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Epigenetic Regulation of Keap1-Nrf2 Signaling

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Abstract

The kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) signaling axis serves as a “master regulator” in response to oxidative/electrophilic stresses and chemical insults through the coordinated induction of a wide array of cytoprotective genes. Therefore, activation of Nrf2 is considered to be an important approach for preventing chronic diseases triggered by stresses and toxins, including cancer. Despite extensive studies suggested that the Keap1-Nrf2 signaling pathway is subject to multiple layers of regulation at the transcriptional, translational, and post-translational levels, the potential epigenetic regulation of Nrf2 and Keap1 has begun to be recognized only in recent years. Epigenetic modifications, heritable alterations in gene expression that occur without changes in the primary DNA sequence, have been reported to be profoundly involved in oxidative stress responses. In this review, we discuss the latest findings regarding the epigenetic regulation of Keap1-Nrf2 signaling by DNA methylation, histone modification, and microRNAs. The crosstalk among these epigenetic modifications in the regulation of Keap1-Nrf2 signaling pathways is also discussed. Studies of the epigenetic modification of Nrf2 and Keap1 have not only enhanced our understanding of this complex cellular defense system but have also provided potential new therapeutic targets for the prevention of certain diseases.

Keywords

Nrf2; Keap1; Epigenetic; DNA methylation; histone modification; microRNAs

1. Introduction

Mammalian cells are constantly exposed to oxidative stresses that are regarded as some of the most important and ubiquitous causes of neoplastic, metabolic, cardiovascular, neurodegenerative, and many other chronic diseases [Molecular Basis of the Keap1-Nrf2 system function by Masayuki Yamamoto in this issue]. To deal with the deleterious effects of oxidative stresses, cells have evolved an elaborate and powerful cellular defense

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machinery against reactive oxygen species (ROS). Central to this cellular defensive machinery is the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and its negative regulator kelch-like ECH-associated protein 1 (Keap1). Under basal conditions, Keap1 acts as an adaptor between Nrf2 and the ubiquitination ligase Cullin-3 (Cul3) and promotes the proteasomal degradation of Nrf2. Upon modification of specific thiols, Keap1 allows Nrf2 to translocate into nucleus and activate the expression of a wide array of antioxidative metabolizing/detoxifying and many other genes by binding to the antioxidant response element (ARE) in their regulatory regions【Structural Basis of KEAP1 Interactions with Nrf2 by Alex Bullock in this issue】[1]. In addition to the Keap1-Nrf2 interaction, the transcriptional activity of Nrf2 is regulated by a complex signaling network, which is discussed in detail elsewhere in the present issue【Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues and energy status, and its contribution to the regulation of antioxidant status, detoxification and metabolism by J.D. Hayes】[2].

The Keap1-Nrf2 signaling axis is the pivotal coordinator of cytoprotective responses towards oxidative/electrophilic stimuli and protects cells against chemical insults. Therefore, activation of Keap1-Nrf2 signaling has been widely accepted as an important strategy to prevent oxidative damage-related chronic diseases, including cancer [3]. However, upregulated Nrf2 activity in cancerous cells leads to resistance to radio- and chemotherapies [4]. Furthermore, activation of Nrf2 confers neoplastic cells with growth and survival advantages during their transformation and progression [5]. Indeed, although Nrf2-knockout mice were more susceptible to chemical-induced carcinogenesis than control mice, high expression of Nrf2 in tumors predicts a poor prognosis, and inhibition of Nrf2 sensitizes cancer cells to chemotherapeutic drugs [6]. Such apparently paradoxical roles of Nrf2 in different stages of cancer initiation and progression are essentially arose from the “double-edged sword” nature of ROS in cancer, and have been extensively investigated and reviewed [5]. However, the roles of Nrf2 in other ROS-related diseases such as neurodegenerative diseases, diabetes, and cardiovascular disease are simply protective. Thus, the mechanisms regulating Keap1-Nrf2 signaling are expected to produce different even opposite outcomes, and the preventive or therapeutic applications of Keap1-Nrf2 signaling modulators have to be carefully evaluated according to the context.

