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NRF2 Rewires Cellular Metabolism to Support the Antioxidant Response

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Abstract

The transcription factor (nuclear factor-erythroid 2 p45-related factor 2, NRF2) is a master regulator of the cellular response to oxidative insults. While antioxidant response enzymes are well-characterized transcriptional targets of NRF2, it is recently becoming clear that NRF2 also supports cellular detoxification through metabolic rewiring to support the antioxidant systems. In this chapter, we discuss the regulation of NRF2 and how NRF2 activation promotes the antioxidant defense of cells. Furthermore, we discuss how reactive oxygen species influence cellular metabolism and how this affects antioxidant function. We also discuss how NRF2 reprograms cellular metabolism to support the antioxidant response and how this functions to funnel metabolic intermediates into antioxidant pathways. This chapter concludes by exploring how these factors may contribute to both normal physiology and disease.

Keywords: NRF2, KEAP1, antioxidant response, metabolism, glucose, glutamine, ROS

1. Introduction

The transcription factor (nuclear factor-erythroid 2 p45-related factor 2, NRF2) is a master regulator of the cellular response to oxidative insults. While antioxidant response enzymes are well-characterized transcriptional targets of NRF2, it is recently becoming clear that NRF2 also supports cellular detoxification through metabolic rewiring to support the antioxidant systems. Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules, including hydrogen peroxide ($H_2O_2$), superoxide, and hydroxyl-free radical. The production of ROS comes from a variety of organelles within the cell, including the mitochondria, the...
endoplasmic reticulum (ER), and peroxisomes. Mitochondria are believed to be the major source of intracellular ROS, as this is where active respiration takes place, which steadily converts oxygen to superoxide [1]. The control of ROS levels in cells is crucial for cellular homeostasis, as Moderate ROS levels in cells can serve as signaling molecules, but excessive ROS production can lead to damage to DNA, proteins, and lipids [2]. Therefore, redox homeostasis is important for cell survival and is achieved by the balance of ROS production and elimination. Several factors have been suggested to affect ROS levels in cells, including hypoxia, ER stress, metabolic alteration, and oncogenes [3].

It has been shown that hypoxia can stimulate ROS production in mitochondria, which activates hypoxia-inducible transcription factor 1 alpha (HIF1α) [4]. Similarly, misfolded proteins that cause ER stress can elicit the unfolded protein response (UPR) and promote ROS accumulation [5]. In addition, metabolic alterations were thought to be a source of oxidative stress, as metabolic reactions are often accompanied by ROS production. Metabolic alterations have often been found in cancer cells, as cancer cells have a higher demand for ATP and other metabolites to sustain their unlimited proliferation and growth, and the higher metabolic activity in cells would lead to more ROS generation due to more mitochondria respiration. Furthermore, oncogenes are originally believed to elevate the ROS levels in cells, supported by the facts that ectopic expression of different oncogenes (e.g., Myc and KRAS) increase ROS levels in cells [6, 7]. However, a more recent study observed that physiological levels of oncogene (KRAS\textsuperscript{G12D} and MYC) expression could trigger a NRF2-dependent antioxidant response to downregulate ROS levels in cells [8]. These different results could be accounted for by both the expression of NADPH oxidase induced by KRAS\textsuperscript{G12D} overexpression, which leads to ROS generation, and an increase in NRF2-dependent ROS scavenging in cells expressing physiological levels of KRAS\textsuperscript{G12D}. In this chapter, we will be discussing the antioxidant programs regulated by NRF2 and how NRF2 supports ROS detoxification through metabolic rewiring. Finally, we will be discussing how these pathways contribute to the physiological function and diseases.

1.1. NRF2 and KEAP1

Nuclear factor-erythroid 2 p45-related factor 2 (NRF2 or NFE2L2) is a stress-responsive cap’n’collar (CNC) basic region leucine zipper (bZIP) transcription factor that directs various transcriptional programs in response to oxidative stress. Kelch-like ECH-associated protein 1 (Keap1) is believed to be the major repressor of NRF2, supported by the evidence that disruption of Keap1 in the mouse increased the abundance and activity of NRF2 [9]. Under basal conditions, NRF2 is kept inactive through binding to its negative regulator KEAP1, which is a redox-regulated substrate adaptor for the Cullin (Cul)3-RING-box protein (Rbx)1 ubiquitin ligase complex that directs NRF2 for degradation [10]. Keap1 is a cysteine-rich protein that can be oxidized by ROS and results in conformational change, which in turn liberates NRF2. The free NRF2 translocates into the nucleus and heterodimerizes with Maf proteins to bind to a specific DNA sequence called an antioxidant response element (ARE, 5’-TGACNNGC-3’) to activate the expression of a group of detoxifying and antioxidant genes, such as \textit{glutathione S-transferase} (GST) and \textit{NAD(P)H:quinone oxidoreductase-1} (NQO1) [11].
It is generally accepted that NRF2 is sequestered by KEAP1 in the cytoplasm under normal conditions and is released from KEAP1 before translocating into the nucleus under oxidative stress (Figure 1). Additionally, it has been demonstrated that both modification on KEAP1 at cysteine 151 and PKC-delta-mediated NRF2 phosphorylation are required for the release of NRF2 from KEAP1 [12]. In addition, there are several other stress kinases that were found to regulate NRF2 nuclear localization by phosphorylation, including the ER stress kinase PERK and the energy sensor AMPK [13, 14]. However, there is also evidence that KEAP1 is not only restricted to the cytoplasm but can undergo nuclear-cytoplasmic shuttling. A study found that the NRF2-KEAP1 complex keeps shuttling constantly between the cytoplasm and the nucleus, and in the nucleus, the nuclear protein prothymosin α competes for NRF2 binding to KEAP1 and enables the liberated NRF2 to activate NRF2 target genes [15]. Additionally, the nuclear export signal (NES) in KEAP1 has been shown to be important for NRF2 regulation, as it was found to facilitate the transport of the NRF2-KEAP1 complex out of the nucleus, which terminates NRF2-mediated antioxidant signaling after stress [16]. Thus, KEAP1 may regulate NRF2 localization through multiple mechanisms, and the model is being constantly refined.

