Study the role of degenerative protein modifications in aging

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Study the Role of Degenerative Protein Modifications in Aging

Guo Xue

SCHOOL OF BIOLOGICAL SCIENCES

2019

Study the Role of Degenerative Protein Modifications in Aging

Guo Xue

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

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Abbreviations

2DE	Two-Dimensional Gel Electrophoresis
ABB	Ammonium Bicarbonate
ACN	Acetonitrile
AUC	Area Under The Curve
BCA	Bicinchoninic Acid
CAT	Catalase
CAM	Carbamidomethylation
CID	Collision-Induced Dissociation
DAVID	Database For Annotation, Visualization And Integrated Discovery
DES	Desmin
DOTP	Dot Product
DPM	Degenerative protein modification
DTT	Dithiothreitol
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assays
EMPAI	Exponentially Modified Protein Abundance Index
ERLIC	Electrostatic Repulsion-Hydrophilic Interaction Chromatography
ESI	Electrospray Ionized
FA	Formic Acid
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetamide
ITRAQ	Isobaric Tags For Relative And Absolute Quantitation
LC	Liquid Chromatography
MRM	Multiple-Reaction Monitoring
MS	Mass Spectrometry
MSMS	Tandem Mass Spectrometry
NH4ACE	Ammonium Acetate
PBS	Phosphate-Buffered Saline
PTM	Post-Translational Modification
ROS	Reactive Oxygen Species
RT	Retention Time
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD	Superoxide dismutase
TCEP	Tris 2-Carboxyethyl Phosphine Hydrochloride
TEAB	Triethylammonium Bicarbonate
TCA	Citric acid cycle
TMT	Tandem Mass Tag
TRIO	Trioxidation
XIC	Extracted Ion Chromatogram

Abstract

Aging remains one of the biggest unsolved problems in modern society. Oxidative stress derived from overwhelmed ROS plays a pathological role in the aging process of all organs especially brain and skin. Protein damages by degenerative protein modifications (DPMs) which refer to spontaneous non-enzymatic post translational modifications (PTMs) such as oxidation and deamidation have long been regarded as mediators of various human degenerative diseases and natural aging. Brain requires exceptionally high energy consumption and thus is extremely vulnerable to energy metabolism protein damage by unexpected accumulation of oxidative DPMs. The affected enzymes can change the metabolic pathway and bioenergy production in the brain and contribute to mental and cognitive decline in the elderly. Skin is another involved tissue worthwhile for discussion because it has highest ROS load among all organs due to the ROS originating from both external and internal sources. Skin proteins are prone to be oxidative modified and the target protein impaired by oxidative stress may result in the reduced barrier function. In order to understand the roles of DPMs in aging brain and aging skin, in this study we conducted a systematic proteomic study in an aging murine model using label free LC-MS/MS proteomics in discovery phase and quantitative multiple reaction monitoring (MRM) LC-MS/MS in validation phase. In brain sample, TCA component enzymes and their key subunits including SULA2, IDH1, IDH2, SDHB, MDH1, FH1 and NDUFS3 were significantly dysregulated in old murine brain compared with younger mice. The deregulation of these enzymes reveals the imbalance of TCA associated energy metabolism. Moreover, the oxidoreductases, thioredoxin (Trx) and glutaredoxin (Grx) that regulate redox signaling by reversing the sulfenic acid (Cys-SOH) and sulfinic acid (Cys-SO₂H) to thiol (Cys-SH) in cysteine were found to be significantly downregulated in old mice brain. The disturbed redox regulation can contribute to oxidative damage in brain proteins by terminal sulfonic acid (Cys-SO₃H). Indeed, in-depth mining the discovery proteomic data identified excessive accumulation of cysteine thiol trioxidation in the key catalytic enzymes MDH1 at Cys137 in the old mice group.

The accumulation of the oxidative damaged Cys137 in MDH1 was confirmed and measured by MRM method. Trioxidation of cysteine residue will generate sulfonic acid $R-S(=O)_2-OH$. Sulfonic acid is a strong acid and accumulation of sulfonic acid will destroy the cell homeostasis and interrupt the optimal pH for enzymes. The 3D simulation showed damaged sites of the enzymes are located at the active zone or the co-factor binding regions, coupled with the kinetic enzymatic activity, indicating the enzymatic activity can be compromised. Catalase (Cat) as a typical antioxidant defense enzyme against high ROS burden is found to be heavily trioxidized at Cys425 in late age stages including 18 and 24 months in skin sample. These irreversible modification of Cat may lead to additional terminal trioxidation (Cys-SO₃H) of other important proteins. Further evaluation the discovery proteomic data identified exclusively accumulation of cysteine trioxidation in two metabolic enzymes associated with cell junction Ctnnb1 at Cys381 and Des at Cys332 in the 18 and 24 months age mice. The high confidence of accumulation of the oxidative damaged Cys381 in Ctnnb1 and Cys332 in Des were confirmed by MRM method. The novel discovery proteomics coupled targeted MRM technique described here have afforded the detection of low-abundant DPMs proteins in mice tissue mixture.

Chapter 1. General Introduction

1.1 Aging and its biological basis

Aging, which represents the process of becoming old in animals, remains one of the biggest unsolved problems in modern society. In biological terms, aging is defined as an irreversible accumulation of deleterious changes in an organism over time which make them more susceptible to diseases and death. In humans, aging is the accumulation of changes in physical, psychological and even social ability. Cognition and knowledge may decline and slow with age, while reaction time and response time may expand. Aging occurs at numerous levels of organization and can be described in many different ways, including modifications and damage to macromolecules, alterations in cellular metabolism and biochemistry, changes in protein expression, harm to organs and the systemic environment, and even alterations to the behavior of the whole system. Aging is a heterogeneous and complicated process and the characteristics of aging vary drastically from the single cell level within an organism to the population level in any one species. Since the patterns of aging differ between individuals, it is very hard to give an accurate description of aging except that mortality is the endpoint of this deleterious and progressive process[2].

The factors proposed to influence biological aging usually belong to two categories,

programmed and error-related [3]. Programmed factors fall under the rule of the biological timetable. Regulation is mainly derived from internal gene expression and affect the system responsible for cellular repair and self-defenses. Based on this principle, Davidovic et al explained that aging is the result of serial activation and inhibition of certain genes. This theory emphasized the role of gene instability of genes in the aging process[4]. In Endocrine theory, which is another sub-category of programmed theory, aging is believed to be hormonally regulated with the key regulator of insulin/IGF-1 signaling pathway[5]. The immunology theory explains that aging follows the decline of the immune system which increases vulnerability to multiple external hazards, ultimately results in diseases [6, 7]. Research has proved that a dysregulated immune response is highly related with cardiovascular

disease, Alzheimer's disease (AD), and cancer. Unlike the programmed theory, which emphasizes the importance of biological clock regulation, damage-related factors in the aging process are environmental factors that may result in an accumulation of damages to an organism. The damage related theories also have multiple sub-theories each with their own explanation. In DNA damage theory, cumulative DNA damages that is not successfully repaired by DNA Polymerases and other repair mechanisms, is thought to be the intrinsic causes of aging [8-10]. Mutations in the DNA sequence or methylation of gene promotor region are causal factors in the disturbance of gene expression. These changes will finally lead to an imbalance of cellular functional molecules and the malfunction cells. Cross linked theories propose that cross-linked proteins get packed into cells and obstruct the normal metabolism of cell compartments [11, 12]. More experiments to determine protein features were performed and provided evidence of the relationship between specific cross-linked proteins and aging. Waste accumulation theories, on the other hand, attributed aging to the build-up of waste or toxic products inside cells. The finding that there is an accumulation of that certain proteins in yeast and lipofuscin cell over time as a byproduct of complex reactions, provides the foundation for this theory [13, 14].

As highlighted by the discussion above, there are many theories and hypotheses that aim to explain the underlying mechanisms of aging at different levels. Across different fields, such as the molecular, cellular and even sociologic field, theories can be formulated at different levels of systematic hierarchy. For example, the "Somatic Mutation Theory" emphasized that mutations on the DNA level results in aging[15], the "Neuroendocrine Theory" focuses more on neuroendocrine secretion[16]. The different theories are not mutually exclusive. Many of the proposed theories interact with each other in complex ways. They often cross-talk with each other as one theory at molecular level may interpret other theories at cellular level. Among the theories that have been put forward to explain aging, "Oxidative Stress Theory" is generally accepted as the explanation of aging that fits well with other theories. This theory holds the view that superoxide and other free radicals, or oxidative stress in general, causes damage to macromolecule, including

nucleic acids, lipids, sugars, and proteins, to give rise to the symptoms of aging [17]. The oxidative stress theory may be integrated into the Somatic Mutation theory in that oxidative stress often causes damage in DNA molecules. Generally, while numerous theories of the cause and development of aging have been proposed, there is still no consensus regarding these processes. However, by communicative consensus and conflict among these existing theories and more and more research focused on this study, we may be heading towards a better understanding of aging and the ability to apply this knowledge to achieve a longer life-span of high quality.

Aging is defined as the greatest risk factor for many human diseases, including cancer, cardiovascular disease and neurodegeneration[18]. For example, Alzheimer disease (AD) is usually diagnosed in people over 65 years of age and has therefore increased in prevalence in recent years. Of the roughly 150,000 people who die each day across the globe, about two thirds die from age-related causes [19]. Successful aging has three major criteria, i) freedom from disease and disability, ii) high cognitive and physical functioning, iii) social and productive engagement, and it is far more attractive than only elongation of human life-span alone [20]. The importance in extending the functional life-span requires that aging-related mechanisms be explored for clinical and practical means of reaching this goal while research continues towards revealing the causes of aging. Understanding aging and how exactly aging promotes the risk of diseases is needed to tackle this growing problem. Discovery of molecular mechanisms involved in the pathology of diseases and the creation of aging models will improve the understanding of the aging process, which will finally contribute to the achievement of healthy aging[20, 21].

The major difficulty in studying human aging is the long duration of this process and as such, model systems are used as a replacement to extrapolate data that can be applied to humans. The selection of models is diverse and closely related with the research aim. Different aging models are controversial to different extents, however they are all able to fulfill the requirements of being small size, having a short life span, and being appropriate for genetic manipulation [22]. There are five common model systems used to study human aging: 1) human cells; 2) unicellular organisms such as the yeast *Saccharomyces cerevisiae*; 3) the roundworm *Caenorhabditis*

elegans; 4) the fruit fly *Drosophila melanogaster* 5) rodents such as mice (*Mus musculus*) and rats (*Rattus norvegicus*). Much of our knowledge about aging is derived from these traditional models and researchers have benefited from their ease of access and cheap procurement and usage. However, the downside of these models is the uncertainty about the universality and generalizability of elucidated mechanisms across different species [23]. Nonetheless, aging studies remains dependent on model systems to shed light on the mechanisms of aging and on potential therapeutic targets for human applications.

At present, research to improve our understanding of aging remains at the initial stage with relatively short-lived and simple creatures such as yeast [14]. Another model organism that is broadly applied to study the biological base of aging is nematode *C. elegans*, which has a life span of 2–3 weeks [24]. Numerous molecular studies conducted on *C. elegans*, encompassing the suppression of gene function by RNA interference or genetic mutation, have benefitted from this model's short life-span [25]. For decades, the use of invertebrate animal models such as those list above has allowed research on aging to provide insight into molecular pathology, with application to improving longevity and the extending the life span. Despite this great progress made by exploring the aging mechanism in simple model organisms, more efforts in recent years have been made to determine the applicability of these findings in the mammalian system. In this regards, mouse model has attracted more attention, particularly as they share many of the same age-related phenotypes of human subjects. As with humans, mice have increased risk for disease like cancer, cardiovascular diseases, and neurodegenerative diseases[26]. However, less knowledge has been obtained regarding mammalian aging. In part, this is due to the difficulties in establishing an aging model. The mouse needs at least two years to be old enough for use in research on aging despite being small mammals [27].

Mice models have been used to study accelerated aging, accelerated senescence, premature aging, premature senescence, and progeria-like syndromes. Mouse mutants with altered life spans are being used to investigate the aging process and underlying mechanism. One of the genetically-modified models is the Athymic nude mouse model, a murine strain bearing spontaneous deletion in the *Foxn1* gene that

results in a deteriorated or absent thymus, a much reduced number of T-cells and ultimately, features of accelerated aging due to this inhibited immune system [28]. The life span of nude mice is normally 6 months to a year. They can live to 18 month or even two years if they live in a controlled, germ free environment and are provided antibiotic treatments. The reduced lifespan is due to accelerated aging of the immune system and the resultant accelerated decline in spleen cell responsiveness to T-cell mitogens such as phytohemagglutinin and staphylococcal enterotoxin [29]. As aging is the main risk factor for the development of many pathological diseases, the use of an animal organism is essential in aging related research to achieve more milestones in the future.

1.2 Oxidative stress

The oxidative stress theory remains one of the most popular explanations of aging at the molecular level. Although the origin of this theory has been modified and is not so clear, it is thought to be developed from an early version of the free radical theory. The free radical theory was first proposed in 1954 by Denham Harman who argued that aging was caused by cumulative damage from free radicals[30]. Free radicals are highly reactive molecules with one or more unpaired electron(s) in their external shell and can be formed when oxygen interacts with certain molecules [31]. However, the term free radical was later proved inexact as some oxidants are not free radicals. H_2O_2 , which is generated from the superoxide anion radical (O_2^-) followed by one-electron reduction with concomitant acceptance of two protons, is not free radical. It is just more aggressively reactive and active due to its chemical formula [32]. Later, a more flexible and general term, reactive oxygen species (ROS), was accepted and used. ROS refer to a collective term for superoxide, H_2O_2 and other highly reactive partially reduced species of oxygen. The somewhat vague definition oxidative stress refers to the imbalance between the production of ROS and the defense against ROS, and its possible role in causing damage to organisms will be discussed [33]. In the oxidative stress theory, oxidative stress, which causes cellular malfunction and waste build-up, is considered a major contributor to the normal aging process and age-related (as well as non-age-related)

pathological diseases.

1.2.1 ROS

Reactive oxygen species (ROS) are highly reactive chemical species containing oxygen, and evidence from different sources have demonstrated that ROS cause cumulative damage to animals. They are generated during oxidative metabolism as well as other cellular response reactions[34]. Different kinds of ROS are known to exist in biological organisms, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), organic (lipid, alkyl, or short chain) hydroperoxides and hydroperoxide radicals ($ROOH$, $ROO^\bullet$) and reactive aldehydes of varying lengths. The term ROS is a broad term that now includes all known radical derivatives of oxygen, and is not restricted to just reactive radicals, [35].

A one-electron reduction of O_2 yields superoxide (O_2^-). It is now known that superoxide is extremely toxic and an intracellular concentration in the picomolar range are lethal. Superoxide spontaneously dismutate to H_2O_2 . This reaction can be considered irreversible as the pH-dependent equilibrium constant for superoxide dismutation to H_2O_2 is $K \sim 10^{18}$ at pH 7.0[36]. Superoxide, will react rapidly with specific radicals involved in metabolism reactions to cause irreversible cellular disorder. Superoxide is a fast oxidizer in causing inactivation of specific enzymes by binding with the catalytic radical, for instance, ribonucleotide reductase.

Hydrogen peroxide is a chemical compound with the formula H_2O_2 . It is more oxidizing than superoxide, but because cells can tolerate micromolar concentrations of hydrogen peroxide, it is biologically less toxic. Hydrogen peroxide is membrane permeable and much more diffusible as H_2O_2 can travel through aquaporins [37, 38]. Although hydrogen peroxide was demonstrated to be a by-product of the mitochondrial respiratory process and can be manipulated by the electron transport chain inside the mitochondria matrix, a high level of matrix H_2O_2 may overwhelm this capacity and some H_2O_2 may escape to the intermembrane space and even the cytosol. The clearance of H_2O_2 that reaches the cytosol relies on redox homeostasis and the balance between the ROS and cellular antioxidant systems. If not cleared,

hydrogen peroxide mainly attacks several specific biological targets like the amino acids cysteine (C) or methionine (M). Thus, a high concentration of hydrogen peroxide will lead to the cysteine and methionine oxidative modifications, like monooxidation, dioxidation and trioxidation.

1.2.2 Pathophysiology of oxidative stress

ROS play a pathological role in many aging related diseases and they are produced by all aerobic cells endogenously or exogenously [39]. Exogenous sources of ROS include environmental pollution, toxic chemicals and radiation, which are converted to free radicals once absorbed inside body [40]. The more biological endogenous sources of ROS include different cellular organs where the oxygen consumption is high such as the mitochondria, peroxisomes and endoplasmic reticulum. The chemical reactions that occur during respiration may field oxidative stress because of the key roles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), lipoxygenase, and angiotensin II [41]. NADPH oxidase is widespread producer of the radical superoxide anion ($O_2^\bullet$) by providing the extra electron to O_2 . Most of $O_2^\bullet$ is extremely unstable and easily dismutated into hydrogen peroxide[42]. H_2O_2 will go through Fenton or Haber–Weiss reaction and form the hydroxyl ion ($OH^\bullet$), which is highly active and reacts with phospholipids especially in membrane proteins. MPO is involved in the catalytic reaction that converts H_2O_2 to the more harmful hypochlorous acid that can severely damage cellular proteins[42].

The ROS that are derived exogenously or endogenously cause oxidative modifications to different cellular carbohydrates, lipids, proteins and DNA. The oxidation of several proteins has already been used as aging markers. The complex oxidation of Low-density lipoproteins (LDLs), the major carriers of cholesterol, together with lipid oxidation cause cholesterol accumulation [43]. Oxidation damage also targets DNA and result in different mutagenic lesions; G-to-T transversion events are observed as 8-dihydro-guaninethe lesions in the most severe cases [44]. With so many harmful consequences caused by ROS at all different level of cells, the antioxidant defense systems are evolved to protect biological metabolism and function.

There are two different ways, enzymatic and non-enzymatic, for antioxidants to rescue or prevent irreversible high levels of oxidative stress. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) are well known antioxidant enzymes. They work independently or collaboratively to prevent the production of very reactive hydroxyl radicals or convert the toxic ROS into unharmed compounds [45]. Besides the primary enzymes that play key roles as antioxidants, more enzymes such as glutathione-S-transferase and glucose-6-phosphate dehydrogenase have relatively newly been studied for their function in more complex reactions in the antioxidant system. Non-enzymatic signaling pathways that are also involved in the system contain molecules, including α -tocopherol (vitamin E), bilirubin, and β -carotene, that can interact with ROS and the resultant products are usually not as dangerous. As these molecules are present in blood and plasma, they claim around 85% of the burden of removing oxidation in plasma [46]. Some of the molecules can also be absorbed from exogenous sources, including vitamin C, vitamin E, phenolic antioxidants and oil lecithins. Each has a different role in the signaling pathway it belongs to; vitamin C helps to remove hydroxyls and vitamin E prevents cell membrane lipid peroxidation [47].

Oxidative stress occurs with a prolonged imbalance of ROS formation and inability of antioxidants to remove accumulated ROS. A large amount of research indicates that chronic, high levels of cellular oxidative stress is the major contributor to aging related dysfunctions and many different kinds of diseases. As ROS and ROS antioxidants can both come from endogenous or exogenous sources, clinical trials indicate that markers of system oxidative stress can largely be reduced by a diet rich in antioxidants and engagement in regular exercise[41]. It is valuable to protect the cell system from oxidative attack together with antioxidant therapy involving SOD and CAT infusions [48-50]. It is currently unknown whether these related methods will reduce the incidence of disease and disease-associated mortality. Nonetheless, elucidating more of the underlying mechanisms in oxidative dysregulation in humans or other organisms will help uncover oxidative targets for therapy and ultimately may even lead to improved longevity.

1.3 Mitochondrial and ROS

1.3.1 Sources of ROS

Ever since the complementary roles of mitochondria and host cells combined for mutual benefit and gave rise to oxidative phosphorylation, this very efficient method of generating ATP from ADP and inorganic phosphate became the major metabolic energy pathway in organisms. However, this symbiotic interaction has grown complicated because of the diverse functions the mitochondria is responsible for. Mitochondria participate in lipid biosynthesis, substrate generation, cellular stress response and so on[51]. Besides these different functions, mitochondria initiate redox signaling in the cell through the release of ROS from the electron transport chain (ETC) under normal conditions. Under pathophysiological conditions, ROS generated by mitochondria can also contribute to the initiation of protein dysfunction[52]. It was demonstrated by experiments that hydrogen peroxidase is a byproduct of mitochondrial anaerobic respiration[53, 54]. Thus, mitochondria are a double-edged sword for aerobic life: necessary for energy production, but also inevitably generate oxidative stress by over-producing reactive oxygen species.

As mentioned in previous paragraphs, the ROS can be produced from either endogenous or exogenous sources. The endogenous sources of ROS include cellular organs such as mitochondria, peroxisomes and endoplasmic reticulum, where oxygen consumption is high. Within these organs, most of intracellular ROS are derived from mitochondria [55]. The superoxide radicals are generated from two primary sites in the electron transport chain (ETC), complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) [56]. Other mitochondrial proteins which not belong to ETC but contribute to the generation of ROS include α -ketoglutarate dehydrogenase in the TCA cycle, monoamino oxidase, glycerol phosphate dehydrogenase and p66shc[55]. In ROS formation in the ETC cycle, electrons are briefly transferred from complex I or II to coenzyme Q or ubiquinone (Q), and Q will be converted to its reduced form as QH₂ after also accepting hydrogen. However, in the Q cycle an unstable semiquinone anion ($\cdot Q^-$)

will be regenerated as an intermediate when QH_2 converts back to Q. The electron carrier $\cdot\text{Q}$ only enjoys a fleeting existence as it immediately transfers the electron to O_2 to produce the superoxide radical. In turn, the superoxide radical can give rise to other types of active ROS as it acts as a base.[57]. Although electron leakage from the ETC is one of the primary sources of ROS generation in mitochondria, other proteins and enzymes are also involved in the production and formation of oxidative stress. For example, p66Shc, an important member of the ShcA protein family, was found to participate in cell apoptosis and act as a mediator in ROS generation [58]. P66Shc contributes to oxidative stress by translocating from the cytoplasm to the mitochondrial intermembrane space where it will react with cytochrome-c [59].

Peroxisomes are another source of ROS. Unlike in mitochondria, in which the generation of ROS is coupled with oxidative phosphorylation to produce ATP, the transfer of an electron to molecular oxygen leads to the release of free energy in the form of heat. Free radicals including H_2O_2 , $\text{O}_2^{\bullet-}$, $\text{OH}\cdot$ and $\text{NO}\cdot$ are formed during β -oxidation of fatty acids, and this is the important metabolic reaction that produces hydrogen peroxide [60, 61]. The endoplasmic reticulum (ER) represents another endogenous source of ROS. Reactions within the ER that are catalyzed by various enzymes, including diamine oxidase, cytochrome p-450 and thiol oxidase enzyme Ero1p, provide the electrons that reduce molecular oxygen to produce ROS [62, 63]. In addition to ROS production in mitochondria, the ER and peroxisomes other endogenous sources of ROS include inflammation, immune cell activation, infection, and prostaglandin synthesis [64, 65].

Mitochondrial theory, derived from oxidative stress theory, posits that mitochondria play a dual role as both major producers and targets of the ROS[66]. Mitochondria theory plays a critical role in explaining the underlying mechanisms of aging. Affected mitochondria are believed to accumulate the damage of mitochondrial functional proteins or mitochondria DNA, with the cumulative damage causing the mitochondria to produce more ROS. The rapid rising amount of ROS will influence subsequent biological processes and this cycle is termed the mitochondrial vicious cycle.

Although later experiments demonstrated that endogenous sources other than the mitochondria can also create oxidative stress, mitochondrial ROS are still considered to be the main producer of oxidative stress that renders proteins vulnerable to dysfunction and modifications that further inhibit their ability to regulate redox homeostasis[67, 68].

1.3.2 Molecular Targets of ROS

When there is a chronic imbalance between ROS generation by endogenous or exogenous sources and ROS removal by the antioxidant system, a high concentration of ROS will accumulate to cause oxidative stress. Since ROS encompass a variety of active and unstable radicals or molecules, all three type of functional organic molecules, lipids, proteins, and nucleic acids, will be affected or damaged as oxidative stress targets.

Nucleic acid comprises of Deoxyribonucleic Acid (DNA) and Ribonucleic acid (RNA). Mitochondrial DNA is more susceptible to oxidative damage compared with nuclear DNA due to its closer proximity with the mitochondrial source of ROS[69]. All components of DNA, including the deoxyribose sugar backbone, and purine and pyrimidine bases can be attacked by ROS as they are chemically available to react with ROS. This subsequently breaks the single or double stranded structure of nucleic acids [70]. Some mutagenic alterations or adducts induced by ROS are tightly related with aging or carcinogenesis [71]. RNA is even more vulnerable to oxidative damages than DNA with its single stranded structure and less extensive repair system [72]. RNA is more likely to carry harmful products of modification, such as 7, 8-dihydro-8-oxo-guanosine (8-oxoG), which is a causal factor for diseases such as Alzheimer disease [73], Parkinson's disease [74] and atherosclerosis [75].

