death [128]. This may explain why targeting TrxR1 has been shown to induce cell death in many cancer cells [129-131]. On the other hand, under physiological conditions, normal cells contain a low level of ROS by balancing the ROS generation and antioxidant capacity. A certain level of ROS elevation is tolerable to normal cells due to their responsive antioxidant capacity [128]. TrxR1 inhibition could even be protective to normal tissues: TrxR1 inhibitor aurothioglucose (ATG) is shown to attenuate lung injury caused by hyperoxic condition due to the protective effects from augmentation of GSH system [132, 133]. Mice with *Txnrd1*-null hepatocytes are more resistant to paracetamol induced liver damage, due to the more robust GSH systems promoted by Nrf2 activation [101]. Thus, targeting TrxR1 by compound inhibition may specifically kill cancer cells and keep normal cells intact, which may serve as an intriguing anticancer strategy and further investigation is needed.

1.3.3.8 Txnip

An important cellular regulator of Trx is thioredoxin-interacting protein (Txnip also named VDUP1 or TBP2). Txnip forms a disulfide bond via Cys247 with Trx catalytic Cys32 to inhibit Trx activity and function [134, 135], thereby playing an essential role in redox signaling in a Trx-dependent manner. It shows that Txnip is able to dissociate Trx from ASK1, thus activating ASK1-JNK (c-jun-N-terminal kinase) signal pathway in inflammatory response [136]. In addition, hyperglycemia induces expression of Txnip through activation of p38 MAPK pathway and carbohydrate response element in Txnip promoter, and promotes ROS generation by inhibiting Trx activity, inducing mitochondrial and Nox4 ROS [135, 137].

However, being one member of arrestin protein family, Txnip has many other Trx independent cellular functions [138]. Studies show that Txnip inhibits adipogenesis

independent of its bind to Trx, however the interaction with Trx is required for the stabilization of Txnip from proteasomal degradation [139]. High concentrations of ROS promote the dissociation between Txnip and Trx, which indicates that the metabolic function of Txnip is redox regulated through Trx dependent stability control [134]. A very important role for Txnip has been proposed in the glucose homeostasis regulation. Txnip deficiency enhances insulin sensitivity with activated Akt signaling and glucose uptake [140-142]. Downregulation of Txnip restores glucose stimulated insulin secretion, attenuated beta cell apoptosis and ROS generation [143]. Further, Txnip null hepatocytes show reduced gluconeogenesis [144]. Taken together, it appears that Txnip is a very promising therapeutic target for type 2 diabetes. Another important role of Txnip is acting as a tumor suppressor. Nucleus translocation of Txnip increases the levels of cyclin dependent kinase (CDK) inhibitor p27 and p16, thus causes G1 cell growth arrest and suppresses tumor growth [145-147].

1.3.3.9 Prxs

Six different types of mammalian peroxiredoxins (Prxs) have been found, which are divided into three types, 2-Cys, atypical 2-Cys and 1-Cys subgroups. Prx I-IV belong to 2-Cys subgroups, whereas Prx V and Prx VI are members of atypical 2-Cys and 1-Cys subgoup respectively. Prxs are distributed in different cellular compartments: Prx I and II are in the cytosol, Prx III locates in the mitochondria, Prx IV is found in ER as well as in the extracellular space, Prx V is expressed in mitochondria and peroxisomes, and Prx VI locates in cytosol. All Prxs are homodimers and contains a conserved Cys at the N-terminal motif, which is the primary H_2O_2 reducing site. Prxs all need Trx as a source of reducing equivalents for their peroxidase activity [148]. The V_{max} of Prx I-III is 6-13 μmol/min^{-}1•mg^{-1} at 37^{o}C, relatively slower than catalase and Gpx [149]. However, since they have higher affinity towards H_2O_2 (K_m values for H_2O_2 are < 20 μM) and are abundant in most cells, Prxs can be very efficient in removing lower levels of H_2O_2 in the cells [148]. In addition to protect the cells from oxidative damage, Prxs are also involved in regulation of H_2O_2 mediated signaling cascades. Overexpression of Prx I and II in cultured cells eliminate growth factor induced ROS production. Overexpression of Prx II inhibites tumor necrosis factor-α (TNFα) induced nuclear factor κB (NFκB) activation [150]. Moreover, mouse Prx-IV overexpression reduces the epidermal growth facor or p53 induced ROS significantly [151]. Taken together, these findings suggest that Prxs regulate intracellular redox signaling through control of cellular ROS.

1.3.4 GSH system

In addition to Trx system, another important NADPH dependent antioxidant system is the glutathione (GSH) system. GSH plays an essential role in this system and serves as an electron donor for glutaredoxin (Grx) and gluathione peroxidase (Gpx). Glutathione is also used by gluathione transferase (GST) conjugating the xenobiotic substrates [152, 153]. In mammalian cells, the GSH concentration is from 0.5 - 10 mM. Most GSH (approx. 90%) is in cytosol, whereas the rest is found in mitochondria, peroxisomes, ER and nuclear [154, 155]. GSH is readily oxidized to glutathione disulfide (GSSG) through reaction with ROS [152]. GR receives reducing equivalents from NADPH to reduce GSSG back to GSH, and to keep the GSH : GSSG ratio in a physiological range, normally > 10 [152]. Interesting, genetically GR deficient are viable [156], and the mice hepatocytes with germline GR knockout showed a normal total GSH level and GSH : GSSG ratio [157]. This could be explained by the fact that the major source of reduced GSH in the cells is from GSH synthesis, but not the GSSG reduction through GR.

1.3.4.1 GSH synthesis

The synthesis of GSH from L-glutamate, L-cysteine and glycine is catalyzed in two consecutive steps by γ-glutamyl-cystien ligase (GCL) and GSH synthase [152, 153]. The formation of the dipeptide γ-glutamylcysteine (γ-GC) is the rate-limiting step in the synthesis of GSH catalyzed by GCS, which involves ATP-dependent condensation of L-glutamate and L-cysteine [158]. GCL is holoenzyme consists of a 73 kDa catalytic heavy subunit (GCLC) and a 31 kDa regulatory light subunit (GCLM), which are encoded by separate genes. It shows that GCLC exhibits catalytic activity and feedback inhibited by GSH, whereas GCLM has no enzymatic activity [159, 160]. Mice with embryonic homozygous knockout of the catalytic heavy subunit GCLC fail to gastrulate with no mesoderm formed and died before E8.5. However, cells isolated from the $GCLC^{-/-}$ blastocysts proliferate normally *in vitro* [161]. On the other hand, genetically disrupt the regulatory light subunit GCLM results in viable and fertile mice with decreased total GSH levels in multiple organs. GCLC with genetic absence of GCLM has approx. 2-fold increase in *K*m for glutamate and significantly increased sensitivity to GSH inhibition. The Gclm deficient fetal fibroblasts show enhanced susceptibility to oxidant such as H_2O_2 [162].

1.3.4.2 Grx

Glutaredoxins (Grxs) belong to the Trx fold family and were first described as glutathione-dependent reductases for the disulfide bond formed in RNR. They are four forms of Grxs in

human cells. Grx1 and Grx2 are major forms of Grxs with dithiol motif; however, Grx3 and Grx5 are monothiol Grxs. Grx1 is a cytosolic protein with the active site Cys-Pro-Tyr-Cys. Grx2 locates in mitochondria and contains the active site Cys-Ser-Tyr-Cys. During catalytic reaction, active site oxidized dithiol Grxs are reduced and reactivated by two GSH molecules, thereafter forming reduced Grxs and one molecule of GSSG. Reduced Grxs serve as general disulfide oxidoreductases and regulate the activity of redox sensitive transcription factor and phosphatase [163-165]. In addition to dithiol mechanism, another important function of Grxs is the monothiol reduction mechanism. *S*-glutathionylation is an important post-translation modification on proteins in redox signaling, which protects the cysteine from irreversible oxidation and is increased during oxidative stress. Most of the deglutathionylation in mammalian cells are carried out by Grxs monothiol reduction [166].

