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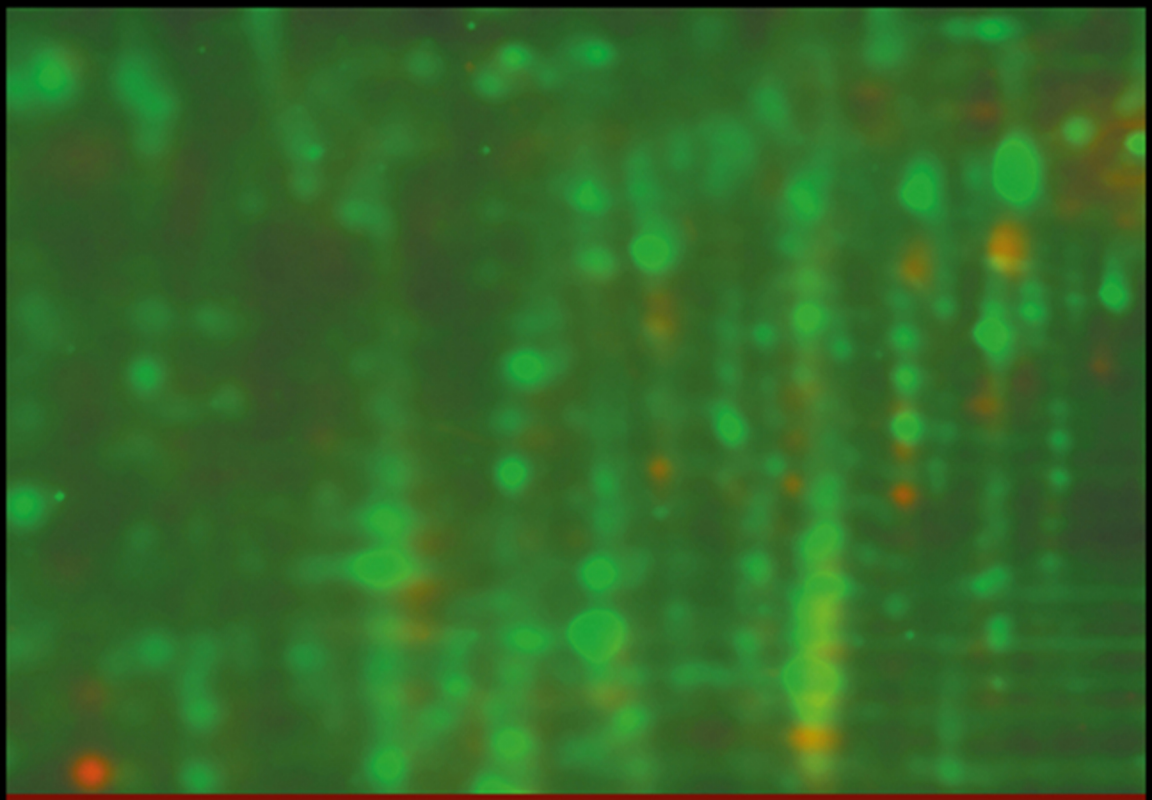

# Protein Carbonylation: Principles, Analysis, and Biological Implications

Edited by Joaquim Ros

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**Protein Carbonylation**

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# Protein Carbonylation

## Principles, Analysis, and Biological Implications

*Edited by Joaquim Ros*

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WILEY

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# Preface

Protein carbonylation has attracted the interest of a great number of laboratories since its pioneering studies at the Earl Stadtman's lab at NIH started in the early 1980s. Since then, detecting protein carbonyls in situations of oxidative stress has become a highly efficient tool to uncover biomarkers of oxidative damage in normal and altered cell physiology. Carbonylated proteins suffer from structural alterations that can impair function or, in certain cases, can have a regulatory role. For these reasons, identification of carbonylated proteins and the site of carbonylation are essential pieces in elucidating the mechanism of altered cellular function occurring under endogenous or exogenous oxidative stresses.

In this book, research groups from several areas of interest have contributed to update the knowledge on the detection, analyses, and identification of carbonylated proteins and the sites where these modifications occur.

I am sure that the scientific community will benefit from these reviews since they deal with specific, detailed technical approaches to study the formation and detection of protein carbonyls. Moreover, the biological impact of such modifications in metabolic, physiologic, and structural functions and how these alterations can help us understand the downstream effects on cell function are discussed.

Finally, I want to express my gratitude to Rodney Levine for his help in designing the book and convincing the authors to contribute a chapter. Without him this book would not have been as successful as the final result shows.

# 1

# Reactive Oxygen Species Signaling from the Perspective of the Stem Cell

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## 1.1 Introduction

Stem cells maintain tissue integrity and homeostasis by regenerating damaged or lost cells throughout life. Impaired stem cell function may promote defective response to stress, aging, and cancer. Work in the past decade has uncovered the critical role that redox signaling plays in the biology of stem cells. A major part of this work has taken place in blood-forming (hematopoietic) stem cells (HSCs) that are broadly used as a model system for adult stem cells. This chapter overviews the investigations of redox regulation of stem cells in the past decade.