Lipid peroxidation is a very important modification as it participates in various pathological conditions. Lipids play key roles in the cell membrane such as in maintaining membrane fluidity, signal reception and cell communication [76]. A series of complicated reactions after the initiation of lipid peroxidation will form the toxic products malondialdehyde (MDA) and 4-hydroxyl nonenal (4-HNA), which can potentially cross react with DNA and cause mutagenesis [77].

Protein is the basic functional molecule that takes part in countless metabolic tasks, and is one of the most important targets attacked by ROS. Proteins can be oxidized both by free radicals such as $O_2^{\bullet-}$, $OH^{\bullet}$, peroxy, alkoxy, hydroperoxy and also by non-radical species such as H_2O_2 , O_3 , $HOCl$, singlet oxygen, $OONO-$ [78]. Oxidative stress with consequential protein oxidation can lead to an alternation in physical and chemical states, including protein conformational changes, loss of enzymatic activity, increased aggregation and fragmentation, formation of unexpected protein crosslinks, etc.[79]. Protein oxidation can be induced at proline, histidine, arginine, lysine, and other amino acid residues. Oxidation will induce different consequences at different amino acids. For example, oxidation of Tyrosine forms 3, 4-Dihydroxyphenylalanine, tryptophan forms nitrotryptophan, asparagine forms aspartic acid, Histidine forms 2-Oxohistidine. Among all the amino acids, the sulfur-containing residues, methionine and cysteine, are more susceptible to ROS oxidation and are able to have multiple oxidation states due to the chemical nature of sulfur [80, 81]. Based on the special biochemical properties of sulfur and the existence of a specific reductase, the reversible modulation of oxidation of these residues is heavily involved in cellular redox regulation [82, 83]. Several proteins have been shown to function as an effector of ROS, which are proteins that are sensitively oxidized by ROS. Biological molecules that are targets of H_2O_2 commonly contain low-potential cysteine residues that are highly reactive to oxidation[84]. There is a well-studied instance where the thiol protein GAPDH (glyceraldehyde-3-phosphate dehydrogenase) undergoes oxidation inside cells when treated with peroxide. H_2O_2 -induced thiol oxidation may drastically alter the normal function of GAPDH proteins by inducing structural and functional modification[85]. A large body of experimental data has demonstrated that lower levels of oxidation, like S-OH and higher level of oxidation like S- O_2H , can be detected and act as a sensor for oxidation stress [86, 87]. H_2O_2 may also be relevant in the redox regulation of certain enzymes, PTP1B (protein-tyrosine phosphatase 1B) is a well-documented example; H_2O_2 has been shown to inactivate recombinant PTP1B in vitro by oxidizing its active site cysteine, most likely to sulfenic acid [88, 89]. Of course, we speculate there are others yet to be discovered.

1.3.3 TCA Cycle

The tricarboxylic acid (TCA) cycle, also known as the citric acid cycle or the Krebs cycle, is the primary metabolic pathway for all aerobic processes in an animal tissue. It is comprised of an array of enzymes located in the mitochondrial matrix, involved in reactions with nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), both of which carry high energy electrons. The reactions of TCA cycle are carried out by eight enzymes that completely oxidize acetate (a two carbon molecule), in the form of acetyl-CoA, into two molecules of both carbon dioxide and water. The reactions of the cycle also convert three equivalents of NAD^+ into three equivalents of reduced NAD^+ (NADH), one equivalent of FAD into one equivalent of FADH_2 , and one equivalent each of guanosine diphosphate (GDP) and inorganic phosphate (Pi) into one equivalent of guanosine triphosphate (GTP). The NADH and FADH_2 generated by the TCA cycle are, in turn, used by the oxidative phosphorylation pathway to generate energy-rich ATP.

The simplified cycle starts with the metabolism of pyruvate, which is derived from the glycolysis of sugars, fats or proteins, into a two-carbon acetate in the form of acetyl-CoA. Acetyl-CoA enters the TCA cycle and with a two-carbon acetyl group transfer to a four-carbon acceptor oxaloacetate this generates citrate (a six-carbon compound). Citrate is converted to the intermediate cis-aconitate as catalyzed by Aconitase and is then further converted to isocitrate. Isocitrate is converted to α -ketoglutarate (five-carbon compounds) by Isocitrate Dehydrogenase (IDH). In addition to this decarboxylation, this step also produces a reduced nicotinamide adenine dinucleotide (NADH_2) and releases one carbon in the form of carbon dioxide (CO_2). After this, the second oxidative decarboxylation steps performed by α -ketoglutarate dehydrogenation complex (OGDC) to convert α -ketoglutarate to Succinyl-CoA and simultaneously release CO_2 . This is then followed by the conversion of succinyl-CoA to succinate, which is catabolized by Succinyl-CoA synthetase. GTP is produced in this reaction. Succinate is converted to fumaric acid by succinate dehydrogenase (SDH). This step uses FAD as a prosthetic group to produce FADH_2 . Malate is formed from fumarate and is then subsequently

dehydrogenated by malate dehydrogenase (MDH) to produce oxaloacetate that can be used in another round of the TCA cycle. During this step, NAD is also converted to NADH₂ [90]. Overall, two carbon atoms are oxidized and released as CO₂, and energy is produced via the GTP, NADH and FADH₂ generated by the TCA cycle. Energy contained in GTP is released in other metabolic processes. NADH will be oxidized in the subsequent oxidative phosphorylation pathway to drive ATP synthesis. FADH₂ is covalently attached to SDH and facilitates electron transfer to coenzyme Q, which is an intermediate in the electron transport chain. Large amounts of ATP are generated by oxidizing NADH and FADH₂ in the respiratory chain. The mitochondrial respiratory reactions are the major energy supply for cellular normal metabolism.

TCA cycle impairment is believed to be promoted by imbalanced ROS homeostasis rather than aging itself [91, 92]. Much research already indicates the interface between excessive ROS and disorders of cellular metabolism, which are implicated in aging and aging related diseases, such as Alzheimer's disease, Amyotrophic Lateral Sclerosis and other neurodegenerative diseases [93-95]. As ROS are easily generated inside mitochondria through sequential reactions, TCA cycle enzymes are inevitably exposed to oxidative stress and can be damaged by ROS. Indeed, prolonged exposure to cellular ROS impact enzymatic function as degenerative protein modifications (DPMs) modify protein structure and function and proteins are prone to carrying oxidation posttranslational modifications (OxPTMs), which are the main type of degenerative protein modifications (DPMs)[96]. Several TCA cycle associated proteins are already found to be susceptible to diverse OxPTMs. For instance, the enzymatic activity of alpha-ketoglutarate dehydrogenase (KGDH) was suppressed after undergoing oxidative modifications caused by exposure of the mitochondrial proteins to hydrogen peroxide [97]. NADP⁺-isocitrate dehydrogenase (NADP⁺-IDH) was also observed to have protein damage after oxidative modification in the kidney of diabetic rats. This subsequently resulted in the perturbation of the cellular antioxidants defense [98]. Protein oxidative modifications made to these TCA cycle enzymes have been implicated in metabolism dysfunction and clinical diseases. However, a

comprehensive analysis of the TCA cycle to determine age-associated changes on the protein cysteine OxPTM level, especially irreversible cysteine trioxidation, has not been previously undertaken due to the difficulty in identifying and quantifying modified proteins that exist in low abundance. Now many reviews have summarized the advantage of applying the proteomics methods for target identification and quantitation [99, 100]. High resolution, high sensitivity and high mass accuracy LC-MS/MS was able to identify novel protein targets. After discovering novel protein OxPTM targets, the MS2 ion based, label free quantitation method MRM was applied for further verification and quantitation. An analysis of the protein OxPTM with high confidence was achieved after double confirmation in both the discovery phase and verification phase. Although an elevated level of ROS is usually counterbalanced by the antioxidant defense mechanism [101, 102], an accumulation of ROS can still occur when the amount of ROS generated, overwhelms and exceeds the limit of the cellular antioxidant defense system and this then leads to pathological consequences [103].

1.4 Degenerative protein modification and Cysteine Oxidation

1.4.1 Degenerative protein modification

DPMs (Degenerative Protein Modifications) are non-enzymatic post-translational polypeptide modifications (PTMs) [96]. DPMs predicted to be most detrimental for protein function and structure include oxidation, carbonylation, carbamylation, glycation, deamidation, isomerization, nitration, and racemization, which yield undesirable structural and functional alternations on proteins, leading to damaged overall organ function. DPMs tightly associated with aging proteome imbalance and aging related diseases [104]. For example, deamidation as a kind of DPM is generated by spontaneous chemical reactions which located at asparagine (Asn) or glutamine (Gln) residues by the respective production of succinimide or glutarimide ring intermediates that following hydrolysis as downstream reaction [105]. Progressive aging is found to be associated with deamidation of various significant proteins even the protein PIMT (L-isoaspartyl methyltransferase), which function as deamidation repair enzymes, finally lead to the function decline of PIMT

and related proteins [106]. Carbamylation is another irreversible, non-enzymatic addition of an isocyanic acid to the free amino groups of lysine (Lys). Proteins modified by carbamylation are found highly accumulated in atherogenesis and neurodegenerative diseases [107]. Carbamylation of apolipoprotein B will contribute to generate low-density lipoproteins (LDL) which was regarded as the main cause of atherogenesis [108]. DPMs are found to be particularly involved in protein-protein interactions and protein turnover, unexpected accumulation of DPMs in elderly individuals indicate the risk for multiple diseases. Although the clinical and scientific importance of exploring DPMs mediated various metabolism and signaling pathways is obvious, the molecular mechanisms behind these modifications remain poorly understood mainly due to the limitation of technical ability. The advancement in LC-MS/MS based proteomics technology has provided the necessary sensitivity and specificity to detect and to quantify DPMs in precise amino acid residues in specific proteins, and therefore open a new avenue to study protein damages in degenerative diseases and aging.

1.4.2 Cysteine

Cysteine (abbreviation as Cys or C) is a sulfur containing proteinogenic amino acid with the formula $\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{SH}$. It is encoded by the codons UGU and UGC. Although classified as a semi-essential amino acid because it can usually be synthesized by the human body with sufficient methionine levels, cysteine can be essential for infants, the elderly and other individuals with malabsorption syndrome. Cys residues within proteins, the least abundant of amino acids (1–2%), have numerous biological functions as a result of their unique features. The cysteine sulfhydryl group is nucleophilic and has pKa values close to neutrality that enable it to significantly influence protein structure and function [109]. Cysteine is easily oxidized into its reactive thiolate form in the cell due to the lower pKa of thiolate [110]. Cysteine is involved in antioxidants as it plays a role as a precursor to the antioxidant glutathione. Synthesis of glutathione relies on its constituent amino acid, cysteine [111]. Cysteine also plays an important role in metal binding, it composites in the protein ion sulfur cluster or may be bound to other metal cofactors in the form of thiolate [112]. Cysteine also plays an important role in maintaining protein

structure stability and in stabilizing protein-protein physical interaction. Most cysteines are covalently bonded to other cysteine residues to form disulfide bonds, which will help with the folding and stability of certain proteins [113].

1.4.3 Cysteine oxidation and redox regulation

The side chain of the cysteine residue contains a terminal thiol (—SH) functional group. Cysteine is commonly tapped into as a nucleophile and serves as an electron-rich donor. Cysteine's reactivity makes it uniquely susceptible to oxidation by reactive oxygen species (ROS). The vacant D-orbital of sulfur atom at the core of the thiol group permits the formation of multiple oxidation levels [114, 115]. These different cysteine oxidation states are recognized as oxidative post-translational modifications (Ox-PTM). Different Ox-PTMs include disulfide bonds (RS-RS), mono-oxidation (also called sulfenylation; S-OH), dioxidation (also called sulfinic acid; SO_2H), trioxidation (SO_3H) and so on (Figure 1.4.3). The different oxidative products of cysteine exhibit distinct chemical properties and biological reactivity. Disulfide bonds (RS-SR) are formed between the thiol radical of 2 independent free thiols in near proximity. This disulfide bond formation is a relatively low level of oxidation and often participates in protein folding and in inducing changes to protein structures[116]. Since proteins are usually located in a cellular reducing environment, the crosslinks based on disulfide bonds are generally unstable.

Cellular homeostasis of ROS is well balanced by regulated production and elimination of ROS. However, a large increase in the level of ROS can overwhelm the cellular antioxidant defense system and trigger oxidative stress. Cysteines in sulfur-containing proteins, which are extremely susceptible to ROS, will be affected by the chronic existence of oxidative stress in the aging process. Firstly, the free thiol of cysteine will be mono-oxidized to sulfenic acid (SOH). Sulfenylation is the lowest level of oxidation induced by ROS hydrogen peroxide or superoxide. It has been long considered to be deleterious protein process, but further studies have suggested that it also emerges in the mediation of cellular redox signaling[117]. The product of the sulfenylation is sulfenic acid, which is unstable and believed to be short lived

in the cell [118], and sulfenic acid can easily be converted to other Ox-PTM statuses because of its highly-reactive nature. Depending on the cellular redox environment, S-OH will be further oxidized by ROS to higher oxidation states or go through a reversible modification. For the reversible modification, it will react with thiols that are in close proximity to form a disulfide bond. This reversible RS-SR formation may protect the original Cys from unexpected over-oxidation [114]. This prevention strategy is feasible as it can reduce the sulfur OxPTMs to free thiol (SH) by the antioxidant defense system. Cysteine plays an important role in cellular redox regulation with its multi direction oxidation. Alternatively, for further oxidation reactions under strong oxidative stress, S-OH will directly convert to S-O₂H (sulfinic acid). OxiPTMs, such as sulfinic acid, were long overshadowed by other canonical representatives, such as the disulfides. Sulfinic acid as a higher oxidation status was once considered as enzymatically irreversible. This view changed with the discovery of sulfiredoxin as it could reduce sulfinic acid to a thiol[119-121]. Although this demonstrated that S-O₂H can be enzymatically reversed, dioxidation is still considered hyperoxidation whose reversible reaction is restricted to only certain conditions and certain organs.

If a sufficient quantity of ROS, like H₂O₂, is available, S-O₂H will undergo further oxidation to reach the highest oxidative state and generate sulfonic acid (S-O₃H). Cysteine trioxidation is a totally irreversible process and there is no known enzyme or molecule capable of reducing sulfonic acid. This irreversible oxidation will lead to irreparable damage to target proteins. The kind of oxidative change that occurs in the protein structure determines whether or not the protein will change its enzymatic activity or subcellular location.

Protein cysteine thiols (R-SH) can undergo a broad range of chemical reactions, like disulfide formation or sulfenation. Redox regulation is defined as the reversible post-translational alteration in the properties of a protein to achieve a change in its oxidation state. It is clearly distinct from terminal oxidation which emphasizes the irreversibility of protein oxidation. The most common disulfide formation occurs between a thiol and another thiol or with glutathione, in a process called

glutathionylation. Both disulfide and glutathione formation can be reduced to thiols by thioredoxin (Trx) and glutaredoxin (Grx). In a relatively higher oxidative stress environment, the thiol group can be oxidized to sulfenic acid. The sulfenic acid state of cysteine oxidation is also reducible. In the presence of a high concentration of ROS, proteins can be sensitive to hyperoxidation, thus yielding sulfinic and sulfonic acid. Sulfinic acid can still be reduced to the thiol state by sulfiredoxin (Srx). However, the trioxidation of cysteine is irreversible. Many oxidative cysteine modifications are reversible through reduction oxidoreductases [122]. The formation of reversible oxidation adducts of proteins are designed and enzymatically controlled to prevent the protein from terminal oxidation, which usually causes protein functional impairment. As cells have developed numerous antioxidant systems or defense systems to protect proteins from hyperoxidation during evolution, the occurrence of irreversible cysteine trioxidation indicates not only a high level of oxidative stress but also defects in the cellular redox regulation signaling pathway. Cysteine trioxidation serves as the hallmark of severe oxidative damage to proteins in the aging process as well as dysfunction in the antioxidant system. Failure of redox regulation, which tries to prevent protein hyperoxidation, will cause irreparable damage to target proteins.

Trioxidation has been reported to significantly modify protein local structure and affect enzymatic activity. Examples of these affected proteins include matrix metallo-proteinase-7 (MMP-7) [123], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [124], and protein-tyrosine phosphatase-1B (PTP-1B) [125]. It is therefore necessary to identify more oxidative target proteins as diseases markers and to reveal the causal factors of dysregulated cellular antioxidation defense. As the molecular mechanism of these processes are still poorly understood despite numerous related experiments, systematic analysis of the redox or termination biology, including of the generation of ROS and identification of oxidative modification targets, will help to reveal the underlying biological mechanism.

Figure 1.4.3. Relevant redox reactions of protein thiols under physiological conditions. Protein cysteine thiols (R-SH) can undergo a broad range of chemical reactions. In the presence of hydrogen peroxide or other reactive oxygen species (ROS), thiol groups can be oxidized to sulfenic acid (S-OH), in which sulfur has the oxidation state 0. Sulfenic acid can either be reduced by thioredoxin (Trx) and glutaredoxin (Grx) to the disulfide state, or become further oxidized to sulfinic acid (S-O₂H). Sulfinic acid can be reduced to the thiol state by sulfiredoxin (Srx). However, oxidation of the sulfinic acid to sulfonic acid, where sulfur has the oxidation state + 4, is irreversible

1.5 Mass spectrometry (MS)-based proteomics in biomarker studies

The entire set of proteins produced or generated by an organism is called the proteome, and proteomics is the application of technologies which enable the identification of the whole proteome or distinct proteins expressed by the genome. The proteome is more complicated than the genome because an organism's actual functional biomolecules differ from cell to cell and from time to time[126]. Besides the differences attributable to variations in gene expression and mRNA translation, many proteins are also susceptible to a wide variety of post-translational modifications (PTMs) under different cellular environments. The proteome represents the actual protein content that constantly changes under different physiological conditions within an organism or a system. The complexity and dynamicity of the proteome requires large scale research methods to identify and monitor protein patterns under different conditions. Methods able to determine the changes of proteome or increasing numbers of proteins in older age or disease sheds

light on biologically meaningful biomarkers. Conventional immunological assays including enzyme-linked immunosorbent assays (ELISA), which employs antibody binding specificity for collateral screening of targeted protein expression, are used for biomarker protein validation and verification[127]. However, the high sensitivity and efficiency of the antibody based assessment method is low-throughput and expensive, while the method development is restricted by different cross- reactivity reactions, unstable biochemical properties of proteins, and liable accessibility of antibodies with high affinity and high specificity for the detected targets[128, 129]. Although these methods are reliable tools to validate specific proteins chosen after short-listing and narrow screening, all the limitations of the traditional immunoassay make these methods impractical for discovery and selection of new biomarkers from the whole proteome of different biological samples [130].

Proteomics provides large scale analysis of proteins and the whole proteome with the assistance of mass spectrometry. Proteomics was initially conducted with the two-dimensional gel electrophoresis (2DE) technique developed in the 1970s [131, 132]. Despite the advantages of this technique, the central concern about its inability to address all the proteins inside a sample limit the application of 2-DE [133]. The field of proteomics benefitted greatly from the development of mass spectrometry (MS) around 20 years later as well as by access to genetic information discovered in various projects, including the publicly available human sequence and uncovered database index. With these changes, the proteomics era began and was able to study a dramatic range of protein expression levels and overall protein composition from samples. [134]. Today, proteomics faces technical challenges with regards to quantitative characterization of proteins. Proteomics must be able to solve issues involving the separation of a complicated biological sample, a limited sample amount and low abundance of proteins, an excessive amount of peptides carried PTM, sample to sample variations, uncertainty in the range of samples, and impurities disturbance [135].

The steadily gained momentum in evolution of MS technologies has made possible the high-output detection and quantification of proteins from a vast dynamic

range of biological systems in an unbiased manner. These progressions have succeeded in dealing with the difficulties discussed previously [136, 137]. Bottom-up proteomics is the method most commonly used method to identify protein with specific amino acid sequences and PTMs. Unlike in top-down proteomics, which analyze intact proteins before digestion and fragmentation, bottom-up proteomics has an alternative workflow [138]. In bottom-up proteomics, crude proteins are first extracted from diverse sample types (.e.g. tissues, cells, human plasma, bio fluids). These proteins are then enzymatically digested into proteolytic peptides that are one dimensionally separated via chromatography. After the fractionation steps, the complexity of the sample mixture will be reduced for better efficiency of peptide detection. Depending on the direction of the study, specific affinity and selection methods will be applied to the sample aiming to enrich desired types of PTMs or specific peptides. The processed peptides are then injected into high pressure reverse-phase liquid chromatography (LC)-coupled to MS (LC-MS), a technique known as shot gun proteomics, for further mass analysis [139]. For mass spectrometry, electrospray ionized (ESI) source is used to ionize peptides and MS1 data is acquired from preliminary mass analysis, followed by dissociation of peptides into short peptide fragments with collision-induced dissociation (CID) in tandem MS (MS/MS or MS2). Subsequently, comparing the masses of the peptide fragment with those listed in the public sequence database is performed for the identification study. The obtained MS1 and MS2 spectra will be used to match the annotated peptide sequence library or spectral library [140]. Based on the consistency of the pair, peptides can be successfully identified and finally proteins with different peptides compositions will also be confirmed. A biological related interpretation will be illustrated and expressed based on the database searched results [141].

After the positive identification and detection of the proteins, the next challenge is to address the relative or absolute quantities of peptides in different samples. Usually there are two different types of mass spectrometry needed to achieve peptide abundance measurements in sample mixtures, one is the isotope label approach and the other is label free approach. Absolute quantitative study refers to the value of protein expression and protein abundance in a sample, whereas relative quantitation

refers to the fold change of protein abundance against internal control proteins. Both are important parameters to shortlist potential biomarkers with high biological relevance. The identification and discovery of novel disease-affected protein occurs when proteins are abnormally expressed in diseases but not in a healthy control. The stable isotope label method is the more accurate and reliable method for protein quantitation and is frequently used in novel biomarker selection studies. One of the big advantages of the isotope label approach is that it reduces the ion suppression in LC-MS and LC-MS/MS when peptides co-elute[142]. The ability for MS to recognize the mass difference of labeled and non-labeled peptides provides the foundation for the peptide labeling strategy. For the labeling, the quantitative ratio is calculated based on the relative signal intensities between labeled (heavy) and non-labeled (light) peptides [143]. The label is introduced to peptides through four major methods, enzymatically, metabolically, chemically, or as a spiked synthetic analog [140].

Isobaric tags for relative and absolute quantitation (iTRAQ) is a commonly used peptide labeling method for in-vitro samples in MS analysis [144]. The iTRAQ labeling usually consists of an N-methyl piperazine reporter group, a balance group function to make the labeled peptide isobaric in each sample, and an N-hydroxy succinimide ester group that is reactive with the primary amines of peptides [145, 146]. There are currently two main reagents used, 4-plex and 8-plex. The big advantage of iTRAQ-based quantitation is that in a single analysis, it can finish 4-plex or 8-plex multiplex all of the different treatments and samples [147]. 8-plex iTRAQ analyses has eight reporter ions derived at 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1, and 121.1 m/z. Despite all the advantages of using this labeling method, including availability of well-developed software for data analysis, this method has some shortcomings, one of which is that the accuracy of the labeling is heavily reliant on the efficiency of labeling [148, 149]. Overall, the iTRAQ technique is cutting-edge and optimized protocols with high sensitivity have successfully been applied in countless biomarker discovery experiments. A wide range of studies on neurodegenerative diseases [150-152], cardiovascular disease [153, 154] and cancer [155-157] have been conducted with this approach.

Stable isotope labels with amino acids in cell culture (SILAC) is a popular method adopted for in-vivo labeling. Usually two populations of cells are cultured, one group is fed with growth medium containing normal amino acids, while the other population of cells is supplied with amino acid deficient growth media with isotopically labeled essential amino acid (e.g. $^{13}\text{C}_6$ -lysine, $^{13}\text{C}_6$ -arginine, $^{15}\text{N}_4$ -arginine). The newly growing cells in this group will incorporate the heavy amino acids into their synthesized polypeptides and proteins. Combined with the analysis in the MS, the mass shift (e.g. 4 Da mass shift for $^{15}\text{N}_4$ -arginine) comparing with the light (unlabeled) counterparts will be able to differentiate the peptides between the two populations [158]. Consistent with the principles of the method, the SILAC application is strictly limited at metabolically active cells while the other tissues, bio fluids and other samples cannot be analyzed. Even within the cell range, not all cell types can be studied as the growth medium does not have sufficient nutrition [159]. When applying this method, the conversion from arginine to proline may impede normal quantitative analysis as the variety and complexity increase [160]. Aside from these limitations, SILAC has been proven as a powerful tool in research about cell and protein dynamics [161, 162]. Up to five study groups can be addressed with heavy labels at one time [163].

Tandem Mass TagTM 6-plex (TMTsixplexTM) Reagents are sets of isobaric compounds which are used for nearly all types of samples in in vitro labeling. TMT6, along with parallel methods like TMT10, the number of which represents the number of heavy isotopes in the reagents, are powerful and robust labeling methods for MS associated analysis. Its application is quite wide, with protein identification and quantitation from multiple samples of cells, tissue or biological fluids possible [164]. The TMT Reagents are amine-reactive and modify lysine residues and the peptide N-termini [165]. Extracted proteins from samples are reduced, alkylated and enzyme digested overnight. Proteolytic peptides are used for labeling with six different labeling tags that each contain different isotopes (i.e., ^{126}Da , ^{127}Da , ^{128}Da , ^{130}Da and ^{131}Da for TMT6) [166]. After labeling of each group with the commercial reagents, the samples from different study groups are pooled together and they form the mixture for later fractionation and desaltation. For each sample, a unique reporter

in the low mass region of the MS/MS spectrum (i.e., 126-131Da for TMT6 Isobaric Label Reagents). The peptides or protein expression level will be analyzed with the relative abundance of the quantifying reporter [167]. the TMT6 labeling methods has been applied for multiple types of disease conditions including dementia [168, 169], heart disease [170], diabetes [171], and pancreatic cancer[172].