Importantly, very different expression levels and activities of Keap1 and Nrf2 have been observed at different stages of different pathological processes. Functional somatic mutations or single nucleotide polymorphisms (SNPs) of Keap1 or Nrf2 occur in many types of cancer and have been utilized to explain the variations in expression and activity of Keap1 and Nrf2【Functional Polymorphisms in NRF2: Implications for Human, by Steven Kleeberger in this issue】[4]. However, although the expression of Keap1 and Nrf2 exhibits significant inter-individual variations in these cancers, somatic mutations exist in only a small portion of cancer tissues [7, 8]. For example, Solis et al. detected nuclear Nrf2 expression in 26% and low or absent Keap1 expression in 56% of non-small cell lung cancers (NSCLCs), and found that this expression correlated with clinicopathologic characteristics; nevertheless, mutations in the NFE2L2 and KEAP1 genes were very uncommon in the examined samples [8]. Therefore, alternative mechanisms must exist to regulate Keap1 and Nrf2 expression. Recently, a body of evidence is emerging that shows

that the Keap1-Nrf2 signaling can be regulated by epigenetic mechanisms in cancers as well as in other diseases, and the present review will focus on the epigenetic regulation of Keap1-Nrf2 signaling as schematically depicted in Figure 1.

2. Epigenetic modifications

The term epigenetics refers to the study of heritable alterations in gene expression that are not due to changes in the primary DNA sequence. DNA-based mechanisms (DNA methylation and histone modification) and RNA-based mechanisms (non-coding RNA) are known to mediate these heritable gene expression alterations [9]. The addition of a methyl group to cytosine bases and covalent modifications of histones at a given promoter can modulate DNA accessibility and chromatin structure, ultimately regulating gene transcription [9]. By targeting mRNA degradation, translation inhibition, and chromatin architecture, non-coding RNAs interfere with various levels of gene expression [10]. Furthermore, interactions between DNA methylation, histone modification, and non-coding RNAs in controlling the epigenome landscape have been recognized; however, the regulatory network remains elusive.

2.1. Epigenetic modifications and human diseases

Epigenetic mechanisms, together with genetic factors, are fundamental for maintaining cellular differentiation and mammalian development [11]. However, the disruption of either epigenetic modifications or genetic functions is associated with abnormalities in various signaling pathways and can lead to the pathogenesis of many human disorders. Unlike genetic changes, aberrant epigenetic marks tend to be acquired in a gradual process [12]. Long-lasting effects of environmental factors and the aging process introduce alterations in the landscape of the epigenome [13, 14]. Thus, studies of epigenetic disruptions are primarily focused on chronic diseases, especially cancer. Studies of epigenetic abnormalities associated with carcinogenesis suggest that epigenetic alterations may interact with genetic dysregulation at all stages to initiate and promote cancer [15, 16]. Global DNA hypomethylation, regional hypermethylation at specific promoters, global reduction of monoacetylated H4K16, and overall microRNA (miRNA) down-regulation are characteristics of cancer cells [17, 18]. In addition, the important role of epigenetic modifications in diabetes [19], autoimmune diseases [20], cardiovascular diseases [21], and neurodegenerative diseases [22] has been recognized.

2.2. Epigenetic therapy

Unlike genetic mutations, epigenetic disruptions in diseases are potentially reversible. For example, genes that have been transcriptionally silenced by epigenetic modifications can be reactivated through epigenetic mechanisms because these genes remain intact, whereas genetic mutations are permanent. Given that epigenetic abnormalities play an important role in human diseases, including cancer, increasing efforts have been focused on the development of agents that target epigenetic mechanisms. Successful examples of the use of epigenetic therapies in the treatment of cancer include hypomethylating drugs and histone deacetylase inhibitors that have been approved by the US Food and Drug Administration (FDA) [23]. In addition, the second generation of novel potential epigenetic therapies, such

as histone methyltrasferase inhibitors and epigenetic reader protein inhibitors, have been discovered and are currently under investigation [24]. Furthermore, numerous studies have suggested that the consumption of dietary phytochemicals may alter epigenetic modifications and reverse abnormal gene transcription, thereby preventing certain diseases, including cancer [25].