1.3.4.3 Gpx

Glutathione peroxidases (Gpxs) are well-known antioxidant enzymes scavenging H_2O_2 or organic hydroperoxides, typically using GSH as the source of reductant [25, 167]. They are eight Gpx isoforms found in mammals: mammalian Gpx 1-4 are selenoproteins with a Sec in the catalytic motif, and Gpx6 contains Sec in human but expresses as a Cys-containing counterpart in mice and rat. Gpx5, 7 and 8 are, however, Cys-containing enzymes in mammals [167]. Gpx1 is the first described selenoprotein and ubiquitously expresses in cytosol and mitochondria [168-170]. Gpx1 is involved in redox regulation of insulin signaling. Gpx1 overexpression causes insulin resistance due to over removal of H_2O_2, which serves as a signaling molecule for insulin signaling transduction [171, 172]. Gpx2 is mainly found in gastrointestinal systems and therefore, also named GI-Gpx [167]. Gpx2 plays a dual role in carcinogenesis: on one side, it prevents oxidative stress induced DNA damage and suppresses malignant initiation; on the other side, Gpx2 is overexpressed in many type of cancers to support antioxidant defense and cancer cell survival [167, 173]. Gpx3 is mainly present in the proximal convoluted tubule of the kidney and plasma. In addition to ROS scavenge, Gpx3 prevents hydroperoxide-mediated activation of lipoxygenases, and thus, suppressing inflammatory responses [174, 175]. Gpx4 is also ubiquitously expressed in cytosol and mitochondria [167]. Specific mitochondrial Gpx4 knockout results in spermatozoa maturation abnormality [176, 177]; however, systematic knockout of Gpx4 is embryonically lethal [178]. Evidence suggests that GPx4 possess a crucial protective function in neuron system by controlling the lipid hydroperoxides level [179]. Gpx5 predominantly expresses in epididymis and is crucial for protecting the spermatozoa from oxidative damages [180]. The knowledge of the function of Gpx6-8 is limited. Gpx6 is found in olfactory system

and may be involved in hearing. Gpx7 and Gpx8 are ER proteins and hypothesized to be important for protein folding [167].

1.3.4.4 GST

Glutathione transferases are also named Glutathione *S*-transferases (GSTs), which comprise a family of phase II detoxification enzymes. GSTs are divided into three major families of proteins, the cytosolic and mitochondrial GST, as well as the microsomal GST. GSTs are well known for catalyzing the nucleophilic conjugation of the reduced GSH to electrophilic carbon, sulfur, or nitrogen atoms of nonpolar xenobiotic substrates, thus preventing their interaction with crucial cellular components. These substrates contain quinones, nitrobenzenes, arene oxides, and α, β-unsaturated carbonyls [181]. In most cases, GSH conjugation on the xenobiotic substrates leads to formation of less reactive compounds, which are subsequently secreted from the cells. However, in certain cases, the GSH conjugated compounds are more reactive than the parent molecule. For instance, GSTs catalyze the conjugation of 1,2-dihaloethanes with GSH, which leads to the formation of genotoxic GSH conjugates with an episulfonium intermediate that can modify DNA [182]. This feature of GSTs has been utilized in cancer chemotherapy to produce reactive metabolites that can target tumor cells with an upregulated expression of transferases [181]. One example is the cytotoxic drug TER286 (also called TLK286) is activated by GST to produce a nitrogen mustard-alkylating agent, which is cytotoxic to murine xenografts [183].

1.3.5 Trx and GSH maintained cytosolic redox homeostasis

The NADPH dependent Trx and GSH systems work together to maintain cytosolic redox homeostasis, DNA replication, ROS scavenge, and cystine reduction in mammalian cells. However, a recent study shows that mammalian hepatocytes have a third, NADPH-independent pathway that can support cytosolic redox homeostasis in the absence of TrxR1 and GR. It demonstrates that this pathway utilizes methionine-fueled *trans*-sulfuration (MTS) to supply necessary cysteine for GSH and protein sysnthesis, which can therefore maintain cytosolic redox homeostasis and cell growth independent of NADPH [157] (Fig. 6).

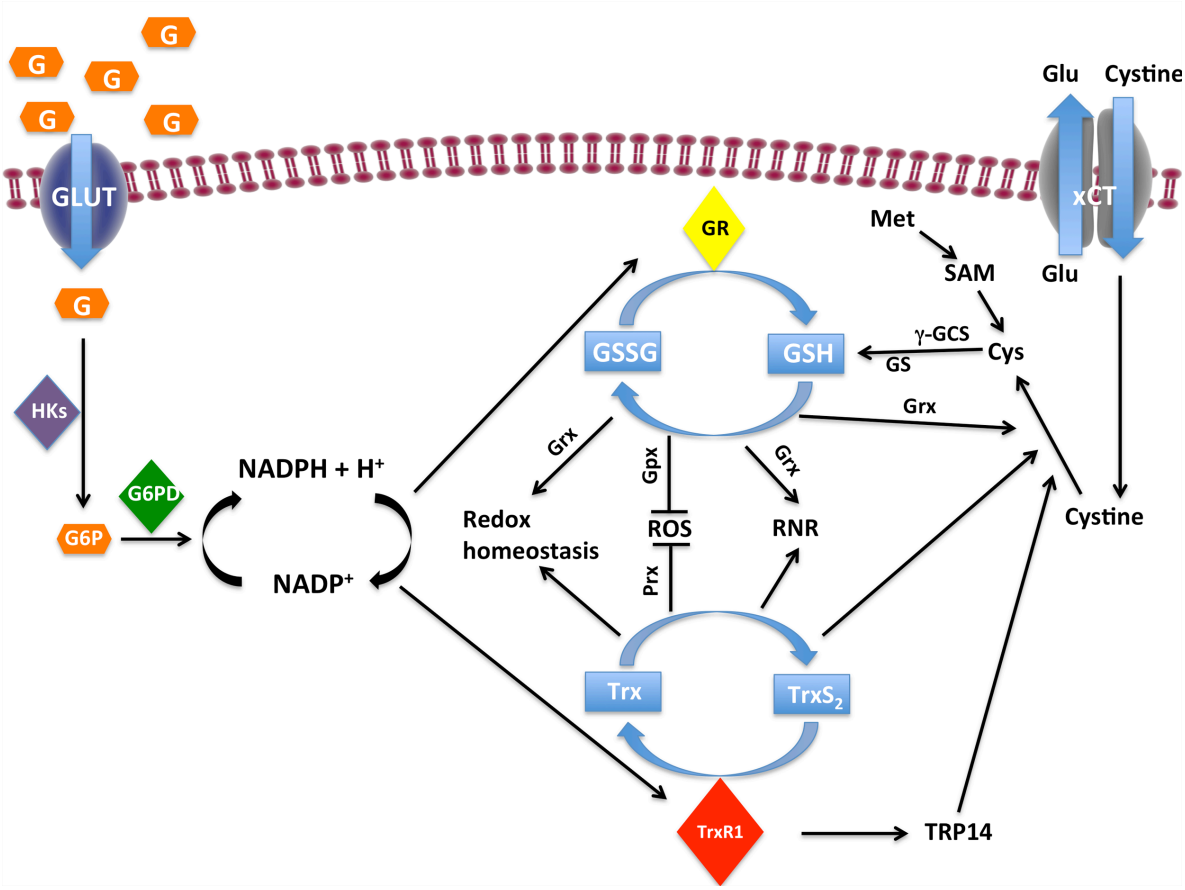

Figure 6. **Trx and GSH maintained cytosolic redox homeostasis**. Mammalian cells take up glucose through glucose transport (GLUT), and then hexokinases convert glucose to glucose-6-phosphate (G6P). G6P undergoes pentose phosphate pathway through the catalytic reaction of glucose-6-phosphate dehydrogenase (G6PD) and generates NADPH. TrxR1 and GR receive reducing equivalents from NADPH to reduce oxidized Trx1 or oxidized GSH (GSSG) respectively, thereby supporting redox homeostasis, ROS scavenging, and RNR reducing. xCT transporter on the membrane imports cystine from extracellular environment and exports glutamate. Cystine is reduced to cysteine (Cys) for GSH synthesis by either the Trx/TRP14 or the GSH system. Methionine can also maintain cytosolic redox homeostasis through MTS pathways to produce Cys, which is then used for GSH and protein synthesis.