Proteolytic ^{18}O labeling is quite a convenient labeling method for enzymatically introduced isotope labeling at the C-terminal of the amino acid chain for MS analysis (e.g. trypsin, lys-C, glu-C)[173]. For two different populations of sample, the first sample pool proteins are incorporated universally with two ^{18}O atoms at the carboxyl termini, and proteins in the second sample pool are cleaved analogously with C-terminals carrying only normal ^{16}O atoms, which is defined as no use of labeling. The two pools of sample are mixed and injected for a single analysis. Relative peptides or proteins abundance is quantified by comparing the paired peptides with their relative intensities to determine the protein expression change [174]. The unpaired peptides may result from the sequence variations of the proteins from the pool [160]. High-resolution mass spectrometry combined with proteomics tools are suitable for the analysis of incorporated ^{18}O atoms [175]. The disadvantages of this method lie in the signal interference from ^{13}C which overlap with with the weaker ^{18}O signals [174]. Nonetheless, this labelling technique has been widely applied in many peptide sequencing studies [165, 166].

The absolute quantitation of protein (AQUA) has replaced the role of chemically synthesized peptides with label free peptides as an internal reference [176]. This approach is used for verification and validation of diverse proteins and peptides on a large scale, especially for peptides carried with specific PTMs e [177]. Targeted MS-based quantification is one of the representative tools that usually gets combined with AQUA. Selected- or multiple-reaction monitoring (SRM or MRM)-MS/MS which is used to define peptides and peptides fragments based on preliminary peptides of interest, are broadly used in the determination and quantitation of targeted peptides [178, 179]. The MRM-MS-based targeted approach is able to simultaneously monitor several peptides and their fragment ions derived

from the LC-MSMS discovery phase with high sensitivity and specificity. MRM is much more advantageous compared to antibody based immune-affinity assays which are heavily restricted by cost and limitation [128]. Thus, it was considered a potential large scale protein verification tool for clinical and scientific usage with reasonable cost and high efficiency [180]. However, unlike using externally synthesized peptides as the standard for quantitative study, the internal reference peptide amounts are affected by variations in sample preparation from time to time [176]. Thus, for more scientific reasons, chemically synthesized peptides may also be applied as an external standard for more accurate peptide identification.

Despite the above introduction about the diverse labeling methods for LC-MSMS study, many experiments are currently conducted with label-free quantification methods. Counting of MS/MS spectra spectral allocated to one peptides and proteins without the help of reporter dye for each label is one of the principles used to measure protein abundance. The observation that more MS/MS spectra are assigned to abundant proteins rather than low abundance proteins support this peptide spectral counting approach [143]. Differential relative quantification for this calculation is estimated by comparing the number of MS/MS spectra of a protein in one study sample group to the same protein in another group. The accuracy of this method was proved in yeast extracts with standard protein abundance highly correlated with the number of MS/MS spectra [181]. Another method that parallels this method is measurement of the peptide chromatographic peak area. When peptides elute in the LC-MSMS during time referred to as retention time, , the chromatographic peaks areas of the extracted ion chromatograms (XIC) value will be acquired [182]. The area under the curve (AUC) will be extracted and it represents the peptide intensity eluted at that time point. This method is biologically relevant as the integrated AUC correlates linearly with protein abundance in many experiments [183, 184]. Label free data processing is a powerful tool for target identification and discovery in multiple samples [185, 186].

Diverse labeling tags and label free approaches, each with their own advantages and restrictions, offer large scale protein identifications and quantitative analysis

when the properties of sample, the availability of MS instrument and the development of analytical software are also considered [187]. Proteomics study with biomarker discovery at both the protein expression and post-modification level can be achieved with reasonable experiment cost and quantitative objectives. MS-based proteomics research is a powerful tool in disease-specific biomarker detection and therefore is of clinical diagnostic and prognostic value. Of course, these techniques could improve with an increase in detection sensitivity when complicated protein mixtures are eluted within a similar retention time, a wider dynamic range of protein abundance that can be differentiated, more emphasis on upgrading the availability and minimizing the variations caused by instrument conditions and so on. Now and in the near future, more advanced MS-based proteomics techniques are likely going to be applied to studies related to systems biology and human plasma samples [188-191].

Identification of ROS targeted protein and their modification sites and species, clearly shows the alteration in protein chemical properties caused by cysteine residue modifications, are critical for understanding the biological mechanism in the oxidative stress induced aging process. However, cysteine residue oxidative modification in proteins identification and quantification are hampered by technical factors. The modified peptides are of pretty low abundance in complex biological samples. The diversity of cysteine oxidation types makes them difficult to recognize. The reactive nature of cysteine thiols makes it an experimental challenge to determine the in vivo cysteine oxidation state of proteins. In terms of technique, there is no distinguishing UV or visible spectrophotometric absorbance/fluoresce property to help in differentiation. With these challenges in cysteine oxidation research, many molecular methods have been developed to solve the problems. For instance, site directed mutagenesis is often employed to determine whether or not specific cysteines have redox-regulated functional roles, but this approach has failed to tell the oxidation status of the target proteins [192].

Although nowadays many assays for generic protein oxidation and breakdown products of protein oxidation are available, only advanced tandem mass

spectrometry approaches can access localization specific PTMs of identified proteins [193]. Mass spectrometry has evolved into a high accuracy methodology for detecting and quantifying protein with posttranslational modifications. Proteomic technology using mass spectrometry (MS) has provided invaluable cutting edge tools for identifying the molecular changes including modification sites and species in target proteins. Proteomics based on MS/MS to identify PTMs, modified sites and to analyze protein sequence by combining protein tryptic digestion and peptide sequencing is valuable technology for discovering novel modified peptides of clinical significance. So far, application of MS in conjunction with other techniques has been able to achieve the identification of more than 900 PTMs, which are listed in the UniMod database (www.unimod.org). Extensively studied phosphorylation, acetylation, more highly abundant ubiquitination, glycosylation, simulation and methylation Cys-modifications, which are all the different major PTMs types occurring inside cells, have been included in the list and this list is rapidly increasing in length.

Due to the low abundance of most PTMs, especially cysteine oxidations, prior enrichment of modified peptides will benefit the large scale systemic analysis of each PTM. However, an enrichment protocol for all cysteine oxidations has not been well developed. Isotope-coded affinity tags (ICAT) based methodology is broadly used to enrich regulatory cysteine modifications. Accurate preservation of specific non-oxidized cysteine will be achieved by three-step differential alkylation labeling; 1) labeled with a tag; 2) chemically reduced; and 3) labeled with a distinguishing tag [194]. In this way, the cysteine sulfhydryl moiety will be enriched by biotin-dimmedone using streptavidin affinity chromatography. But this method has limited utility when detecting targeted moderate proteins due to the instrument sensitivity and sample complexity. Another limitation in the use of this approach because disruption of protein structure with numerous ICAT adducts often occurs after alkylation. More reliable and applicable methods to overcome the limitations listed above is essential for characterizing the dynamic changes in redox regulation.

An alternative to stable isotope labeling approaches is a label free quantitation

method which combines high mass accuracy, resolution and sensitivity mass spectrometry with high reproducible targeted multiple reaction monitoring (MRM). The introduction of Q Exactive mass spectrometers with high mass accuracy, sensitivity and resolution together with nano-HPLC, creates a powerful tool to discover and measure peptides based on MS and MS/MS ion intensity. Proteome-wide analysis of biological mixtures is performed using reverse phase column coupled with EASY spray to separate different types of modified peptides. Cysteine-modified peptides with biological significance were identified with high-chromatographic resolution in the discovery phase and then functioned as targets in the verification phase. After selecting the novel cysteine oxidation sites, the label free quantitation method MRM is adapted to perform ion intensity based quantitative study to further shortlist the proteins and peptides. The open source, fast updated software, Skyline, provided the visualization of ion ranking and co-elution, which serves as positive or negative validation of the targets.

1.6 Thesis overview and research aims

Aging, a major demographic phenomenon of our time, is the main risk factor for the occurrence of many pathological processes. Aging related diseases, such as cancer, cardiovascular disease and neurodegenerative diseases, are the most common cause of death. Aging associated functional losses, such as the energy decline followed by TCA cycle impairment, in different tissues are believed to be due to the accumulation of oxidative damage of macromolecules caused by ROS. The mitochondria is the major source of endogenous ROS production during respiratory chain, or other, reactions. While ROS are counterbalanced by the cellular defense system, oxidative stress appears when there is an imbalance between production of ROS in mitochondria and the activity of the antioxidant defense system. Protein oxidation products that are formed by exposure to oxidative stress are believed to play key roles in chronic damage processes. Protein cysteine residue oxidation has gained more attention due to its unique feature of stepwise oxidations and therefore, its involvement in redox regulation. Numerous research has demonstrated that in vitro exposure of proteins to ROS elicits oxidative cysteine

modification that results in changes in enzymatic catalytic activity, heat stability, and proteolytic susceptibility. As mitochondria is the major location where proteins are exposed to high concentration of ROS, especially during the TCA cycle, proteins are more prone to be oxidatively modified. However, comprehensive analysis of the TCA cycle and other metabolism processes to determine age associated changes on the protein cysteine oxidation level, especially irreversible cysteine trioxidation, has not been previously undertaken. Identification of ROS target proteins with their modification species and sites, including the biochemical properties of reactive Cys residues, which are the major targets of ROS, are critical for understanding the molecular mechanisms in the ROS-induced aging process.

As previously described, MS-based proteomics approaches have proven to be useful in aging biomarker studies, not merely just in the identification of candidate markers, but also in shedding light into the pathophysiology of ROS-induced pathological processes. In addition, the improvement of various effective fractionation strategies and proteome enrichment techniques, have achieved highly sensitive detection of attractive low abundance PTM proteins by MS. The studies presented in my thesis aim to improve current approaches and will address at least the following three aspects: 1) the lack of mouse brain and skin based screening assays that effectively discriminate underlying PTM modified protein levels between young and old mice; 2) the incomplete understanding of the TCA cycle enzyme pathological processes underlying different aging conditions; and 3) poorly understood mechanisms underlying ROS induced aging including cysteine terminate oxidation and redox regulation. In order to achieve better exploration in the abovementioned issues, my PhD research work has been divided into two parts. The first part focuses on the exploration of oxidative protein damage of TCA cycle proteins in the brains of mice of three different age. The second part emphasizes the underlying mechanism of ROS damage of skin proteins from mice of four different ages. The workflow and major focuses are list in the Figure 1.6 in below.

In part I, accelerated aging-specific protein biomarkers were identified in pooled mice brain samples using the TMT6-labeled based quantitative approach combined

with label free proteomics. Subsequent verification of changes in protein expression level was achieved with specific western blot. Functional pathological crosslink analyses suggested the involvement of age specific markers in TCA cycle and other important signaling pathways. The oxidative stress damaged proteins with trioxidation DPM are identified and confirmed.

In part II, assessment of label free proteomics data of skin tissue from four different age stages of mouse, 1 month, 6 month, 18 month and 24 month, is performed to prove the good correlation. Age dependent dynamic profiling of proteins change tendency with fuzzy c-mean clustering assigned proteins to different clusters. The representative metabolism process are determined for each cluster. Protein targets under ROS attack are identified with both discovery LC-MS/MS and verification MRM. Tight junction proteins are found to be the major targets of ROS with trioxidation DPMs exclusively in late age groups.

Figure 1.6. Schematic overview of PhD research work

**Chapter 2. Oxidative damage to the TCA
cycle enzyme MDH1 dysregulates bioenergetic
enzymatic activity in the aged murine brain**

2.1 Abstract

Protein damages by spontaneous degenerative protein modifications (DPMs) such as oxidation and glycations have long been recognized as mediators of various human degenerative diseases and natural aging. Energy metabolism is well-known to be responsible for the production of reactive oxygen species (ROS) and critical enzymes can be damaged by imbalance redox reactions. Brain has exceptionally high-energy requirements and thus is susceptible to damage by ROS and accumulation of DPMs in proteins especially enzymes. The affected enzymes can change the metabolic pathway and bioenergy production in the brain and contribute to mental and cognitive decline in the elderly. In order to understand the roles of DPMs in aging brain, in this study we conducted a systematic proteomic study in an aging murine model using label free LC-MS/MS proteomics in discovery phase and quantitative multiple reaction monitoring (MRM) LC-MS/MS in validation phase. Several TCA functional enzymes and their key subunits including SULA2, IDH1, IDH2, SDHB, MDH1, FH1 and NDUFS3 were significantly dysregulated in old murine brain compared with younger mice. The deregulation of these enzymes can generate excessive ROS and free electron. Moreover, the oxidoreductases, thioredoxin (Trx) and glutaredoxin (Grx) that regulate redox signaling by reversing the sulfenic acid (Cys-SOH) and sulfinic acid (Cys-SO₂H) to thiol (Cys-SH) in cysteine were found to be significantly downregulated in old mice brain. These two processes can contribute to oxidative damage in brain proteins by terminal sulfonic acid (Cys-SO₃H). Indeed, in-depth mining of the discovery proteomic data identified excessive accumulation of cysteine thiol trioxidation in the key metabolic enzymes MDH1 at Cys137 in the old mice group. The accumulation of the oxidative damaged Cys137 in MDH1 was confirmed and measured by MRM method. The MDH1 trioxidation is exclusively occurs in old age group. The damaged sites of the enzymes are located at the active zone or the co-factor binding regions, indicating the enzymatic activity can be compromised. Enzymatic kinetic analysis confirmed that MDH1 function was declined by > 50% in the old mice brain.

2.2 Introduction

Aging leads to brain changes on physical volume, cognitive, vasculature and molecular level [195, 196]. The effects of aging on brain and cognition have compound aetiologies and aging causes change to multiple aspects of brain. The volume of the brain and/or its weight has been widely found to shrink at a rate of around 5% per decade after age 40 [197]. As mammal brain ages the vasculature increase the possibility of stroke and high blood pressure. The most obviously occurred cognitive change related with aging is memory decline. Aging has been identified as a major risk factor for most common neurodegenerative diseases, including Alzheimer's disease (AD), cerebrovascular disease (CVD), Parkinson's disease and Lou Gehrig's disease [198, 199].

Evolutionary studies indicated that the higher efficiency of brain cognitive required increased expression of energy expression gene as the energy demand of brains are high. Aging associated cerebral energy metabolism decline in the brains has long been connected with neural cognition damage and dysfunction [200]. TCA cycle, as the final common path for aerobic oxidation of any molecule that can be transformed into an acetyl group, plays central role in the regulation of brain energy homeostasis. The high energy demand of brain render it sensitive to disturbance in the fuel supply and thus the deficit of TCA cycle is a well-known hallmarks of aging and dementia [201]. Numerous experiments proved that increased ROS leakage is responsible for the impairment of TCA cycle during aging process [202]. The elevated level of intrinsic ROS occurred in the electron transport chains inside mitochondria during aging process. As TCA cycle enzymes and proteins are continuously exposed to high concentration of ROS, they are inevitably to be affected by the ROS induced damage. Some of TCA cycle proteins are found to be heavily damaged by ROS induced changes. Such as some TCA enzymes with Iron-sulfur (Fe-S) clusters which were oxidized and iron is liberated, reacting in Fenton-Haber-Weiss reactions with SOA and HPO to generate more reactive ROS[203]. However, the exact mechanism of these decline is not well understood. As protein constantly exposed to various form of ROS, it will lead to protein oxidation which

may involve in cleavage of the polypeptide chain, modification of amino acid side chains, and conversion of the protein to derivatives. TCA cycle proteins are subject to alternations caused by oxidative modifications. Protein damages by spontaneous non-enzymatic post translational modifications (PTMs) or degenerative protein modifications (DPMs) such as oxidation and glycation have long been recognized as mediators of various human degenerative diseases and natural aging [95, 96]. However, the conventional biochemical methods could only quantify the total damaged proteins in biological or clinical samples. Recent advancement in LC-MS/MS based proteomics technology has provided the necessary sensitivity and specificity to detect and to quantify DPMs in precise amino acid residues in specific proteins, and therefore open a new avenue to study protein damages in degenerative diseases and aging.

Among all the amino acid which are susceptible to oxidative stress, cysteines are sulfur containing residue which can apply multiple oxidative states. The thiol group in cysteine residue can be stepwise oxidized to sulfenic acid (Cys-SOH) by adding one oxygen and further di-oxidation to yield sulfinic acid (Cys-SO₂H). These two level of oxidation are reversible by oxidoreductases Thioredoxin (Trx) and Glutaredoxin (Grx). However, further oxidation of sulfinic acid to sulfonic acid (Cys-SO₃H) is considered irreversible as till now no known enzymes can reverse this reaction. Although cysteine oxidation is enzymatically regulated process which play key role in redox signaling pathway, the trioxidation are spontaneously generated responding to extreme oxidative environment. In this way the trioxidation, representing the failure of redox regulation which try to prevent the proteins hyperoxidation[115], will cause unrepaired damage to target proteins. It has been reported that the trioxidation has significantly modified the protein local structure and affected the enzymatic activity, such as matrix metallo-proteinase-7(MMP-7), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and protein-tyrosine phosphatase-1B (PTP-1B). It is therefore necessary to identify more oxidative target proteins as diseases marker and to reveal the causal factors of dysregulated cellular antioxidant defense.

Several TCA cycle associated proteins are already found to be susceptible to diverse OxPTMs. For instance, enzymatic activity of alpha-ketoglutarate dehydrogenase (KGDH) was suppressed after undergo oxidative modifications derived from the exposure of mitochondrial to hydrogen peroxide. Another NADP⁺-isocitrate dehydrogenase (NADP⁺-IDH) was observed protein damage after oxidative modification in the kidney of diabetic rats, subsequently result in the perturbation of cellular antioxidation defense. Protein oxidative modifications to these TCA cycle enzymes have been implicated in metabolism dysfunction and clinical diseases. However a comprehensive analysis of TCA cycle to determine age associated changes on the protein cysteine OxPTM level, especially irreversible cysteine trioxidation, has not been previously undertaken due to the difficulty in identifying and quantifying of low abundance of modified proteins. After discovering novel protein OxPTM targets, the MS2 ion based, label free quantitation method MRM is applied for further verification and quantitation. High confident analysis of the protein OxPTM was achieved after double confirmation in discovery phase and verification phase.

We present here, a novel and sensitive proteomics study combined with targeting molecular assay, a high accuracy analysis of whole brain proteome of three different ages in mouse. Our investigation firstly demonstrated the TCA cycle defects in aging process with differential expressed proteins in three age groups. We also performed in depth analysis on the peptides PTM level with both the discovery phase LC-MS/MS and verifying phase LC-MRM/MS. Two TCA cycle dehydrogenase MDH1 was identified as irreversible oxidation targets. The PTM level detection and confirmation demonstrated the proficiency of our proteomic strategy to quantitatively study site specific oxidation states. The comprehensive evaluation of the dynamic changes in TCA proteins expression and modification would allow us to characterize the TCA cycle defects during aging and to understand the distribution of redox status in different physiological states.

2.3 Material and method

2.3.1 Reagent and chemicals.

All reagents were purchased from Sigma-Aldrich unless state otherwise. Protease inhibitor cocktail tablet were purchased from Roche (Basel, Switzerland). Sequencing grade-modified trypsin was purchased from Promega (Madison, WI).

2.3.2 Tissue Preparation

Table 2.3.2: Frozen post-mortem mouse brain tissue

Group	Mouse type	Age (weeks)	Average weight(g)	number
Young	Male NCr nude mice	8	26.33	4
Middle		19-22	36.23	4
Old		60	34.14	4

The 12 mice (listed in Table 2.3.2) were euthanized with excess isoflurane anesthesia. Within reasonable manipulation time, the brains were carefully collected and snap-frozen in liquid nitrogen and then stored at -80 °C until use. All the procedures were approved by the Institutional Review Board at Nanyang Technological University (NTU, Singapore) and performed in accordance with the ethical guidelines.

2.3.3 Brain homogenate preparation

Four young mice brain, four middle mice brain and four old mice brain (In total 12 mice) were applied in the sample preparation. Use pestle to grind every individual mouse brain to powder with adding the liquid nitrogen to the mortar simultaneously in cold environment (4°C). The continuous supply of liquid nitrogen guarantees the necessary hardness of tissue for grinding. All the mice brains performed the same grinding procedure separately. After that, the powder for each individual mouse brain was stored in -80°C for later usage. The grinding strategy which mix all the

different areas of brain reduces the impact of biological variation by reporting the averaged quantity of proteins associated with age-specific changes.

2.3.4 SDC-Assisted In-Solution digestion mass spectrometry

2.3.4.1 Brain Tissue Homogenization

Each mouse brain tissue powder (100mg) supplemented with complete protease inhibitor were homogenized in 600 μ l of 50mM Triethylammonium Bicarbonate (TEAB) buffer containing 1% (w/v) Sodium dodecyl sulfate (SDS). The homogenization process was achieved at the max speed of bullet blender homogenizer (Next Advance, NY) with the 100mg metabolic beads (0.9-2.00 mm particles) mixing with tissue and homogenization buffer. The tissue homogenate was centrifuged at 100,00 x g for 10 minutes and the supernatant was collected. 400 μ l of homogenization buffer was added to the precipitated beads and tissue mixture and another round of homogenization is progressing until the pellet got totally homogenized. All the supernatants are combined as the mouse brain tissue homogenate. All the steps were strictly performed in cold room (4 °C). After get the individual mouse brain homogenates, BCA protein quantification assay was performed to measure the protein concentration of each subject.

25mg of brain tissue from each individual mouse brain homogenate was pooled together and a total 100mg pooled brain homogenate sample was formed. The homogenization buffer is 2% SDS in 50 mM TEAB, this approach is commonly used in quantitative mass spectrometry study for maximum extraction of the proteins. Proteins of the three groups were purified by two times acetone precipitation. The aim of two-time acetone precipitation is to remove the contaminations of the sample including SDS which will affect the column in the injection steps. First precipitation step total 4 times of the homogenate volume of chilled acetone was added to the homogenate and the mixture was stored at -20°C for 2-3 hours and following a 5000 X g 4 °C 30 min centrifuge was performed. The pellet was collected and dissolved in dissolution buffer (2% SDC in 50 mM TEAB), around 2 ml dissolution buffer is needed as SDC is not a strong detergent as SDS). Then another 4 times volume cold acetone was added to the re-dissolved homogenate and stored in -20 for another 3

hours. Centrifuge at 5000 g for 30 minutes and the pellets are dissolved in 1ml 50mM TEAB containing 2% SDC. Clean magnetic beads can be used to help the dissolved process.

2.3.4.2 Reduction, Alkylation and Trypsin digestion

The mouse brain proteins were reduced by dithiothreitol (DTT) which maintain a final concentration at 10mM for 30 min at 60 °C and subsequently alkylated with iodoacetamide (IAA) which maintained a final concentration at 55mM for another 30 min at room temperature and the reaction was processing in dark. After this the proteins were enzymatically digested by sequencing-grade trypsin at 37 °C overnight with the ratio of proteins to trypsin 40:1 (W/W). Adding formic acid (FA) to a final concentration of 0.5% and pH of the solution is under 3 to quench the trypsin catalytic work and precipitate the remaining SDC. Following centrifuge at speed 12000 x g for 10 min, collect the supernatant in a new tube and keep on ice. Add 50 mM TEAB to dissolve the pellet then add 0.5% FA to form pellet again, the repeat of the centrifuge step aims to extract all the proteins that may precipitate together with SDC. The supernatant combination will perform desalting step using C18 Sep-pak 50 mg cartridge. The purified peptide will be eluted down in Elution Buffer (50% ACN, 0.1% FA) and concentrated by the vacuum spin. Finally, the Eppendorf tubes which contain dry peptide at the bottom will be stored at -80 °C until use for next step.

2.3.4.3 TMT six-plex isobaric label based spectrometry

Table 2.3.4.3: TMT 6plex tag corresponding to the specific age sample

Isobaric Tag	126	127	128	129	130	131
Mouse group	Young	Middle	Old	Young	Middle	Old

Six plex TMT 6 reagent multiplex kit was used for the labeling of the different age group. The manufacturer's protocol was used for this experiment. As show in

the Table 2.3.3.3, two independent replicate was performed in one set of the sixplex reagent. The different age groups of sample were labeled with specific isobaric tag. The young group was labeled with 126 and 129 isobaric tag, the middle group was label with 127 and 130 isobaric tag, and finally the old group was labeled with 128 and 131 isobaric tag. The labeled six sample were pooled together and concentrated to dry by spin-vacuum. The dry peptides were dissolve in 0.1% TFA and keep adding 0.5% TFA until the solution pH was less than 2. Then the acidic pooled sample was performed desalting using the C18 Sep-Pak cartridge. The eluted purified peptides were concentrated to dry by spin-vacuum and the dry peptides were ready for the following steps.