2.3. Epigenetic modifications and oxidative stress

Oxidative stresses are involved in almost all chronic diseases including ageing. Interestingly, epigenetic mechanisms have been reported to be profoundly involved in oxidative stress responses. ROS, such as hydroxyl radicals, can cause serious DNA lesions and lead to mutagenesis; such lesions can also result in global DNA hypomethylation [26, 27]. For instance, the DNA oxidization product 8-OHdG strongly inhibits the methylation of adjacent cytosines and impairs the binding of methyl-CpG-binding domain proteins (MBDs) [27]. Moreover, demethylation of methyl-CpG by Ten-eleven translocation (TET) enzymes, a family of Fe(II)/α-ketoglutarate-dependent dioxygenases, is a highly oxidative stress labile process. It involves serial oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, which inhibits DNA methyltransferase 1 (DNMT1) recognition, then to 5-formylcytosine and 5-carboxylcytosine, and finally be excised from DNA by glycosylases [26]. Furthermore, histone modifications can also be modulated by oxidative stresses [28]. In addition, many Nrf2-activating chemopreventive compounds have been identified as epigenetic modulators, and the expression of several Nrf2-target genes has been found to be regulated epigenetically [29-32]. Therefore, it is expectable that complex interactions exist between Keap1-Nrf2 signaling and epigenetic modifications.

3. Regulation of the Keap1-Nrf2 signaling pathway by DNA methylation

DNA methylation is widely observed in organisms ranging from prokaryotic bacteria to vertebrates; however, in vertebrates, heritable methylation only occurs at the 5 position of the cytosine pyrimidine ring in CpG dinucleotides. CpG methylation serves as an epigenetic mechanism to memorize the transcriptional state [33]. In mammalian cells, DNA methylation patterns are established during embryogenesis and development or under certain physiological and pathological conditions by de novo DNMT3a and DNMT3b, then maintained by DNMT1 during DNA replication. On the other hand, DNA demethylation can occur through active demethylation by TET enzymes or through passive demethylation caused by the absence of DNMT1 activity during DNA replication [34]. Approximately 60% of human genes contain clusters of CpG sites called CpG islands in their GC-rich promoter regions, and their expression can be epigenetically regulated by DNA methylation [35]. The methyl moiety lies in the major groove of the DNA helix and can potentially interact with many DNA-binding proteins. CpG methylation in the binding sequences can inhibit the binding of transcription factors and the initiation of transcription, or it can attract MBDs, such as MBD1, MBD2, and MeCP2, which can recruit co-repressor complexes to silence gene transcription [35]. In addition, DNA methylation also collaborates with histone modifications to regulate chromatin accessibility and gene transcription. MBDs often associate with histone deacetylases (HDACs) and histone lysine methyltransferases (HKMTs) to regulate histone modifications [33].

The hypermethylation of several genes regulated by Keap1-Nrf2 signaling has been investigated for decades. For example, the hypermethylation of CpG islands in the promoter region and expression silencing of pi class glutathione S-transferase (GSTPi) have been observed in prostate cancers [31]. NAD(P)H:quinone oxidoreductase 1 (NQO1), UDP-glucuronosyltransferases 1A1 (UGT1A1), glutathione peroxidase (GPX), and manganese superoxide dismutase (MnSOD) have also been reported to be regulated by promoter methylation [30, 36-38]. Regulation of Keap1 and Nrf2 expression by DNA methylation has been investigated in recent years and is discussed below and summarized in Table 1.