1.3.6 Other antioxidant enzymes

Superoxide is produced by a single electron reduction of an oxygen molecule and causes many types of oxidative damages (see section 1.2.2). To be protective, superoxide dismutases (SODs) are ubiquitously expressed and catalyzes the dismutation of superoxide ($O2^{\cdot-}$) to hydrogen peroxide (H_2O_2), which is less reactive and can be degraded by other enzymes, such as Prxs, Gpxs or catalase (see below). There are three forms of SODs present in humans. SOD1 or CuZn-SOD is a copper and zinc-containing homodimer and is found almost

exclusively in the cytosol. SOD2 or Mn-SOD is a maganeses-containing tetramer and is exclusively located in the mitochondrial matrix. SOD3 is a recently characterized tetramer enzyme, with copper and zinc in its active center and present extracellularly [184].

Catalase is widely distributed to bacteria, fungi, plants and animals that exposed to oxygen [185]. Catalases are divided into three types: typical or mono-functional catalases, bifunctional catalase-peroxidases, and pseudo catalase. The mammalian catalase is a homotetramer mono-functional catalase with molecular weight of each subunit approx. 60 kDa. Each subunit contains a heme, ferriprotoporphyrin IX group in its active center [186]. This heme-containing protein is the most efficient enzyme, that can degrade millions of hydrogen peroxide molecules every second ($\sim 10^7$ per sec) into molecular oxygen and water [187].

1.3.7 Low molecular weight antioxidants

In addition to enzyme dependent antioxidant defense system, low molecular weight antioxidants are also important to humans, especially to blood and other biological fluids, where antioxidant enzyme systems are deficient or even absent. Vitamin A consist of a group of unsaturated hydrocarbons, including retinol, retinal, retinoic acid and several provitamin A carotenoids [188]. Vitamin A is able to directly quench singlet oxygen [189-191], it also inhibits NO production through inhibition of iNOS gene transcription [192]. Vitamin C (ascorbate) is the most important water-soluble low molecular weight antioxidant. It directly scavenges different type of ROS, especially $O_2^{\cdot -}$ and $OONO^-$ [193]. Vitamin E is the mixture of different isoforms of tocopherol and tocotrienol, among which α-tocopherol is the most biological form. Vitamin E is the major lipid-soluble antioxidant and plays an essential role in protecting the cellular membrane from lipid peroxidation [191, 194, 195]. Tocopheroxyl free radical can be recycled back to α-tocopherol by ascorbate, thus acting together to prevent lipid peroxidation [196]. Oxidized ascorbate can be recycled back to reduced form through either the Trx or the GSH system [7]. In addition to its enzymatic related reactions discussion above (see section 1.3.4), GSH itself directly reacts with lipid peroxyl radical, peroxynitrite and H_2O_2. Further, GSH conjugates with NO, forming S-nitroso-glutathione adduct [152]. α-Lipoic acid (LA) is also an important antioxidant. The reduced form of Lipoate, dihydrolipoate, can react with various forms of ROS. It also protects cell membranes from oxidative damage by interacting with vitamin C and GSH, which may in turn recycle vitamin E [197].

1.4 HIGH GLUCOSE AND OXIDATIVE STRESS

High glucose circumstance has been shown to induce oxidative stress and hyperglycemia induced oxidative damage has been believed to be an essential mechanism of diabetic complications. In diabetic patients, particular cell types are more susceptible to hyperglycemia than other cell types, such as endothelial cells and mesangial cells. The reason is that most cells are able to reduce glucose uptake when they are exposed to high glucose environment to keep a constant glucose levels inside the cells. However, cells that are damaged by hyperglycemia are those cannot reduce the glucose uptake efficiently, that lead to high glucose concentration inside the cells [10, 198]. How high intracellular glucose levels cause oxidative damage will be discussed below:

1.4.1 Polyol pathway activation

Polyol pathway is mainly catalyzed by aldose reductase (AR) reducing toxic aldehydes to inactive alcohols. However, when the glucose concentration is high in the cell, aldose reductase also starts reducing glucose to sorbitol [10]. Under normal condition, polyol pathway is only in charge of approx. 3% glucose metabolism [199], whereas it increases to 30% under hyperglycemia condition [200]. This process consumes NADPH, and thus affecting the activities of NADPH dependent antioxidant systems such as Trx and GSH systems [10]. As a result, hyperglycemia would increase susceptibility of the cells to oxidative stress. Indeed, endothelial cells cultured in high glucose medium are shown to have increased sensitivity to H_2O_2 treatment with a reduced GSH level [201]. Aldose reductase is found in nerve, retina, glomerulus and vascular tissues, which are all hyperglycemia sensitive [202]. Increased NADPH consumption in these cells through reducing high amount of glucose to sorbitol causes oxidative stress. Study shows that diabetic dogs decrease nerve conduction velocity over time as it does in humans. However, five years aldose reductase inhibitor treatment prevents this defect in the diabetic dogs [203].

1.4.2 AGE formation

Advanced glycation end products (AGEs) are formed by glycation reaction, which refers to the addition of glucose or other glycating products from glucose to proteins or lipids through non-enzymatic reaction [204, 205]. Under hyperglycemia, the intracellular production of AGE precursors, such as glyoxal, methylglyoxal and 3-deoxyglucosone [206], is increased, which leads to glycation of intracellular proteins and causes cellular dysfunction. Further, intracellular AGE precursors can diffuse out of the cells, modify and cross-link the extracellular matrix [207]. The circulating proteins modified by AGE precursors bind to

receptors of AGE (RAGE), inducing ROS production and NF-κB activation. This subsequently causes series pathological changes in gene expression [208].

1.4.3 Protein kinase C activation

Protein kinase C (PKC) is widely expressed in mammalian cells and phosphorylates a number of target proteins. It is well known that high glucose level enhances the *de novo* synthesis of diacylglycerol (DAG), which activates PKC [209-212]. PKC dependent activation of NADPH oxidases (NOX) and ROS production have been reported in different studies. High glucose exposed smooth muscle cells and endothelial cells produce significant higher amount of ROS than those cultured in low glucose. Either PKC inhibitor or NOX inhibitor completely blocks the elevated ROS production [213]. Endothelial cells exposed to high glucose reveal enhanced formation of nitrityrosine and 8-hydroxydeoxyguanosine (8-OHdG) with overexpressed NOX components as well as increased apoptosis. PKC inhibitor reduces nitrotyrosine levels, decreases 8-OHdG concentrations and attenuates cell apoptosis under such condition [214]. These findings indicate a high glucose induced PKC-dependent NOX activation, which enhances ROS production.

1.4.4 Mitochondrial superoxide production

As introduced in section 1.2.1, when glucose is metabolized through mitochondrial oxidative phosphorylation, it undergoes tricarboxylic acid (TCA) cycle and generates electron donors, such as NADH and $FADH_2$. Electrons are transferred from the donors through the complexes of mitochondrial electron transport chain, which generates proton gradient across the membrane. The energy from this gradient voltage will either be used by ATP synthase for ATP synthesis or dissipated by uncoupling proteins (UCPs) for heat generation. In cells exposed to high glucose, there is overload of TCA cycle and over production of NADH and $FADH_2$. The excessive electron flow will saturate the electron transfer capacity in the complexes of mitochondrial electron transport chain and increase the voltage gradient to reach a critical threshold. At this point, the complex III will be blocked, and single electron leakage from complex I, coenzyme Q or complex III will occur to reduce O_2 to superoxide [10, 198]. Expressing UCP1 or MnSOD in the vascular endothelium blocks ROS production under hyperglycemia condition [215]. Depletion mitochondrial DNA from endothelial cells results in functional deficiency on mitochondrial electron transport chain and causing the loss of ROS production in these cells under hyperglycemia [10]. It also shows that inhibitors of mitochondrial electron transport chain complex I, complex II or mitochondrial pyruvate transporter inhibit high glucose promoted ROS production in rat kidney proximal tubular cells [216].