2.3.4.4 Electrostatic repulsion-hydrophilic (ERLIC) HPLC fractionation

TMT sixplex labeled sample and label free sample both performed electrostatic repulsion-hydrophilic interaction chromatography (EARLIC) fractionation by High-performance liquid chromatography (HPLC). 60 µl of buffer A (85% acetonitrile, 0.1% formic acid) was added to the Eppendorf tubes which contains the TMT-labeled or non-labeled peptides at the bottom, vortex to promote it totally dissolve. Then the sample was fractionated by the PolyWAX LP column (4.6 x200 mm, 5 µm, 300 Å) (polyLC Columbia, MD, USA) at flow rate of 1ml/min. Different composition of Buffer A (85% acetonitrile, 0.1% formic acid) and Buffer B (30% acetonitrile, 0.1% formic acid) form different reversible mobile phase during the whole process. The one hour gradient process works as follows: 100% buffer A for 5 min, 0-36% buffer B for 25 min, 36-100% buffer B for 20 min and 100 % buffer B for 10 min (Table 2.3.3.4). The peptides distribution and intensity was checked under the 280nm ultraviolet (UV) absorption chromatograms. Total 60 tubes containing 1 ml of liquid were collected and combined to 15 tubes. The samples were dried in the concentrator and stored in -80 for the liquid chromathography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

Table 2.3.4.4: Buffer composton for the 60 minute ERLIC HPLC gradient

Time point	Percentage of Buffer A	Percentage of Buffer B
0 th min	100%	0%
5 th min	99%	1%
30 th min	64%	36%
50 th min	1%	99%
60 th min	0%	100%

The dried fractionated peptides were reconstituted in 3% ACN, 0.1% formic acid (FA) and injected to Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to online microflow MDLC system. The TMT sixplex labeled or label free peptides were separated on an EASY-Spray DIONEX PepMap C18 column (75 μm ID $\times$ 15 cm, 3 μm ES800). Each fraction was injected duplicate and was analyzed by the LC-MS/MS using 60 min gradient. 400-1200 m/z full scan will be presented.

2.3.5 In Gel Digestion for label free Mass Spectrometry

60 μg of each brain subject was pooled to formed approximately 250 μg combination group. This approach was designed to decrease the possible mistake caused by individual variation and get more confident results.

~250 μg of protein from each experiment group was loaded into the 10% SDS-PAGE and the full lanes of each sample was visualized by Coomassie Brilliant Blue short staining (10 min staining). After around one-hour destaining during which the blue area except protein containing parts turned to transparent again, rinse the entire slab of one dimensional gel and excise the full lanes. Cut excised gel lane of each group into 1 mm^2 gel cubes. Note that too small pieces could clot the pipet tips and too big ones may cause time consuming for the destaining. After collected all the gel cubes in a 1.5ml tube and washed with miliQ water. Added 400 μl of Buffer A (25mM Ammonium bicarbonate) and incubated with occasional vortexing for 10

minutes. Removed buffer A and added Buffer B (25mM ammonium bicarbonate, 50 % acetonitrile) with the same vortex time. Depending on the staining intensity, following by the 2-3 cycles of Buffer A and Buffer B until the gel pieces become transparent. Totally distained gel pieces were dehydrated using acetonitrile 400 μ l and removed all the remaining liquid with 10minute spin-vacuum using the concentrator.

Added 400 μ l DTT buffer (DTT final concentration 10mM in 25mM ammonium bicarbonate) and incubated at 60 °C water bath for 30 min, then removed the DTT buffer and added 400 μ l IAA buffer (IAA final concentration 55mM in 25mM ammonium bicarbonate), incubated at room temperature and protected from light for another 30 min. After removed all the IAA buffer and repeated two more AB cycle, gel pieces were dehydrated with acetonitrile again and shrinked gel cubes were subjected to protein digestion at 37 °C in sequencing grade modified trypsin (Promega, Madison, WI, USA). After 12-hour overnight trypsin digestion, the peptides inside the gel were extracted out by Buffer D (50% acetonitrile, 5% acetic acid) multiple times with vortex 10 min each time. The extracted peptides were concentrated by vacuum concentrators (Eppendorf AG, Hamburg, Germany) before processing LC-MS/MS.

2.3.6 Mass Spectrometric Data Search and Analysis

The peak list generation, protein identification, and peptide quantification were performed using in-house Mascot server (version 2.4, Matrix Science, MA) and peaks studio version 7.5 (bioinformatics Solutions, Waterloo, Canada) with a precursor ion tolerance of 10 ppm and fragment ion tolerance of 0.05 Da[204]. Peptide PTMs were identified by PEAKS PTM algorithm[205]. The false discovery rates (FDRs) for both the protein and peptides were adjusted to be < 1%. For Mascot database searches, trioxidation of Cys(Cysteine), Met(methionine), Tyr(Tyrosine), Trp(tryptophan) and Carbamidomethylation at Cys were set as variable modifications and peptides with Mascot score > homology or identity score were used to generate the peptide list. The UniProt mouse database downloaded on June 23, 2015 and was used for the protein database search. User defined parameters were

(i) sample type: TMT 6-plex (peptide-labeled) or label free; (ii) digestion: trypsin (iii) Max missed Cleavages: 2 (iv) Fix modification: no (v) Variable Modifications: trioxidation(CMWY) carbamidomethylation. Protein and peptide identification and their respective quantification were also carried out using an in-house Mascot server (version 2.3.02, Matrix Science, MA) and PEAKS Studio version 7.5 (Bioinformatics Solutions, Waterloo, Canada). The precursor tolerance was set as 10 ppm and fragment ion tolerance at 0.05 Da for PEAKS. For Mascot database search, trioxidation of Cysteine, deamidation of Asparagine and Glutamine, oxidation of Methionine and Carbamidomethylation of Cysteine were set as variable modification. All the generated spectra were searching against the UniProt mouse data base.

2.3.7 LC-MRM-MS Quantitative Analysis and Verification

The same set of mouse brain sample was individually digested, desalted and vacuumed dried with in solution digestion. Rather than peptide labeling and HPLC fractionation, the sample was directly triplicate injected in the VSQ Vantage triple quadrupole mass spectrometry coupled to an EASY-nLC 10000 nanoflow UHPLC system (Thermo Scientific Inc., Bremen, Germany). The targeted peptides will be specifically assayed based on the double selection process including peptide selection (Q1) and fragmentation selection (Q3) after peptide fragmentation (Q2). The retention time (RT) for each peptide eluted in QE was obtained from the 60min full MS/MS analysis. The predicted RT for target peptides eluted in vantage was estimated with approximately ± 5 error and a 10min time range was determined as isolation range. The 4-6 most abundant peptide fragmentation ions were selected as transitions for analyzing method. A total of 2 μ g peptides was injected to an Acclaim PepMap100 trap column (75 μ m $\times$ 2 cm; nanoViper C18, 3 μ m, 100 Å) and resolved on an Acclaim PepMap RSLC C18 column (75 μ m $\times$ 15 cm; nanoViper C18, 2 μ m, 100 Å) (Thermo Scientific, USA). The peptidome was conducted with mobile phase A (0.1% FA in HPLC water), and mobile phase B (0.1% FA in ACN) at a flow rate 300 nl/min. Liquid chromatography was performed using a 60-min gradient starting with 5 % mobile phase B and 95 % mobile phase A, 5-30% mobile phase B for 45 min. 30-50% mobile phase B for 4 min. the gradient was then increased to 80%

mobile phase B over 6 s, maintained under isocratic conditions for 1 min, then reverted to initial conditions over 6 s, and finally re-equilibration at 5% B for 3 min. An electrospray potential of 1.5 kV and capillary temperature of 250 °C were used for ionization. Transition scan times were 20ms for isolation. The tolerance of selectivity for Q1 and Q3 were set at 0.7 Da. A collision gas pressure of 1mTorr of argon was applied for Q2.

2.3.8 Skyline Quantitation

The spectral library was created from the Mascot and PEAKS protein identification search results for the three age stages mouse brain sample. For quantification, we adapted a platform-independent software named Skyline and Skyline v4.1 was applied to extract quantitative information from multiple LC-MS/MS data. For the verification of peptides, at least 3 transitions coeluting within the predicted time window will be considered positive identification of the targeted peptides. The dotp value representing relative abundance of each transition need to pass the threshold of the software (0.85). For the quantitative study of peptide, Skyline ranked the y and b fragmentation ions from the targeted peptide based on transitions intensity. The most intense transitions from the coeluting peak will be selected for calculating peak area with the following criteria: precursor charge state 2, 3, 4, 5; ion charge state 1, 2; ion type b, y; always select N-terminal to proline for special ions. Skyline extracted the area under the ion chromatogram curve through the duration of eluting by interpolating peak height between the raw data points. The extracted areas of selected transitions were summed to generate the peptides area. Second derivative transform was used for demonstrating purpose. The modification rate was calculated based on each modified peptide normalized to corresponding unmodified same peptide, function as internal reference.

2.3.9 Western blot validation

Protein lysis from tissue was collected and protein concentration was quantified using the bicinchoninic acid (BCA) assay. 20 µg proteins was resolved in 10% SDS-PAGE and then transferred to membrane. Following blocking with 5% skim milk in TBS-T washing buffer (TBS-T, 1mM Tris, 0.15M NaCl, 0.1% Tween) for 1h at

room temperature. Then the membrane will probe with specific primary antibody with optimal dilution overnight 4 °C. The loading control was β -actin (1:10000) (EMD Millipore, USA). Horseradish Peroxidase (HRP)-conjugated anti mouse or HRP-conjugated anti rabbit (cell Signaling Technology, USA) were used correspondingly as secondary antibody. Then the bands will be visualized on the Carestream Medical X-ray Green/MXG film (Carestream Health, USA) by developing in SuperSignal™ West Pic Chemiluminescent Substrate (Thermo Fisher Scientific, USA).

2.3.10 Enzymatic Activity Assay

Enzymatic activity assay for Malate Dehydrogenase 1 (MDH1)

Abcam MDH1 activity assay kit (ab200009) was applied for the sensitive and accurate measurement of target enzyme function. The enzyme activity is determined by the production of NADH in the following MDH1 catalyzed reaction:

The generation of NADH is coupled to the 1:1 reduction of a reporter dye to yield a colored (yellow) reaction product whose concentration can be monitored by measuring the increase in absorbance at 450 nm. In each well, only native MDH1 is immunocaptured from the sample. The well was designed to remove all other enzymes, including MDH2 from the activity measurement.

All reagents and assay plate are equilibrated to room temperature (18-25°C) prior to use. Lyophilized NAD⁺, Sodium Malate, Reagent Dye and coupler were provided in powder followed with resuspending with certain amount of nanopure water to make 100 X stock solution. The Wash buffer and Incubation buffer were diluted to 1 time for use.

Same amount of minced mice brain tissues were collected from three different age groups with three subjects in each age. The brain tissues were thoroughly rinsed with PBS for three times and blood vessel clot was removed. 50 mg of wet tissue was homogenized with 500 μ L Extraction Buffer accordingly in bullet blender (Next

Advance, NY, USA). The tissues were incubated on ice for 20 min followed by centrifugation at 18,000 x g for 20 minutes at 4°C. The supernatant were immediately processed BCA for protein quantification. Based on the protein quantification result and the protein concentration range requirement for optimal assay. 30 µg of proteins were collected from each mouse and three mouse brain sample of the same age were combined together. ~90 µg of proteins for each age stage were diluted with 1X Incubation Buffer to generate sample solution with protein concentration 900 µg/mL which located at the optimal working range of 15 – 1,000 µg/mL.

The reaction buffer was prepared immediately prior to use with 1X Lyophilized NAD⁺, 1X Sodium Malate, 1X Reagent Dye and 1X coupler in base buffer. 100 µL of brain protein homogenates from three different ages was added into each well. The MDH1 was specifically immunocaptured in each well after incubating for 2 hour followed by removing impurities with three times of wash. 200 µL of Reaction Buffer was added to the well and yellow color development was immediately record with kinetic setting for 50 minutes with the following settings.

Mode	Kinetic
Wavelength:	450 nm
Time:	up to 45 min.
Interval:	20 sec.
Shaking:	Shake before and between readings

2.4 Results

2.4.1 Aging mice brain proteomics analysis workflow

Comparative quantitative proteomics analyses of brain tissue from different ages are useful for biomarker discovery and verification on both proteins expression level and PTM level. TMT6 chemical labeling approach that affords both quantitation and multiplexing analyses in a single reagent was employed in this study. As demonstrated in Figure 2.4.1, in order to decrease the variation of detecting aging

stage-specific targets that defines aged condition from young stage, mice brains are grouped into Young (Y), middle (M) and old (O) and were preliminary regarded as part of the experimental design. For each age group, equal concentration of pooled brain proteins (200µg) were proteolytically digested with trypsin. Tryptic peptides obtained from Y, M and O groups were labeled with TMT six-plex isobaric tag or directly label free. The in solution digested peptides were assigned for HPLC fractionation. The in gel digested peptides were cut into nine fractions according to protein abundance. Separated peptides were analyzed in duplicates by LC-MS/MS and relative abundance of specific peptide among age groups is determined by comparing the intensities of the TMT6 reporter fragment ion in the 126-131 m/z region of the peptide product ion spectra. The analysis of proteins expression difference among each age groups are combined of both TMT labeling group and label free group to generate potential targets. The protein targets of expression difference are verified by western blot. The modified proteins of biological meaning were verified by LC-MRM-MS. Three technical replicates were performed in this study to increase the reliability of differences in quantitative changes in PTM level.

Figure 2.4.1. Work flow of TMT6, label free and MRM-based approach. Schematic demonstration of the experimental design for discovery through verification quantitative proteomics on mice brain from different age using LC-MSMS and MRM-based approach.

2.4.2 Assessment of quantitative MS data quality

To assess the reliability of label free proteomics data in this study. The correlation of measured pairwise emPAI of replicate A and replicate B for each protein were examined on scatter $\log_2$ plots depicted in Figure 2.4.2. The data was examined in all three age groups separately. The generated Pearson correlation coefficients (r) and accompanied p values for each scatter $\log_2$ plots of measured emPAI values between Y_A and Y_B (Figure 2.4.2 A, $r = 0.9720$, $p < 0.0001$), representing consistency of measured emPAI between the triplicates in young group. Correspondingly, good reproducibility was also observed in scatter $\log_2$ plots of measured emPAI values between M_A and M_B (Figure 2.4.2 B, $r = 0.9719$, $p < 0.0001$). Likewise, statistically significant correlation of measured emPAI between O_A and O_B (Figure 2.4.2 C, $r = 0.9748$, $p < 0.0001$) was detected among the triplicates. The use of label free mice brain processing protocols and statistical analytical system for proteomics lead to the low system and quantitation variability observed here, thus confirming the reliability and confidence of our presented label free dataset.

Figure 2.4.2 Correlation assessment between mouse label free duplicate experiments. Correlation assessment of measured emPAI values for each protein (FDR < 1%) between duplicates. (A) Scatter $\log_2$ plots of measured emPAI values of proteins identified in Y_A and Y_B. (B) Scatter $\log_2$ plots of measured emPAI values of proteins identified in M_B and M_B. (C) Scatter $\log_2$ plots of measured

emPAI values of proteins identified in O_A and O_B. Y_A, Y_B, replicate A, B and young age mice; M_A, M_B replicate A,B in middle age mice; O_A, O_B, replicate A, B and C in old age mice; r, Pearson correlation coefficient; ****, extremely significant.

2.4.3 Proteomic profile of mouse whole brains in different age stages

To investigate the brain proteomics response to aging, we employed LC-MS/MS based proteomic approach combined with LC-MRM/MS to detect the alternations in proteome level. The workflow for label free scheme and proteomic analysis is described in Figure 1A. Briefly, the mice brain were firstly pooled according to age and performed in-gel digestion assisted LC-MS/MS to discover protein targets. Novel targets were further verified by in-solution assisted MRM analysis with individual sample. A total 5156 proteins were identified in old mouse group. 5498 proteins were identified in young group mouse and 5417 proteins were identified in middle group. Comparative analysis of proteins between young and old are presented in Figure 2.4.3 B and C. 591 proteins were significantly up-regulated ($O/Y > 2$) and 681 proteins were down-regulated ($O/Y < 0.5$) in old group. We further analyzed proteins those significantly up-regulated or down-regulated in old age group by gene ontology approach using David Functional Annotation. We found that TCA cycle were actively expressed in both up-regulated proteins and down-regulated proteins, respectively. As we are interested to identify the incidence and potential impact of DPMs within key TCA cycle components. We focused on the analysis in the TCA cycle associated proteins instead of whole global proteome.

Figure 2.4.3. LC-MS/MS based proteomics analysis on mice brain of different age. A. Schematic demonstration of the experimental workflow from discovery-phase to verification phase by proteomic analysis of mice brain using label free MS/MRM-based approach. B. Summary of proteins identified in all three age groups and differentially expressed in middle and old groups related to young group. C. Distribution of the O/Y ratios. D-E. Gene ontology of different expressed proteins among different age groups.

2.4.4 Imbalance of TCA proteins expression

In the proteomics data, the EMPAI (Exponentially Modified Protein Abundance Index) value of specific TCA enzymes in old group shows significant alternation comparing against young mice group. A total of eight important component enzymes in TCA cycle were found to be up- or down- regulated in old brain tissue compared with young age control group (Figure 2.4.4). Of these TCA enzymes, five mitochondrial proteins are playing vital roles in the ATP production through the mitochondrial respiratory chain, while the remaining three cytosolic proteins are

involved in the diverse metabolism and transportation of amino acid and other nutrients.

The proteins identified as being modulated in old mouse were subjected to pathway analysis which demonstrated important roles in cellular metabolism and energy production. A total of five proteins were found to be up-regulated in old brain sample. The malate dehydrogenase 1 (MDH1) which catalyze the reversible oxidation of malate to oxaloacetate was found under high up-regulation in aged post-mortem brain tissue by above 3 fold (Figure 2.4.4). MDH1 was localized to the cytoplasm and also plays core role in the malate-aspartate shuttle between cytosol and mitochondria. Fumarate dehydrogenase 1 (FH1) catalyzes the hydration/dehydration of fumarate to malate and mutation in FH is associated with renal cell carcinoma [206]. In our study, FH1 expression was found to be significantly increased in old group relative to young group. NADH dehydrogenase [ubiquinone] iron-sulfur protein 3 (NDUFS3) as one of the iron-sulfur component of complex I in electron transport chain protein was detected 2 fold increased abundance in old group. The NDUFS3 is closely related with mitochondrial dysfunction and ROS leakage [207]. Another TCA associated protein which plays a dual role in ETC is succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB). We also observed SDHB 2 fold up-regulation. Finally, the enzyme pyruvate dehydrogenase E1 component subunit beta (PDHB) was found to be up-regulated by 2 fold change in old brain subjects compared with young ones. PDHB links the glycolytic pathway to the TCA cycle through conversion of pyruvate to acetyl-CoA and CO₂ [208].

Besides TCA cycle proteins significantly up-regulated which list as before, meanwhile we also found other three TCA associated proteins demonstrated obvious down-regulation in old brain tissue comparing against young groups. Succinyl-CoA ligase [ADP-forming] subunit beta (SUCLA2) will hydrolyzes ATP to convert succinyl-CoA to succinate and form ADP [209]. This protein represent the only step of substrate-level phosphorylation in the TCA and was decrease on expression level in old group above 2 fold. While at the same time, Isocitrate dehydrogenase 1 (IDH1)

and Isocitrate dehydrogenase 2 (IDH2) was both decreased, IDH1 and IDH2 catalyzes the reversible oxidative decarboxylation of isocitrate to yield α -ketoglutarate (α -KG) as part of the TCA cycle.

Figure 2.4.4 Fold change of TCA proteins in old group related to young age. TCA cycle associated proteins with significantly increased abundance in old mouse brain samples include FH1, SDHB, MDH1 and NDUFS3. The significantly down-regulated enzymes includes IDH2, IDH1 and SUCLA2. B. The differently expressed proteins in TCA cycle. The upregulated proteins are labelled in red arrow and the down-regulated ones are labelled in blue arrow.

2.4.5 TCA associated proteins expression level validation by western blot

Based on the reported experience in the proteomics data field, only a few fractions of proteins targets will identify biological expression alternation when applying western blot. To further refine the candidate list, proteins show inconsistence expression change tendency with western blot with the proteomics data will be excluded.

Western blotting results showed that increased expression of SDHB was readily recognized by an antibody against SDHB (Figure 2.4.5 A). Notably, the protein up-

regulation which verified by western blot indicated the similar fold change with the corresponding mass spectrometry EMPAI value. SDHB was gradually up-regulated in an age dependent manner with finally reach 2 fold increased in old group. To validate the EMPAI values for protein NDUF3 demonstrated obvious increase in old brain sample that, we applied an antibody specifically recognizing NDUF3 and this protein was confirmed with increased in old group above 2 fold change (Figure 2.4.5 B). Moreover, the expression of IDH1 reduced in an age dependent manner is displayed by both the proteomics result and specific antibody detection (Figure 2.4.5 C). Different with SDHB and NDUF3, for IDH1 the obviously decrease on expression level was showed at middle stage, which indicated the disturbance of the protein expression was appeared at earlier age stage. The expression of MDH1 was increased in an age dependent manner (Figure 2.4.5 D).

Figure 2.4.5 Functional categorization of TCA cycle associated proteins that were differentially expressed in accelerated aging groups compared with young controls. A. Western blotting detection of protein SDHB demonstrates increased expressed with two fold up-regulation is corresponding to mass spectrometry data. B. NDUFS3 expression showed above threefold increase in old group. C. IDH1 decrease expression was proved with more than two times down-regulation. D. MDH1 expression increased in an age dependent manner. Unpaired T-test ***p<0.003, **** p<0.0001.

2.4.6 Imbalance of cellular redox regulation

In addition to the dysregulation of TCA proteins, glutaredoxin (Grx) and thioredoxin (Trx) that are key enzymes in the redox regulation were significantly downregulated in the old mice group. Grx and Trx regulate this thiol-based redox signaling by reverse the oxidized sulfenic acid (R-SOH) back to thiol group. Thus, downregulation of these enzymes can contribute to imbalance in cellular redox regulation and accumulation of oxidative damaged proteins. Cysteine thiol group of signaling proteins can be oxidized by reactive oxygen and nitrogen species that function as physiological messengers in redox signaling. As the cysteine trioxidation is irreversible oxidation which may cause unrepaired damages to certain proteins. The microenvironment develop several ways to stabilize the cysteine sulfenic acid as this form could in turn be sensitive to hyperoxidation yielding sulfinic (R-SO₂H) and sulfonic acid (R-SO₃H)[119]. So the accumulation of Cysteine trioxidation indicated the disturbed of redox signaling pathway which emphasize on the two enzyme which can revert cysteine sulfinic acid to the thiol form. When we analyze the expression of this two enzyme. We found the Grx was decreased in old group in the expression by 2 fold and Trx was down-regulated 2 times. (Figure2.4.6). The significantly down-regulation of these two redoxin enzymes may indicated the impairment of the redox regulation during aging. The dysregulated process can contribute to oxidative damage in brain proteins by terminal sulfonic acid (Cys-SO₃H).

Figure 2.4.6. Redox regulation of cysteine modification and decreased expression of GRX and TRX. A. Stepwise oxidation of cysteine thiol group. The mono-oxidation and di-oxidation can be reversed by Grx, Trx and sulfiredoxin (Srx). B. Grx expression decreased in an age dependent manner and was not detected in the old mice. C Trx expression showed significantly decreased in old group comparing to young group. **** $p < 0.0001$.

2.4.7 Detecting and quantitating Cysteine trioxidation peptide with TCA cycle enzyme MDH1

After demonstrating that several targets proteins in TCA cycle had altered expression levels in aged mice, we analyzed the composition of these protein on the modification level. As the use of reverse phase column displayed a high-resolution chromatographic separation that allowed detection of low abundance PTMs, we next focused on the assessing of the oxidation status of endogenous proteins. Among those proteins, MDH1 is one of the core enzymes which catalyzes the reversible

oxidation of malate to oxaloacetate and actively participates in malate-aspartate shuttle between cytosol and mitochondrial. Interestingly, MDH1 was identified to be exclusively trioxidized at amino acid cysteine in old mice brain tissue. The tryptic peptide sequence VIVVGNPANTNCLTASK containing Cysteine137 (Cys137) as trioxidation residue was determined by MS1 precursor and MS2 spectrum. MS/MS spectrum allows not only the confirmation of the modified site localization and target peptides sequence. Meanwhile it also provides the retention time and m/z ratio for the extraction of ion chromatogram based quantitation of trioxidation with high mass accuracy. The targeted peptide VIVVGNPANTNCLTASK observed the retention time 29' in the mass spectrometry elution and the precursor m/z ratio was 879.9460.