3.1. Regulation of Nrf2 expression by DNA methylation

The protein expression of Nrf2 and the Nrf2-targeted gene heme oxygenase 1 (HO-1) was abolished in skin tumors in a skin cancer mouse model [39]. Similar results were obtained in a transgenic adenocarcinoma of mouse prostate (TRAMP) model, in which the expression of Nrf2 and its downstream target genes, such as UGT1A1, glutathione S-transferase Mu 1 (GSTM1), and NQO1, were gradually down-regulated in prostate tumors during tumorigenesis [40]. Frolich et al. also reported that the expression of Nrf2 and GST mu family genes was significantly decreased in TRAMP prostate tumors [41]. More importantly, the expression of Nrf2 and several downstream genes such as GST and NQO1 has been found to be decreased in human prostate cancers compared with normal epithelia or localized adenoma [41, 42]. Yu et al. identified CpG islands in the promoter regions of human, rat, and mouse NFE2L2 genes and demonstrated that the suppression of Nrf2 expression in TRAMP prostate tumors and TRAMP C1 cells was mediated by the hypermethylation of specific CpG sites in the Nrf2 promoter [41, 42]. Further study by Khor et al. using human prostate cancer samples identified three specific CpG sites in the Nrf2 promoter that were hypermethylated during prostate cancer progression [43]. Moreover, treatment of TRAMP cells with the DNMT inhibitor 5-aza-2′-deoxycytidine (5-aza) and the HDAC inhibitor trichostatin A (TSA) could restore Nrf2 expression, which was accompanied by the dissociation of MBD2, MeCP2, and methylated histones [42].

Interestingly, inhibition of methylation or demethylation of the Nrf2 promoter has been found to be involved in the action of many chemopreventive chemicals. Dietary feeding of a γ-tocopherol–rich mixture of tocopherols (γ-TmT) dose-dependently suppressed prostate tumorigenesis and hypermethylation of the Nrf2 promoter in TRAMP mice and was associated with higher Nrf2 and NQO1 protein levels. γ-TmT treatment inhibited the protein expression of DNMT1, DNMT3a, and DNMT3b in the prostate of TRAMP mice, suggesting that γ-TmT inhibited both de novo and sustained methylation [44]. It has been well documented that many dietary cancer chemopreventive compounds, including curcumin [45], isothiocyanates [46], tea polyphenols [47], and genistein [48], are epigenetic modifiers [29]. Some of these compounds, such as curcumin, sulforaphane, 3,3′-diindolylmethane, and Z-Ligustilide (from the traditional Chinese medicine Radix *Angelicae Sinensis*), were also found to demethylate the Nrf2 promoter and re-activate Nrf2 signaling in the prostate of TRAMP mice or TRAMP C1 cells, possibly through the inhibition of DNMT and HDAC expression [49-52]. The CpG sites in the promoter region of Nrf2 are heavily methylated in mouse skin epidermal JB6 P+ cells and could be demethylated by sulforaphane, apigenin, or Tanshinone IIA. Such demethylation was associated with suppression of TPA-induced

transformation, reactivation of Nrf2 signaling, and expression of Nrf2 target genes, along with the inhibition of protein expression of DNMTs and HDACs [53-55]. These findings suggest that Nrf2 expression during carcinogenesis can be epigenetically regulated through DNA methylation at specific CpG sites in its promoter and that such mechanisms could be targeted for cancer prevention. However, given the paradoxical roles of Nrf2 in the process of carcinogenesis, the exact impact of Nrf2 modulators on cancer would be context-sensitive.

3.2. Regulation of Keap1 expression by DNA methylation

Loss of Keap1 function has been observed in many cancer tissues and is regarded as the main cause of Nrf2 over-activation. In addition to somatic mutations, the epigenetic regulation of Keap1 has been investigated in human tissues and cells of different diseases. Wang et al. first showed that Keap1 is highly expressed in BEAS-2B human normal bronchial epithelial cells but is down-regulated in a series of lung cancer cell lines and human lung cancer tissues. This down-regulation was accompanied by the hypermethylation of CpG sites in the Keap1 promoter region and could be restored by 5-aza treatment [56]. Further studies by the same group suggested that hypermethylation of the Keap1 promoter abrogated the binding of stimulating protein-1 (SP-1), and 5-aza treatment restored SP-1 binding to the Keap1 promoter [57]. In another study, using 47 pairs of NSCLC tissues and normal specimens, promoter methylation was detected in 47% of NSCLCs but in none of the normal tissues, whereas somatic mutations were detected in 15% of NSCLCs; patients harboring both alterations had the worst prognosis [58].