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## 1.2 ROS Regulation

ROS are generated from the reduction of molecular oxygen by one electron. ROS species are composed mainly of superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH^-$). The superoxide anion is highly reactive and is rapidly reduced to $H_2O_2$ by the antioxidant enzyme superoxide dismutase (SOD) [1]. $H_2O_2$ can be further reduced to $H_2O$ and $O_2$ by cellular antioxidants. ROS react adversely with and damage DNA, lipids, and proteins, the cumulative effects of which may cause cellular alterations or death. Overall ROS-mediated damage to macromolecules is thought to contribute to the physiological effects of aging [2]. ROS are also considered to be essential components in multiple biological processes as second messengers intimately implicated in the physiological regulation of signaling pathways [3]. Alterations of ROS generation versus scavenging, that is creating the redox milieu, may lead to disease as a result of either too much direct ROS damage (e.g., DNA mutations) or perhaps by impaired function of physiologically relevant ROS-dependent signaling pathways (e.g., myeloproliferative disorder; see succeeding text).

The main source of ROS in the cell is mitochondrial respiration. The generation of proton motive force by the electron transport chain—which leads to ATP production through ATP synthase in a process known as oxidative phosphorylation—is responsible for mitochondrial respiration. However, a small fraction, approximately 0.1–0.2% of $O_2$, consumed by mitochondria form ROS through the premature electron flow to $O_2$ mainly through complexes I and III [4]. The cell type, the environment, and ultimately the activity of mitochondria can influence greatly the precise proportion of ROS generated from mitochondrial respiration [5]. Thus, modulations of mitochondrial activity as well as metabolism in general regulate ROS levels; for instance, reduced ROS levels are achieved by decreasing the rate of mitochondrial respiration via minimizing oxidative phosphorylation. Furthermore, processes that regenerate oxidized glutathione, such as the pentose phosphate pathway, repress ROS levels. Another major source of ROS, in addition to mitochondria, is the membrane-bound protein NADPH oxidase (NOX), which consumes NADPH to generate $O_2$ and subsequently $H_2O_2$. NOX generation of ROS has antimicrobial effects in host defense. In addition, NOX are also important for producing ROS in non-phagocytic cells to influence cellular signaling including growth factor (GF) signaling [6]. This includes increased NOX4-mediated ROS production in stem cells [7]. Notably differentiation of mesenchymal stem cells (MSCs) toward adipocytes or neuron-like cells has also been shown to employ NOX4-mediated $H_2O_2$ signaling as well as mitochondrial ROS [8, 9]. Elevated ROS in MSCs on the other hand reduces their engraftment potential and induces apoptosis after transplantation [7, 10].

Under normal physiological conditions, the generation of ROS is tightly regulated by the ROS scavenging system. ROS scavengers are antioxidant

enzymes that can neutralize ROS by directly reacting with and accepting electrons from ROS. When ROS production outpaces ROS scavenging, an excessive accumulation of ROS occurs, leading to oxidative stress and adverse effects on multiple cellular components including proteins, lipids, and nucleotides. To counteract this, the cell contains multiple types of antioxidants specific to different species of ROS, which helps to prevent pathological levels of ROS and to repair oxidative damage to cellular components. These include SOD, catalase, peroxiredoxins (PRX), thioredoxin (TRX), glutathione peroxidase (GPX), and glutathione reductase (GR). Glutathione, a tripeptide, is one of the most abundant antioxidants synthesized by the cell. Oxidized proteins and $H_2O_2$ are reduced by glutathione through the glutaredoxin and TRX system. Other key antioxidants include SOD and catalase, which reduce $O_2^-$ and $H_2O_2$, respectively. The subcellular localization of antioxidants at areas of high ROS generation, such as within the mitochondria, may further enhance the efficiency of ROS scavenging.

## 1.3 ROS Signaling

Despite their deleterious properties, cumulating evidence in the past three decades has established ROS as pivotal signals in cell fate regulation [11, 12]. There is little doubt that oxygen radicals serve as signaling messengers that variably influence cellular behavior [13, 14]. ROS reaction with proteins such as transcription factors, kinases, and phosphatases alters processes that regulate cell cycle, apoptosis, quiescence, or differentiation [15–17]. GF and oncogenic signaling [18–23] are some examples of ROS signaling. ROS also influence transcriptional activity and likely epigenetics [24–26]. The main ROS species involved in intracellular signaling are Hydrogen peroxide ($H_2O_2$) mostly due to their relatively longer half-life and ability to easily diffuse through membranes relative to other types of ROS [27]. $H_2O_2$ is also among ROS species with substrate specificity that generates reversible oxidation that is likely to trigger signaling cascade in *in vivo* physiological settings [12].