Figure 1. KEAP1-mediated NRF2 regulation. Under normal condition, NRF2 binds to KEAP1 dimers through the DLG and ETGE motifs within the Neh2 domain in NRF2, which leads to the constitutive ubiquitination and proteasomal degradation of NRF2. ROS, which induces oxidative stress, can oxidize the Cys residues on KEAP1, leading to the conformational change of KEAP1 and thus releasing NRF2. NRF2 can translocate into the nucleus, heterodimerize with MAF proteins, and bind to the specific DNA sequence termed antioxidant response element (ARE) to activate the downstream antioxidant genes, including the enzymes required for GSH production, ROS elimination, phase II metabolism, and the export of xenobiotics.
Recently, there is a theory explaining how NRF2 is stabilized under stress conditions, called the “two-site substrate hinge and latch model.” It is known that each of the two dimeric KEAP1 molecules will bind NRF2 separately through their respective Kelch repeat domain to either the ETGE or DLG motifs in NRF2. It is proposed that the dimeric KEAP1 captures NRF2 first through the ETGE motif, which has 100-fold more affinity to KEAP1, followed by DLG motif docking onto the other Kelch repeat domain. The stress-induced KEAP1 conformational change leads to the release of the DLG motif from KEAP1, preventing NRF2 from ubiquitination by Cul3. It is proposed that the non-ubiquitinated NRF2 would remain bound to the KEAP1-Cul3 complex and therefore prevent the newly translated NRF2 from inhibition by the E3-ubiquitin complex, thereby promoting NRF2 accumulation in the nucleus [17]. In addition, the other mechanisms have been reported to stabilize NRF2 through interfering with the formation of the NRF2-KEAP1 complex. Those KEAP1-binding competitors, such as p21Cip1/WAF1 and BRCA1, were found interacting with NRF2 and thus block the association of KEAP1 with NRF2 [18, 19]. Interestingly, another mechanism was proposed as an alternative way to stabilize NRF2 through the interaction between the autophagy adaptor protein p62/sequestosome-1 (p62/SQSTM1) and KEAP1, which leads to the sequestration of KEAP1 in the autophagy-deficient cells and enables NRF2 accumulation [20].

2. The antioxidant response program regulated by NRF2

The elimination of xenobiotics from cells is an important process for preventing cell toxicity and can be divided into three stages: phases I, II, and III. Phase I involves a transfer of a hydroxyl, a carboxyl, or an amino group to the toxic compound, which is often mediated by the cytochrome P450 enzymes. The modified metabolites generated by phase I enzymes will further be modified by phase II conjugation enzymes, which are mostly transferase enzymes that can transfer the endogenous hydrophilic molecules to the metabolite to increase the solubility and promote excretion. The small hydrophilic molecules include glucuronic acid, glutathione, sulfate, amino acids, and a methyl group, which are catalyzed by glucuronyl transferases, glutathione transferases, sulfotransferases, amino acid transferases, and N- and O-methyltransferases, respectively [21]. NRF2 is known to participate in the regulation of several phase II enzymes, such as heme oxygenase-1 (HO-1) and UDP-glucuronosyltransferase (UGT). While phase I and phase II enzymes are majorly responsible for chemically modifying the hydrophobic toxin to make it more hydrophilic, phase III is mostly related to the function of drug efflux performed by those drug transporters, such as the multidrug resistance (MDR) pump.

NRF2 coordinates with several antioxidant pathways in response to oxidative stress (Figure 2). The best-known example is the regulation of the synthesis and regeneration of glutathione (GSH), which is the most abundant antioxidant molecule in cells. GSH production is supported by NRF2 through controlling several enzymes responsible for GSH synthesis, such as the glutamate-cysteine ligase (GCL) complex composed of two subunits, the modifier (GCLM) subunit and the catalytic (GCLC) subunit, which catalyze the rate-limiting step of GSH synthesis that converts cysteine and glutamate into GSH [22]. NRF2 also controls the
expression of glutathione S-transferases (GST) and glutathione peroxidases 2 (GPX2), which are enzymes known for detoxifying epoxide and \( \text{H}_2\text{O}_2 \), respectively. Additionally, NRF2 also regulates the expression of the solute carrier family 7 member 11 (SLC7A11), which encodes the cystine/glutamate transporter XCT [23]. XCT can import cystine (CySS) into the cell, which can be reduced to cysteine (Cys) using GSH or thioredoxin reductase 1 (TXNRD1). Cys can then be used to support GSH production. GSH is utilized to convert \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \), accompanied by the production of oxidized glutathione (GSSG), which can be reduced back to GSH through glutathione reductase 1 (GSR1). This step is particularly important as cells need to replenish the pool of reduced GSH to keep carrying on the next reduction cycle [24].
Furthermore, NRF2 was also found to regulate the expression of thioredoxin 1 (TXN1) and thioredoxin reductase 1 (TXNRD1), which can reduce oxidized protein thiols [25, 26]. TXN contains two redox-active cysteine residues and can be oxidized when reducing the disulfides within the oxidized proteins. The oxidized TXN can be reduced by NADPH-dependent TXNRD1. The TXN system is crucial for normal cellular function as TXN knockout mice were reported to be embryonic lethal [27]. TXN helps modulate the activity of many transcription factors through oxidoreductive modification of the protein. For example, a DNA repair protein, redox factor Ref-1, was reported to promote the DNA-binding activities of several transcription factors, including HIF-1α and p53, which confers cytoprotective function under hypoxia. The reducing environment promotes the DNA binding of HIF-1 in the nucleus, which is achieved by TXN-mediated Ref-1 reduction [28, 29].

Another important function of NRF2 in mediating antioxidant response is through promoting NADPH production. NRF2 promotes the expression of NADPH-generating enzymes, including the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) in the pentose phosphate pathway (PPP); the isocitrate dehydrogenase (IDH), which converts isocitrate to α-ketoglutarate; and the malic enzyme 1 (ME1) that converts pyruvate to malate [30]. NADPH is a major source of reducing power in cells, which can be used to regenerate glutathione and thioredoxin. Additionally, other NRF2-regulated enzymatic processes, such as quinone detoxification by NQO1 and aldo-keto reductase (AKR), also require NADPH.