Similar results have been obtained in other cancers, including malignant gliomas and breast, colorectal, prostate, thyroid, and head and neck cancer cells. Frequent promoter hypermethylation and correlated down-regulation of Keap1 expression were observed in malignant gliomas and contributed to resistance to therapies and disease progression [59]. Aberrant Keap1 promoter methylation was detected in more than half of primary breast cancers and pre-invasive lesions but not in normal breast tissues, whereas no Keap1 mutations were detected in examined breast cancer cases. Methylation was more frequent in ER-positive, HER2-negative than in triple-negative breast cancers, and Keap1 promoter hypermethylation predicted higher mortality risk in triple-negative patients [60]. Keap1 promoter methylation was also observed in 53% of colorectal cancer tissues, in 25% of adjacent normal mucosa, and in 8 out of 10 colorectal cancer cell lines analyzed [61, 62]. Loss of Keap1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. In addition to point mutations of Keap1 in various prostate cancer cell lines, down-regulation of Keap1 expression by promoter hypermethylation was identified in DU-145 prostate cancer cells [63]. Hypermethylation is the major inactivating mechanism of Keap1 in thyroid cancers (70.6%) and head and neck cancers (29.3%) and is associated with a worse prognosis [64]. Here again we saw the uncertainty of the outcomes produced by epigenetic regulation of Keap1-Nrf2 signaling in cancers: while silencing of Nrf2 by DNA methylation is implicated in carcinogenesis, activation of Nrf2 signaling by hypermethylation of Keap1 promoter is also associated with tumor progression and resistance to therapies.

On the other hand, Keap1 promoter hypermethylation in oxidative stress-related diseases other than cancers plays mainly protective roles. Increased oxidative stress during chronic aging is a major pathological factor of age-related cataracts (ARCs), especially in diabetes patients; therefore, impaired Keap1-Nrf2 signaling is proposed to be involved in the pathogenesis of ARCs [65]. Although no SNPs in Nrf2 or Keap1 were found to be associated with Alzheimer's disease or age-related cataracts [66], demethylation of the Keap1 promoter accompanied by increased Keap1 and decreased Nrf2 expression was identified in cataractous lenses with increasing age or in diabetes patients, which may lead to failure of the cytoprotective system and increased oxidative stress [67, 68]. Exposure to homocysteine resulted in endoplasmic reticulum (ER) stress and the suppression of Keap1-Nrf2 signaling by ER-associated degradation (ERAD) and demethylation of the Keap1 promoter, elevated ROS generation and lens oxidation [65]. Treatment of human lens epithelial cells (HLEC) with acetyllcarnitine prevented the effects of homocysteine and significantly increased the levels of Nrf2 and downstream antioxidant genes [69]. Sodium selenite has been employed to induce cataracts in animal models and can suppress Keap1-Nrf2 signaling in HLECs by ERAD and Keap1 promoter demethylation, possibly by both reducing DNMT1/3a protein levels and inducing Tet1 expression [70]. Methylglyoxal and valproic acid promote lenticular protein oxidation and cataract formation by almost the same mechanisms as selenite [71, 72]. In addition, demethylation of the Keap1 promoter and increased Keap1 expression have been observed in diabetic cardiomyopathy, thus suppressing Nrf2 activity and disturbing the redox balance [73].

According to the observations described above, it would be of therapeutic interest to determine whether demethylation of the Keap1 promoter in neoplastic tissues could suppress tumor progression and resistance to therapies or whether the activation of Nrf2 signaling in lens epithelial cells could prevent cataract formation or the onset of other oxidative stress-initiated diseases.