ROS signal via direct modification of proteins by amino acid oxidation, the most common of which is oxidation of cysteine residues [28]. ROS signaling to amino acids can cause functional changes in a range of proteins. Proteins directly modified by ROS—known as redox sensors—undergo a conformational change as a result of oxidative modification that influences their function, stability, subcellular localization, interactions with other proteins, and other critical processes. A major example is provided by ROS modulation of protein tyrosine phosphatases (PTP) [1]. It has been shown recently that ROS-mediated inhibition of PTP1B (encoded by PTPN1) in oncogenic-induced senescent cells results in the upregulation of cell cycle inhibitor $p21^{CIP}$, cell cycle arrest, and senescence by a mechanism involving miRNAs. These studies showed that argonaute that regulates miRNA loading is a target of PTP1B

whose repression results in tyrosine phosphorylation of argonaute and reduced loading of miRNAs targeting $p21^{CIP}$ leading to cell cycle arrest and senescence [29]. These studies illustrate the extent of ROS signaling impact and further reiterate the function of ROS as rheostat in cell signaling [30]; in addition by establishing a link between ROS, inhibition of phosphatases, and regulation of miRNAs, these studies expand the scope of ROS-mediated modulations of signaling pathways.

ROS regulation of protein function is complicated by many feedback loops. While ROS can modify protein function, a growing network of proteins modulates ROS levels. These include PTEN and sirtuins (SIRTs) (specifically SIRT1 and SIRT3), ataxia telangiectasia mutated (ATM), p38 mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), and protein kinase B (AKT) protein kinases as well as the multifunctional apurinic/apyrimidinic (AP) endonuclease1/redox factor-1 (APE/Ref-1) protein. Transcription factors such as nuclear factor kappa B (NFκB) mediate ROS transactivation of the hypoxia-inducible factor 1 alpha (HIF-1α) [31]; Forkhead box O (FOXO) family; nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or NRF2; PR domain containing 16 (PRDM16); and p53 tumor suppressor [32–37]. Among these, many proteins considered as redox sensors that also modulate ROS levels have key functions in the regulation of stem cell fate (*reviewed in* [13, 38]) (Figure 1.1). For instance, changes of ROS and p53 activity by thioredoxin-interacting protein (TXNIP) may be implicated in hematopoietic stem cell (HSC) function specifically with age [39]. The polycomb group

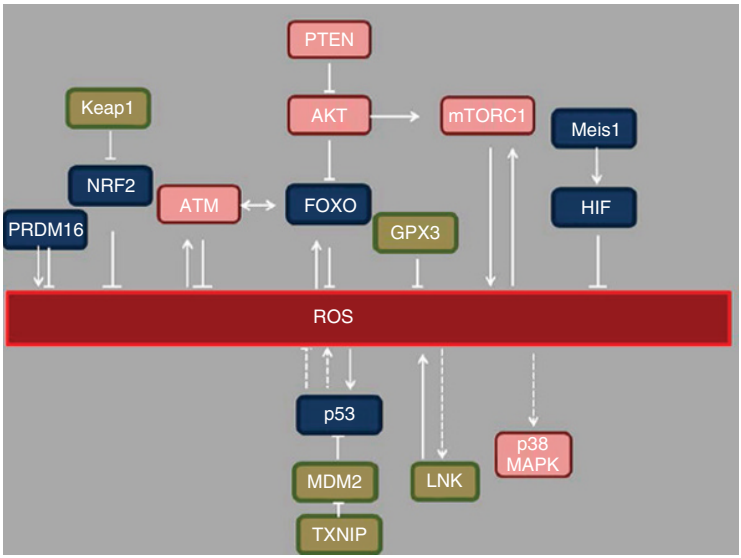


**Figure 1.1** Redox sensors critical for stem cell fate: ROS regulation of signaling molecules and transcription factors and their effect on ROS regulation.

member BMI1 also regulates stem cell function, modulates ROS levels, and is implicated in regulating mitochondrial function [40–42]. Some of these have also been implicated in the regulation of mitochondrial biogenesis or metabolism.

## 1.4 ROS and Stem Cells

Current findings raise the possibility that ROS modulations influence signaling pathways that ultimately impinge on key transcription factors. In turn these factors readjust ROS levels by regulating the expression of antioxidant, metabolic, and mitochondrial genes. Transcription factors that are essential for stem cell machinery and critical for cellular redox state include HIF, FOXO, PRDM16, NRF2, and p53. This model postulates that ROS function as rheostat especially in cells that are highly sensitive to levels of ROS [30] such as stem cells that maintain low ROS levels.