Glucuronidation is another process involved in xenobiotic metabolism, which involves conjugation of glucuronic acid, derived from UDP-glucuronic acid to a substrate, which is mediated by UDP-glucuronosyltransferase (UGT) enzymes. It is considered to be the major way to eliminate most of the xenobiotics. For example, the end product of heme metabolism, bilirubin, is eliminated through this pathway, which is catalyzed by the enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1). In addition, various types of amino acids, such as taurine and glycine, can also be attached to the molecules to assist excretion. Furthermore, it has been found that S-adenosyl-l-methionine (SAM) can provide a methyl group for the conjugation reaction. For example, arsenic, which is known to generate oxygen-based radicals, can be detoxified through the reaction mediated by As(III) S-adenosylmethionine methyltransferases [31]. Another kind of detoxification pathway, called sulfation or sulphonation, is catalyzed by a group of enzymes, termed sulfotransferases (SULTs), which are responsible for transferring sulfonyl group donated by 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the hydroxyl or amine groups [32]. The major function of sulfonation was known to modulate the receptor activity of estrogen and androgen and steroid biosynthesis [33, 34]. The inorganic sulfate used for the phase II sulfation pathway is derived from a process called sulfoxidation, which converts those sulfur amino acids (e.g., cysteine and methionine) into the sulfate.

Quinones are chemicals that contain quinoid ring, which can be converted to semiquinones by NADPH: cytochrome P450 reductase. Semiquinones react with oxygen and generate ROS, which leads to oxidative damage to cells. To prevent ROS production from semiquinones, the cytosolic, NRF2-regulated flavoproteins NAD(P)H: quinone oxidoreductases (NQOs) and aldo-keto reductase (AKR) can compete with P450 reductases and convert quinones to a relatively stable hydroquinones (quinols) [35]. In addition, NRF2 also regulates the expression
of ferritin subunits to promote free Fe\(^{2+}\) sequestration, which can inhibit Fe\(^{2+}\)-dependent free radical generation, and thus reducing the Fe\(^{2+}\)-mediated toxicity [36].

3. The regulation of cellular metabolism by reactive oxygen species

Reactive oxygen species have pleiotropic effects on cellular processes, including the modulation of cellular signaling and metabolism (Figure 3). Following oxidative insult, cellular metabolism is reprogrammed to increase the production of cellular building blocks to replace damaged cellular components. Changes in the cellular redox state can alter the oxidation state of metabolic enzymes, resulting in changes in their activity and altered flux through upstream and downstream pathways. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most oxidized proteins in response to hydrogen peroxide [37]. It has been proposed that this serves to reroute metabolic intermediates into the pentose phosphate pathway to facilitate NADPH generation and adaptation to oxidative stress [38]. Indeed, GAPDH mutants that cannot be oxidized impair the cell’s ability to generate NADPH following oxidative insult [39]. Curiously, oxidized GAPDH interacts with RNA- and DNA-binding proteins, suggesting that these adaptation mechanisms may not be limited to alterations in enzyme activity [40]. Glycolytic intermediates also build up further downstream in response to oxidative insults. Pyruvate kinase M2 (PKM2) is also inhibited by oxidation [41], which enhances flux through the pentose phosphate pathway and oxidative stress resistance. PKM2 also controls flux into the serine biosynthesis pathway [42], which supports glutathione production [43, 44]. The glycolysis modulator TIGAR, a fructose-2,6-bisphosphatase (Fru-2,6-BP), also plays a key role in the modulation of metabolism in response to oxidative stress. TIGAR is induced in response to ROS [45] and protects cells from ROS-induced cell death [46]. TIGAR modulates the intracellular levels of fructose-2,6-bisphosphate (Fru-2,6-BP), thereby regulating the activity of PFK1 and flux through glycolysis. PFK1 inhibition diverts upstream metabolites into the PPP, thereby increasing production of NADPH to support the antioxidant response [46–49]. TIGAR also inhibits ROS by binding to hexokinase 2 (HK2) and promoting its activity at the mitochondria during hypoxia [50]. This binding is independent of the bisphosphatase activity of TIGAR, suggesting that the regulation of the PPP by TIGAR is both phosphatase dependent and independent. Thus, modulation of glycolytic enzyme activity by ROS supports the antioxidant response.

Oxidation of enzymes in the TCA cycle, electron transport chain (ETC), and β-oxidation pathway also occurs following oxidative stress. Oxidation can either be reversible or irreversible, depending on the degree of oxidative stress. Reversible oxidation is temporary and occurs through disulfide bond formation, S-glutathionylation, or oxidation of cysteine residues to sulfenic acid. Irreversible oxidation occurs through oxidation of cysteine residues to sulfenic or sulfonic acid. Temporary S-glutathionylation of enzymes in the TCA cycle, ETC, or β-oxidation pathway protects them from irreversible oxidation when ROS is high but may also hypothetically serve as a mechanism to diminish mitochondrial ROS production by reducing their activity [51].
Figure 3. Modulation of cellular metabolism by ROS. ROS have pleiotropic effects on cellular metabolism, including the inhibition of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase M2 (PKM2), which results in accumulation of upper glycolytic intermediates and increased flux through the pentose phosphate pathway (PPP). ROS also promote PPP flux through the activation of TIGAR and by lowering cellular NADPH levels, thereby increasing the activity of glucose-6-phosphate dehydrogenase (G6PD). ROS inhibit reactions in the mitochondria, including the TCA and β-oxidation, which may serve to lower the production of ROS in already stressed cells. Furthermore, ROS induce transcriptional changes that lead to metabolic alterations through the activation of the transcription factors HIF and NRF2.