Table 2.4.7 Selected Peptides and MS2 Transitions for MRM quantitation

protein/modified cysteine	peptide sequence	precursor m/z	product m/z
MDH1 Cys137 sulfonic acid	VIVVGNPANTN <u>C</u> LTASK	874.9460++	1167.531(y11+),670.3076(y6+),519.3137(y5+), 406.2296(y4+),305.1819(y3+),312.2282(b3+)
internal reference	VIVVGNPANTNCLTASK	879.4644++	1176.5677(y11+),1347.6321(y13+),1008.4779(y9+), 406.2296(y4+),312.2282(b3+)

To further increase the confidence of the final selection of high level oxidation targets, we apply high specificity MRM analysis combined with reverse phase column to verify the potential targets in discovery phase. The precursor ion m/z value (874.9460++) was extracted from MS1 while MS/MS data were obtained for selected fragmentations ions (transitions). Five most intense transitions with one extra (y6 670.3076) which indicated modified site for candidate peptide were acquired from the MS/MS data (Table 2.4.7). The retention time with around ± 5 minutes (24-34.5) was regarded as the duration of new time window. As MRM will perform double selection based on the MS1 filter as well as the transitions m/z value, another batch of trypsin digests sample of individual subject from each age group (young, middle and old) were analyzed triplicate in vantage based on the MRM filtering.

Figure 2.4.7.1 Positive identification of MDH1 trioxidized peptide VIVVGNPANTNCLTASK. A. Visualization of co-eluted peak of six transitions of peptide VIVVGNPANTNCLTASK in ~10 minutes time window. B. Visualization of co-eluted peak of six transitions of peptide VIVVGNPANTNCLTASK.

After the MRM experiment, the MDH1 Cys137 trioxidation peptides was positively verified in old group through match with the MS1 filter (+47Da) and the formation of all six transitions co-eluted peak within expected eight minutes time window. The detection also passes the skyline program threshold standard with high dotp value 0.95 which indicated high similarity with the library peak format (Figure

2.4.7.1). The representative transitions showed strongest signal and high reproducibility. However, in the young and middle group, no cysteine trioxidation peak was detectable above noise level. Meanwhile, the corresponding non-cysteine trioxidation peptide derived from the same protein was set as internal reference (879.4644). As cysteine is liable and easily modulated, the non-trioxidized cysteine residue will be carbamidomethylated (+57 Da) during the alkylation step in the IAA environment. The trioxidation prevent cysteine from carbamidomethyl modification will lead to the 10 Da difference between trioxidized peptide and internal reference peptide. Same procedures were applied to study the internal reference in all 3 age groups, 5 most intense transitions were selected as the representative ions for the measurement as described before. The co-elution peak of all five transitions with high dotp value (0.92) were identified in all three groups (Figure 2.4.7.2).

Figure 2.4.7.2 Identification and quantitation of MDH1 trioxidized peptide and corresponding unmodified peptide. A. MS/MS spectrum of MDH1 peptide including the Cys trioxidation site displaying exclusively expressed in old group. The spectrum shows the peptide well fragmentation pattern and is labeled with both b and y ions. B. Dotp value of the modified peptides showing good match on relative abundance of each fragmentation ion between library and sample. C. MS/MS spectrum of MDH1 peptide including the Cys carbamydomethyl. D. High dotp value of the unmodified peptides showing high similarity between on library and sample based on relative abundance of each fragmentation ion.

After the positive verification of target oxidative peptide and non-oxidative peptide, the quantitation analysis was further studied to determine the age dependence of irreversible trioxidation level. The area under the curve (AUC) was

used to determine the abundance of each ions and sum up for the intensity of the peptide. The AUG value of each representative transitions were extracted to generate the peptide abundance of trioxidized VIVVGNPANTNCLTASK. As we focusing on the oxidation level of specific amino acid rather than general protein expression level itself, to render the data more reasonable and comparable among each research populations, we demonstrated both the area of modified peptides and corresponding unmodified peptides derived from the same protein. After the detailed statistic study, the MDH1 trioxidation level in old group was highly accumulated while no irreversible oxidation was identified in other young and middle age groups. The absolute value of modified peptides is extracted in each biological replicate. The response curve was generated by analyzing three technical replicated at each biological replicate in each age. (Figure 2.4.7.3)

Figure 2.4.7.3 Quantitation of MDH1 trioxidized peptide and corresponding unmodified peptide. A-F representing MDH1 peptide VIVVGNPANTNCLTASK trioxidation and carbamidomethyl modified version MS2 ion intensity. Fragment ion intensities are displayed from all three different ages for sulfonic acid. Cys137 trioxidation peak is described as upper panel and Cys137 Carbamidomethyl is described as down panel. A and B in first row represent sulfonic acid is not presented in young group. C and D in the second row indicated no trioxidized peak identified in middle age when the carbamidomethyl peptides is well present. E and F positively verified the existence of Cys137 trioxidation in old group. Differences in vertical axis for each panel show the change in ion intensity as a result of age increase. The retention time indicates Cys137 sulfonic acid modified peptides elute at similar time points. Second-Derivative transform used in display. G. AUG of trioxidized peptides in each technique replicates of three biological replicates. H. Statistical analysis shows the highly accumulation of trioxidation in old group. ****, $p < 0.0001$, extremely significant.

2.4.8 Cysteine trioxidation affect protein normal expression and alternated structure

In our label free proteomics study, mouse MDH1 was identified to be upregulated more than 3 times in old group comparing against young group. In order to validate the expression tendency in aging process, we applied the specific MDH1 antibody for detection. We take three subjects for each age group and perform antibody detection individually to avoid the biological variation. The results explicitly indicated that MDH1 expression was increasingly modulated in an age dependent manner (Figure 2.4.5). The western blot result was consistent with mass spectrometry analysis which proved high quality proof. We can regard the over expression of MDH1 as one of the compensate mechanism for the enzymatic function lose.

To detect the potential structural and functional conformation alternation caused by the presence of abnormally accumulated oxidized cysteine residues. We use software Pymol to probe possible functional effect of the trioxidized residue in MDH1. We download the MDH1 model from PDB database as template [PDB: 5MDH]. The significance of selective C137 oxidation was proved through the observation that the modified residue is located at the active zone of the enzyme. As

Arg131 is the active site of MDH1 which will bind with hydrogen to initiate the enzymes function. The Cys137 and Arg131 in the same alpha helix and Cys137 is obviously adjacent to the active site. The modified site at Cys137 shows the close vicinity with enzymatic active zone. The modification has high chance to interfere with the binding and finally leads to dysfunction based on the proximity of modified site and active site (Figure 2.4.8.1). We used the plugin function of pymol, pyTM[210], to simulate the conformational property alternation after the modification. The pyTM is a tool that allows structural insight into how introduced PTM potentially can exert an experimentally observed effect. The software introduce mono- and di-oxidation which shows the structure after adding one or two oxygen at the modification site. The visualization of predicted PTM carrying conformation indicated high chance of interference the vdW clashes within vicinity space. The simulation of mono- and di-oxidation of the target peptides provide the conformational change with adding oxygen atoms. Combined with the knowledge that the modified sites is in proximity with the active center of the enzymes, the structural alteration provided the base for functional changes.

Figure 2.4.8.1 3D structure simulation of MDH1. A. Overview of 3D structure of MDH1 based on [PDB: 5MDH]. B. A close up view of the modified peptides. The substrate binding site at Asn131 labeled red and the modified site at Cys137 labeled

yellow. Cys137 was in a close proximity with the substrate binding site at Asn131. The Cys137 was at the active zone of the enzyme. C. the PyTM simulation of the alternation of modified peptides. The modified Cys137 shows in sphere representation with nitrogen labeled in blue and oxygen labeled in red. Adding oxygens at the Cys137 change the structure and charge status of the peptide. The structure modulation is displayed by pyTM software.

2.4.9 Accumulation of cysteine trioxidation at C137 inhibit the MDH1 catalytic activity

C137				
H.sapiens	126	VIVVGNPANTN	CLTASK	142
M.musculus	126	VIVVGNPANTN	CLTASK	142
S.scrofa	126	VIVVGNPANTN	CLTASK	142
D.rerio	126	VIVVGNPANTN	CLTASK	142
D.melanogaster	129	VGNPANTNALV	CSSYAP	145

Figure 2.4.9.1 conserved Cys137 in different species. The Cys137 is a highly conserved residue.

As 3D simulation of the target MDH1 provided the structural basis for its functional impairment. Sequence alignments from diverse species showed that Cys137 of MDH1 is a highly conserved residue (Figure 2.4.10.1). We then assessed the endogenous MDH1 enzymatic activity through specific assay kit. MDH1 activity assay kit is applied for the sensitive and accurate measurement of MDH1 activity. Based on the MDH1 catalytic reaction: Malate + NAD⁺ ↔ oxaloacetic acid + NADH, the generation of NADH from this reaction is coupled to the 1 : 1 ratio of a reporter dye to yield a yellow color whose concentration can be measured by the absorbance at 450nm. Three subjects from each age group were pooled together and the pool sample used for the MDH1 activity assay. Colorimetric enzyme kinetic analysis and calculation were performed to reveal the activity difference of different age groups. After 50min kinetic measurement, MDH1 activity was significantly decreased in old brain tissue comparing with middle and young ones. Initial reaction showed little difference among three age groups. However, the aged group demonstrated lower activity starting from 10 minutes to the end of the reaction. The middle group also slightly decrease but still maintain at the reasonable level

comparing with young group. This real time measurement correspond well with the trioxidation level of each age group. We also calculated the reaction rate from the real time kinetic curve. The rate/slope is extracted from the liner range of the time point data. It is expressed as rate (mO.D./min), it clearly demonstrated the decrease tendency of MDH1 activity in an age dependent manner. This result suggested that cysteine trioxidation negatively regulate MDH1 activity.

Figure 2.4.9.2 Colorimetric MDH1 enzymatic activity assay. MDH1 activity is significantly reduced in old brains. Enzyme kinetic analysis was performed for tissue samples from three different age groups. A. Schematic diagram of specific MDH1 activity assay. B. O.D absorption at 450nm recorded every ten minutes, showing that the old brain group had the lowest enzymatic activity throughout. C. O.D. absorption at 450nm recorded for a 45 min graph demonstrated a decrease in the MDH1 enzymatic activity in the old brain group. The assay was performed with three technical replicates. Two-way ANOVA, * $p=0.005$, ** $p=0.003$, *** $p<0.0001$.

2.5 Discussion

Impairment of cellular energy production systems including increasing dysfunction of the TCA cycle is considered a hallmark of the aging process. Here, we used high-resolution tandem mass spectrometry coupled with high-performance MRM-based quantification to identify specific proteins that are susceptible to homeostatic disturbance induced by aging. Moreover, focusing on the key TCA enzyme MDH1, we define the nature, frequency and functional impact of trioxidation DPMs in the aging brain.

With evidence pointing toward a vital role of TCA cycle in aged phenotype and neurodegenerative diseases, an understanding of the changes to the proteome is warranted. While here we focused on DPMs in MDH1, we also uncovered more widespread changes in expression level of several other TCA functional enzyme proteins or their key subunits: SULA2, IDH1 and IDH2 were significantly less abundant in the old mouse brain compared with young mouse brains. IDH1 has multiple roles in cellular metabolism; in particular it protects against age-induced oxidative stress by producing NADPH and α -KG, both of which function in cellular detoxification processes in response to oxidative stress [211, 212]. Decreasing levels of IDH1 might indicate excessive oxidative stress with less efficient ROS clearance. Evidencing more extensive deregulation of TCA associated proteins, some, such as SDHB, MDH1, FH1 and NDUFS3 showed drastic up-regulation in old compared to young brains. Together, the changes in expression level of the various TCA cycle components are likely to result in the accumulation or depletion of the intermediate metabolites of the TCA cycle, which could result in accumulation of toxic metabolites and ROS in the cells. Imbalanced ROS homeostasis is an important mechanism by which dysfunctional mitochondria contribute to aging. The enzymes that lead to ROS generation are broadly distributed in the TCA cycle, and include electron transport chain (ETC) complexes and other dehydrogenases. ROS can be generated in the mitochondria by these deregulated enzymes resulting in the transfer of a single electron to an oxygen molecule. The up-regulated proteins in the ETC are potentially responsible for the rising ROS level in the old brain; in particular,

increased levels of SDHB, which is the way-station of electron transportation from the TCA cycle to the Ubiquinone (Q) of the ETC complex, may represent an increase in electron leakage and result in the accumulation of ROS. In line with these findings, extensive review analysis of brain proteomics literature in the context of aging was included. Proteomic profile of brain mitochondria during aging as well as the functional bioenergetic correlates are evaluated by different studies[213]. IDH1 and IDH2 are reported to be decreased expression from 12 to 24 months. PDHB and FH1 are found to be elevated in expression till 12 months in isolated mitochondrial proteomics experiments[214]. These results suggest that dysregulated expression of TCA cycle enzymes may contribute to a decline in mitochondrial bioenergetics during aging. Collectively, the expression changes of these proteins induce mitochondrial dysfunctions that lead to cellular stress in the aging process

Given that various redox regulatory mechanisms converge upon mitochondria to modulate ATP synthesis and ROS production, it is important to consider how redox signals mediate these metabolic processes. The cellular redox balance is ordinarily maintained through diverse kinds of reversible cysteine modifications, such as S-glutathionylation which protects enzymes from irreversible oxidation. [215]. The function of Grx and Trx is to reverse the Cys oxidation (S-OH) back to reduced states and thus prevent hyperoxidation. Our mass spectrometry data showed that Grx and Trx are significantly downregulated in the old brain group; this low level of expression in the aging process may be reflective of a severe perturbation in the redox regulation system that can lead to irreversible oxidative damage of key proteins.

The functions of proteins in the TCA cycle are regulated not only by their expression level but also by protein modifications. Protein acetylation and methylation as enzymatic PTMs have been reported to emerge in the processes of mitochondrial metabolism[216, 217]. Spontaneous DPMs are emerging as important markers of aging and aging-related neurodegeneration, notwithstanding their presence and roles in TCA cycle from aging brain tissues have yet to be explored. TCA cycle proteins are particularly susceptible to oxidation as they are in

a microenvironment of high concentration of ROS. Our study reveals an excessive accumulation of cysteine thiol trioxidation in the MDH1 enzyme. As thiol trioxidation at cysteine residues is irreversible and cannot be repaired by any known enzymes, protein damage by thiol trioxidation can alter the structure and function of the protein. In the discovery data-dependent LC-MS/MS experiment, we identified Cys137 trioxidation in MDH1 exclusively in the old mouse brain, then verified and quantified this finding by targeted MRM MS. Together these data show that MDH1 is one of the metabolic enzymes that is affected at both the level of expression and by DPMs. As reduced activity of MDH1 is related to senescence [218], the trioxidation of MDH1 provides a mechanism by which this could occur. The 3D model of MDH1 showed that the trioxidation site is within the active zone and in close proximity to the substrate binding site, Asn131, thus the conformational alternation after Cys137 trioxidation could significantly impact MDH1 activity. Cysteine trioxidation generated sulfonic acid, which is a strong acid and it is commonly cited as being around a million times stronger than the corresponding carboxylic acid. The accumulation of sulfonic acid may imbalance the cellular homeostasis and destroy the environment. More MDH1 enzymatic activity will be further impaired in the imbalanced microenvironment. Consistent with this prediction, we found significant decrease in MDH1 activity in protein extracts from aged brains compared to young brains. Based on the results from both assays, we conclude that Cys137 trioxidation of MDH1 negatively impacts the activity of MDH1.

Overexpression of MDH1 has been observed in a wide spectrum of cancers[219]. MDH1 functions in both malate-aspartate shuttle and Gln metabolism[220]. Some researches revealed that the enzymatic arginine methylation of suppresses glutamine metabolism in pancreatic cancer[217]. In our study, we reported that the function of MDH1 is compromised by oxidative damage during the aging process of the brain. For the upregulation of MDH1, we hypothesis that the increased expression of the protein MDH1 may be induced to compensate the malfunctioned MDH1 due to trioxidation. The expression of genes encoding the specific steps of the TCA cycle come under increasing control of the regulatory

pathway as mitochondrial respiratory capacity is reduced. In this way, expression of those genes can be maintained to compensate for the loss of certain components [221]. Early researches indicate that the expression of MDH1 was regulated so as to compensate the TCA energy deficit in rat[222]. The maintenance of bioenergetic function via compensatory mechanisms including alterations in mitochondrial remodelling or enzyme expression was showed to contribute to preserve function[214]. Further exploration required more molecular experiments to validate. The decline of cellular energy production due to TCA cycle impairment is considered a hallmark of the aging process; the accumulation of irreversibly oxidized TCA cycle proteins is likely to contribute to declining ATP generation in the brain tissue of old mice. Identification and quantification of oxidative modifications provides insight into protein functional outcomes in response to cellular oxidative stress in the aging process. As Grx and Trx are the only enzymes known to revert sulfenic acid back to the thiol state, dysfunction of these enzymes leads to imbalanced redox regulation and irreversible trioxidation of certain core TCA enzymes. MDH1 was identified in our study as a potential ROS sensor that is impaired by Cys137 trioxidation in the brain tissues of old mice. Our experiments provide new insights into how redox signals mediated through cysteine oxidation and the potential mechanisms for cellular energy decline in the aging process.

2.6 Conclusion

In this study, TMT6 labeling and label free LC-MS/MS methods were applied using mouse brain obtained from young, middle and old groups. Proteome analysis focus on TCA cycle proteins and cellular cysteine redox regulations are performed in the discovery phase. Eight important proteins including SUCLA2, IDH1, IDH2, NDUFS3, MDH1, PDHB, SDHB and FH1 as subunit of TCA cycle enzymes are found to be up- or down-regulated in expression level. The imbalance of TCA cycle proteins are further confirmed with the western blot validation. Among the ROS affected proteins, MDH1 was identified with trioxidation at Cys137 exclusively in old group. The 3D simulation and predication provided the structural foundation on the functional loss which if subsequently proved by the enzymatic activity assay. As

cells build up various redox regulation to maintain the ROS and antioxidants balance, the severe trioxidation illustrated the failure of redox regulation. Grx and Trx as the desired enzymes to reduce the reversible cysteine oxidation and prevent hypderoxidation were found to be obviously down regulated in aged groups. The decreased expression on these two proteins indicated the dysregulation of cellular antioxidants defense system. Our study provides new insight into the cellular redox regulation and TCA induced energy decline in aging process. The novel trioxidized peptides has great diagnostic values and possible utility in the treatment and management of ROS associated diseases, warrants further examinations in a valid outcome study.

**Chapter 3. Accumulation of irreversible
trioxidation in aged skin dysregulates
antioxidants enzymatic activity and cell-cell
junctions**

3.1 Abstract

Oxidative stress in skin plays a major role in intrinsic aging and even more for extrinsic aging. Skin is the unique tissue worthwhile for discussion because it has higher ROS content comparing with other organs due to the ROS originating from both external and internal sources. A reduced barrier function observed in aging is regarded as the predominant molecular syndrome in skin. The barrier of the skin disruption induced by loss of specific cell adhesion proteins during aging is due to modification in cell junction structure or function. Skin proteins are prone to be oxidative modified and the target protein impaired by oxidative stress may result in the reduced barrier function. Protein damages by degenerative protein modifications (DPMs) which refer to spontaneous non-enzymatic post translational modifications (PTMs) such as oxidation and deamidation have long been regarded as mediators of various human degenerative diseases and natural aging. In order to understand the roles of DPMs in aging skin, in this study we conducted a systematic proteomic study in an aging murine model using label free LC-MS/MS proteomics in discovery phase and quantitative multiple reaction monitoring (MRM) LC-MS/MS in validation phase. In skin sample, dynamics studies assigned skin proteins into different cluster with corresponding tendency. Several critical enzymes including Cat, Ckm and Lgals1 are identified as ROS target proteins which carried high level protein oxidation. Cat as the typical antioxidant defense enzyme against high ROS burden are found to be heavily trioxidized at Cys425 in late age stages including 18 and 24 months. These irreversible modifications of this Cat may leads to affected antioxidant ability and subsequently cause more terminal sulfonic acid (Cys-SO₃H) of other important proteins. Further evaluation of proteomic data identified exclusive accumulation of cysteine trioxidation in two metabolic enzymes associated with cell junction Ctnnb1 at Cys381 and Des at Cys332 in the 18 and 24-month age mice. The significant accumulation of the oxidative damaged Cys381 in Ctnnb1 and Cys332 in Des were confirmed by targeted MRM approach.

3.2 Introduction

3.2.1 Function of skin

Skin is the most obvious and largest tissue of the body. Skin accounts for approximately 15% of the total adult body weight as it covers the whole external surface of the body and the mucous membranes lining the body's orifices. The first and main role of skin is to build a barrier between the body and the outside environment. This barrier prevents not only entry of toxins and bacteria from the external but also water and electrolyte loss from within the body. The necessity of the skins barrier is already fully demonstrated in conditions where the barrier is ruptured in premature babies where the barrier is incomplete [223] or in victims of severe burns [224]. These individuals are at significant risk of dehydration and infection due to the dysfunction in the barrier. Correspondingly, the skin has established a robust immune response and a remarkable ability to repair the barrier quickly once the breaches occurred. Besides its barrier function, skin also has several other important functions including regulation of temperature, sensation, excretion and synthesis of vitamin D. Skin is also important in a social context as it is part of the mechanism by which we recognize people.

Aging studies has concentrated on a series of milestone finding that dates back to 1956. During this year Denham Harman raised that reactive oxygen species (ROS) accumulate over time and are a major contributor to the aging process [225]. By identifying mitochondria as the main source of endogenous ROS, this concept was further broadened 16 years later. This finding regarding the roles of mitochondria and ROS formed the foundation for the free radical theory of mitochondrial [226]. However, opposing evidence does exist, especially as the experiment's results showed that SOD+/- mice had a clear increase in the ROS load but quite a normal lifespan[227]. It is not surprising that in the last decade, serious doubts arose over whether or not ROS are in fact the most important factor driving aging. However, if the oxidative stress theory of aging is to be held true in any organ of the human body it is in the skin. Besides the fact that ROS load in the skin is higher than in any other organ, the skin is also the only organ where damage caused by exogenous ROS is at

least as significant as endogenous ROS[228]. For the skin, damage accumulated in the extrinsic aging process accounts for a high percentage of the total deleterious effects.

Extrinsic aging is described as a result of environmental influences, while intrinsic aging focuses on genetic factors, biochemical and physical changes that occur during the normal aging process [229]. The skin is the organ where extrinsic aging is more important than intrinsic aging, as only three percent of all skin aging factors have a genetic background [230]. UV-radiation is one amongst many other environmental factors that influence skin aging [231]. The photoaging derived from UV-radiation in skin shared the similar biological meaning with extrinsic aging. Aging leads to many changes to the skin in both epidermal as well as dermal skin layers: the skin becomes rougher, dryer and slacker, the loss of the elastic tissue (elastin) in the skin with age causes the skin to hang loosely, the skin is more likely to develop lesions such as various skin diseases and skin tumors. The sensitivity of skin also drops due to the downregulated production of sex hormones and a decreased number of nerve-endings. In effect, the skin gradually loses its function as the first barrier protecting us from the environment [232-234].

Human skin essentially consists of two layers: The dermis below and epidermis on above. These two layers are attached to each other via the basal lamina. The consequences of aging are presented differently on the dermis and epidermis. In the dermis, the disorder of extracellular matrix (ECM) derived from either extrinsic aging or intrinsic aging obtained the most attention as the key factor in aging related dermis disruption. ECM enzymes have different functions in the processing of elastic fibers, collagens, and proteoglycans [235, 236]. Elastic fibers are bundles of protein that exist in the ECM and they are formed by the elastic microfibril, which consists of numerous proteins such as microfibrillar-associated glycoproteins, fibrillin, fibullin, the elastin receptor, and amorphous elastin[237]. Fibrillins and fibrillary collagens are glycoproteins with branched oligosaccharides whereas proteoglycans consist of one long, unbranched glycosaminoglycan side chain. The dermal network is formed with all glycoproteins connected with each other via hyaluronic acid[238].

Collagen I and III produce long collagen fibrils that are intertwined to form an intra-dermal net which is anchored to the dermal-epidermal junction by collagen VII [239, 240].

Extrinsic skin aging results in the drastic loss of collagens I, III and VII, and the functional network formed by long collagen, elastic fibers and glycoproteins becomes unorganized and unstructured. This uncoordinated dermal cluster is further exacerbated by inflammation or UV exposure that cause neutrophils to relocate to dermis [241]. The protein matrix metalloproteases (MMPs) play important roles in collagen and elastic fiber degradation, which are major components of the ECM. The heavy upregulation of MMPs and corresponding downregulation of their inhibitors, TIMP1 and 3, are hallmarks of skin aging [242, 243]. The degradation of ECM components are promoted by ECM-turnover [244]. Exogenous UV-radiation generates ROS and also activates surface receptors including MAP-kinase p38, c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) [245]. MMP1, the only enzyme to cleave collagen, MMP9, which is able to degrade elastic fiber, and MMP3 is highly expressed as a downstream effect of activation of the transcription factor activator protein 1 (AP-1). AP-1 is also responsible for inhibiting the collagen production catalyzed by TGF- β [246]. The AP-1 mediated signaling pathway leads to the overall degradation of the ECM including the different type of collagen and elastic fibers. Photoaging is also the biggest risk factor for various kind of skin diseases. Extrinsic stimuli such as UV-irradiation may induce skin cancer through mutations in the tumor suppression gene, p53 [247]. Another age-associated skin disease is a vitiligo-similar appearance. The visible white spot results from idiopathic guttate hypomelanosis and lighter tanned skin is a common symptom in aged patients [248, 249]. The ROS imbalance in skin causes the impairment of either melanin transport to keratinocytes or impairment of the differentiation and survival of melanocytes, subsequently leading to skin disease [250-252].