### 1.4.1 Adult Stem Cells

Adult stem cells including stem cells of the hematopoietic system, skin, muscle, brain, and intestine share two key properties: (i) they are capable of self-renewing divisions to generate other stem cells and (ii) are multipotent, able to give rise to all cells within their tissue of origin. Adult stem cells replace differentiated cells and replenish damaged and lost tissue during fetal life and throughout life after birth. Adult stem cells with very few exceptions are mainly quiescent under homeostatic conditions as has been definitively shown for stem cells of the skin and hematopoietic system [43–46] (reviewed in Ref. [30]). Quiescence of stem cells is critical for their self-renewal property. In response to damage or loss and in contrast to homeostasis, stem cells proliferate extensively to regenerate their tissue of origin. To adapt to either quiescence or the highly proliferative state, stem cells have adopted metabolic plasticity. While the precise nature of the stem cell metabolic program remains elusive, levels of ROS appear to both reflect the stem cell metabolic state and have profound effects on stem cell behavior [13]. This is of major importance since perturbations in stem cell properties are associated with degenerative diseases and aging.

Multipotent hematopoietic progenitors in *Drosophila* exhibit higher ROS levels relative to their downstream progenies [47]. This property is shared with mammalian myeloid blood progenitors relative to their upstream HSC. In this *in vivo* drosophila model, burst of endogenous ROS in hematopoietic progenitors primes the larval lymph gland for differentiation [47]. In agreement with an *in vivo* ROS function in mediating hematopoietic cell fate, accumulated ROS in primary hematopoietic progenitors in the context of loss of transcription factor FOXO3 leads to myeloproliferation [48]. In mammals, stem cells of the hematopoietic system contain low ROS levels [49]. Among major known

HSC regulators of ROS are transcription factors FOXO (FOXO3) and ATM protein kinase. FOXOs are evolutionarily conserved regulators of redox state that inhibit oxidative stress in quiescent cells by direct transcription of antioxidant genes including SOD and catalase [50–56]. FOXO's control of the redox homeostasis is also via the pentose phosphate pathway [57]. The redox control contributes to FOXO regulation of aging and longevity [53–55]. In the hematopoietic system, in addition to stem cells, FOXO3 regulates redox state in primary erythroblasts and myeloid progenitors [48, 58].

Increased ROS in HSC is associated with HSC differentiation and increased production of their immediate progenitors [49]. Notably, HSC are highly enriched in glutathione *S*-transferase enzymes that mediate detoxification of xenobiotics and defense against environmental stress and cellular damage [59]. Dormant HSCs are acutely sensitive to oxidative stress, a cellular state instigated by an imbalance between the generation and the detoxification of ROS [33, 36, 37, 60–62]. In many cases unbalanced ROS accumulation is associated with impaired HSC function *in vivo* [60, 63, 64]. Some of the main examples are provided by ATM kinase $(Atm)^{-/-}$ HSC, loss of *Foxo1/3/4* (Forkhead box O 1/3/4) transcription factors, or just *Foxo3* deletion [33, 36, 37, 60]. In many cases such as in $Atm^{-/-}$ HSC, increased ROS levels mediate defects of stem cell activity [60]. However, in contrast to $ATM^{-/-}$ HSC, elevated ROS do not mediate the defective long-term repopulation activity—that is, the ultimate measurement of *in vivo* blood stem cell activity—of $Foxo3^{-/-}$ HSC [60, 65]. ATM and FOXO3 are in a cross talk in which ATM enzymatic activity and expression are regulated by FOXO3 [48, 66]; FOXO3 is required for HSC mitochondrial metabolism [65], while the role of ATM in mitochondrial regulation of HSC is less clear. Control of redox balance and metabolic gene transcription by FOXO3 is also implicated in the maintenance of neural stem cells (NSCs) [57, 67]. However NSCs require high ROS to maintain their self-renewal and the regulation neurogenesis properties [68]. Although FOXOs are also critical for embryonic stem cell (ESC) pluripotency, this function does not seem to be through regulation of oxidative stress in ESCs [69].

NRF2 is a ubiquitously expressed transcription factor and a master regulator of antioxidant response and mitochondrial biogenesis. Loss of NRF2 results in relative expansion of HSCs and increased generation of their progenitors without any impact on HSC self-renewal. This has been attributed to cell intrinsic hyper-proliferation and is associated with modulations of cell migration and homing [70]. Unexpectedly the defective HSC function in these mice is associated with normal ROS levels; on the other hand ROS levels are increased upon restoration of $NRF2^{-/-}$ HSC function [71]. In addition, enhanced NRF2 signaling increases hematopoietic stem and progenitor cell function [70, 71] and mitigates irradiation-induced myelosuppression and mortality [71]. These studies suggest that despite the association that is commonly observed between ROS levels and HSC function [63, 72–77], elevated ROS do not always result in HSC defective function; these conclusions are analogous to that derived from