Reactive oxygen species also affect the activity of transcription factors that control metabolic enzyme expression. Two of the best-known examples of ROS-regulated transcription factors are NRF2 and hypoxia-inducible factors HIF1α and HIF2α. There are many parallels between these transcription factors. First, their regulation is very similar. Under basal, unstressed conditions, their levels are kept low due to targeted degradation by the proteasome. For HIF transcription factors, this occurs by hydroxylation at conserved proline residues by prolyl hydroxylases under normoxia, allowing their recognition and ubiquitination by the VHL E3 ubiquitin ligase, which targets HIFs for proteasomal degradation. Under hypoxia, the activity of the prolyl hydroxylases is inhibited, due to both lower oxygen availability and ROS generation by the mitochondria. ROS induce oxidative dimerization of the HIF prolyl hydroxylase, leading to its inactivation and HIF stabilization [52]. HIF activation reprograms cellular metabolism by upregulating glycolytic gene expression in inhibiting pyruvate entry into the TCA cycle [53], thereby mediating a shift from oxidative phosphorylation to glycolysis. This metabolic reprogramming has been shown to suppress ROS production in cells [54].
4. NRF2 reprograms cellular metabolism to funnel metabolic intermediates into antioxidant pathways

While NRF2 regulates the expression of antioxidant and NADPH-generating enzymes, metabolic intermediates are required to support these pathways. Recent studies have demonstrated that NRF2 also regulates the expression of metabolic enzymes that funnel intermediates into pathways that support antioxidant function (Figure 4). In this section, we will discuss how NRF2 reprograms cellular metabolism to support cellular detoxification and repair.

**Figure 4.** NRF2 reprograms cellular metabolism to support the antioxidant response. NRF2 induces many enzymes (denoted in purple) to increase the flux through metabolic pathways that support the antioxidant response, including the PPP (G6PD, PGD), nucleotide biosynthesis pathway (PHGDH, MTHFD2, PPAT), glucuronidation pathway (UGDH), enzymes that metabolize TCA cycle intermediates (ME1, IDH1), and enzymes in the glutathione biosynthesis pathway (GLS, SLC7A11, GCLM, GCLC). NRF2 also concomitantly represses lipid biosynthesis (ACLY, ACC, FASN, SCD1) to spare NADPH for detoxification reactions. The alterations in cellular metabolism serve to enhance the NRF2-induced transcriptional upregulation of metabolic enzymes by providing substrates for the enzymatic reactions.

### 4.1. Glucose metabolism

Many NRF2-regulated detoxification pathways rely on a supply of glucose for their activity. Thus, it is logical that NRF2 would not only regulate the enzymes in those pathways but the supply of metabolites that feed into them. Indeed, NRF2 activation leads to increased glucose uptake and glucose addition in fibroblasts [55]. Furthermore, interference with the supply of
glucose was found to inhibit NRF2-mediated detoxification of reactive species. In this section, we will discuss NRF2-regulated pathways that utilize glucose.

4.1.1. The pentose phosphate pathway

Following phosphorylation by hexokinase, glucose enters glycolysis as glucose-6-phosphate (G6P). At this step, G6P can either continue through glycolysis or be diverted into pentose phosphate pathway, which consists of the oxidative and non-oxidative branches [56]. The oxidative branch produces both ribose-5-phosphate (R5P), which is precursor for nucleotide synthesis, and NADPH, which is not only required for biosynthesis reactions but also for antioxidant response function. The oxidative branch of the PPP consists of three irreversible steps and is regulated by cellular NADPH levels. Importantly, the first committed step of the pathway, catalyzed by glucose-6-phosphate dehydrogenase (G6PD), is inhibited by NADPH [57]. The steps of the non-oxidative branch of the PPP are reversible and serve to funnel intermediates, such as F6P and G3P, between glycolysis and the PPP. The reversible nature of the non-oxidative PPP branch enables the PPP to adapt to the metabolic demands of cells. Following oxidative insult, the activity of the oxidative branch increases to direct the non-oxidative branch toward resynthesizing F6P, which is then converted back to G6P to replenish the oxidative branch, allowing enhances for NADPH generation. However, in proliferating cells with a high demand for nucleotides, both the oxidative and non-oxidative branches serve to generate R5P.

NRF2 regulates the expression of enzymes in both the oxidative (G6PD and PGD) and non-oxidative (TKT and TALDO1) arms of the PPP [58]. Thus, NRF2 supports both NADPH and nucleotide production. Indeed, it was recently demonstrated that the pentose phosphate pathway (PPP), a major contributor of NADPH for the maintenance of glutathione in its reduced state, is critical for NRF2-induced proliferation [58, 59].

4.1.2. Nucleotide biosynthesis

The de novo synthesis of purine nucleotides proceeds from R5P in a 10-step pathway to produce inosine monophosphate (IMP), which is subsequently metabolized to AMP and GMP. The first step is the synthesis of phosphoribosyl pyrophosphate (PRPP), in which the enzyme PRPS1 transfers a pyrophosphate group onto R5P. Next, PPAT, a NRF2-regulated enzyme [59], catalyzes the displacement of the pyrophosphate with an amide nitrogen from glutamine. The subsequent steps involve the incorporation of glycine, 2 N_{10}-formyl-THF units, aspartate, and glutamine-derived amide groups. In contrast, the synthesis of pyrimidines starts from ammonia and bicarbonate and proceeds in a six-step pathway that involves in the incorporation of aspartate and PRPP. By regulating the activity of the PPP and the levels of R5P, NRF2 may influence the production of both purines and pyrimidines. NRF2 also influences cellular levels of glycine, N_{10}-formyl-THF, and glutamine, which will be discussed in the following sections. Thus, NRF2 supports nucleotide biosynthesis through direct transcriptional mechanisms and by rewiring cellular metabolism to funnel metabolic intermediates into the pathways.
4.1.3. The serine biosynthesis pathway

The amino acids, serine and glycine, are required for many biological processes. Beyond protein synthesis, serine is essential for the synthesis of sphingolipids and most phosphati-
dyserine head groups, an important component of cellular membranes [60, 61]. Glycine
contributes one nitrogen and two carbon atoms to the purine ring for nucleotide biosynthesis. Serine and glycine also charge the folate pool with one-carbon units, including \( \text{N}_{10}\text{-formyl-THF} \), that are ultimately used to produce nucleotides, methionine, and 5-adenosylmethionine, the latter of which is the major methyl donor for methyltransferase reactions including those of DNA and histones [62, 63]. Serine is also required for the transsulfuration pathway that generates cysteine. Furthermore, both cysteine and glycine are incorporated into the tripeptide glutathione, and thus serine is critical for both cellular redox homeostasis and xenobiotic metabolism processes [64, 65]. Consequently, intermediary metabolism is organized so that serine and glycine are positioned to play essential roles in biomass accumulation, DNA replication, epigenetics, and redox homeostasis.