4. Histone modifications and the Keap1-Nrf2 signaling pathway

Eukaryotic DNA is wrapped by octomers of four core histone proteins into repeating nucleosomes, which are further folded into chromatin fibers [74]. This highly organized and dynamic protein-DNA complex has two structurally and functionally distinguishable configurations, namely, heterochromatin and euchromatin. Heterochromatin represents a highly condensed structure with repressed gene transcription as a result of low accessibility of transcription factors and RNA polymerase II to their recognition sequences, whereas euchromatin is loosely packed and more easily transcribed [75]. It is suggested that posttranslational modifications of specific residues on the N-terminal tails of histones play a pivotal role in the modulation of the chromatin structure; ultimately, they regulate the transcriptional activity of a wide variety of genes. Here, we review and discuss the mutual effects of histone modifications and the Keap1-Nrf2 signaling pathway. Histone modifications shown to regulate the Keap1-Nrf2 signaling are summarized in Table 2.

4.1. Histone acetylation and the Keap1-Nrf2 signaling pathway

There is compelling evidence that the acetylation of histones neutralizes the positive charge, destabilizes the nucleosome structure, and promotes the accessibility of transcriptional factors to a genetic locus, thereby activating gene transcription, whereas histone deacetylation leads to gene silencing [76]. Histone acetyltransferases (HATs) and HDACs, which add and remove the acetyl groups, respectively, constitute a group of enzymes that dynamically regulate histone acetylation/deacetylation and gene transcriptional activity. Liu et al. reported that class 1 HDACs (1, 2, and 3) inhibit ARE-dependent gene expression. Furthermore, this study demonstrated that the Nf-kB subunit p65 suppresses the Nrf2-ARE pathway via selective deprivation of CREB-binding protein (CBP, a member of HAT) from Nrf2 and promotion of the recruitment of HDAC3 to ARE. Specifically, p65 enhances the interaction of HDAC3 with MafK (a known dimerization partner with Nrf2), facilitates the recruitment of endogenous HDAC3 to the ARE element, helps to maintain the histone hypoacetylation state in the local chromosome and hence represses ARE-dependent gene expression [77]. This mechanism provides direct evidence regarding the involvement of HDAC3 in the negative regulation of the Nrf2 pathway by NF-κB in response to inflammatory-related stimuli. Similarly, the impact of HDACs on the inhibition of Nrf2-mediated antioxidant defense in neuroinflammation has been investigated. Exposure to conditioned medium from lipopolysaccharide (LPS)-treated microglia (MCM_{10}) induced HDAC activity in astrocyte-rich cultures, which correlated with decreased acetylation in histones (H3 and H4) and reduced expression of Nrf2 and its target gene γ-glutamyl cysteine ligase modulatory subunit (γGCL-M). Notably, treatment with HDAC inhibitors, such as valproic acid and TSA, markedly elevated acetylation in H3 and H4, restored the Nrf2-mediated antioxidant responses, and thus resulted in an increased resistance to oxidative stress (H_2O_2) in astrocyte-rich cultures exposed to MCM_{10} [78]. In addition to protecting against neuroinflammation, the HDAC inhibitor also exhibited a promising effect in the protection of neuronal cell viability from oxygen-glucose deprivation and the attenuation of cerebral ischemic injury in the ischemic stroke mouse model via Nrf2 activation. Experimental evidence clearly showed that HDAC inhibitors activated the Nrf2 signaling pathway and up-regulated the Nrf2 downstream targets HO-1, NQO1, and glutamate-cysteine ligase catalytic subunit (GCLC) by suppressing Keap1 and promoting dissociation of Keap1 from Nrf2, Nrf2 nuclear translocation, and Nrf2-ARE binding. Importantly, the protective effect of HDAC inhibitors in cerebral ischemia was abolished in Nrf2-deficient mice [79]. Therefore, activation of Nrf2 through HDAC inhibition may provide a promising therapeutic strategy for preventing neural damage in ischemic stroke.