Foxo3$^{-/-}$ HSC studies [65, 70, 78]. Current findings point to unhealthy mitochondria rather than ROS as potential mediators of stem cell defects [65, 79] in the case of Foxo3$^{-/-}$ HSC. Given the importance of both NRF2 and FOXO3 for mitochondrial function [65, 72–75, 80], it is conceivable that lack of association between ROS elevation and defective HSC function phenotype might indicate active involvement of mitochondria in NRF2$^{-/-}$ HSC as has been proposed for Foxo3$^{-/-}$ HSC [65]. Similar NRF2 functions are described in lung stem cells. In mouse and human airway basal stem cells (ABSCs), intracellular flux from low to moderate ROS levels is required for stem cell self-renewal and proliferation. The stem cell self-renewal involves modulations of ROS levels that activate NRF2 and Notch pathways [81]. NRF2 bears interesting functions in cancer stem cells that involve its interactions with the cell cycle inhibitor p21 (Cdkn1a) that competes with Keap1 for NRF2 binding [82, 83] and stabilizes NRF2 in TGF-beta-responsive squamous cell carcinoma stem cells [84]. This binding increases glutathione metabolism and NRF2 antioxidant response that render cells drug resistant. Decreasing NRF2 increases drug-induced apoptosis in these cancer stem cells without significantly modifying their low cycling profile [84]. In resting drosophila intestinal stem cells, NRF2 (CncC) is constitutively active in maintaining low ROS levels [85]. Increased degradation of NRF2 by Keap1 enhances intestinal stem cell proliferation. Loss of NRF2 increases ROS levels and accelerates age-related degeneration of the intestinal epithelium.

These studies raise the possibility that HSC defects are not directly mediated by ROS elevation when mitochondrial function is defective [70, 71, 78, 86–92]. In these settings as observed in *Foxo3*$^{-/-}$ and *Nrf2*$^{-/-}$ HSC, ROS elevation might only be secondary to changes in mitochondrial function, a signal that might be indicating the unhealthy state of mitochondria and mediating only some (e.g., DNA damage) of stem cell defects [65, 70, 71, 93]. ROS elevation in hematopoietic progenitors induces myeloproliferation *in vivo* [48]. Importantly, scavenging ROS *in vivo* improves myeloproliferation in the context of human leukemias [94, 95].

### 1.4.2 Embryonic Stem Cells

ESCs originate from the inner cell mass of the mammalian blastocyst and possess the ability to differentiate all three germ layers of the embryo under defined *in vitro* conditions [96]. ESCs are highly resistant to oxidative stress [97] but, undergo apoptosis when exposed continuously to high ROS levels. Their genomic integrity and clonal recovery is maintained when cultured under physiological oxygen levels (2%) [98], whereas prolonged hypoxic environment leads to increased ROS and apoptosis [99].

ESCs have a shortened G1 cell cycle phase which enable them to self-renew rapidly. ESC self-renewal relies mainly on glycolysis and the pentose phosphate pathway, with oxidative phosphorylation clearly suppressed [100–104]. The rapid generation of ATP and the precursors for nucleotide biosynthesis by

glycolysis and the pentose phosphate pathway, respectively, enable the rapid DNA replication and ESC growth [105]. Undifferentiated pluripotent ESC in contrast to their lineage-committed progenies relies on enhanced lactate production and an uncoupling of electron transport chain flux from ATP production, suggesting their dependence on glycolysis. This is associated with an immature mitochondrial morphology and a more reduced redox environment, further supporting the notion that ESC avoids dependence on mitochondrial metabolism [104, 106]. Forced activation of oxidative phosphorylation by knockdown of uncoupling protein 2 (UCP2), that limits pyruvate entry into the mitochondrial oxidative phosphorylation pathway, as well as by metabolites that activate this pathway leads to loss of stem cell properties and increased differentiation or apoptosis [104]. Enhancing glycolysis or inhibition of oxidative phosphorylation may also be achieved through hypoxia-induced HIF activation that results in improved proliferation and maintenance of ESCs while repressing differentiation similar to experiments described earlier [107, 108]. In all cases, improved stem cell maintenance is associated with decreased ROS levels. The high sensitivity of mouse ESC to endogenous ROS is in part mediated by deacetylase sirtuin 1 (SIRT1) coordination of p53 activity toward (inhibition) antioxidants with its regulation of pluripotency factor Nanog expression [109]. These functions might also be related to SIRT1 regulation of ESC mitochondria [110]. These findings support the idea that ESC fate may be directly modified by ROS modulation of metabolism. They also suggest that in ESC as in cancer cells glycolysis supports the biosynthetic demands of highly proliferative cells [105].