Taken together, high intracellular glucose concentration induces ROS production through polyol pathway activation, AGE formation, PKC activation and mitochondrial respiration (Fig. 7).

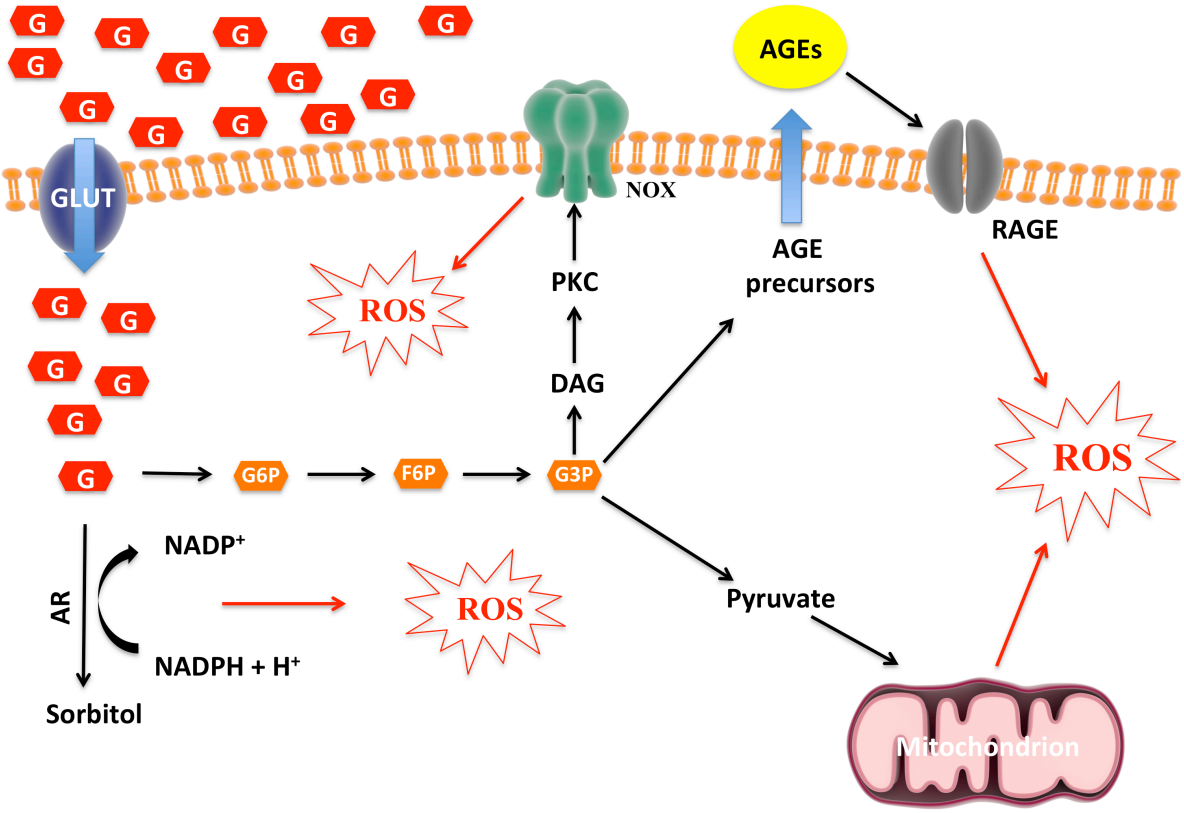

Figure 7. **High glucose induced oxidative stress**. High glucose circumstance or hyperglycemia causes high intracellular glucose concentration in certain cell types and activates four pathways to increase ROS production. Activated polyol pathway catalyzed by AR (aldose reductase) reduces excessive amount of glucoses to sorbitols and over consumes NADPH, which inhibits NADPH-dependent antioxidant systems and causes ROS production. Increased glucose metabolism promotes the formation of glyceraldehyde 3-phosphate (G3P), which enhances the *de novo* synthesis of DAG (diacylglycerol), thereby leading to PKC-dependent NOX activation and ROS production. AGE precursors formed by glucose metabolism diffuse into extracellular environment and glycate circulating proteins to form AGEs, which subsequently binds to RAGE (receptors of AGE) and induces ROS formation. Electron leakage from mitochondrial electron transport chain serves as an important source of ROS and will be exaggerated with access to high glucose.

1.5 ADIPOCYTE DIFFERENTIATION

1.5.1 Adipose tissue and adipocyte differentiation

Traditionally, adipose tissue is considered to be an inert mass that passively stores energy. However, adipose tissue is now identified as a complex and highly active metabolic organ with essential endocrine functions. Adipose tissue secrets many circulating factors including plasminogen activator inhibitor-1, leptin, adiponection, resistin, and protein of renin-angiotension systemm etc. These hormones regulate variety of processes as homeostasis, blood pressure, immune function, angiogenesis and energy balance [217, 218]. Metabolic defects caused by either excessive or deficient adipose tissue illustrate the importance of endocrine function derived from adipose tissue. Obesity is usually associated with morbidities such as insulin resistance, hyperglycemias, dyslipidemia, proinflammatory and hypertension, which are known as metabolic syndrome [219]. On the other hand, adipose deficiency also correlates with features of metabolic syndrome [220].

Adipose tissue is composed of connective matrix, nerve tissue, stromo-vascular cells, immune cells and most importantly, adipocytes [217]. Adipogenesis refers to the process that mesenchymal stem cells differentiate into adipocytes. This differentiation process is divided into two phases. The first phase is known as determination, results in conversion of multipotent cells into pre-adipocyte, which maintains its original morphology but loses the potential to differentiate into other cell types. The second phase is known as terminal differentiation, in which the pre-adipocytes acquire the mature adipocyte characteristics [218]. There are two types of adipocytes in mammals, named white and brown adipocyte respectively. Compared to white adipocytes, brown adipocytes accumulate smaller lipid droplets and contain a much higher number of mitochondria, which make it look brown [218]. In addition to the genes that are expressed in white adipocytes, brown adipocytes also express some distinct genes such as uncoupling protein-1 (UCP1), which uncouples the proton potential in mitochondria respiration and facilitates energy to be dissipated as heat without ATP synthesis [221].

Adipocyte differentiation involves in activation of a series of transcription factors and signaling events that work in orchestra to drive the initiation and maturation of adipocytes.

1.5.2 PPARγ

PPARγ belongs to the nuclear receptor superfamily and acts as a master regulator of adipocyte differentiation [218]. Overexpression of PPARγ is capable of inducing adipocyte

differentiation in cultured fibroblasts, on the contrary, genetic disruption of PPARγ impairs the development of adipose tissue in mice dramatically [222, 223]. So far, no factor has been identified to promote adipocyte differentiation in absence of PPARγ, and most adipogenesis inducers are found to at least partially activate PPARγ expression or activity [218]. PPARγ is shown to be not only important for adipogenesis, but also crucial for maintenance of adipocyte maturation. Overexpression of dominant-negative PPARγ into mature adipocytes results in de-differentiation with decrease of lipid content and adipocyte marker expression levels [224]. There are two isoforms of PPARγ, γ1 and γ2. Both γ1 and γ2 mRNA are expressed abundantly in adipose tissue and found in skeletal muscle, whereas γ1 is also detected in liver, spleen and heart [225].

The endogenous PPARγ ligand remains unclear. Earlier studies suggest different metabolites having ligand activity for PPARγ activation, including 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), certain polyunsaturated fatty acids, nitro unsaturated fatty acid, and oxidized low-density lipoprotein (oxLDL) etc [226-230]. However, these biological ligands bind to PPARγ with relatively low affinity (Kd = 2-50 μM), compared with the affinity (normally Kd < 1 nM) for most endogenous ligands for nuclear receptors [231]. Similar to other nuclear receptors, PPARγ binds to DNA together with retinoid X receptor as a heterodimer. It shows that PPARγ-responsive element consist of two repeats of "AGGTCA" separated by a single nucleotide [231].