3.2.2 ROS production in skin

Enzymatic ROS production occurs from the mitochondrial electron transport chain, NADPH oxidases, xanthine oxidoreductase (XOR), several peroxisomal oxidases, enzymes in the cytochrome P450 family, cyclooxygenases, and lipoxygenases. These enzymes are an important source of ROS in all organs including the skin. The generation of ROS, enzymatically or non-enzymatically, in the cell are manifold. Endogenous ROS generation as discussed in the general introduction is exemplified in skin tissue. In addition to the endogenous ROS production the skin ROS induced by UV irradiation are the main contributors to the aging of skin. UVA, in the range of 320–400 nm, drive most of the ROS generation due to its ability to penetrate the deeper layer of epidermis. The alterations caused by UVA in skin dermis are responsible for the deleterious progression of photoaging [253]. UVA light will be absorbed by cellular chromophores like urocanic acid, riboflavins, melanin, bilirubin, heme, porphyrin, and pterins [254, 255]. Two reactions will follow after the appearance of chromophores in the excited state: further excitation to a triplet excited state or falling back to ground state. The triplet state will actively react with molecular oxygen to produce ROS or modify DNA [256]. These reactions will result in cellular mtDNA damage and initiate the mitochondrial vicious cycle [257]. Besides UVA, UVB is another contributor to photoaging. Unlike UVA which can reach the deeper dermis, UVB only has the capacity to penetrate till the epidermis and thus mainly affects keratinocytes and melanocytes. However, the UVB can directly react with DNA and cause damage to cells without acting via photosensitizers [254]. Moreover, the near- infrared light also affect both the epidermis and dermis. Near- infrared light is even involved in the mitochondrial ETC and triggers the complex IV ROS leakages. Based on these molecular mechanisms, UV and infrared light lead to cellular energy impairment and other morphological and functional alterations.

3.2.3 Antioxidants defense system in aging

Skin has developed various ways to prevent ROS damage with its own antioxidant system. The formation of the cornified envelope is a useful way and its function is fully demonstrated with the fact that the ROS load is higher in the

epidermis than in the dermis[258]. The cornified envelope gradually increases its density to be fully assembled in the stratum corneum. The antioxidant ability of cornified envelopes depends on SPRR proteins that quench ROS by forming intramolecular disulfide bonds with its over-proportional enrichment in cysteines. The non-enzymatic antioxidants including vitamin C, Vitamin E, Beta-Carotene and CoQ10 also play an important role in the skin. The water soluble vitamin C is an electron donor and thus can react with a potential dangerous free radical to oxidize itself and form “semidehydroascorbic acid”. In this way, vitamin C can convert ROS to less reactive products and prevent the oxidative damage caused by UV-radiation [259]. Another vitamin with antioxidative capacities is α -tocopherol/vitamin E, which exists in the cell membrane [260]. Due to its lipophilicity, Vitamin E can stop lipid peroxidation by reducing the lipid peroxy radical to hydroperoxide. α -tocopherol is also involved in mediating different signaling pathways, including the protein kinase C pathway[261]. Vitamin E that is absorbed by skin is responsible for the decrease of MMP transcription levels and photocarcinogenesis risk. Beta-carotene is a typical antioxidative carotene in skin which cannot be synthesized by primates and has to be taken up by food from plants. It has the ability to reduce ROS production by inhibiting lipoxygenases. The last antioxidant discussed here is CoQ10. Lipid soluble CoQ10H₂ is also involved in the prevention of lipid peroxidation, but in a less effective way [262].

The antioxidant defense system involves the regulation of reactive oxygen species by different enzymes: superoxide dismutases, catalases, glutathione peroxidases, ferritin, and peroxiredoxins. Superoxide dismutases are the most influential enzyme in the control of ROS with its role in converting dismutate superoxide to hydrogen peroxide. Three isoforms of this protein, SOD1, SOD2 and SOD3, are present in different subcellular locations. SOD1 is in the cytosol, SOD2 is in the mitochondria and SOD3 is found in the extracellular space. Experiments have demonstrated that a deletion of superoxide dismutase will cause severe skin aging with dramatic skin atrophy and is even lethal in mice. Sod1^{-/-} and Sod2^{-/-} mice both showed the obvious skin aging phenotype comprised of a reduced amount of procollagen I, a thinning of the epidermis, clear atrophy of the dermal connective

tissue, and atrophy of the subcutaneous fat tissue [263-265]. The tripeptide glutathione GSH bearing thiol group and a special gamma peptide linkage are the next antioxidants. As mentioned in the previously under cysteine mediated regulation, the formation of GSSG from two GSH via disulfide bonds under high oxidative stress prevents further hyper oxidation. GSSH and GSH are both reversible and can be recovered with the catalysis of glutathione reductase. The higher GSSG: GSH ratio in aged mice skin compared to young skin indicate the role of GSH antioxidants in maintaining the skin ROS balance [266]. The protein ferritin, which stores iron, is the main contributor to concealing iron ions from the Fenton reaction. The level of ferritin in the epidermis or dermis is increased to match increased UV irradiation. Thus, ferritin plays key role in the regulation of redox homeostasis[267]. The next class of enzymes with antioxidative capacity is the peroxiredoxins which identify six isoforms. PRXD6 expression is induced after UV-irradiation and the increased PRXD6 will help to decrease lipid oxidation. Meanwhile the loss of prxd6 will increase the skin tumor risk [268].

Catalase (Cat) is a very prominent enzyme involved in detoxifying hydrogen peroxide to generate water and molecular oxygen. The catalase expression is located in the stratum corneum of in skin and the absolute amount of this enzyme is greater than that of the SOD enzymes. The Cat protein has four identical polypeptide chains that each contain a heme group. The first step of the detoxifying reaction involves formation of an oxoferryl porphyrin cation radical after the heme group reacts with hydrogen peroxide. A water molecule is also produced during this process. The cation radical is very reactive and immediately undergoes the second reaction to react with another hydrogen peroxide and produce water and molecular oxygen. The heme group is also regenerated after finishing the task [269]. A remarkable experiment has shown that the activity of this enzyme in the dermis and epidermis alters the aging process in the opposite direction. The catalase activity increases in epidermis with an increased load of ROS in the skin. However, the reduction of enzymatic activity in the dermis is still not well understood [270].

3.2.4 Cell Junction

Cell junctions consist of multiprotein complexes that enable communication between neighboring cells or between a cell and the extracellular matrix. They also build up the paracellular barrier of epithelia and control the paracellular transport. Cell junctions are especially abundant in epithelial tissues, such as skin. In vertebrates, there are three major types of cell junction: Adherens junctions, Gap junction and tight junction.

Tight Junctions (TJs) is branching network of sealing strands. Much of what is currently known about Tight Junctions has come from work in simple epithelia such as the gut. In simple epithelia, adjacent cells are linked together by the junctional complex which consists of desmosomes and the adherents complex, as well as TJs. Adherents junctions and desmosomes are responsible for cell adhesion whilst TJs are responsible for intercellular sealing. Tight junctions exist at the most apical end of the junctional complex where they form a physical barrier to free passage of molecules through the so called paracellular pathway i.e. the pathway between epithelial cells. This barrier maintains normal organ function and also prevents systemic contamination by microbes and toxins existing in the surrounding environment [271].

3.3 Materials and Methods

3.3.1 Reagent and chemicals

Unless individually indicated in the text, all the other chemicals and HPLC-Graded Solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade-modified trypsin was from Promega (Promega).

3.3.2 Animal

C57BL/6NTac mice of four different age group (1 month, 6 months, 18 months & 24 months) were purchased from InVivos Pte Ltd (SG, Singapore). The animals were housed in temperature and humidity-controlled cages under a 12h dark/light cycles at 21 °C and fed with standard commercial chow. The animals were allowed to adapt to these conditions for a minimum of two weeks prior to use in the

experiments. All procedures performed were approved by the Nanyang Technological University Institutional Animal Care & Use Committee (NTU-IACUC) and were carried out in strict accordance with the International Guiding Principles for Animal Research.

3.3.3 Sample Preparation

Mice of both genders from four different age groups were used in this research. The mice were anaesthetized by administering isoflurane by inhalation for the duration of the experimental procedures (IsoFlo, VeterinariaEsteve, Bologna, Italy). Skin tissue samples of 50mg were extracted from the dorsal region of each mice with the fur being thoroughly removed prior to washing with 1X Phosphate-Buffered Saline (PBS). Liquid nitrogen was used to flash-freeze the tissue samples before being mechanically fragmented into powder with the use of mortar and pestle. The samples were then dissolved in lysis buffer (2% Sodium dodecyl sulfate (SDS); 100mM Ammonium Acetate (AA); pH 7) and then homogenized using the bullet blender homogenizer (Next Advance) with mixed beads of size 0.9 to 2.0 mm. Most proteins were extracted after repeating three to four cycles of homogenization until no visible pellets could be observed. Acetone precipitation was performed to extract the total protein from the dissolved samples and remove any interfering substances. Chilled Acetone (-20°C) was added into the sample in a 4:1 (v: v) acetone-sample volume ratio and incubated at -20°C for 4 hours to precipitate the protein content from the samples. The samples were then centrifuged at 12,000g for 20 mins at 4°C to pellet the precipitated protein. The protein pellets were re-dissolved in buffer B (1% Sodium deoxycholate (SDC); 100mM AA) via the use of bullet blend homogenizer and the protein concentrations were measured using the BCA protein Assay (Thermo, Rockfold, IL). Protein samples of 1.5mg were pooled based on protein concentration from mice of the same age group and subsequently used for in-solution tryptic digestion.

3.3.4 Sodium Deoxycholate assisted In-solution tryptic digestion

In-solution tryptic digestion was performed as previously described except for minor modifications [272]. The samples were diluted five times with buffer B and

the skin tissue proteomes were reduced in the presence of 10mM Dithiothreitol (DTT) for 30 mins at 60°C. Iodoacetamide was added for a final concentration of 20mM to facilitate alkylation of the proteomes while samples were incubating at room temperature for 1 hour in the dark. The samples were further diluted with 100mM AA to lower the concentration of SDC to 0.5%. Tryptic digestion was carried out overnight at 37°C at a 1: 50 (w/w) enzyme-to-protein ratio. 10% formic acid (FA) was added to the sample to quench the reaction as well as to precipitate SDC out from its acidic environment. The precipitated SDC was pelleted by centrifugation (12,000 x g; 20 mins; room temperature) and the supernatant containing the peptides was transferred into a new tube. The pellets were re-dissolved in 100 mM AA and 10% FA was added again to re-precipitate the SDC content prior to another centrifugation to facilitate and improve peptide recovery. This process was performed two more times and all supernatant was collected into a single tube. Desalting was performed using the C-18 Sep-pack 100mg cartridge (waters, Milford, MA, USA) to remove any impurities from the samples and the elutes were dried using a vacuum concentrator (Eppendorf, Hamburg, Germany) to collect the peptide.

3.3.5 High-pressure Liquid Chromatography (HPLC) fractionation

Desalted peptides were fractionated by high-pressure liquid chromatography (HPLC) as previously described. The dried peptides collected were reconstituted in 200µl mobile phase A (85% ACN, 0.1% Acetic Acid) and fractionated using a Luna® 3 µm NH₂ 100 Å, LC Column 250 x 4.6 mm column (Phenomenex, Torrance, CA, USA) on a Shimadzu Prominence UFLC system (Dionex, Sunnyvale, CA, USA) with UV monitoring of peptide intensities at 280nm performed throughout. Peptide separation was carried out at 1ml/min using a 60 min gradient as follows: 100% mobile phase A for 5 min, mobile phase B (0.1% FA in HPLC water) was increased until achieving 16% in 30 min, 16-50% for 10 min, 50 – 100% for 10 min, and finally maintenance d at 100% B for 5 min. Fractions were collected at 1min intervals and combined by concatenation. Concatenated fractions were then completely dried in the vacuum concentrator.

3.3.6 LC-MS/MS

The dried peptide fractions were reconstituted in mobile phase A (3% ACN, 0.1% FA) to a final peptide concentration of approximately 1.0 ug/ul prior to peptide analysis by LC-MS/MS and a Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA). Peptide separation were carried out at a flowrate of 300 nL/min using a Dionex EASY-Spray 75 $\mu\text{m} \times 10\text{ cm}$ column packed with PepMap C18 3 μm , 100 Å (Thermo Fisher Scientific) while maintaining a working temperature of 35 °C. Liquid chromatography was performed using a 60min gradient established by mobile phase A (0.1 %FA in HPLC water) and mobile phase B (0.1% FA in ACN) as follows: 3-35 % B for 40 min, 35-50 % B for 7 min. 50-80 % B for 1 min, 80% B for 2 min, and finally re-equilibration at 3% B for 10 min. Peptide separations were analyzed at an electron-spray potential of 1.5 kV. A full MS scan (350-1,600 m/z range) was obtained at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set at 15 s. The resolution of the higher energy collisional dissociation (HCD) spectra was set to 17,500 at m/z 200. The automatic gain control (AGC) setting of the full MS scan and the MS2 scan were 3E6 and 2E5, respectively. The 10 most intense ions with a threshold of 2,000 counts were fragmented in HCD mode with a maximum ion accumulation time of 100 ms and normalized collision energy of 28 %. An isolation width of 2 was used for MS2. The mass spectrometry data acquisition was performed in positive mode to identify peptides and post-translational modifications (PTM). Single and unassigned charged ions were excluded from MS/MS. For HCD, the underfill ratio was defined as 0.2%.

3.3.7 Mass Spectrometric Data Analysis

Proteome and peptide spectra were identified and their respective quantifications were calculated out using an in-house Mascot Server (version 2.6.02, Matrix Science, MA). The fragment ion tolerance was set as 0.05 Da. Variable modifications of the Mascot database search were as follows: Trioxidation of Cysteine; Deamidation of Asparagine and Glutamine; AGEP_Carboxyethyl of lysine and Carbamidomethylation of Cysteine; The MS/MS spectra in raw files were converted to mascot generic files generated and that were searched against the

UniProt mouse database (91089 sequences and 38788886 residues) for protein identification and DPMs analysis.

3.4 Results

3.4.1 Workflow D Workflow for label-free quantitative proteomics of skin derived from mice at different ages.

For the accurate determination of global protein and oxidation protein profiles of mice skin, the proteomics sample preparation is a major factor that needs to be well-thought-out. Some studies have shown that some types of artificial modification of proteins does not occur at low pH (below 6), yet can be induced by sample preparation in the presence of urea. In efforts to minimize artifactual trioxidation and deamidation, the use of urea and high pH in sample protein preparation in this study was avoided. Briefly, as depicted in Figure 3.4.1, skin samples were collected from the dorsal region of mice from four different age groups. The skins were fragmented mechanically into powder via the use of mortar and pestle with the assistance of liquid nitrogen to flash-freeze the samples first. The proteins were extracted after multiple rounds of bullet blender homogenization with beads. Skin proteins were solubilized in SDS containing lysis buffer. Acetone precipitated skin proteins were again solubilized in SDC containing lysis buffer and were reduced, alkylated and subsequently enzymatically digested into peptides with trypsin. The whole process was performed at pH lower than 6. The tryptic peptides were fractionated with low pH ERLIC HPLC with a NH_2 column, followed by desalting using a C18 cartridge. The desalted elutes were dried under vacuum. Finally, dried peptides were analyzed in triplicates by LC-MS/MS and data generated was analyzed using bioinformatics tools. After data analysis, several protein targets were selected as trioxidation candidates and were further verified and quantified by MRM.

Figure 3.4.1 Workflow of label-free quantitative proteomics. Schematic workflow of label-free quantitative proteomics workflow of protein extraction from pooled skin samples obtained from mice of different ages including 1 month, 6 months, 18 months and 24 months.

3.4.2 Assessment of skin label free proteomics data quality

To assess the reliability of label free data quantitation in this study, the respective emPAI value for each protein, which indicates the abundance of each protein, was calculated. The correlation of measured emPAI for each protein between replicates of each age group was examined on scatter $\log_2$ plots as depicted in Figure 3.4.2. The data was examined in all four age groups separately. The generated Pearson correlation coefficients (r) and accompanied p values for each scatter $\log_2$ plot of measured emPAI values between 01A and 01B (Figure 3.4.2 A,

$r = 0.9720$, $p < 0.0001$), between 01B and 01C (Figure 3.4.2 B, $r = 0.9711$, $p < 0.0001$), and between 01A and 01C (Figure 3.4.2 C, $r = 0.9743$, $p < 0.0001$), indicate consistency of measured emPAI between the triplicates. Correspondingly, good reproducibility was also observed in scatter $\log_2$ plots of measured emPAI values between 06A and 06B (Figure 3.4.2 D, $r = 0.9789$, $p < 0.0001$), between 06B and 06C (Figure 3.4.2 E, $r = 0.9209$, $p < 0.0001$), and between 06A and 06C (Figure 3.4.2 F, $r = 0.9224$, $p < 0.0001$). Likewise, statistically significant correlations of measured emPAI between 18A and 18B (Figure 3.4.2 G, $r = 0.9789$, $p < 0.0001$), between 18B and 18C (Figure 3.4.2 H, $r = 0.9786$, $p < 0.0001$), and between 18A and 18C (Figure 3.4.2 I, $r = 0.9838$, $p < 0.0001$) were detected among the triplicates. Last but not least, statistically significant correlations of measured emPAI between 24A and 24B (Figure 3.4.2 J, $r = 0.9802$, $p < 0.0001$), between 24B and 24C (Figure 3.4.2 K, $r = 0.9806$, $p < 0.0001$), and between 24A and 24C (Figure 3.4.2 L, $r = 0.9834$, $p < 0.0001$) were detected among the triplicates. The use of low pH SDC assisted skin processing protocols and use of a stable analytical system for mice skin proteomics led to the minor system and quantitation variability observed here, thus confirming the reliability and confidence of our presented label free dataset.

Figure 3.4.2 Correlation assessment between skin label free triplicate experiments. Correlation assessment of measured emPAI values for each protein (FDR < 1%) between triplicates. (A) Scatter log₂ plots of measured emPAI values of proteins identified in 01A and 01B. (B) Scatter log₂ plots of measured emPAI

values of proteins identified in 01B and 01C. (C) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 01A and 01C. (D) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 06A and 06B. (E) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 06B and 06C. (F) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 06A and 06C. (G) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 18A and 18B. (H) Scatter $\log_2$ plots of measured emPAI values of proteins identified in 18B and 18C. (I) Scatter $\log_2$ plots of emPAI values of proteins identified in 18A and 18C. (J) Scatter $\log_2$ plots of emPAI values of proteins identified in 24A and 24B. (K) Scatter $\log_2$ plots of emPAI values of proteins identified in 24B and 24C. (L) Scatter $\log_2$ plots of emPAI values of proteins identified in 24A and 24C. 01A, 01B and 01C replicate A, B and C in 1 month mice; 06A, 06B and 06C replicate A, B and C in 6 month mice; 18A, 18B and 18C replicate A, B and C in 18 month mice, 24A, 24B and 24C replicate A, B and C in 24 month mice. r , Pearson correlation coefficient, ****, extremely significant.

3.4.3 Expression profiling of the skin proteins in different age group

The expression of quantified skin proteins obtained from the combined search results were profiled to gain an understanding of skin protein involvement in different ages of mice, which is at present unknown. Firstly, we analyzed protein expression in the young group (1 month) and the old group (24 month) as these two groups have the most obvious age difference. The volcano plot, which displays and uses both the FC and t-statistic applies the reliable double filtering criterion corresponding to cutting out of two rectangular corners away from the origin (Fig.3.4.2). The proteins labeled with black could not be selected as even proteins with large fold-change but nevertheless an insignificant test result may be outliers with very large values in one group. The proteins in the gray shaded area were also not considered because proteins with a significant test result (small t-test p-values) but low fold change could be a false signal caused by low variance. This would mean that these genes are not of biological significant and not in need of further examination. The y-axis is an adjusted p-value based on $-\log_{10}$ calculated from the t-test and the cut-off line is the p-value equal to 0.05. The x-axis represents fold change and is presented in $\log_2$ format. Protein are considered as upregulated with the $\log_2$ FC more than or equals to 1 and downregulated with the value less or equal to -1. After the analysis of the volcano scatter plot for proteins in skin from mice

aged 24 months and 1 months, 851 proteins were located in the right upper corner labeled in red and 163 proteins were located in left upper corner labeled in green. This result indicates that more proteins are upregulated with age.

Figure 3.4.3 Volcano plot showing differential expression in 24 months and 1 month old mice skin. The x-axis is the log₂ ratio of gene expression levels between these two stages; the y-axis is the adjusted p-value based on $-\log_{10}$. The colored dots (L1=green) and right (L3i=red) represent the differentially expressed genes compared to $p < 0.05$ (Represented by black horizontal line) and 2-fold expression difference (represented by two black vertical lines).

3.4.4 Age dependent dynamics of mice skin protein changes associated with specific pathways

The dynamic profiles of proteins from four different age groups were grouped using a fuzzy c-means clustering approach. Two of the profiles groups of the proteins did not display any significant changes among ages. The proteins that displayed profile changes were grouped into three different profiles as shown in Figure 3.4.4 A–C. Figure 3A–N depicts each of the profiles along with examples of several

proteins within each. The protein abundance changes identified were considered to be present as a consequence of aging. There were a number of proteins from specific pathways or certain cellular mechanisms that were assigned to the same cluster. To discern functional processes or cell-type specific patterns of change, we grouped proteins into a total of five clusters using the fuzzy c-mean method using R studio software. It is found that age dependent changes in proteome composition affect a range of signaling pathways and mechanical processes. As some proteins were not significantly changed on the expression level, two of the clusters which did not show a clear tendency for expression changes were not discussed.

The significantly changed proteins were assigned to three clusters. The first cluster in Figure 3.4.4 A, is comprised of proteins associated with older age. The expression levels of these proteins drastically increased at the age of 24 months. This first cluster of proteins are predominately associated with carbon metabolism including in the TCA cycle, oxidative phosphorylation, and other related processes. For example, pyruvate dehydrogenase E1 alpha 1 subunit (Pdha1) are the proteins involved in catalyzing the overall conversion of pyruvate to acetyl-CoA and CO₂, and they provide the primary link between glycolysis and the tricarboxylic acid (TCA) cycle. The expression change curve of Pdha1 is shown in Figure 3.4.4 F. Catalase (Cat) serves to protect cells from the toxic effects of hydrogen peroxide as it converts hydrogen peroxide to water and molecular oxygen. It also promotes growth of cells, including T-cells, B-cells, myeloid leukemia cells, melanoma cells, mastocytoma cells and normal and transformed fibroblast cells. Cat expression is increased in an age dependent manner. Transaldolase (Taldo1) is involved in step 2 of the sub-pathway that synthesizes D-glyceraldehyde 3-phosphate and beta-D-fructose 6-phosphate from D-ribose 5-phosphate and D-xylulose 5-phosphate[273]. The expression of Taldo1 is increased as shown in Figure 3.4.4 G.

The second cluster in Figure 3.4.4B, is comprised of proteins that changed in an age dependent manner. These proteins showed a constant high increase in expression level as age increased. Proteins associated with skeletal muscle development and other regions are also present in this cluster. Four and a half LIM domains of protein

1 (Fhl1) are involved in muscle development or hypertrophy. Diseases associated with FHL1 include Myopathy, X-Linked, With Postural Muscle Atrophy and Scapuloperoneal Myopathy, X-Linked Dominant. Fhl1 was shown to not be present in one month old mouse skin but showed a large increase in an age dependent manner (Fig 3.4.4 I). Galectin-1 (Lgals1) expressed in skeletal muscle is another protein in this cluster and the curve shows that it is significantly upregulated especially in 18 months and 24 months old mice skin.

Last but not least, the third cluster in Figure 3.4.4 C also demonstrate protein change in an age dependent manner, however, the differences between group are not as large as in the second cluster. Many of these proteins are extracellular matrix proteins involved in cell junctions, including the gap junction, tight junction and cell adherent. Catenin beta-1 (Ctnnb1) is one of the important proteins in tight junctions. It plays a role in the regulation of cell adhesion as a component of the E-cadherin: catenin adhesion complex. The decline in Ctnnb1 is consistent with an age-related deterioration of cell to cell adhesion after 6 months (Fig 3.4.4 M). Annexin A7 (Anxa7) is a calcium/phospholipid-binding protein that promotes membrane fusion and is involved in exocytosis. Anxa7 is upregulated continuously as age increases (Fig 3.4.4 K). Integrin beta-4 (Itgb4) is another component of cell junctions. Itgb4 is a receptor for laminin and integrin and mediates cell-matrix or cell-cell adhesion. Its expression level was found to be increased 18 months but was slightly decreased in 24 months old mice (Fig3.4.4L). Laminin Subunit Beta 2 (Lamb2) belongs to a family of extracellular matrix glycoproteins. Laminin are the major non-collagenous constituents of basement membranes. They have been implicated in a wide variety of cell adhesion processes. Lamb2 was shown to be upregulated until 18 months of age and then maintained a constant level for the following months of life (Fig 3.4.4.N). This unstable alternation of cell junction proteins indicates the disturbance in cell communication between neighboring cells via specialized proteins.