The study of ROS and metabolism in stem cell fate regulation has led to improved differentiation and reprogramming protocols including induced pluripotent stem cell (iPSC) generation [111, 112]. The reprogramming process reverts a fully differentiated somatic cell to a pluripotent stem cell state. The degree of activation of mitochondrial metabolism is implicated in mouse ESC fate determination. Differentiation of ESCs toward the cardiac lineage has specifically benefited from metabolic and ROS studies [102, 113, 114]. During the iPSC reprogramming process, metabolic rewiring from oxidative phosphorylation to glycolysis might precede the activation of other required steps [100] consistent with transcriptional regulation of multiple metabolic genes by the key reprogramming factor OCT4 [115]. Further in support of this, iPSC generation via small molecules modulates the transition to aerobic glycolysis [116]. In addition, conditions that support low $O_2$ levels improve the efficiency of reprogramming and continued maintenance of iPSCs [117]. Glycolysis may also reduce the ROS levels: in iPSCs many ROS scavenging pathways are enhanced, and mitochondrial $O_2$ consumption is suppressed under hypoxia, leading to diminished levels of ROS. Collectively these findings raise the possibility that increased ROS levels interfere with reprogramming efficiency [118]. Consistent with this notion, increased ROS levels during reprogramming cause damage to DNA [119].

## 1.5 ROS, Metabolism, and Epigenetic Influence

Many metabolic intermediates influence posttranslational modifications of histones and therefore the epigenetic landscape of stem cells. ROS-mediated changes in the concentrations of various metabolic intermediates modulate glycolysis and oxidative phosphorylation metabolic activity and therefore might influence epigenetic regulation [24–26]. This may be relevant to the regulation of stem cell fate [120–125]. For instance, the methylation of CpG islands in DNA requires *S*-adenosyl methionine (SAM) that is generated through threonine metabolism upregulated in ESCs. Demethylation ensues through a series of hydroxylation of the methyl group catalyzed by ten-eleven translocase (TET) enzymes that requires alpha ketoglutarate (αKG) and $O_2$ as substrates [126, 127]. Acetylation of histone-tail lysines requires acetyl-CoA, the TCA cycle metabolite. Similarly, the SIRTs that are deacetylases for histones and other proteins require nicotinamide adenine dinucleotide (NAD). The tight regulation of generation of metabolites might employ ROS that directly influence the interactions of transcription factors and histone acetyltransferases [15]. Given the SIRTs and TET critical enzymatic functions in HSCs, ROS-mediated regulation of metabolites might be implicated [122, 123, 128–130]. How mechanistically nutrient availability and metabolic flux control stem cell histone and epigenetic landscape remains relatively unknown.

Manipulating metabolic pathways with either genetic approaches or pharmacological interventions can directly influence stem cell quiescence, self-renewal, or differentiation [87, 104, 131]. Direct modulation by ROS of metabolic enzymes or other proteins that are implicated in nutrient sensing pathways determines the metabolic flux [132–134]. In these contexts ROS signaling may mediate cross talk between metabolism and pathways that determine stem cell fate decisions. In addition, ROS-independent mechanisms via abundant metabolites may change the epigenetic landscape. Metabolic enzymes may also exert functions other than catalyzing metabolic reactions [24, 25, 135–137]. These alternatives have been poorly characterized in stem cells. Collectively, these studies highlight the intricate relationship between ROS and mitochondria in regulating stem cell fate.

## 1.6 Stem Cells and Mitochondria

Increasing evidence suggests that mitochondria are central to the regulation of stem cell fate. HSCs have relatively high numbers of mitochondria that are overall inactive. As a result mitochondrial respiration is low in HSCs relative to downstream progenitors [138, 139]. A key function of mitochondria in HSCs was recently demonstrated by studies of mitochondrial permeability transition pore (mPTP) [140]. The closure of mPTP in the heart embryo accelerates myocyte differentiation [141] and is associated with decreased ROS levels.