1.5.3 C/EBP family

Six isoforms of C/EBP proteins have been identified and all of which act as homo and/or heterodimers via a bZIP domain [232]. Many of them are involved in adipocyte differentiation, among which C/EBPβ and C/EBPα are the most essential players. The expression pattern of these transcription factors during the progress of adipocyte differentiation indicates a signaling cascade whereby expression of C/EBPβ in early stage of differentiation leads to induction of C/EBPα [233].

C/EBPβ gene produces different isoform proteins; liver-enriched activator protein (LAP) is the transcriptional active form, whereas liver-enriched inhibitory protein (LIP) is a truncated form that negatively regulates the LAP activity [234]. Ectopic expression of C/EBPβ in 3T3-L1 preadipocytes is able to induce adipocyte differentiation without hormonal treatment [235]. Further, forced expression of C/EBPβ in NIH-3T3 fibroblasts induces PPARγ expression and promotes adipogenesis upon differentiation induction [236]. Mitotic

clonal expansion (MCE) refers to the process that post-confluent cells undergo one or two rounds of cell division upon treatment of differentiation inducing agents, which is a prerequisite for *in vitro* differentiation of mouse embryonic fibroblasts (MEFs) and mouse preadipocytes 3T3-L1 [237, 238]. C/EBPβ is shown to be essential for MCE, MEFs with C/EBPβ knockout neither undergo MCE nor differentiate to adipocytes upon induction. However, overexpression of C/EBPβ (LAP) but not dominant-negative C/EBPβ (LIP) in the knockout MEFs restores MCE and adipogenesis [238]. C/EBPβ and C/EBPδ deficient MEFs have severely defected adipocyte formation with no C/EBPα and PPARγ expression. Mice lacking C/EBPβ developed normal WAT with reduced lipid accumulation in BAT. On the other hand, mice with C/EBPβ and C/EBPδ double knockout have impaired adipose tissue development [239].

C/EBPα also plays an important role in adipogenesis and directly induces adipocyte genes. Ectopic expression of C/EBPα in various mouse fibroblasts promotes adipocyte differentiation without hormonal induction [240]. Expression of C/EBPα antisense RNA in 3T3-L1 preadipocytes suppresses adipocyte genes expression, lipid accumulation and adipogenesis in these cells [241]. Homozygous C/EBPα knockout causes early lethality after the birth of mice, due to defective liver gluconeogenesis and subsequent hypoglycemia [242, 243]. Restoration of hepatic C/EBPα expression rescues the mice and shows limited effects on BAT. However, the WAT is almost absent in these mice with excessive lipid levels in serum, suggesting that C/EBPα is required for WAT development *in vivo* [243].

1.5.4 Insulin signaling in adipocyte differentiation

Insulin signaling has remarkable effects on adipocyte differentiation. It stimulates glucose uptake and lipid synthesis, which are important for adipocyte metabolism [244]. Mouse fibroblasts and brown preadipocytes that have inactivated insulin receptor (IR) or IR deficiency show dramatically impaired ability to differentiate into adipocytes [245, 246]. However, the link between insulin signaling and adipocyte differentiation is complex, with many downstream signaling events involved. The knockout of individual insulin receptor substrates in brown preadipocytes demonstrates that IRS-1 is the most important IRS for adipogenesis with a severe defect in this process upon depletion. IRS-3 knockout also shows moderate influence on adipogenesis, but no effect observed from IRS-2 or IRS-4 knockout [247]. Phosphatidylinositol-3 kinase (PI3K) and protein kinase B (Akt/PKB) pathway plays an essential role in downstream of insulin signaling cascades, and are also crucial for adipogenesis. Mice lacking Akt2/PKBβ exhibit an age-dependent loss of adipose tissue and a

dramatic reduction of adipose depots by 22 weeks of age [248]. Another downstream player, mammalian target of rapamycin (mTOR) is also involved in adipocyte differentiation. Using rapamycin to inhibit mTOR kinase activity blocked adipocyte differentiation through inhibition of the transcription activity of PPARγ [249]. Insulin signaling can also activate and phosphorylate cyclic AMP response element-binding (CREB) protein, which is reported to be a primary transcription factor that promotes the initiation of adipogenesis [250, 251].

The phosphatases that negatively regulate insulin signaling also have enormous impact on adipocyte differentiation. Protein-tyrosine phosphatase 1B (PTP1B) is shown to directly inhibit insulin signaling by suppressing the phosphorylation of insulin receptor [252]. In addition, PTP1B deficient brown preadipocytes undergo an accelerated adipogenesis with upregulation of PPARγ and adipogenic markers [253]. Phosphatase and tensin homologue (PTEN) dephosphorylates and converts phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to phosphatidylinositol (4,5)-bisphosphate (PIP2), so that inhibiting the activation of Akt/PKB. Liver specific PTEN knockout results in increased phosphorylation of Akt/PKB, fatty liver and induction of adipocyte-specific genes, implying adipogenic transformation in the liver [254, 255].

1.5.5 Redox regulation in adipocyte differentiation

Many studies have shown that intracellular ROS is important modulating factor in adipocyte differentiation. Evidence shows that NADPH oxidase contributes as a major source of ROS during adipocyte differentiation in 3T3-L1 preadipocytes [256]. Inhibiting NADPH oxidase using inhibitor or si/shRNA attenuates ROS production and adipocyte differentiation [257, 258]. In human mesenchymal stem cells, an early increase of ROS derived from mitochondrial metabolism is shown to be essential for adipocyte differentiation. Antioxidants that target mitochondria suppress adipocyte differentiation, which is then rescued by exogenous H_2O_2 treatment [259]. Further, inhibiting mitochondrial activity by rotenone or siRNA knockdown of the mitochondrial transcription factor A (TFAM) causes significant adipocyte differentiation suppression [260]. NO also has a vital role in adipocyte physiology. Rat preadipocytes are found to produce 50% more NO during the initiation of adipocyte differentiation. Deprivation of endogenous NOS activity by NOS inhibitor suppresses the differentiation process [261].

There are different aspects from which ROS can regulate adipogenesis (Fig. 8). Accumulated evidence shows that ROS, especially H_2O_2 may act as secondary signaling messengers to enhance signaling transduction via oxidation and inactivation of phosphatases, which are

negative regulators of phosphorylation signaling cascades [19, 262-264]. ROS can also directly oxidize and activate Src tyrosine kinases, thereby activating Akt/PKB dependent signaling [265-267]. As discussed above, MCE is an essential process for *in vitro* differentiation of MEFs and preadipocytes 3T3-L1. H_2O_2 treatment accelerates cell cycle progression during MCE and facilitates adipocyte differentiation in 3T3-L1 cells. Further, oxidative stress increases the oxidation and dimerization of C/EBPβ, which subsequently enhances its DNA binding capacity [268]. Another redox regulated adipogenesis signaling molecule is the master regulator PPARγ, of which the activation is ligand dependent. Nitro-modified unsaturated fatty acids are recently found to be relatively potent endogenous PPARγ ligands that activate PPARγ at sub-micromole concentration with increased production under oxidative stress [269].