However, inhibition of HDAC does not always lead to Nrf2 activation. Mercado et al. reported that Nrf2 activity is impaired as a result of decreased Nrf2 stability in the presence of TSA (an HDAC inhibitor) in BEAS2B (human airway epithelial) cells or in HDAC2-knockdown cells. TSA treatment also significantly ameliorated the elevation of HO-1 expression in mice exposed to cigarette smoke. In addition, a significant correlation between the expression of HDAC2 and Nrf2 was found in monocyte-derived macrophages obtained from chronic obstructive pulmonary disease (COPD) patients [80]. Thus, a vicious circle in the pathogenesis of COPD is proposed: reduced HDAC2 activity observed in COPD as a result of oxidative stress could suppress Nrf2 stability and activity, thereby increasing

oxidative stress due to limited antioxidant responses, which then further impairs HDAC2 activity [80]. In fact, several studies have suggested that Nrf2 plays an important role in lung inflammation by modulating HDAC activity. For example, Nrf2-deficient mice were found to have diminished HDAC2 levels in the lungs and increased susceptibility to chronic cigarette smoke- and LPS-induced lung inflammation, which were not reversed by steroid therapy [81]. HDAC6, a critical regulator of autophagy-mediated airway inflammatory responses, was elevated in the lungs of Nrf2-deficient mice in response to cigarette smoke exposure [82]. Taken together, these findings show that the activity of the Keap1-Nrf2 signaling pathway is epigenetically regulated by HDACs; conversely, Nrf2-mediated oxidative stress responses may have an epigenetic impact on other signaling pathways through the modulation of HDAC activity. The involvement of the Nrf2-HDAC axis in the pathogenesis of human disorders, especially in inflammatory diseases, requires further investigation.

The detailed mechanisms underlying the regulation of the Keap1-Nrf2 pathway by HDAC/HAT are not yet fully understood. It appears that HDAC and HAT and their inhibitors not only regulate Nrf2 activity and ARE-dependent gene expression via the adjustment of histone acetylation in the promoter regions [77, 78] but also selectively modulate the acetylation of Nrf2 independently of histones [83, 84]. Lysine residues within the Nrf2 Neh1 DNA-binding domain can be acetylated directly by HAT (p300/CBP) in response to sodium arsenite-induced stress, and this acetylation is followed by the elevated expression of ARE-dependent genes [84]. hMOF, the HAT required for histone H4K16 acetylation, can acetylate Nrf2 at Lys^{588}. In human NSCLC tissues, hMOF-mediated acetylation of Nrf2 increased its nuclear retention and the transcription of its downstream genes, subsequently modulating tumor growth and drug resistance [85]. In addition, the acetylation of Lys^{588} and Lys ^{591} in the Neh3 domain by a selective inhibitor of sirtuin 1 (SIRT1, a class III HDAC) favors the nuclear localization of Nrf2, resulting in enhanced binding of Nrf2 to ARE and thereby increasing Nrf2-mediated gene expression. By contrast, a SIRT1 activator induces deacetylation, suppressing Nrf2 signaling accordingly [83].

The regulation of phase II detoxification enzymes by histone acetylation/deacetylation has also been investigated. It was previously reported that the acetylation of histones H3 and H4 on the chromatin of the promoter regions of the glutathione S-transferase placental form (GSTP) and GSTP enhancer 1 (GPE1) occurred in the H4IIE hepatoma cell line, where GSTP expression is activated, but not in the normal liver [86]. Monocytic leukemia zinc-finger protein (MOZ), a member of HAT, stimulates GSTP promoter activity in the presence of Nrf2. Although the precise mechanism by which histone acetylation regulates the gene transcription of GSTP remains unclear, the elevation of both MOZ and Nrf2 levels may be required [87]. UGT1A is another example of phase II enzymes regulated by histone acetylation. Gender-specific repression of UGT1A is controlled via chromatin remodeling through the recruitment of estrogen receptor alpha (ERα), HDAC1, and HDAC 2 to the xenobiotic response element (XRE) sites [88].