Figure 3.4.4 Skin protein dynamics profiles from four different age groups of mice. (A–C) Three distinct time-course clusters identified by fuzzy c-means clustering of the time series data for all proteins. The selected proteins and their change tendency in D–N are typical of the three clusters found in panels A–C. A represents the carbon metabolism associated proteins cluster and others. D-Cat, E-Dsp, F-Pdha1 and G-Taldo1 are the example proteins in this cluster. B represents the

skeletal muscle cluster proteins and others. H-Des, E-Fhl1 and I-Lgals1 are the example proteins in this cluster. C represents cell junction proteins and others. K-Annax7, L-Itgb4, M-Ctnnb1 and N-Lamb2 are the example proteins in this cluster. Warm (red) and cold (blue) colors indicate low and high deviation from the consensus profile, respectively. Two-way ANOVA, * $p=0.005$, *** $p=0.003$, **** $p<0.0001$.

3.4.5 Detection of trioxidation skin proteins with LC-MS/MS

In the sample preparation process, alkylation of IAA induce carbamydomethyl modification at the cysteine site. If the cysteine residue is irreversibly oxidized in the biological system, it will not be able to be further modified by IAA. Cysteine trioxidation (+48 Da) and cysteine carbomydomethyl (+57.0106 Da) have a mass difference of approximately 9 Da, and this mass shift can easily be differentiated in LC-MS/MS based proteomics analysis. Advances in proteomic techniques have afforded the assessment and quantitative evaluation of site-specific trioxidation on specific protein targets for peptide biomarker studies. Following these criteria, some unique trioxidized peptides and trioxidized sites in proteins were detected in skin of mice in the late age stage. Several proteins were found to be exclusively modified in 24 months old mice and the others present only in 18 and 24 months old mice.

Catalase (Cat) is one of the proteins found to carry trioxidation in the late age stages, 18 months and 24 months) but are not present in 1 and 6 months old mice skin. Cat serves to protect cells from the toxic effects of hydrogen peroxide as it converts hydrogen peroxide to water and molecular oxygen. The result indicates that the damage of Cat which is a key antioxidant can induce a vicious cycle of oxidative stress and accelerate the tissue dysfunction. It also promotes growth of cells, including T-cells, B-cells, myeloid leukemia cells, melanoma cells, mastocytoma cells, and normal and transformed fibroblast cells. According to the cluster analysis in chapter 3.4.4, Cat expression is increased in an age dependent manner. Cat was found to be trioxidized at Cys425, which is consistent in mice of both 18 and 24 months. The MS/MS spectrum allows for the determination of the modified site location as well as of the target peptide sequences (Fig 3.4.5.1). Meanwhile, it also provides the retention time and m/z ratio for the extraction of ion chromatogram based quantitation of trioxidation with high mass accuracy. The Cat targeted peptide,

A

Figure 3.4.5.1 MS2 spectrum of Cat peptide SALEHSVQCAVDVCR under trioxidation or carbamydomethyl. A. SALEHSVQCAVDVCR trioxidation at Cys425, which is identified by y7. B. SALEHSVQCAVDVCR non trioxidation peptide spectrum as reference. The spectrum were generated from a mascot search result.

Galectin-1 (Lgals1) is another protein found to be trioxidized in the late age stages, 18 months and 24 months, but not in skin from mice 1 and 6 months of age. Lgals1 is the lectin that binds beta-galactoside and a wide array of complex carbohydrates. Lgals1 plays an important role in regulating apoptosis, cell proliferation and cell differentiation. It also inhibits phosphatase activity of the CD45 protein and therefore, the dephosphorylation of Lyn kinase [274]. According to the cluster analysis in chapter 3.4.4, Lgals1 expression is increased in an age dependent manner and the slope representing change increases at late age stages. Protein Lgals1 was found to be trioxidized at Cys43, which is consistent in both 18 and 24 months old mice. The MS/MS spectrum allows for the determination of the modified site location and of the target peptide sequence (Fig 3.4.5.2). It also provides the retention time and m/z ratio for the extraction of ion chromatogram based quantitation of trioxidation with high mass accuracy. The Lgals1 targeted peptide, DSNNLCLHFNPR, had a retention time of 34.3 min during mass spectrometry elution, and the precursor m/z ratio was 739.3281 with two charges. The spectrum indicates that DSNNLCLHFNPR was well-fragmented with correct cleavage site.

A

B

Figure 3.4.5.2 MS2 spectrum of Lgas1l peptide DSNNLCLHFNP under trioxidation or carbamydomethyl. A. DSNNLCLHFNP trioxidation at Cys43 which is identified by y7. B. DSNNLCLHFNP non trioxidation.

3.4.6 MRM validation of trioxidation peptides in late age stages

To further increase the reliability of the final candidate list of high level oxidation targets, we combined high specificity MRM analysis with reverse phase column to verify potential targets from the discovery phase. The precursor ion was set for the Q1 selection. At least four of the most intense transitions were extracted to set the Q3 double filtering selection for MRM. The retention time with ± 5 minutes was used to determine the final time window. After the MRM verification and confirmation experiments, peptides not able to pass the criteria for the Q1 and Q3 will be excluded. The protein and peptides information used to MRM validation was presented in table 3.4.6.

The first protein positively verified by MRM was Cat, and the precursor ion m/z value (563.9457+++) was extracted from MS1 while MS/MS data was obtained for selected fragmentations ions (transitions). Four of the most intense transitions, including y7 838.4087, which indicated the modified site for the candidate peptide, were acquired from the MS/MS data. The retention time with ± 4 minutes was regarded as the duration of new time window. As MRM performs double selection based on the MS1 filter as well as the transitions m/z value, another batch of trypsin

digest samples from individual subjects from each age group were analyzed in vantage based on the MRM filtering. The Cat Cys425 trioxidation peptides was positively verified in the 18 months and 24 months age group, which successfully matched with the MS1 filter (+47Da) and formed a co-eluted peak of four at the retention time around 30.7'. The detection also passed the skyline program threshold standard with a high dotp value of 0.89, which indicated high similarity with the library peak format. The representative transitions showed the strongest signal and high reproducibility (Fig3.4.6 AB). In the 1 and 6 months age group, no cysteine trioxidation peak was detectable above noise level. However, the corresponding non-cysteine trioxidation peptide derived from the same protein was set as the internal reference (566.9579). Four of the representative transitions were selected as the representative ions for the measurement as described before. The co-elution peak of all five transitions with a high dotp value (0.92) was identified in all three groups.

Another protein that was verified by MRM was Lgals1. The analysis process was similar to that with Cat. Briefly, the precursor ion m/z value (739.3281++) was extracted from MS1. The non-cysteine trioxidation peptide had an m/z value as 743.8464 as reference. Five transitions, with one y7 838.4087, which represented the modified site, were acquired from the MS/MS data. The retention time of ± 5 minutes was regarded as the duration of the new time window. The Lgals1 Cys43 trioxidation peptides were positively verified in the 18 months and 24 months age group, which simultaneously matched with the MS1 filter and formed a co-eluted peak of four at the retention time around 35.2 and resulted in a high dotp value of 0.88 being obtained. The representative transitions showed the strongest signals and high reproducibility (Fig3.4.6 CD). However, in the 1 and 6 months age group, no cysteine trioxidation peak was detectable above background noise level. Meanwhile, the corresponding non-cysteine trioxidation peptide was positively identified with five most intense transitions selected as the representative ions for measurement as described before.

Creatine kinase M-type (Ckm) plays a central role in tissue energy transduction with its function to reversibly catalyzes the transfer of phosphate between ATP and

various phosphogens. The MS1 precursor ion m/z value (577.3113+++) was obtained from the preliminary proteomics data. Four representative fragmentation ions y_6 793.4454+, y_5 664.4028+, y_4 535.3602+, y_3 422.2762+ are extracted from MS2 spectrum to set as Q3 filtering criteria. The retention time 25' with ± 5 minutes was regarded as the duration of new time window. The Ckm Cys254 containing peptide FCVGLQKIEEIFKK was successfully verified after MRM double selection in only late age stages include 18 and 24 month. The trioxidized peptide co-eluted peak is not formed in 1 and 6 month age mouse skin tissue. The verified peptide is of high confidence and reproducibility with its dotp value 0.87 which indicated the high similarity with the data base library (Figure 3.4.6 EF).

There are more proteins that were also verified with double confirmation, including Fructose-bisphosphate aldolase (Aldoart1), Desmin (Des), and Desmoplakin (Dsp). The determination of specific trioxidation sites on peptides is practical and enables sensitivity of standardized assays that can be developed for clinical usage.. The trioxidation proteins can serve as aging biomarkers, and more importantly, further functional studies based on the candidate list will shed light on the failures of aging related redox regulation and other issues with metabolism that may be studied.

Figure 3.4.6 The MRM verification result in 24 month mice skin. A. The positive co-eluted peak of Cat y7, y5, b5 and b8 at retention time 31.7 was confirmed in the 24months group. B. The dotp value of peak identified in Cat is 0.89 in the 24 months group. C. The positive co-eluted peak of Lgals1 y7, y6, y4, y3 and b5 at retention

time 35.2 was confirmed in 24 months group. D. The dotp value of peak identified in Lgals1 is 0.88 in the 24 months group. E. The positive co-eluted peak of Ckm y6, y5, y4 and y3 at retention time 25.2 was confirmed in the 24months group. F. The dotp value of peak identified in Ckm is 0.87 in the 24 months group.

Table 3.4.6 List of significantly regulated trioxidized peptides and proteins in mice skin

Gene symbol	Protein name	Peptide sequence	Transitions	Age group	Retention time
CAT	Catalase	SALEHSVQC#AVDVKR	C [y7] - 838.4087+ V [y5] - 616.3777+ H [b5] - 538.2620+ Q [b8] - 852.4210+	18, 24 month	30.01
		SALEHSVQCAVDVKR	S [y10] - 1161.6045+ Q [y8] - 975.5040+ C [y7] - 847.4454+ A [y6] - 687.4148+ D [y4] - 517.3093+ V [y3] - 402.2823+ V [b7] - 724.3624+ Q [b8] - 852.4210+	Alkalages	24.7
CKM	Creatine kinase M-type	FC#VGLQKIEEIFKK	E [y6] - 793.4454+ E [y5] - 664.4028+ I [y4] - 535.3602+ F [y3] - 422.2762+ G [b4] - 455.1595+	18, 24 month	25.4
		FCVGLQKIEEIFKK	L [y10] - 1275.7671+ Q [y9] - 1162.6830+ E [y6] - 793.4454+ E [y5] - 664.4028+ I [y4] - 535.3602+ F [y3] - 422.2762	Alkalages	19.1
DES	Desmin	HQIQSYTC#EIDALKGTN DSLMR	K [y9] - 1037.5044+ T [y7] - 852.3880 N [y6] - 751.3403+ S [y4] - 522.2704+ I [b3] - 379.2088+ S [b5] - 594.2994+ T [b7] - 858.4104+	18, 24 month	31.3
		HQIQSYTCEIDALKGTND SLMR	D [y12] - 1320.6576+ A [y11] - 1205.6307+ L [y10] - 1134.5936+ K [y9] - 1021.5095+	Alkalages	29.8

			G [y8] - 893.4145+ L [y3] - 210.1254++ E [b9] - 1147.4837+ A [b12] - 1446.6318+		
DSP	Des moplakin	LTGEC#LGWMR	G [y4] - 565.2551+ W [y3] - 254.6205++ T [b2] - 215.1390+ G [b3] - 272.1605+ E [b4] - 401.2031+ G [b7] - 722.3025+ E [b4] - 201.1052++	18 ,24 m onth	27 .1
			T [y9] - 1109.4867+ E [y7] - 951.4175+ C [y6] - 822.3749+ G [y4] - 549.2602+ W [y3] - 492.2387+ G [y8] - 504.7231++ W [y3] - 246.6230++ G [b3] - 272.1605+ E [b4] - 401.2031+	1, 6 month	28 .2
Ald oartl	Ald oartl - Fructose- bisphosp hate aldolase	ALANSLAC#Q GK	S [y7] - 754.3400+ L [y6] - 667.3080+ A [y5] - 554.2239+ C [y4] - 483.1868+ S [b5] - 457.2405+	18 ,24 m onth	22 .9
		ALANSLACQ GK	A [y9] - 949.4408+ N [y8] - 878.4036+ S [y7] - 764.3607+ L [y6] - 677.3287+ A [y5] - 564.2446+ C [y4] - 493.2075+ A [b3] - 256.1656+ A [b7] - 641.3617+	Al l ages	20 .3
Ctn nbl	Cate nin	LVQNC#LWT LR	W [y4] - 575.3300+ Q [b3] - 341.2183+ C [b5] - 606.2552+ Q [b3] - 171.1128++ N [b4] - 228.1343++	18 ,24 month	31 .4
		LVQNCLWTLR	V [y9] - 1189.6146+ Q [y8] - 1090.5462+ N [y7] - 962.4876+ C [y6] - 848.4447+ W [y4] - 575.3300+ N [b4] - 228.1343++	1, 6 month	31 .4

Myl pf	Myo sin light chain protein 2	NIC#YVITHGDAK	C [y10] - 1154.5146+ Y [y9] - 1003.5207+ I [y7] - 741.3890+ T [y6] - 628.3049+ H [y5] - 527.2572+ G [y4] - 390.1983+ C [b3] - 379.1282+ Y [b4] - 542.1915+ Y [b4] - 271.5994++	18 ,24 month	27 .6
		NICYVITHGDAK	C [y10] - 1163.5514+ Y [y9] - 1003.5207+ V [y8] - 840.4574+ I [y7] - 741.3890+ T [y6] - 628.3049+ H [y5] - 527.2572+ D [y3] - 333.1769+ C [b3] - 388.1649+ V [b5] - 650.2967+	1, 6,18 month	25 .1
		C#NGVLEGIR	L [y5] - 294.1792++ E [y4] - 237.6372++ G [y3] - 173.1159++ V [b4] - 422.1340+	24 month	36 .85
Myh 15	Myo sin 15	CNGVLEGIR	G [y7] - 743.4410+ L [y5] - 587.3511+ E [y4] - 474.2671+ G [y3] - 345.2245+ G [y7] - 372.2241++ L [y5] - 294.1792++ G [y3] - 173.1159++	Al l ages	27 .41
		DSNNLC#LHFNPR	C [y7] - 934.4200+ L [y6] - 783.4260+ H [y5] - 670.3420+ F [y4] - 533.2831+ N [y3] - 386.2146+ N [b4] - 431.1521+ L [b5] - 544.2362+	18 ,24 month	34 .9
Lgal s1	Gale ctin	DSNNLCLHFNPR	N [y10] - 1284.6266+ L [y8] - 1056.5407+ L [y6] - 392.2167++ N [b4] - 431.1521+ L [b5] - 544.2362+ C [b6] - 352.6370++	Al l ages	24 .0
Fhl1	Four and a half LIM	C#LHPLASETFVSKDGK	T [y8] - 881.4727+ F [y7] - 780.4250+ V [y6] - 633.3566+ K [y4] - 447.2562+ A [b6] - 683.3181+	18 ,24 month	32

	domains protein 1	CLHPLASETFVSKDGK	E [y9] - 1010.5153+ V [y6] - 633.3566+ S [y5] - 534.2882+ K [y4] - 447.2562+ D [y3] - 319.1612+ D [y3] - 160.0842++	1, 6 month	24
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3.4.7 Detection of cell junction proteins trioxidation in old (24 month) group

The Adherens junction (AJ) and Tight junction (TJ) provide important adhesive contacts between neighboring epithelial cells. Although these junctions comprise different proteins, there are similarities in the roles of specialized transmembrane proteins informing extracellular adhesive contacts between cells, and intracellular links to the actin cytoskeleton and signaling pathways including the regulation of gene transcription. In the discovery phase, we found several protein associated with cell junction are oxidative targets as they exclusively trioxidized in late age stages including 18 and 24 month. After further refined the list of targets attacked by ROS with quantitative MRM analysis approach, cell junction associated protein catenin beta-1 (CTNNB1) and desmin (DES) are confirmed as oxidative sensor or targets.

CTNNB1 protein, which belongs to the catenin family together with p120-catenin and α -catenin, is identified in label free LC-MS/MS. β -catenin is a subunit of the cadherin protein complex and acts as an intracellular signal transducer in the Wnt signaling pathway[275]. CTNNB1 binds the C-terminal cytoplasmic domain of E-cadherin, the core transmembrane protein of the AJ, to connect with the actin cytoskeleton and form the mature cell-cell contacts. Those cell-cell adhesion complexes are necessary for the establishment and maintenance of epithelial cell layers and barriers. As a component of the complex, CTNNB1 can regulate the cell adhesion to influence the communication [276]. We apply high specificity MRM analysis combined with reverse phase column to verify the target CTNNB1 in discovery phase.

The MS1 precursor ion m/z value (647.3346++) was extracted while MS/MS data were obtained for selected fragmentations ions (transitions) from the discovery mass spectrometry. Five most intense transitions plus b5 606.2552 which indicated modified cysteine for candidate peptide were acquired from the MS/MS data. The retention time with ± 5 minutes was regarded as the duration of new time window. As MRM will perform double filtering with Q1 based on the MS1 filter as well as the Q3 transitions m/z value, a new batch of trypsin digests sample of individual

subject from four age group were analyzed in vantage based on the MRM filtering. The Ctnnb1 Cys381 trioxidation peptides was positively verified in 18 month and 24 month age group which successfully match with the MS1 filter (+47Da) and form co-eluted peak of six fragmentation ions at the retention time around 31.3'(Figure 3.4.7.1C). For the similarity and consistency evaluation, the high dotp value 0.93 which represent the high consistency with library data proved the identification also pass the skyline program threshold standard (Figure 3.4.7.1B). The representative transitions showed strongest signal and high reproducibility. At the same time in the 1 and 6 month age group, no cysteine trioxidation peak was detectable above noise level. However, the corresponding non-cysteine trioxidation peptide derived from the same protein was set as internal reference (651.8530++). Four most intense transitions were selected as the representative ions for the measurement as described before. Transitions y6 848.4447 which located the unmodified C is also included in the peak.(Figure 3.4.7.1) The co-elution peak of all five transitions with high dotp value (0.93) were identified in all four age groups.

Figure 3.4.7.1 CTNNB1 trioxidized peptide and corresponding unmodified peptide identification by MRM. **A.** MS/MS spectrum of CTNNB1 peptide LVQNC(TriO)LWTLR including the cysteine trioxidation site. Spectra of ions used to confirm identity and site of modification. **B.** Dotp value of the trioxidized peptide showing good match on relative abundance of each fragmentation ion between library and peaks identified in old group. **C.** Positive identification of CTNNB1 trioxidized peptide LVQNC(TriO)LWTLR. Visualization of co-eluted peak of six transitions of target peptide ~10 minutes time window. **D.** MS/MS spectrum of CTNNB1 peptide LVQNCLWTLR which is regarded as unmodified peptides. Potential fragments for further MRM verification are provided. **E.** High dotp value of the unmodified peptides showing high similarity between on library and sample based on relative abundance of each fragmentation ion. **F.** Positive identification of CTNNB1 trioxidized peptide LVQNCLWTLR. Visualization of co-eluted peak of four transitions of target peptide ~10 minutes time window. Skyline Second-Derivative transform applied in display.

The second target protein associated in the cell junction and cell-cell communication is desmin (DES). Intermediate filaments composed of desmin are attached to membrane-associated attachment proteins that form a dense plaque on the cytoplasmic face of the membrane. Desmin was linked to desmosomal adhesion molecules which may regulate the muscle contraction and movement. Interestingly, DES was identified to be exclusively trioxidized at amino acid cysteine in 18 and 24 month mice skin tissue. The tryptic peptide sequence HQIQSYTCEIDALKGTNDSLMR containing Cysteine332 (Cys332) as trioxidation residue was determined by MS1 precursor and MS2 spectrum. MS/MS spectrum allows not only the confirmation of the modified site localization and target peptides sequence. Meanwhile it also provides the retention time and m/z ratio for the extraction of ion chromatogram based quantitation of trioxidation with high mass accuracy. The targeted peptide HQIQSYTCEIDALKGTNDSLMR observed the retention time 31' in the mass spectrometry elution and the precursor m/z ratio was 863.0636++.

After the MRM experiment, the DES Cys332 trioxidation peptides was positively verified in 18 months and 24 months age group through match with the MS1 filter (+47Da) and the formation of all six transitions co-eluted peak within expected ten minutes time window. The six transition ions are listed y9 1037.5044+,

y6 751.3403+, y4 522.2704+, b5 379.2088+, b5 594.2994+, b7 858.4104+. The detection also passes the skyline program threshold standard with high dotp value 0.91 which indicated same relative intensities with the library peak format (Figure 3.4.7.2). However in the 1 month and 6 month group, no cysteine trioxidation peak was detectable above noise level. Meanwhile, the corresponding non-cysteine trioxidation peptide derived from the same protein was set as internal reference (860.7442+++). Same procedures were applied to study the internal reference in all four age groups, six most intense transitions were selected as the representative ions for the measurement as described before. The co-elution peak of all five transitions with high dotp value (0.89) were identified in all three groups (Figure 3.4.7.2)

Figure 3.4.7.2 DES trioxidized peptide and corresponding unmodified peptide identification by MRM. **A.** MS/MS spectrum of DES peptide HQIQSYTC(TriO)EIDALKGTNDSLMR including the cysteine trioxidation site. Spectra of ions used to confirm identity and site of modification. **B.** Dotp value of the trioxidized peptide showing good match on relative abundance of each fragmentation ion between library and peaks identified in old group. **C.** Positive identification of DES trioxidized peptide HQIQSYTC(TriO)EIDALKGTNDSLMR. Visualization of co-eluted peak of six transitions of target peptide ~10 minutes time window. **D.** MS/MS spectrum of DES peptide HQIQSYTCEIDALKGTNDSLMR which is regarded as unmodified peptides. Potential fragments for further MRM verification are provided. **E.** High dotp value 0.89 of the unmodified peptides showing high similarity between on library and sample based on relative abundance of each fragmentation ion. **F.** Positive identification of DES trioxidized peptide HQIQSYTCEIDALKGTNDSLMR. Visualization of co-eluted peak of six transitions of target peptide ~10 minutes time window. Skyline Second-Derivative transform applied in display.

3.5 Discussion

Aging is an age-dependent obligatory and progressive event that occurs in all tissues of an organism. However, skin is probably the organ most significantly influenced by aging as skin is subjected to two forms of aging which occur concurrently. Intrinsic ageing is mainly derived from the endogenous oxidative stress just like aging in other tissues. Extrinsic aging is due to the ROS produced in interactions with the environment. As well as changes in appearance, a decline in several functions has been recognized in aged skin. These include: decreased epidermal barrier function; reduced mechanical resistance; reduced sensory perception; delayed wound healing; reduced immunological reactions; altered thermoregulation and reduced vitamin D production. With the increased focus on age-dependent alterations in skin structure and function, there is an emerging interest in the impact of age on epithelial barriers. The experiments will focus on the impact of aging associated ROS on the redox regulation and cell junctions of skin.

In our study based on the skin proteomics approach from 1, 6, 18 and 24 months age mice, age dependent dynamic analysis revealed the relation between protein change tendency clusters with the associated metabolism process. Many cell junction proteins are found to be upregulated or downregulated in an age dependent manner, including Ctnnb1, Anxa9 and Itgb4 and others. Ctnnb1 was one of the important protein in tight junction and plays a role in the regulation of cell adhesion as component of catenin adhesion complex. The Ctnnb1 decline consistent with an age-related deterioration of cell to cell adhesion after 6 months. Integrin beta-4 (Itgb4) is another component of cell junction. Integrin beta-4 is a receptor for laminin and integrin mediate cell-matrix or cell-cell adhesion. It was found to be increased in the expression level when the skin reach 18 month and slightly decreased in 24 month mice. The unstable alternation of cell junction proteins indicated the disturbance in the normal cell-cell communication between neighboring cells via specialized proteins.

Our study further reveals an unrestrained accumulation of trioxidation-associated S-O₃H residues in the cell junction protein Ctnnb1 and Des. Cell junction

proteins in skin are under high concentration of ROS derived from both endogenous and exogenous source. During the aging process they are prone to be modulated in both expression level and DPMs level. Several cell junction proteins are found to be irreversible trioxidation which indicated the extremely high oxidative stress as well as the dysfunction of cell antioxidants defense system. Ctnnb1, or name as β -catenin, was one of the protein targets identified with cysteine trioxidation by both discovery LC-MS/MS and verification MRM. Upon unbiased full screening mascot MS/MS spectrum, Cys381 containing peptide LVQNC(TriO)LWTLR of Ctnnb1 was exclusively trioxidized in late age groups including 18 and 24 months mouse. Ctnnb1 was identified as the oxidative sensor with the double confirmation from MRM coeluting peak formation. This experiment indicate Ctnnb1 is one of the cysteine containing ROS attack proteins during the skin aging process which is alternated by both expression level and DPMs level. Ctnnb1 is also expected to be consider as sensor for cellular oxidative stress. As it is reported that declined function of Ctnnb1 was related with dysregulation of cell junction. As Ctnnb1 is the key downstream component of the canonical Wnt signaling pathway, the functional loss due to unrepaired damage caused by cysteine trioxidation may also contribute to the inactivation of Wnt responsive genes. Ctnnb1 binds with the core adherens junction protein E-cadherin and the interactions between Ctnnb1 and E-cadherin result in a large increase in the affinity of the interaction. The conformational change caused by adding three oxygens atoms at the Cys381 of Ctnnb1 will negatively affect the functional assembly of the cadherin core complex and initiation of cell–cell adhesion. As the cell-cell adhesion is the structure base for multiple cell-cell communication and transmembrane signaling pathways.