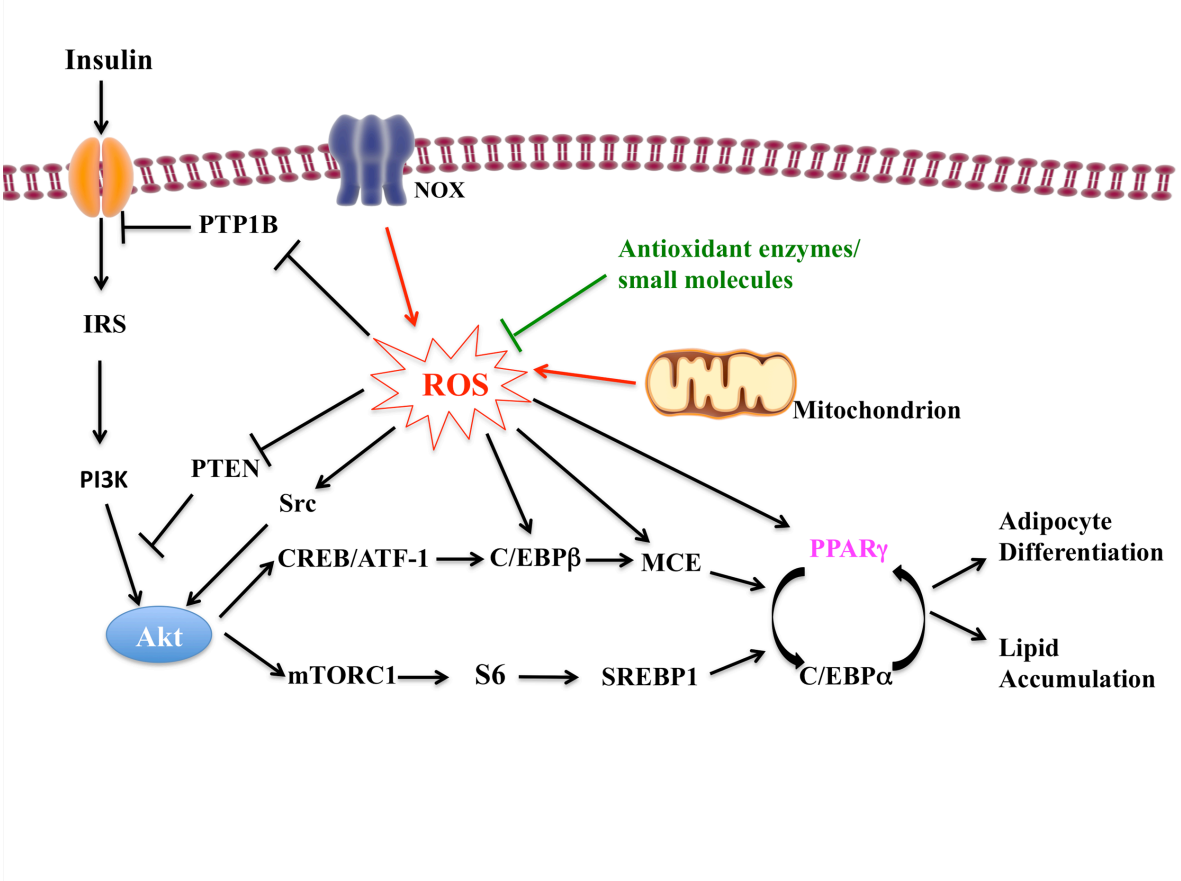

Figure 8. **Redox regulation in adipocyte differentiation**. Insulin signaling activates downstream PI3K/Akt pathway, which further activates downstream events that connected to PPARγ, the master regulator of adipocyte differentiation. Intracellular ROS promotes adipogenesis from different aspects. ROS oxidizes and inactivates phosphatases, such as PTP1B and PTEN, to enhance the insulin signaling cascades. In addition, ROS also oxidizes and activates Src kinase, thereby activating Akt/PKB. Moreover, ROS enhances the activity of C/EBPβ and accelerates the MCE, which are important factors for the initiation of adipogenesis. Endogenous PPARγ ligands production can also be promoted by ROS accumulation.

2 AIMS OF THIS THESIS

Mammalian TrxR1 is importantly involved in DNA synthesis, antioxidant defense and maintenance of cellular redox homeostasis. However, the necessity of Sec in TrxR1 is not clear. Here we first wish to study the unique cellular functions and properties of the natural Sec-containing TrxR1 compared to its Cys-containing counterpart. In addition, TrxR1 has been implied to play a crucial role in redox regulation; we thereby wish to investigate the impact of TrxR1 in redox sensitive signaling events. Moreover, many cancer cells have upregulated antioxidant systems including TrxR1 to adapt to the increased oxidative stress, and several anticancer compounds used clinically or under clinical trails are shown to target TrxR1 as at least part of their cytotoxicity mechanism. Therefore, we wish to further investigate the role of TrxR1 as an anticancer target.

Specific objective in each paper is to:

Paper I

Investigate the targeting effects of the mutant p53 activator and anticancer drug lead named APR-246 (PRIMA-1^{MET}) on mammalian TrxR1.

Paper II

Study how the Sec content of mammalian TrxR1 modulates the cytotoxicity of drugs that target TrxR1 and the glutathione dependence of the cells.

Paper III

Explore the importance of Sec-containing TrxR1 to the growth and survival of mammalian cells.

Paper IV

Elucidate the role of TrxR1 in regulation of insulin signaling and adipocyte differentiation.

3 RESULTS AND CONCLUSIONS

3.1 PAPER I

Tumor suppressor p53 is for DNA repair, cell cycle arrest and apoptosis initiation. Several different types of DNA damage can activate p53, such as double-strand breaks in DNA produced by γ-irradiation, ultraviolet irradiation and chemical damage to DNA. In response to this, the transcription activity of p53 is activated to induce downstream genes, such as p21 that causes cell cycle arrest. In addition, p53 can also initiate apoptosis in response to oncogene activation, and thus serves as a tumor suppressor [270]. It shows that p53 is inactivated in most human cancers, and direct gene mutations in p53 contribute to half of the inactivation [271]. Therefore, mutant p53 becomes a promising target for novel anticancer therapy.

APR-246 (PRIMA-1^{MET}) is converted to methylene quinuclidinone (MQ), a Michael acceptor that can covalently binds to Cys in p53, promotes the correct folding of mutant p53 and restores the wild-type p53 activity [272, 273]. Since mammalian TrxR1 has a Sec on its active site, which is much more reactive than Cys, we therefore wished to study the targeting effects of APR-246 on TrxR1.

We found that APR-246 (active compound MQ) inhibited the activity of both recombinant and cellular TrxR in cells, with different p53 background. However, Sec-to-Cys mutant TrxR1 is resistant to this inhibition, suggesting that the targeting residue is the Sec in wild-type TrxR1. Inhibited TrxR1 still maintains its NADPH oxidase activity, which causes massive ROS production. Indeed, knockdown TrxR1 attenuated APR-246 induced ROS and cell death independent of p53 status.

This study demonstrates that targeting TrxR1 is an additional cytotoxicity mechanism of APR-246. The ability of targeting TrxR1 and restoring wild-type p53 activity in the tumor could produce a synergic effect that disrupts the cellular redox and activates apoptosis signaling cascades. Targeting TrxR1 in addition to mutant p53 will reduce the risk of resistance development, since long-term treatment with APR-246 may cause loss of mutant p53 expression. Taken together, this finding reveals the mechanism that APR-246 could potently and specifically target tumor cells in relative to normal tissues.

3.2 PAPER II

In mammalian cells, $SePO_3$ is used as the selenium source for Sec synthesis and incorporation into selenoproteins. However, it is reported that SPO_3 might compete with $SePO_3$ as a substrate of SecS, that leads to Cys synthesis and incorporation in the site of Sec residue on selenorptoens. This normally occurs under selenium deficient conditions [52, 53]. Study shows that using SPO_3 to treat NIH 3T3 cells converts up to 96% of the TrxR1 to Cys-containing variant. Based upon this, we wished to titrate the Sec content in TrxR1 using SPO_3 or selenite supplementation. We wondered how this would modulate the cytotoxicity of drugs that target TrxR1 and the glutathione dependence of the cells.

In this study, we supplemented cells with different concentration of SPO_3 or selenite, and subsequently measured total and specific cellular TrxR activities. It revealed that SPO_3 increased Cys incorporation into TrxR1. On the other hand, selenite promoted the expression of Sec-containing TrxR1. Meanwhile, we found that high concentrations of SPO_3 (> 300 μM) were highly toxic to the cells. However, lower concentrations of SPO_3 (100 - 300 μM), attenuated the cytotoxicity of cisplatin on both A549 and HCT116 cells. On the contrary to the cancer cells, which have increased cellular TrxR activity, NIH 3T3 cells showed low basal TrxR activity and were highly susceptible to GSH depletion. We thus used selenite to boost TrxR activity in these cells, and found the resistance to BSO treatment increased, whereas the resistance to cisplatin decreased. The obtained resistance to GSH depletion is in agreement with the literatures that there are complementary functions between Trx and GSH pathways, and under many conditions, either Trx or GSH system is sufficient enough to support the cellular redox homeostasis [103, 106, 157, 274].