Modulations of ROS-independent mPTP also impact myocyte differentiation, indicating that the effect of mPTP might be mediated by ROS. Interestingly, the induction of mPTP is also involved in increased ROS upon exposure of bone marrow stem cells to ambient air and found to be the source of reduced stem cell harvest for bone marrow transplantation [140]. The induction of mPTP is thought to be at the source of "ischemia–reperfusion damage" that is initiated by a burst of oxygen radicals rapidly produced by mitochondria [142, 143], resulting in mitochondrial swelling and OXPHOS uncoupling [144], leading to necrosis [145]. Transient mPTP opening may function in a regulatory capacity and induce stem cells to differentiate. ROS regulation of mPTP is mediated by the control of cyclophilin D and p53. Oxidative stress facilitates recruitment of mitochondrial CypD to the inner membrane and promotes mPTP. Moreover, p53 also induces mPTP opening. Under normal physiological conditions, low amounts of p53 suppress ROS, whereas high amounts of p53 induce ROS accumulation in response to cellular stress. Thus, these opposing responses might depend on the cellular levels of p53 [146]. The p53 regulation of ROS also occurs in stem cells [140]. The generation of p53-induced genes (PIGs) leads to ROS production, mitochondrial oxidative damage, and apoptosis. Another p53 target gene, phosphate-activated mitochondrial glutaminase (GLS2), protects against oxidative stress and regulates energy supply. GLS2 converts glutamine to glutamate, regulates GSH synthesis and energy production, and is key to glutamine metabolism. By promoting GSH-dependent antioxidant defense, p53-induced GLS2 controls intracellular ROS levels. Therefore by linking glutamine metabolism, energy supply, and ROS levels, p53 plays a relatively unique function in cellular metabolism that might be important in oncogenesis [147]. HSCs show low levels of ROS and are enriched for glycolytic metabolites [138, 139, 148]. Similar analyses in NSCs and MSCs also revealed a preference for aerobic glycolysis and repression of oxidative phosphorylation [57, 76, 77]. Multiple factors are implicated in glycolytic and pentose phosphate pathway dependence of adult stem cells and more specifically of HSCs, including the low energy requirements of quiescence, the need to minimize oxidative stress from mitochondrial ROS, and their location within a hypoxic niche [49, 149]. Evidence of this comes from the genetic ablation of HIFs, which causes activation of oxidative phosphorylation and an increase in ROS, resulting in the subsequent loss of quiescence and the self-renewal properties of HSCs [150, 151]. In HSC, Meis1 regulates both HIF1α and HIF2α [63, 139]. Data suggest that Meis1 is an important regulator of HSC metabolism upstream of HIF [63]. Conditional deletion of M2 isoform of pyruvate kinase (PKM2) or lactate dehydrogenase (LDH)A, that are critical enzymes of glycolysis, further underline the importance of glycolytic metabolism for normal HSC and leukemic stem cells and regulated by ROS [78]. Increased ROS as a result of loss of LDHA partially mediates *Ldha*$^{-/-}$ blood stem and progenitor cells' defective functions [78]. Activated HSCs exit from quiescence to replenish downstream blood lineages that coincides with a shift from

glycolysis to oxidative phosphorylation. This metabolic requirement is illustrated by functional studies of key regulators of pyruvate oxidation and entry to mitochondria, such as pyruvate dehydrogenase kinase (PDK) and PTEN-like mitochondrial phosphatase (PTPMT1) [87, 131]. Loss of PDK in mice results in increased activation of oxidative phosphorylation, loss of HSC quiescence, ROS accumulation and exhaustion of the HSC pool, highlighting the importance of PDK and glycolysis for maintaining HSC function [131]. Deletion of PTPMT1, which favors glycolysis, leads to their expansion of the HSC pool in mice but prevents differentiation into downstream lineages [87]. These studies underscore how the balance between oxidative phosphorylation and glycolysis is essential for HSC maintenance and differentiation. They also point to mitochondria as a critical regulator of HSC activity [152].

Mitochondrial involvement in stem cell fate is likely to act beyond a switch to oxidative phosphorylation from aerobic glycolysis. Mitochondria are highly dynamic organelles at the center of major signaling pathways. They control cellular processes such as $Ca^{2+}$ signaling, ROS production, iron metabolism, and apoptosis. Mitochondrial morphologies, oxidative phosphorylation, and subcellular localizations are influenced by and reflect their activity. Normally, actively respiring mitochondria elongated shapes and are densely packed with cristae. Folded cristae provide increased surface to accommodate electron transport chain complexes [153]. In ESCs, the mitochondrial network is punctate, with individual mitochondrion that is small and rounded in shape with low numbers of swollen cristae [101, 103, 104], indicating an immature and inactive mitochondrial network. ESC mitochondria have a low respiratory capacity but a relatively high mitochondrial membrane potential, an important component of the proton motive force [103, 104, 154]. High mitochondrial membrane potential can be an indicator of increased electron transport chain activity, whereas low mitochondrial membrane potential is associated with lower amounts of respiration, and complete loss of mitochondrial membrane potential can trigger apoptosis [155]. Similar to ESCs, HSCs also contain relatively immature mitochondria, suggesting low mitochondrial activity in HSC. As a consequence, HSCs exhibit lower respiratory rate and a low mitochondrial membrane potential when compared with downstream progenitors [139, 156]. The difference in mitochondrial membrane potential between ESCs and HSCs may represent the proliferative and primed to differentiate nature of ESCs, in contrast to HSCs that are mostly quiescent. Although adult stem cells' mitochondria relative to more differentiated cells are metabolically inactive and produce limited ATP, functional mitochondria are required for adult stem cells' proper maintenance. Deficiencies or mutations in genes important for stem cell mitochondrial function are associated with loss of HSC quiescence and *in vivo* repopulation capacity [86, 88–90, 157, 158]. Interestingly, in almost all these models, ROS levels are relatively increased and rescued with *N*-acetylcysteine (NAC), a glutathione precursor that reduces ROS levels. Collectively, these results identify ROS as a key (although not unique) sensing mechanism

for stem cell to gauge mitochondrial health and activity. The need to constantly survey and maintain the health and numbers of mitochondria within stem cells may be central to stem cell biology. This function is partially filled by the mitophagy machinery that ensures clearance of damaged mitochondria, by transcription factors such as PGC1α that control mitochondrial biogenesis [159], and potentially by mitochondrial dynamics that are intimately linked to mitochondrial metabolism [153]. In agreement with this model, human stem cell-like mammary cells segregate young versus old mitochondria asymmetrically in their progenies to maintain stem cell properties [160].