Cells obtain serine and glycine through import from the extracellular space or de novo synthesis. Amino acids are transported by systems which are broadly defined based on their physiochemical properties and substrate specificity [66]. Multiple systems import serine, including the commonly expressed ASC system that mediates the symport of alanine, serine, or cysteine with sodium [67]. De novo synthesis of serine from the glycolytic intermediate 3-
phosphoglycerate (3-PG) occurs through a pathway consisting of three sequential steps: phosphoglycerate dehydrogenase (PHGDH) oxidizes 3-PG to 3-phosphohydroxy pyruvate (pPYR) using an \( \text{NAD}^+ \) cofactor, phosphoserine aminotransferase (PSAT) transaminates pPYR to phosphoserine (pSER) using glutamate as the nitrogen donor, and finally phosphoserine phosphatase (PSPH) dephosphorylates pSER to produce serine [68]. Importantly, the serine thus produced can be converted to glycine via serine hydroxymethyltransferases concomitantly charging the folate pool with one-carbon units.

Recently, we found that NRF2 regulates the serine biosynthesis pathway through ATF4 and PHGDH, which led to an independence from exogenous serine sources [43]. We observed that serine-derived glycine was found to support glutathione metabolism. While most of the cysteine for glutathione was derived from exogenous source in nutrient replete culture, serine-derived cysteine may become more important under nutrient-limiting conditions. Additionally, glycine and intermediates of the folate cycle were found to support the biosynthesis of both purines and pyrimidines, which synergized with the regulation of the pentose phosphate pathway to supply ribose for nucleotides. By regulating both the production of serine and glycine, as well as the enzymes that utilize these amino acids, NRF2 may better influence their fate by funneling them into antioxidant response pathways.

The serine biosynthesis pathway may support the NRF2-regulated antioxidant program in multiple ways. First, the ability to supply glutathione through the availability of the metabolite substrates for de novo GSH synthesis supports ROS detoxification. Furthermore, the production of nucleotides supports the replenishment of the oxidized nucleotide pool and facilitates DNA repair. Additionally, serine-derived metabolites such as glutathione, glycine, cysteine,
and SAM support phase II metabolism because they are utilized for conjugation with toxins for toxin metabolism and excretion.

4.1.4. Glucuronidation pathway

In the process of glucuronidation, UDP-glucuronosyltransferases, such as the NRF2-regulated enzyme UGT1A1 [69], transfer the glucuronic acid component of UDP-glucuronic acid onto substrates, including drugs, bilirubin, hormones, and other molecules. This serves to increase the solubility of these molecules for their excretion. UDP-glucuronic acid is generated from G6P in a multistep process. First, phosphoglucomutase converts G6P to glucose-1-phosphate (G1P). Next, the UDP molecule is attached by UDP-glucose pyrophosphorylase to generate UDP-glucose. Finally, UDP-glucuronate is generated from UDP-glucose by UDP-glucose dehydrogenase, a NRF2-regulated enzyme [70, 71]. Thus, NRF2 promotes the expression of enzymes that synthesize UDP-glucuronate, as well as glucose uptake to promote entry of G6P into this pathway.

4.1.5. The TCA cycle

Following pyruvate entry into the mitochondria, pyruvate is metabolized in the TCA cycle to generate NADH and FADH2 to fuel the electron transport chain but also metabolic intermediates for biosynthesis and NADPH production. Following the generation of acetyl-CoA by pyruvate dehydrogenase (PDH), acetyl-CoA condenses with oxaloacetate to form citrate. Citrate can be exported to the cytoplasm, where it is used as precursor for lipid biosynthesis or metabolized in the mitochondria to isocitrate by aconitate. In the next step, isocitrate dehydrogenase (IDH) metabolizes isocitrate to α-ketoglutarate (α-KG), which is further metabolized to succinyl-CoA by α-KG dehydrogenase complex. Succinyl-CoA is then transformed to succinate by the succinyl-CoA synthetase. Succinyl-CoA is oxidized to fumarate by the succinate dehydrogenase (SDH) complex and then hydrated to malate by fumarate hydratase (FH). Oxidation of malate, catalyzed by malate dehydrogenase, finally regenerates oxaloacetate, and the cycle continues.

There are several points of the TCA cycle that are critical for NRF2-regulated processes. First, NRF2 promotes PDH flux to increase substrate entry into the TCA cycle and also promotes cellular glucose oxidation [58]. This results in increased TCA cycle activity and sufficient substrate availability for NRF2-regulated enzymes that require TCA cycle intermediates. These enzymes include malic enzyme 1 (ME1) and IDH1, which generate NADPH, and thus the levels and subcellular compartmentalization of malate and isocitrate dictate the activity of these enzymes. ME1 is a mitochondrial enzyme that metabolizes malate to pyruvate to generate NADPH. IDH1 is a cytosolic enzyme that metabolizes isocitrate to α-KG to generate cytosolic NADPH. Furthermore, pyruvate carboxylase was identified as a potential NRF2-MafG target gene [72]. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate. In cells that have significant malic enzyme activity, this enzyme could play a critical anaplerotic role by replenishing oxaloacetate so the TCA cycle can progress.
4.2. Glutamine metabolism

Glutamine is the most abundant free amino acid in human serum, with average concentrations of around 500 μM. Proliferating cells in culture are highly dependent on glutamine, as it is both a key nitrogen donor and carbon supply for the TCA cycle. NRF2 controls the uptake, metabolism, and fate of glutamine in cells in addition to its role in regulating glucose metabolism. As mentioned previously, NRF2 regulates the expression of ATF4, which controls the expression of the glutamine transporter SLC1A5 [73], and asparagine synthetase (ASNS), which generates both asparagine and glutamate from aspartate and glutamine. Indeed, we found that NRF2 controls the binding of ATF4 to the ASNS promoter [43]. Additionally, glutaminase (GLS), which metabolizes glutamine to glutamate, is a NRF2 target gene [74]. Here, we will discuss NRF2-regulated pathways that utilize glutamine and their role in supporting the antioxidant response.