In summary, these results suggest that the selenium status of cells can affect the Sec incorporation into TrxR1, and thereby modulating the cytotoxicity of drugs that target TrxR1 and the glutathione dependence of the cells.

3.3 PAPER III

Since mammalian TrxR1 is synthesized as a Cys-containing counterpart under selenium starvation conditions, together with the fact that some organisms express a closely related Cys-containing TrxR, such as *D. melanogaster* [42], we wonder if the Sec is necessary for the cellular function of mammalian TrxR1, and whether there are any unique properties or cellular functions of Sec-containing TrxR1 compared to its Cys variant.

In this study, we utilized a MEF cell line with TrxR1 knockout (*Txnrd1*$^{-/-}$ MEFs). Further, we stably overexpressed different variant of TrxR1 in the knockout cells. Immunoblotting, ^{75}selenium radioactive labeling and cellular enzymatic assay confirmed the successful construction of the knockout and mutant expressing TrxR1 MEF cell lines. We found only the TrxR1 null MEFs had significant activation of Nrf2, with unregulated Trx and GST activities as well as total GSH content. Therefore, it is not surprising that the TrxR1 deficient cells are highly susceptible to GSH depletion with massive cell death. However, the TrxR1 mutant cells showed more resistance to BSO treatment than the knockout cells.

We subsequently observed that when seeded in high density (> 8000 cells/cm^2), all the cells grew normally regardless of the TrxR1 status. However, when seeded at lower density (< 1000 cells/cm^2), only cells that express Sec-containing TrxR1 could survive and proliferate, whereas the other cells underwent extensive cell death. Intriguingly, both conditioned medium from high-density culture and catalase supplementation were able to completely rescue the cell death of the knockout MEFs at low-density. This indicates that H_2O_2 is the toxic factor that kills the cells. Extracellular H_2O_2 measurement revealed that the TrxR1 deficient MEFs secreted fourfold H_2O_2 compared with the parental wild-type MEFs (*Txnrd1*$^{fl/fl}$ MEFs). Expressing Sec-containing TrxR1 but not the Cys mutant enzyme in the knockout cells reverted the H_2O_2 release.

DMEM medium with high-glucose (25 mM) is the standard medium for MEFs culturing [275], which is also the medium we use here. High glucose and hyperglycemia have been linked to induction of excessive amount of H_2O_2 and cause of oxidative damage [10]. We thus used DMEM with low-glucose (5 mM) and found that in sharp contrast to what was observed in high-glucose medium, the knockout MEFs survived and grew very well in the low-glucose medium. In addition, the oxidized Trx and activated JNK in the knockout MEFs in high-glucose was completely reversed when the low-glucose medium was used. This study shows that Sec-containing TrxR1, but not the Cys variant, is exclusively required for self-sufficiency of MEFs by control of glucose metabolism derived H_2O_2, which is not compensated by elevated GSH system.

3.4 PAPER IV

Recent studies show that liver-specific deletion of *Txnrd1* results in alterations of glycogen and lipid storage in liver [101, 102], which motivate us to investigate the nutrient storage in the knockout MEFs. We observed that *Txnrd1*$^{-/-}$ MEFs possess 2-folder higher triglyceride contents compared to the *Txnrd1*$^{fl/fl}$ MEFs. In addition, we are also curious about the adipocyte differentiation capacity in the MEFs with TrxR1 knockout. We found knocking out TrxR1 in the MEFs promotes adipocyte differentiation dramatically upon hormone induction with strongly upregulated PPARγ expression. The essential process for *in vitro* differentiation of MEFs, MCE, spontaneously occurred in the knockout MEFs without any treatment and became accelerated upon induction. However, complete cell cycle arrest without any indication of MCE was observed in the parental *Txnrd1*$^{fl/fl}$ MEFs. Insulin related signaling cascades Akt, CREB/ATF1 and S6 were more activated in the knockout cells upon differentiation stimulation. We thereafter found a reduced PTEN activity in the *Txnrd1*$^{-/-}$ MEFs, which may contribute to the Akt activation. Through overexpression wt but not active site mutated PTEN in the knockout cells, we were able to attenuate the Akt activation and adipocyte differentiation when subjected to hormone treatment.

We showed that p27, a cell cycle arrestor, was downregulated and destabilized in the knockout cells, which is due to the activated Akt that inhibits the expression and induces the degradation of p27. The reduced p27 thus stimulated MCE and adipocyte differentiation in the knockout cells. NAC treatment inhibited Akt activation, stabilized p27, and thus blocked MCE and adipocyte differentiation. We found NAC treatment did not further increase but even decrease the total GSH levels in the knockout cells. Therefore, the effects of NAC are likely come from its intrinsic antioxidant activity.

Another mechanism contributes to enhanced adipocyte differentiation in the knockout MEFs is their 30-fold increased PPARγ mRNA levels compared to the parental cells. Recent studies show that unsaturated fatty acids modified by NO might be potent endogenous PPARγ ligands that can be furthermore produced upon oxidative stress [229, 269, 276]. Therefore, it is plausible that the *Txnrd1*$^{-/-}$ MEFs with higher oxidative stress have much higher PPARγ expression. Here we found nitrooleate (AONO2) indeed induced adipocyte formation in the knockout cells. To validate our findings in MEFs, we isolated pre-adipocytes from human and differentiated them *in vitro*. We observed that knockdown TrxR1 significantly increased adipogenesis in these human cells as well. We conclude that TrxR1 suppresses adipocyte differentiation through inhibiting insulin signaling cascades, MCE and PPARγ expression (schemes summarizing these findings are shown in Fig. 9 of Paper IV).

3.5 DISCUSSION AND FUTURE PERSPECTIVE

This thesis further explored the role of TrxR1 as an anticancer target, and investigated the modulation effects of the Sec residue on the cytotoxicity triggered by TrxR1 inhibitor. We also unveiled that Sec-containing TrxR1 is required for the self-sufficiency of MEFs under high-glucose condition due to the control of glucose derived H_2O_2, which is not compensated by upregulated GSH system. This is, to our knowledge, the first time a crucial Sec-dependent role of TrxR1 identified for mammalian cells. We subsequently showed that TrxR1 suppresses adipocyte differentiation through negatively regulating insulin signaling, MCE and PPARγ expression, which indicates an important role of TrxR1 in glucose homeostasis and physiology of adipose tissue *in vivo*.

In **paper I**, we found that the mutant p53 activator APR-246 (PRIMA-1^{MET}) also targets and inhibits TrxR1, converts the enzyme to a dedicated NADPH oxidase. It reports earlier that a wild-type p53 activator RITA, which prevents the interaction between MDM2 and p53, also inhibits TrxR1 and contributes to its apoptosis induction. Long-term treatment with a single drug may apply a specific selection pressure on the tumor and results in resistance development. Combination treatment of APR-246 and RIPA would activate mutant p53 and prevent p53 degradation at the same time, together with TrxR1 inhibition, this treatment might have synergic effects on killing cancer cells and minimize the resistance development.

In addition, this study further strengthens the principle that targeting TrxR1 seems to be an intriguing anticancer strategy. As discussed before, TrxR1 contains a Sec in its C-terminal active site, which is more reactive than Cys with a much higher nucleophilicity. Further, the C-terminal Sec-containing active site of TrxR1 is exposed and easily accessible [277], whereas the Sec residues of the other major selenoprotein family Gpxs are embedded [278]. In addition, cancer cells normally have increased ROS levels compared to their normal counterparts [122, 123], which require upregulation of antioxidant systems including TrxR1 to maintain the cellular redox balance. Targeting Sec residue on TrxR1 inhibits its enzymatic activity and converts this enzyme to a NAPDH oxidase with increased ROS production. Since cancer cells are more reliable on cellular antioxidant capacity and more susceptible to oxidative stress, inhibiting TrxR1, especially selectively targeting the Sec residue, is likely to be much more toxic to cancer cells than healthy tissues. Based upon this, we screened approx. 400,000 compounds and identified a novel TrxR1 inhibitor with high potency and specificity. This inhibitor showed anticancer efficacy in different mouse models *in vivo* without having apparent toxicity in normal tissues [Stafford et al., manuscript in preparation]. Further development on this compound is in progress.