Additional metabolic checkpoints likely to regulate stem cell fate include mitochondrial fatty acid oxidation mediated by the PML–PPARδ axis. Fatty acid oxidation promotes HSC asymmetrical cell division [161]. In HSC, fatty acid oxidation supports the generation of acetyl-CoA [161], which is fed into the TCA cycle whose production of citrate leads ultimately to the generation of NADPH. In turn, NADPH refills the reduced glutathione pools to further control ROS levels [64]. Collectively, recent findings [71, 78, 162] raise the possibility that unbalanced ROS accumulation, independent of deteriorating HSC functions, might be an indicator of the unhealthy state of mitochondria in HSC.

## 1.7 ROS and Stem Cell Aging

Aging is a progressive loss of physiological integrity and is considered the primary risk factor for many late-onset diseases [163, 164]. Stem cell decline is thought to be a major contributor to the aging process [165, 166]. Long-lived stem cells accumulate damaged molecules with age that compromise their repair processes and function and impair their capacity to regenerate lost or injured tissues. Although highly complex mechanisms are in play, the discovery of evolutionarily conserved developmental pathways that might mitigate aging effects have heightened the hope that healthy aging and delaying age-related diseases might be an achievable goal.

The free radical theory of aging posits that aging is caused by ROS-mediated damage to macromolecules, cells, and tissues [2]. Increasing evidence however has implicated mitochondria rather than ROS in the aging process [167–169]. Although mitochondria have been implicated in the regulation of stem cells, the role of mitochondria in stem cell aging remains unclear. Mitochondrial DNA mutations alter HSC function but do not appear to mediate the HSC aging [138]. Additional work on mitochondrial metabolism in stem cells should illuminate regulation of stem cell function by mitochondria and its relationship to stem cell aging and malignant transformation.

The NAD that serves as a redox regulator has been implicated in the organismal aging process. NAD could also potentially be involved in stem cell aging [167–169]. The NAD–NADH ratio is a measure of cellular redox status and

implicated in the maintenance of the glycolytic flux. NAD activates several enzymes including silent information regulator 2 (Sir2) that is a deacetylase for histones and other proteins [170]. Sir2 is a key regulator of life span in several organisms. SIRT1 of the SIRT family is the closest homolog of yeast Sir2 in mammals and has critical functions in the regulation of metabolism, genome stability, DNA repair, chromatin remodeling, and stress response [170, 171]. Specifically, SIRT1 is key in controlling mitochondrial homeostasis by regulating the expression of oxidative phosphorylation enzymes and PGC1 that is critical for mitochondrial gene expression [168]. SIRTs are also implicated in blood-forming stem cells and their aging [122, 123, 129]. One of SIRT1 protein substrates is FOXO3 [172, 173]. Particularly, loss of SIRT1 leads to a phenotype associated with hallmarks of stem cell aging, some of which are mediated by relative loss of FOXO3 activity in *Sirt1* mutant HSC [122]. SIRT1 has many additional substrates including p53 and HIF1 that are critical for stem cell function; thus SIRT1 may regulate stem cells through a panel of key stem cell proteins [174]. Another SIRT family member (of 7 SIRTs in mammals), SIRT3, protects old HSC from stress-induced damage [129]. Finally, SIRT7 is required for mitochondrial protein folding stress response and HSC regenerative capacity [130]. These studies identify SIRTs as major regulators of HSC and ROS and/or mitochondria in HSC [122, 129, 130] and collectively raise the possibility that SIRT regulation of ROS and/or mitochondria might be implicated in HSC aging and NAD modulations might influence this process.

## 1.8 Concluding Remarks

Redox modulations in stem cells may provide a means to coordinate stem cell fate with metabolism and mitochondria. A greater understanding of mechanisms that control redox state in stem cells, their relation to stem cell mitochondrial metabolism, and fundamental stem cell processes might lead to novel approaches and potential compounds for therapeutic interventions in aging and diseases of stem cells. Development of improved probes and tools for detection and measurements of ROS species and metabolites in highly limited numbers of adult stem cells will provide a major step in that direction.

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# 2

# Analysis of Protein Carbonylation

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## 2.1 Introduction

Posttranslational modifications (PTMs) play a major role in cell signaling; one of the better known cases being kinase and phosphatase-mediated phosphorylation of proteins. Redox signaling involves numerous PTMs as well, but in this instance modifications are initiated by oxidation and reduction of methionyl and cysteinyl residues (Figure 2.1) at the surface of specific proteins [1, 2],

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