4.2.1. Glutathione biosynthesis

As glutamate is one of the three amino acid constituents of glutathione and can also supply the TCA cycle through the production of α-KG, NRF2 may support both the antioxidant response and NADPH production through the control of glutamine metabolism. Indeed, when Nrf2 is inhibited, the carbon flux from glutamine into GSH biosynthesis is severely diminished [59]. Furthermore, glutamate is an obligate exchange molecule for the NRF2-regulated cysteine transporter SLC7A11, an obligate antiporter. Cysteine is also an amino acid constituent of glutathione, and glutamate production may support glutathione metabolism by increasing the intracellular availability of cysteine. Glutamine and glutamate are also the nitrogen donors for many key metabolic pathways. Glutamate donates the nitrogen for the transamination step in serine biosynthesis, which can supply both glycine and cysteine for glutathione production.

4.2.2. Other biosynthesis

As described earlier, glutamine is required for two steps in purine biosynthesis, whereas aspartate, which acquires its nitrogen from glutamate, is required for a step in purine biosynthesis and a step in pyrimidine biosynthesis. Additionally, glutamine is a major source of TCA cycle carbon. Glutamine-derived α-KG enters the TCA cycle and in many cell lines contributes more carbon to TCA cycle intermediates than glucose. Thus, glutamine entry into the TCA cycle can provide metabolic intermediates for NRF2-regulated NADPH-producing enzymes, such as IDH1 and ME1.

4.3. Lipid metabolism

The effects of NRF2 on lipid metabolism are less well characterized. Studies have found that increased NRF2 signaling in the mouse liver is associated with repression of lipogenesis [75]. Suppression of lipogenesis plays an important role in the NRF2-regulated antioxidant response. While NRF2 activates NADPH production through the upregulation of the pentose phosphate pathway, malic enzyme, and isocitrate dehydrogenase, NRF2 also supports cellular
NADPH levels by suppressing NADPH-consuming processes. Lipid biosynthesis is one of the most NADPH-consuming processes in the cell. Wu et al. found that NRF2 suppresses both lipid biosynthesis and desaturation genes in the mouse liver, including NADPH-utilizing enzymes fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1) [30]. Additionally, high-fat diet-induced lipid biosynthesis enzyme induction, including Acly, Acaca, and Fasn, is more pronounced in the livers of Nrf2−/− mice [76]. Suppression of lipogenesis thereby increases the availability of NADPH for use by the antioxidant response.

Activation of NRF2 also promotes fatty acid oxidation. NRF2 knockdown was found to suppress the expression of fatty acid oxidation genes ACOX1, ACOX2, CPT1, and CPT2 [77], and NRF2 activation in the mouse lung induces the expression of fatty acid oxidation genes and lipases. Activation of fatty acid oxidation may serve several functions. First, it may induce the degradation of damaged lipid molecules so they are not utilized for membrane synthesis. Second, fatty acid oxidation has been shown to provide NADPH for ROS detoxification [78].

4.4. Heme and iron metabolism

Another critical function of the NRF2 antioxidant program is the regulation of iron metabolism. NRF2 regulates heme degradation, thus removing excess amounts of the prooxidant molecule heme from the cellular pool. Heme oxygenase metabolizes heme to iron and biliverdin. Free iron is reactive and dangerous to cells, and NRF2 regulates the expression of ferritin heavy and light chains (FTL and FTH1), which immediately bind free iron, as well as ferroportin, which exports it [79]. The NRF2 target biliverdin reductase metabolizes biliverdin to bilirubin [30]. In the body, bilirubin is excreted as waste, but bilirubin can serve as an antioxidant molecule, and the bilirubin-biliverdin redox couple has direct antioxidant function [80]. Furthermore, bilirubin inhibits NADPH oxidase, which serves to keep ROS levels low.

Although it may seem counterintuitive, NRF2 also regulates enzymes involved in heme biosynthesis. Ferrochelatase (FECH) is a direct NRF2 target gene [36] that is responsible for the final step in heme biosynthesis. Importantly, the first step in heme biosynthesis requires the amino acid glycine, the production of which is NRF2 regulated. While the heme may have prooxidant functions in large quantities, heme is constantly turned over and excreted from cells as bilirubin [81]. The synthesis of biliverdin and bilirubin actually protects cells from oxidative stress, possibly due to the antioxidant functions described above.

4.5. Autophagy and the proteasome

Large intracellular particles, including damaged mitochondria and protein aggregates, are degraded by a process known as autophagy. Intracellular components are first engulfed in double membrane vesicles called autophagosomes, which subsequently fuse with lysosomes for digestion of their contents and recycling of their building blocks. The autophagy adapter molecule p62 is a direct target of NRF2 [82]. While p62 may play a role in the stress-responsive degradation of damaged intracellular components, it remains to be determined whether autophagy is critical for the NRF2-regulated antioxidant response. Rather, the relationship between p62 and NRF2 is much more complex. Studies have identified that autophagy
Deficiency leads to NRF2 activation due to p62 accumulation [83]. p62 was found to bind to the NRF2-binding site on KEAP1, thereby competing with NRF2 for KEAP1 binding [84]. However, not only does p62 prevent NRF2 binding to KEAP1, it induces autophagic degradation of KEAP1, resulting in a positive feedback loop of NRF2 activation [82]. This process is regulated, as mTORC1-dependent phosphorylation of p62 increases its affinity for KEAP1, thereby activating NRF2 [85]. Furthermore, there may be other components of this process. Sestrins, which have long been linked to protection from oxidative stress, were found to interact with p62 and KEAP1 to promote KEAP1 autophagic degradation [86]. Sestrin 2 is a leucine sensor that inhibits mTORC1 signaling [87], providing an interesting link between amino acid sensing, mTOR, and NRF2-KEAP1 pathway.

NRF2 also regulates the removal of damaged and misfolded proteins through the regulation of the proteasome. Unwanted proteins are degraded by the 26S proteasome, which consists of a 20S core and a 19S regulatory subunit. NRF2 regulates the 20S proteasome subunits PSMA1, PSMA7, PSMB3, PSMB5, and PSMB6 [88–90], as well as the proteasome activator PA28αβ [73]. Importantly, induction of the proteasome is required for adaptation to oxidative stress [91] and can extend the life span of human fibroblasts [92].