In **paper II**, we found that increased Sec incorporation into TrxR1 sensitized the cells to cisplatin treatment, whereas elevated expression of the Cys variant of TrxR1 render the cancer cells resistant. The connection between body Se status and cancer has been implicated for many years [279]. It appears that to reach the serum selenium levels that are optimal for the activity and expression of Gpx3 and selenoprotein P could reduce the incidence of various types of cancer [280]. Long-term selenium supplementation in participants from the Chinese region of Qidong with low selenium in the soil reduced the incidence of primary liver cancer [281]. The Nutritional Prevention of Cancer (NPC) trial showed that only the men with low basal levels of plasma selenium had a decreased prostate and colorectal cancer when subjected to selenium supplementation [282]. In another Phase III trial, the prostate cancer incidence was decreased (non-significantly) in the selenium-supplemented men with the lowest group of basal plasmas selenium levels [283]. A Spanish study found significant lower selenium levels in the patient group with high incidence of colerectal adenomas or colerectal cancer having the age below 60 years [Spanish]. On the contrary, the famous Selenium and Vitamin E cancer Prevention Trial (SELECT) showed neither selenomethionine nor vitamin E prevented prostate cancer with the age above 50 years. It is likely due to the considerably high basal plasma levels of selenium in the SELECT participants with a mean value above the optimal concentration for the activity and expression of the plasma selenoproteins Gpx3 and selenoprotein P [280, 284]. The possible mechanism of the selenium protection effects might be due to its modulation on the activities of selenoproteins, such as selenoprotein P, TrxR and Gpxs, which suppress the ROS mediated Akt signaling and DNA mutation [280].

Likewise, early epidemiological studies showed that the selenium levels in serum, plasma, and urine are lower in various types of cancer patients compared to that in the controls [285]. Thus, the selenium deficiency in cancer patients is likely to induce Cys variant TrxR1 expression, which may cause resistance to electrophilic compound treatment that has TrxR1 inhibition as part of its efficacy mechanism. Chemotherapy in combination with selenium supplementation might be a solution to this type of resistance. To approve this, additional *in vivo* studies are needed: mice with tumor xenografts can be fed with either selenium sufficient or deficient diet, and then the anticancer efficacy of cisplatin will be compared. Besides, the cytotoxicity on normal tissues should also be taken into consideration; for example, the nephrotoxicity triggered by cisplatin will also be evaluated under different selenium status.

In **paper III**, we observed that Sec-containing TrxR1 is required for the survival and growth of MEFs under high glucose condition when cultured at low density, due to the important

elimination of glucose-derived H_2O_2. This is, to our knowledge, the first time a unique and essential Sec-dependent role of TrxR1 identified for mammalian cells. Insulin resistance caused by genetic variants, aging or obesity results in high blood glucose in the body. To maintain glucose homeostasis, expansion of pancreatic β cell mass by cell growth and enhancement of β cell function usually occur to compensate insulin resistance through increase of insulin secretion. Nevertheless, after long time exposure to hyperglycemia, β cells become defective and ultimately failed mainly due to the oxidative damage triggered by hyperglycemia. The onset of β cell failure accompanied with insulin resistance indicates the development of type 2 diabetes [286]. The results from this study imply that TrxR1 might play a key role in antioxidant defense of pancreatic β cells against this oxidative damage. Direct modulation of TrxR1 expression level and selenium supplementation will be used in culture of primary pancreatic islet and the β cell lines, thereafter the cell growth and cell death will be evaluated under high glucose condition. In addition, (db/db) mice that are lacking of leptin receptor and usually used as mouse model for study of obesity-induced diabetes. By constructing pancreatic β cell-specific *Txnrd1* knockout (db/db) mice, we will be able to investigate if TrxR1 is involved in the protection of β cells against hyperglycemia induced oxidative damage *in vivo*. β cell failure will be assessed by monitoring insulin secretion in pancreatic β cell-specific *Txnrd1* knockout (db/db) mice compared to control (db/db) mice. Measurement of β cell mass and detection of cell death markers will further validate and characterize the β cell failure. Thus, this project has a potential to provide new insights into the role of TrxR1 in antioxidant defense in the development of type 2 diabetes, and to yield innovative prevention solutions to type 2 diabetes.

In **paper IV**, we found TrxR1 suppresses adipocyte differentiation through inhibiting insulin signaling cascades, MCE and PPARγ expression. Early studies show that germline depletion of *Txnrd1* results in embryonic lethality [96, 97]. This is less likely caused by the deficiency in cell proliferation since the embryos underwent several rounds of proliferation and accumulated thousands of cells before they died. It is believed that the alteration of cellular signaling events in TrxR1 deficient cells induced this lethality. Recent studies demonstrate that liver-specific TrxR1 knockout influences glycogen and lipid storage in this organ, indicating a metabolic alteration upon *Txnrd1* disruption [101, 102]. The present study reveals that TrxR1 plays an essential role in redox regulation of insulin signaling cascades and adipocyte differentiation. In addition to these signaling pathways, more cellular signaling cascades can be affected upon removal of TrxR1. Thus, it is plausible that the embryonic

lethality triggered by depletion of *Txnrd1* is due to the disordered redox cellular signaling that causes the defects in embryonic development.

It appears that TrxR1 has dual roles in cancer. Accumulated indications imply that TrxR1 serves as a tumor suppressor in normal tissues preventing transformation and cancer initiation. High ROS levels cause DNA damage, thereby triggering DNA mutation and oncogenetic transformation [287]. Moreover, ROS can oxidize Src kinases and activate Akt signaling, which promotes cell survival, cell proliferation and facilitate carcinogenesis. Through ROS scavenging, TrxR1 could prevent DNA mutation and transformation. Further, as discussed before and also showed in this study, TrxR1 is important for the maturation and function of p53, which is a strong tumor suppressor that induces cell cycle arrest and apoptosis. PTEN is another important tumor suppressor that negatively regulates Akt pathway [288]. The present study demonstrates that depletion of *Txnrd1* causes reduction of PTEN activity and activation of Akt signaling, suggesting that TrxR1 also supports cellular PTEN function. Taken together, in additon to removal of ROS, TrxR1 suppresses cancer initiation through supporting the functions of tumor suppressors. This theory is endorsed by recent study showing that tissue-specific knockout of TrxR1 increases the chemical induced hepatocarcinogenesis [102].

To our knoledge, it is the first time that a role of TrxR1 in regulation of adipocyte differentiation and insulin signaling cascades identified, however, animal studies are requried to further analyze how TrxR1 regulate glucose homeostasis and adipose physiology *in vivo*. Since TrxR1 knockout mice are embryonically lethal, adipose- and/or muscle-specific knockout of TrxR1 can be used to study the roles of TrxR1 on adipogenesis and insulin sensitivity *in vivo*. The mice will be feeded with standard chow diet (SCD) or high fat diet (HFD), and subsequently total body mass, fat mass, glucose tolerant test (GTT), insulin tolerance test (ITT), fasting glucose level, and fasting insuin level etc. will be compared between wild-type and knockout mice. In the knockout MEFs with adipocyte differentiation, we found small lipid droplets accumulated with upregulation of UCP1 expression, indicating brown adipocyte features. However, *in vivo* data are needed to further validate and confirm whether it is white or brown adipocyte induced by TrxR1 knockout. Through these animal studies, we will obtain deeper insights into the role of TrxR1 in regulation of glucose homeostasis and adipose physiology, which might yeild to novel strategies for treatment of type 2 diabetes and prevention of obesity.

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