Des is another cell junction protein identified to be irreversible oxidized at Cys332 in 18 and 24 month mice skin. Desmin is a muscle-specific, type III intermediate filament which integrates sarcolemma and many other biological structure. Intermediate filament composed desmin are attached to different membrane proteins to build a compacted network located in cytoplasmic. This cytoplasmic plaque will bind with cadherin and extend through the membrane to finally form the actual rivets which is defined as Desmosome. Mutations within the

desmosome are the main cause of arrhythmogenic right ventricular cardiomyopathy (ARVC). The Des was found to hold cysteine trioxidation by both discovery LC-MS/MS and verification MRM with Cys332 containing peptide HQIQSYTCEIDALKGTNDSLMR. The unbiased full scan shows the well fragmentation of the peptide with correct cleavage site. The proper fragmentation ions y9 1037.5044+, y6 751.3403+, y4 522.2704+, b3 379.2088+, b5 594.2994+, b7 858.4104+ are set as the second filtering in MRM. The relative ion intensity among representative transitions are compatible to the database library. The modification of Des in late age groups are firmly verified based on the standard criteria. As the desmin filament are linking to desmosome which plays key role in cell-cell adhesion and muscle movement, it is plausible that the highly oxidative damaged Des lead to decline or dysfunction in cell signal transmit about contractions and muscle movement. Cell adhesion is a vital component of many organs especially skin. Loss of this adhesion effects cell structure, cellular functioning and communication with other cells and the extracellular matrix and can lead to severe health issues and diseases.

The irreversible protein oxidation due to accumulation of excessive oxidative stress is a hallmark of aging. Cellular homeostasis of ROS is well balanced by regulated production and elimination of ROS. In skin endogenous and exogenous ROS can be generated via various ways, meanwhile the oxidation state of proteins can be reversibly alternated through multiple redox regulation. The formation of reversible oxidation adducts of proteins are designed and enzymatically controlled to prevent the protein from terminal oxidation, which usually result in protein functional impairment. Such as the disulfides can add another covalent attachment, glutathione or another cysteine, through a mixed disulfide bond. This process which is known as glutathionylation aims to stabilized cysteine and prevent further oxidation. The peroxisomal localized catalase (Cat) which is able to detoxifies hydrogen peroxide. The catalase enzyme is very prominently expressed in the skin and plays key role in elimination of ROS. However, Cat itself is identified as ROS target with Cys425 trioxidized exclusively in old age stage including 18 and 24 month. The modification site containing peptide SALEHSVQCAVDVCR peptide is

characterized with MS/MS spectrum and fragmentation ions are extracted for MRM. The trioxidation is positively confirmed with both full screen proteomics and targeted MRM. The irreversible oxidation of Cat may cause unrepaired damage to its normal detoxification function. Cat as the prominent enzyme is an important component of skin antioxidant defense system. The heavily accumulation of hyperoxidation in the enzyme is high possible to be responsible for the partially dysregulation in cellular defense. Extensive amounts of ROS will finally damage cells or proteins with unrepaired hyperoxidation. As cells have developed numerous antioxidant systems or defense systems to protect proteins from hyperoxidation during evolution, the occurrence of irreversible cysteine trioxidation indicates not only a high level of oxidative stress but also defects in the cellular redox regulation signaling pathway. Vicious cycle appeared when the antioxidants enzyme itself is also the targets of ROS attack. Cysteine trioxidation serves as the hallmark of severe oxidative damage to proteins in the aging process as well as dysfunction in the antioxidant system. Failure of redox regulation, which tries to prevent protein hyperoxidation, will cause irreparable damage to target proteins.

3.6 Conclusion

In this study, unbiased full scan LC-MS/MS coupled targeted high performance MRM methods were applied using mouse skin tissue from 1, 6, 18 and 24 months age groups. Proteome analysis focus on whole proteome dynamics change tendency and fuzzy c mean cluster was performed based on the analysis. Carbon metabolism, skeletal muscle movement and cell junctions are found to be dysregulated in expression level. Cat as the desired enzymes to detoxify H_2O_2 and reduce the cellular ROS content and prevent hyperoxidation were found to be obviously damaged by oxidative DPMs in aged groups. The finding reveals the plausible explanation of ROS mediated oxidative damage signaling pathway. The decline on Cat and other critical enzymes indicated the dysregulation of cellular antioxidants. Among the DPM modified target protein of ROS, cell junction associated protein Ctnnb1 and Des are representative oxidative sensor identified in our study. Ctnnb1 was identified with trioxidation at Cys381 exclusively in late age groups. Des is another ROS target

protein with irreversible oxidation at Cys332. Our research provides new insight into the ROS mediated initiation of oxidative damage to cells and cell junctions proteins induced cell adhesion decline in aging process.

Chapter 4. Conclusion and Future work

Alongside with global population aging, the burden of aging related diseases or aging itself will remain significant. Aging is a time-dependent obligatory and deleterious process that occurs in all tissues of an organism. Rather than aging in general, oxidative stress derived from overwhelmed ROS accumulation plays a major role in the aging associated pathology. ROS originating from different sources including endogenous and exogenous discussed in different tissues basically affect all cell compartments. Although DNA and lipids are also targets to be attacked, the modification and potential subsequent alternations of proteins poses a major problem for cells. Degenerative protein modifications (DPMs) are non-enzymatic post-translational polypeptide modifications (PTMs). DPMs yield undesirable structural and functional change of proteins tightly associated with aging proteome imbalance. Cysteine trioxidation is a hyperoxidation which generate sulfonic acid (S-O₃H) step wisely from reduced thiol. Cysteine trioxidation is a totally irreversible process and there is no known enzyme or molecule capable of reducing sulfonic acid. The irreversibility of this DPM will assigned proteins to unrepaired damages on structure and function. It was defined as terminal oxidation which is clearly different with redox regulation and is implicitly understood to impair protein function. Redox regulation typically refers to the reversible alternation of properties of proteins as a result of change in the oxidation state. The redox regulation is one of the important component of cellular antioxidants defense system. As increase in ROS levels over time is a common feature of all human tissues, cell evolved many attempts were made to quench these ROS by multiple antioxidants defense system. Redox regulation and terminal oxidation include the whole process for generation of cellular ROS, oxidative DPMs in ROS target proteins regarding modification species and specific sites, their enzymatic activities and structures, and the effects on downstream signaling pathways. The relationship between physiological changes in aging and underlying mechanisms involving oxidative stress remain poorly understood. Identification of ROS target proteins and their modification sites and species, including the chemical properties of reactive Cys residues which are the major targets of ROS, are critical for understanding the molecular mechanisms in oxidative stress involved signaling pathways in aging process. Although the clinical

and scientific importance of exploring oxidative DPMs is obvious, the molecular mechanisms behind these modifications remain poorly understood mainly due to the limitation of technical ability. The advancement happened recently in LC-MS/MS and LC-MRM/MS based proteomics technology has provided the necessary sensitivity and specificity to detect and to quantify DPMs in precise amino acid residues in specific proteins, and therefore open a new avenue to study protein damages in degenerative diseases and aging. In order to address the aforementioned issues, chapter 2 of this thesis studies aging affected proteins in three different age mice brains, chapter 3 studies the aging dynamics of skin tissue in four different age mice.

In chapter 2, we developed a multi-targets panel consisting of novel proteins oxidative targets in TCA cycle that reliably predicts aging from both expression level and oxidative DPMs level. We use brain tissue extracted from mice from three different ages. This chapter explores the role of oxidative stress in the general aging and in the neurodegeneration process of brain. Cellular energy production system defect partially derived from TCA cycle dysfunction was regarded as one of the well-known hallmark of brain aging. Identification and quantification of ROS attacked proteins elucidate the protein functional outcomes in response to age-dependent cellular stress. Using high-sensitivity tandem mass spectrometry together with high-performance MRM-based quantitation, susceptible proteins which are vulnerable to ROS homeostasis imbalance occurred in ageing process with alteration in expression level and DPMs level. Corresponded to their functional categorization, the biological consequences and the role of protein mediators in mechanism remain under exploration individually.

Gene Ontology analysis predicted that TCA functional enzyme proteins or their key subunits were remarkably dysregulated in old mouse brain compared with younger stages. SULA2, IDH1 and IDH2 are found to be significantly downregulated. Expression level change of IDH1 is of high confidence which were double validated by MS/MS detection and western blot, the NADP⁺-dependent IDH1 function as multiple roles in cellular metabolism. They protect the cells from

damages of oxidative stress derived from aging since the generation of NADPH and α -KG as the product of their enzymatic reaction. NADPH and α -KG are well known antioxidants function in cellular detoxification processes in response to oxidative stress. The largely downregulation of IDH1 and IDH2 expression may be a potential indicator of the excessive oxidative stress with less efficient ROS clearance. The results in this study further support the reported information that IDH1 expression decrease over time in the brain of aging mouse. Several other TCA associated proteins showed drastically up-regulation, such as SDHB, MDH1, FH1 and NDUFS3. SDHB represents the iron-sulfur subunit of succinate dehydrogenase (SDH) that is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q). NDUFS3 is the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. The molecular explanation of up-regulation requires more experiment to find out and here we can just give some predication. As imbalance of ROS is an important mechanism by which mitochondrial was proposed to result in aging. ROS can be generated by numerous containment of redox enzymes capable of transferring single electron to oxygen. The enzymes involved in electron leakage are mainly distribute to TCA cycle, ETC complexes or other metabolism realated dehydrogenases. The increased proteins which participate in the electron transduction accounted for a potential responsibility for the rising ROS level in old group. Such as SDHB, which is the way-station of electron transportation from TCA cycle to ETC complex Ubiquinone (Q), the up-regulation may represent an increase of electron leakage which resulting in accumulation of ROS production. These expression amount change of targets proteins does provide supporting evidences of mitochondrial disturbance due to cellular stress in aging process. In general view, the dysregulation of TCA component proteins should result in the accumulation or overconsumption of the intermediates in between. The imbalance in the intermediates is plausible to destroy the stable lasting elements supply or healthy cell microenvironment due to the packed gathering of toxic chemicals.

DPMs refers to non-enzymatic PTMs which yield spontaneous structural and functional change of proteins biologically related to aging proteome defects. Our study further reveals an excessive accumulation of trioxidation-associated S-O₃H residues in the MDH1 enzyme. Trioxidation at cysteine is one of the important DPMs which is irreversible hyperoxidation in extreme oxidative condition. TCA cycle proteins are under high concentration of oxidative stress and in the aging process more likely to be the modulated in not only expression level but also DPMs level. As high level irreversible oxidation cannot be repair by any known enzymes, it is responsible for the forever impairment of protein function through modulating the structure. The study presented here concentrated on the analysis of irreversible oxidation affected proteins. TCA cycle enzyme MDH1 was further studied for the irreversible oxidation residues accumulations with quantitative proteomics analysis. With the unbiased full screening mascot LC-MSMS discovery, we identified Cys137 containing peptide VIVVGNPANTNCLTASK in MDH1 was exclusively trioxidized in old mice brain. The existence of Cys137 trioxidation in aging was positively confirmed in old group for both discovery phase and MRM verification phase but not presented in any young or old group. MDH1 is proposed to be one of the cysteine containing ROS attack proteins during the aging process which is affected by both expression level and modification level. It would also be expected to be consider as an aging biomarker and ROS sensor for cellular oxidative stress. The simulation of 3D model study of MDH1 provided the solid structural basis for the conformational change, the trioxidation site is within the active zone and in proximity to the substrate binding site Asn131. The conformational alternation after cysteine trioxidation is prone to influence on the interaction between enzyme and substrate, in this way to interfere the enzymatic initiation process. As the plausible conformational change after carrying oxygen molecules is simulated with the plugin PyTM and provided the structural basis for functional loss.

As the impairment of the cellular energy production system including TCA decline was considered as one of the principle hallmark of aging process in brain, the gathering of irreversible oxidation of TCA proteins may to some extent explain the decline of the normal ATP generation. Thus cause cognition decline in elderly

brain as central nerve system has extremely high energy demand. Identification and quantitation of protein oxidative modifications shed light on protein functional outcomes in response to cellular oxidative stress in aging process.

As cell evolved various redox regulatory mechanisms converge upon mitochondria to modulate ATP synthesis and ROS production, it is important to consider how redox signals modulate mitochondrial processes. MDH1 identified as ROS sensor contain thiol groups which are most likely to be oxidized due to their unique features or specific interactions. Further exploring the mechanism may will help to relationship between physiological changes and molecular mechanisms involving aging redox biology. As Here, our experiments provide the new sight on how redox signals mediated through cysteine oxidation and the potential mechanism for cellular energy decline in aging process. The cellular redox hemostasis can be balanced through diverse reversible cysteine mediated reaction, such as reversible S-glutathionylation serves the role to protect enzymes from irreversible oxidation. S-glutathionylation of SOH can also involve in plethora of side reactions to protect cysteine residues from further oxidation when ROS is highly accumulated. Notably Grx, Trx and Srx as reductase enzymes which are found to be able to convert low level of cysteine oxidation to thiol or other reduced states, are in charge to prevent the irreversible oxidation and balance the redox state. As cell developed multiple ways to prevent the proteins from hyperoxidation. Irreversible trioxidation of core TCA enzymes stipulated the imbalance of cellular ROS environment as well as the dysfunction of orderly redox regulation. Our mass spectrometry data which showed the Grx and Trx significantly downregulation in aging process. As the Grx and Trx are the only enzymes found to revert the sulfenic acid back to thiol state. The downregulation in aging process may indicate the severe disturbance of redox regulation system.

The chapter 3 aims to give an overview of the role of oxidative stress in the general pathology and in the aging process of skin. As brain is the leading organ that serves as the center of the nervous system in almost all vertebrate animals, skin is another important tissue worth discussion because the ROS load in this organ is

higher than in any other organ due to the ROS originating from external and internal sources. Skin is probably the organ most significantly influenced by aging as skin is subjected to two forms of aging which occur concurrently. Intrinsic ageing is mainly derived from the endogenous oxidative stress just like ageing in other tissues. Extrinsic ageing is due to the ROS produced in interactions with the environment. Besides the quite difference in appearance including rougher and slack skin, wrinkles and more fragile properties, decline and dysregulation in molecular metabolism and functions has been characterized in aged skin. The underlying molecular disturbs including decreased mechanical resistance, delayed wound healing ability and reduced immunological reactions, reduced sensory perception and so on. Recently more attention has been attracted on age-dependent modifications in skin structure and function, there is an arising need in the impact of age on epithelial barriers. Our studies focus on the influence of aging associated ROS on the redox regulation and cell-cell communication of skin.

Our skin study performed the high sensitivity label free proteomics approach for skin sample from 1, 6, 18 and 24 months age mice, 18 and 24 month are well known late age stages. Fuzzy c-mean cluster was applied for age dependent dynamics evaluation and relation between protein change tendencies with the allocated metabolism process are successfully revealed. Proteins participates in carbon metabolism, skeletal muscle construction and movement, cell communication and cell junctions are assigned to different clusters. Pdha1 involved in catalyzing the general conversion of pyruvate to Acetyl-CoA and CO₂ and provides the primary link between glycolysis and the TCA cycle are found to be consistently increased in an age dependent manner. Pdha1 plays cross talk role in many energy and carbon metabolism. Cell junction proteins are found to be dysregulated in an age dependent manner, including Ctnnb1, Anxa9 and Itgb4 and others. Ctnnb1 was one of the core protein in tight junction and plays a role in the regulation of cell adhesion as component of catenin adhesion complex. The Ctnnb1 expression decline consistently with an age-related deterioration of cell to cell adhesion after 6 months. Integrin beta-4 (Itgb4) is another component of cell junction. Integrin beta-4 is a receptor for laminin and integrin mediate cell-matrix or cell-cell adhesion. It was

found to be increased in the expression level when the skin reach 18 month and slightly decreased in 24 month mice. The unstable alternation of cell junction proteins indicated the disturbance in the normal cell-cell communication between neighboring cells via specialized proteins.

The peroxisomal localized catalase (Cat) which participants in the process of carbon metabolism is identified in the first cluster with upregulated tendency. Cat is able to detoxify hydrogen peroxide as the role of antioxidants enzyme. Skin cellular homeostasis of ROS is well balanced by regulated production and elimination of ROS in time. Although high loads of endogenous and exogenous ROS can affect the skin protein via various ways, skin developed defence mechanism that the oxidation state of proteins can be reversibly alternated through multiple redox regulation. The formation of reversible oxidation adducts of proteins are enzymatically manipulated to prevent the protein from terminal oxidation, which subsequently result in protein functional impairment. For example, the existing disulfides or reduced cysteine can add another covalent attachment, glutathione or free cysteine, to form a complicated disulfide bond. This process named glutathionylation aims to stabilized cysteine and prevent further oxidation. Of course various other enzyme such as Superoxide Dismutases, Cat, Ferritin, and Peroxiredoxins aplay important role in quenching ROS. Catalase which mentioned before are able to catalyze the reduction of H_2O_2 , the major contributor to cellular oxidation stress. The Cat is able to detoxify hydrogen peroxide with the reaction as follows: $2 \text{H}_2\text{O}_2 = 2 \text{H}_2\text{O} + \text{O}_2$. The catalase enzyme is very prominently expressed in the skin and plays key role in elimination of ROS. However, Cat itself is identified as ROS target with Cys425 trioxidized exclusively in old age stage including 18 and 24 month. The modification site containing peptide SALEHSVQCAVDVQR peptide is characterized with MS/MS spectrum and fragmentation ions are extracted for MRM. The trioxidation is positively confirmed with both full screen proteomics and targeted MRM. The irreversible oxidation of Cat may cause unrepaired damage to its normal detoxification function. The heavily accumulation of hyperoxidation in the enzyme is high possible to be responsible for the partially dysregulation in cellular defense. Extensive amounts of ROS will finally damage cells or proteins with unrepaired

hyperoxidation. Research reported that it remains mysterious that catalase activity decreases in the dermis in aging process. Our finding of exclusively Cat trioxidation in late age stages may to some extent provide the plausible explanation to the queries. As cells have developed numerous antioxidant systems or defense systems to protect proteins from hyperoxidation during evolution, the occurrence of irreversible cysteine trioxidation indicates not only a high level of oxidative stress but also defects in the cellular antioxidants defense system. When the antioxidants enzyme itself become the targets of ROS attack, a visible vicious cycle may appear which elucidates the possible initiation of ROS induced large scale protein damages. Our findings help to understand relationship between physiological changes and molecular mechanisms involving ROS.

Interestingly, our study further reveals an unrestrained accumulation of trioxidation-associated S-O₃H residues in the cell junction protein Ctnnb1 and Des. As the downstream reactions and damages of the vicious cycle mentioned previously, the dysfunction of detoxify enzyme will lead to enlarged influence on proteins. Cell junction proteins in skin are under high concentration of ROS derived from both endogenous and exogenous source. During the aging process they are easily to be modulated in both expression level and oxidative DPMs level. Several cell junction proteins are found to be irreversible trioxidation which indicated the extremely high oxidative stress as well as the dysfunction of cell antioxidants defense system. Ctnnb1, or name as β -catenin, was one of the protein targets identified with cysteine trioxidation by both discovery LC-MS/MS and verification MRM. Ctnnb1 plays dual roles in both tight junction and focal adhesion. Proteomic technology using mass spectrometry (MS) is the invaluable cutting edge tools for identifying the modification sites and species in Ctnnb1. Cys381 containing peptide LVQNC(TriO)LWTLR of Ctnnb1 was trioxidized in late age groups including 18 and 24 month mouse. Ctnnb1 was identified as the oxidative sensor with the double confirmation from MRM co-eluting peak formation. This experiment indicate Ctnnb1 is one of the cysteine containing ROS attack proteins during the skin aging process which is alternated by both expression level and DPMs level. As it is reported that declined function of Ctnnb1 was related with dysregulation of cell

junction, our data shed light on the exploration of Ctnnb1 mediated cell cell communication disorder. Ctnnb1 binds with the core adherens junction protein E-cadherin and the interactions between Ctnnb1 and E-cadherin result in a large increase in the affinity of the interaction. The conformational change caused by adding three oxygens molecules at the Cys381 of Ctnnb1 can negatively influence the functional assembly of the cadherin core complex and initiation of cell-cell adhesion. As the cell-cell adhesion is the structure base for multiple cell-cell communication and transmembrane signaling pathways. Desmin is the second cell junction protein identified to be irreversible oxidized at Cys332 in 18 and 24 month mice skin. The Des was found to hold cysteine trioxidation by both discovery LC-MS/MS and verification MRM with Cys332 containing peptide HQIQSYTCEIDALKGTNDSLMR. The full scan shows the well fragmentation of the peptide with clear cleavage site. The proper fragmentation y and b ions y9, y6, y4, b3, b5, b7 are applied for the method of second filtering in MRM. The relative ion intensity among representative transitions are compatible to the database library. The modification of Des in late age groups are firmly verified based on the standard criteria. Desmin is a type III intermediate filament which forms the sarcolemma and many other biological structure. Desmin are attached to different membrane proteins or transmembrane proteins to build a compacted network located in cytoplasmic. This cytoplasmic plaque will bind with cadherin and extend through the membrane to finally form the actual rivets which is defined as Desmosome. As the desmin filament are linking to desmosome which plays key role in cell-cell adhesion and muscle contraction, it is plausible that the highly oxidative damaged Des lead to decline or dysfunction in cell signal transmit about contractions and muscle movement. Meanwhile, the disease associated with mutations within the desmosome arrhythmogenic right ventricular cardiomyopathy (ARVC). Cell adhesion is an important component of different organs especially skin. Loss of this adhesion effects cell structure, cellular functioning and communication with other cells and the extracellular matrix and can lead to severe health issues and diseases. Our study of irreversible cysteine trioxidation as a degenerative modification provide novel perspective in future related research.

The application of high-sensitivity tandem mass spectrometry together with high-performance MRM-based quantitation method is not limited to the identification and evaluation of biomarker candidates, it also plays important role in detection and quantitation of susceptible proteins which are vulnerable to ROS homeostasis imbalance occurred in aging process demonstrated obviously alter in expression level and DPMs level. However, some underlying mechanism remained unsolved. Such as what is the mechanism that the target MDH1 expression elevated after being trioxidized? Which signaling pathways involved in the process that cells sense the dysfunction of trioxidized MDH1? How ROS mediated redox regulation contribute to aged skin? Future molecular investigations are essential and important for the confirmation of the hypothesized pathophysiological roles undertaken by candidate proteins in the progression of aging. The development of the novel proteomics technique described in this thesis, can be readily applied to any type of sample and contribute to the identification of novel DPMs at cysteine and other amino acid residues. The findings in this thesis would permit the better understanding of the role of oxidative stress in different tissues with the damage caused by irreversible cysteine trioxidation and the imbalance in cellular antioxidants defense system. These studies will have meaningful influence on the achievement of continued healthy aging. Regardless of the proteomics strategy that was used, we are optimistic that with rationalized and systematic workflow in which couples oxidative sensor discovery and validation, mass spectrometry and molecular functional studies, protein diagnostic will eventually find clinical utility in near future.

Chapter 5. References

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Appendix A: Publications

1. Jung Eun Park, SW Tse, **Guo Xue**, Christina Assisi, Serra A, Gallart X, JK Low, OL Kon, CY Tay, Jamms P Tam, Sze SK, Pulsed SILAC-based proteomic analysis unveils hypoxia- and serum starvation-induced de novo protein synthesis with PHD finger protein 14 (PHF14) as a hypoxia sensitive epigenetic regulator in cell cycle progression *Oncotarget*. 2018 Dec 27
2. Gallart-Pallu X, Serra A, Lee BST, **Guo X**, Sze Sk, Brain ureido degenerative protein modifications are associated with neuroinflammation and proteonopathy in Alzheimer's disease with cerebrovascular disease *J Neuroinflammation*. 2017 Sep 2;14(1):175
3. Aida Serra, XavierGallart-Palau, Wei Yi Koh, Zoey Jia YuChua, **Xue Guo**, Chase Jia JingChow, Wei Meng Chen, Jung EunPark, TianhuLi, James P.Tama, Siu KwanSze, Prooxidant modifications in the cryptome of beef jerky, the deleterious post-digestion composition of processed meat snacks *Food Journal International*. 2019 July

Appendix B: Conference Presentation

Poster

1. 66th ASMS Conference on Mass Spectrometry & Allied Topics 2018, June 3 - 7, 2018 San Diego, California

Title: Xue Guo, Jung Eun Park, Gallart Palau Xavier Ramon, Siu Kwan Sze
Discovery Proteomics Uncovered Damaged Proteins in TCA Cycle Induced
Bioenergetic Decline in Aging Mice.

2. 2nd Peptides and Proteins Symposium Singapore 2016 December 8th – 9th,
2016

Title: GuoXue, Park Jung Eun and Sze Siu Kwan Quantitative proteomics
study of brain tissues of aging mouse model reveals cellular energy decline due to
cysteine trioxidation in citric acid cycle proteins.