5. Consequences for tissue homeostasis and disease

In the previous section, we have discussed how NRF2-regulated cellular metabolism supports the antioxidant program. In this section, we will focus on how these programs contribute to both normal physiology and disease.

5.1. NRF2 and tissue homeostasis

In normal cells exposed to oxidative insults, these pathways work in concert to produce building blocks for the replacement and repair of damaged components. Recently, several studies demonstrated that the NRF2-regulated antioxidant response is crucial for tissue homeostasis. The recent work by Telorack et al. unraveled the function of GSH in keratinocytes and showed that GSH deficiency affects keratinocyte survival and wound repair. By using mice deficient in glutamate-cysteine ligase (GCL) in keratinocytes, the authors found that GSH deficiency results in more DNA damage and cell death, suggesting a crucial role for the antioxidant capacity in skin integrity maintenance [93]. In addition, NRF2 was reported to confer radioprotection to human lung fibroblasts through upregulating miR-140 expression, which is involved in the regulation of lung fibroblast self-renewal [94]. In the following sections, we will discuss how the regulation of metabolism and ROS levels by NRF2 contributes to tumor development, aging, and stem cell function.

5.2. NRF2 and cancer

NRF2 is frequently mutated in many types of cancers, with the mutations clustered around the DLG (43%) and ETGE (57%) motifs, which are the two critical KEAP1 interaction domains [95]. Furthermore, mutations in KEAP1 that abolish KEAP1 function were also found to activate...
NRF2 and promote the growth of lung cancer cells [96]. A recent study characterizing the genomic alterations in squamous cell lung cancers has found the NRF2-KEAP1 pathway that altered in 34% of the 178 tumor samples examined [97]. Constitutive activation of NRF2 has been considered as a resistance mechanism to chemo or radiation therapy because the NRF2-mediated antioxidant response eliminates therapy-induced ROS, thereby promoting cell survival. For example, NRF2 activation was found to protect cells against ionizing radiation toxicity and confer radioresistance [98].

In addition, aberrant activation of NRF2 also serves to increase the production of building blocks to sustain uncontrolled proliferation in cancer. The activation of the serine biosynthetic pathway may represent a significant contribution to the pro-tumorigenic activity of NRF2. We and others have observed that enhanced de novo synthesis of serine and glycine from glucose reduces the reliance of cells on extracellular sources [43, 99]. Additionally, many NRF2-regulated metabolic pathways were found to confer growth advantages to cancer cells, such as the upregulation of the PPP, nucleotide synthesis, and NADPH production. Mitsuishi et al. have shown that NRF2 promotes nucleotide production via upregulation of PPP and glutamine utilization in response to proliferating signals, which in turn accelerates tumor growth [59]. Interestingly, another study showed that NRF2 overexpression increased human telomerase reverse transcriptase (hTERT) levels and upregulated the expression of two PPP enzymes, G6PD and TKT, which could promote the progression of glioblastoma [100]. Moreover, NRF2-regulated TKT expression has also been found to promote cancer development via counteracting oxidative stress [101].

Furthermore, the NADPH-producing metabolic enzyme ME1 which links glycolysis with the TCA cycle was reported to promote the metastatic potential of HCC and correlated with poor prognosis [102]. Another study has observed that ME1 could promote tumor growth, potentially through increasing NADPH production, which is required for GSH regeneration [103]. Additionally, another NADPH-generating enzyme IDH1 was found to be crucial for the anchorage-independent growth, a property shared by many malignant cells. It was shown that cell detachment promotes ROS production in the mitochondria. The oxidative stress in mitochondria could be counteracted by IDH1-driven reductive carboxylation in the cytosol, followed by the transfer of citrate into the mitochondria, which was to be oxidized by IDH2 to generate NADPH [104]. Moreover, research published by Saito et al. has found that p62-mediated NRF2 activation in HCC could promote tumor malignancy via redirecting glucose into the glucuronate pathway and glutamine toward GSH synthesis [105]. These studies demonstrate that multiple NRF2-regulated metabolic enzymes play important roles in cancer.

5.3. NRF2 and aging

Oxidative stress has been suggested to cause several pathologies, and many of which are age related. The oxidative stress theory of aging came early in 1956 from Denham Harman, who proposed that aging is attributed to the side effects of free radicals on cell constituents and that mitochondria are the key organelles associated with aging [106]. This is supported by the finding that aging leads to more oxidation of the GSH and more damaged mtDNA in the mitochondria, which can be prevented by oral antioxidant administration. In fact, the levels
of oxo-8-deoxyguanosine, which is considered to be the major oxidative damage to DNA, have been found correlated linearly with oxidation of glutathione [107]. Moreover, aging was associated with the decline of antioxidant response, as some of the antioxidant genes induced by oxidative stress declined with age, such as GSH levels [108]. However, some of the changes of the antioxidant genes in the aged mice are inconsistent, and the phenotype might be tissue specific. Overall, the age-dependent changes of the antioxidant genes suggest that ROS levels might contribute in part for the aging processes. As mentioned in the previous section, NADPH production is one of the important antioxidant responses to protect the cell from ROS-induced damage. Interestingly, the key enzyme G6PD, which catalyzes the rate-limiting step of the PPP has been found to extend the life span of transgenic Drosophila melanogaster [109]. A similar finding was observed in mice, in which G6PD overexpression could protect the transgenic mice from oxidative damage and improve their life span through the elevation of NADPH levels, thereby lowering the level of ROS-induced damage (8-OHdG) [110].

Because increased oxidative stress is thought to be a major characteristic feature of aging and NRF2 has long been considered as the major regulator of the cellular antioxidant response, the potential role of NRF2 in aging is intriguing. Indeed, there have been several studies connecting NRF2 with aging. The study from Hirota et al. showed that Nrf2−/− mice accelerated UVB-induced photoaging, including the phenotypes of loss of skin flexibility and wrinkle formation [111]. Additionally, another group observed that the hearing ability of NRF2-deficient mice was more impaired and the number of hair cells was significantly reduced compared to the wild-type mice [112]. Furthermore, progressive loss of NRF2 activity was seen with aging, which may be explained by studies showing that the expression of NRF2 positive regulators P62 and BRCA1 decreased with age, while NRF2 negative regulators, KEAP1 and BACH1, increased with age [113].

Another potential mechanism for age-dependent loss of antioxidant genes was provided by one of the studies showing that the presence of transcription repressor BACH1 along with the absence of coactivator CREB-binding protein (CBP) in the aged mice may convert NRF2 binding from an active ARE to an alternative ARE [114]. Interestingly, a recent paper published by Kubbén et al. found that repression of NRF2 antioxidant pathways served as a driver mechanism for the genetic premature aging disease Hutchinson-Gilford progeria syndrome (HGPS) [115], which is caused by constitutive progerin production, a mutant version of the nuclear protein lamin A. Progerin was known responsible for many cellular defects, one of which is the attrition of mesenchymal stem cells (MSCs) that leads to tissue defect [116]. This study demonstrated that sequestration of NRF2 by progerin, which results in protein mislocalization and the impairment of NRF2-mediated antioxidant response, contributes to progerin-associated nuclear aging defects [115]. Additional studies are still required to further support the role of NRF2 in the aging processes.

5.4. NRF2 and stem cells

Regulation of ROS levels in cells is important, as excessive ROS can lead to cellular damage. In contrast, a modest level of ROS is crucial for maintaining proper biological functions. The redox status has been suggested to regulate stem cell self-renewal and differentiation, and
accumulating evidence indicates that stem cells undergo self-renewal in the environment with low ROS levels, while ROS levels increase in differentiated stem cells [117, 118]. Because embryonic stem (ES) cells give rise to all the tissues in an organism, mechanisms are in place to avoid the accumulation of DNA to prevent mutations that would transmit to tissues and subsequent generations. To achieve this, ES cells have higher antioxidant defense mechanisms, resulting in a lower mutational frequency than differentiated cells [119, 120]. Furthermore, downregulation of ROS generation in stem cells is achieved through a reduced dependence on oxidative phosphorylation [121, 122]. This leads to a greater reliance on glycolysis, which serves to not only generate ATP but also metabolic intermediates to feed the pentose phosphate pathway. Thus, the metabolic reprogramming of stem cells to support antioxidant responses supports ROS detoxification and stem cell health.

As a redox sensor, NRF2 might also contribute to the regulation of stem cell function. Indeed, NRF2 was found constitutively active in intestinal stem cell (ISC) in *Drosophila*, and KEAP1-mediated NRF2 repression was required for ISC proliferation. The redox balance controlled by NRF2-KEAP1 is critical for intestinal homeostasis, as evidenced by NRF2 loss leads to age-related degeneration of the intestinal epithelium [123]. Moreover, NRF2 is required for the survival of hematopoietic stem progenitor cells (HSPCs) and the development of myeloid cells in mice, as NRF2 KO bone marrow had defect in stem cell function and showed reduction of chimerism after transplantation [124]. A recent study has found that the deacetylase SIRT6 maintained the homeostasis of human mesenchymal stem cells (hMSCs) via regulation of redox metabolism through coactivating NRF2. SIRT6-null hMSCs showed a premature cellular attrition and could be rescued by overexpressing HO-1, suggesting that an imbalance of ROS levels could result in stem cell decay [125]. Another recent published work demonstrated that reduced NRF2 expression is responsible for the decline of neural stem/progenitor cell (NSPC) function, as it is found that NRF2 expression was suppressed in the aged NSPC, and overexpression of NRF2 in those old cells could render them to a similar state as the young NSPCs, by showing increasing cell survival and regeneration [126].

In addition, NRF2 activation was found crucial for the reprogramming of induced pluripotent stem cell (iPSC), which was known to be an inefficient process and required the metabolic shift from the oxidative phosphorylation to glycolysis as a major way to generate ATP. It is shown that at the early phase of iPSC reprogramming, ROS levels increase, which activate NRF2, and it is important for navigating the transition [127]. Additionally, high glutathione and glutathione peroxidase 2 levels were found critical for maintaining genomic integrity of human pluripotent stem cells, which were known highly sensitive to ROS-induced cell death [128].

Besides modulating the function of normal stem cell, it is reported that redox balance could also affect cancer stem cell (CSC) function. CSCs or tumor-initiating cells are small populations of cells with stem-like properties, which were considered responsible for drug resistance and tumor relapse. A study has found that NRF2 is responsible for maintaining the self-renewal function of glioma stem cells (GSCs), supported by the evidence that NRF2 deficiency attenuated the tumorigenicity of GSCs in the xenograft model [129]. Interestingly, another study using the lung cancer model observed a downregulated expression of 26S proteasome in lung cancer stem cells. As 26S was known to target NRF2 for degradation, it is postulated...
that reducing NRF2 degradation might result in high levels of NRF2 expression which confers the growth advantage to CSCs [130].

6. Conclusions

In this chapter, we have discussed how NRF2 controls the antioxidant response and reprograms cellular metabolism to support antioxidant function, as well as the role of these pathways in tumorigenesis, stem cell function, and aging. Taken together, these studies demonstrate that cellular modulation of ROS levels is highly important for maintaining the proper function of many cellular processes. Understanding how NRF2 coordinates different metabolic pathways to support cellular detoxification opens new avenues for therapeutic intervention. Proper tissue homeostasis occurs when the right amount of NRF2 activity is achieved. Too much NRF2 activity is associated with uncontrolled proliferation and neoplasia. Too little NRF2 activity results in a decline in stem cell function and aging. Restoring the balance could help treat these diseases. Because cancer cells hijack the protective capability of NRF2 to promote drug resistance and proliferation, specific NRF2 inhibitors would be desirable for the treatment of cancers that have constitutively active NRF2. In contrast, for those diseases caused by NRF2 loss of function or a defect in one of the NRF2-regulated pathways, reactivating an alternative pathway that could also contribute to ROS detoxification might be a way to resolve the problem. Intensive studies are still required to unravel the crosstalk between different NRF2-regulated metabolic pathways and diseases and enable the development of a better strategy for combating human diseases.

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