4.2. Histone methylation and the Keap1-Nrf2 signaling pathway

Histone methylation is also a critical player in the regulation of chromatin compaction and gene expression. The methylation of histones occurs on all basic residues, including arginines, lysines, and histidines. Different lysine sites can be mono (me1), di (me2) or tri (me3) methylated [89]. Depending on which residue is methylated and the degree of methylation, histone methylation can lead to either gene activation or suppression. For example, enhancer of zeste homolog 2 (EZH2) specifically catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) and leads to transcription repression [90], whereas histone-lysine N-methyltransferase (SetD7) monomethylates histone H3 lysine 4 (H3K4me1) and favors the binding of the transcription factor [91]. Abnormal expression of histone methyltransferases (HMT) and histone demethyltransferases (HDMs) may write an aberrant epigenetic mark on the histone tail, influencing gene expression and resulting in disease. The proteasome inhibitor MG132 increases the degradation of EZH2 through a compensatory Nrf1- and Nrf2-dependent increase in the proteasome subunit level [92]. Li and coworkers showed that decreased EZH2 expression significantly correlated with the elevated expression of Nrf2, NQO1, and HO1 in lung cancer tissues and cell lines, which was mainly attributed to a decrease in H3K27me3 in the Nrf2 promoter but not the NQO1 or HO1 promoter. Interestingly, the inhibitory effect of EZH2 on lung cancer growth in vitro and in vivo was abolished by Nrf2 deficiency [93]. Collectively, these data suggest that EZH2 suppresses lung cancer growth by inhibiting Nrf2 expression via H3K27 trimethylation in the promoter region. However, the up-regulated EZH2 level in a number of cancers, including prostate cancer, breast cancer, lymphomas, gastric cancer, hepatocellular carcinoma, and bladder cancer (see review [90]), suggests that EZH2 can be tumorigenic. The correlation of overexpression of EZH2 and the Keap1-Nrf2 signaling pathway in cancer tissues needs to be investigated. Furthermore, given the promising effect of EZH2 inhibitors in the treatment of cancer [94, 95], it will be interesting to explore the effect of EZH2 inhibitors on the Nrf2 pathway in cancer cells.

The modification of the Keap1-Nrf2 signaling pathway by methylation of histone 3 lysine 4 in diabetic retinopathy has been identified [96-98]. The diabetic environment induces oxidant production in the retina and its capillary cells and decreases the antioxidant response [99]. Mitochondrial superoxide dismutase (Sod2), an Nrf2 downstream target, becomes subnormal in diabetes due to lysine-specific demethylase-1 (LSD1)-mediated reduction of H3K4me1 and - me2 levels at the retinal Sod2 promoter. Sod2 inhibition may result in increased mitochondrial superoxide and the development of diabetic retinopathy [97]. In addition to Sod2, suppression of GCLC, an enzyme that is important for the biosynthesis of GSH, has been implicated in the progression of diabetic retinopathy. Specifically, reduced H3K4me1 and H3K4me3 and increased H3K4me2 at Gclc-ARE4 in the retina in diabetes results in impaired binding of Nrf2 at Gclc-ARE4. One possible reason for such an increase in H3K4me2 could be that activated JARID family protein (KDM5A), an H3K4me3 demethylase, demethylates H3K4me3, resulting in elevated H3K4me2 [98]. As an upper regulator, the association of Keap1 and Nrf2 could be regulated through epigenetic mechanisms, further impairing the antioxidant responses in diabetes. Indeed, hyperglycemia increases the binding of Sp1 at the Keap1 promoter through enrichment of H3K4me1 due to the activation of SetD7. In line with this finding, SetD7 knockdown leads to a lower

H3K4me1 level at the Keap1 promoter and reduced Sp1 binding, accompanied by the restoration of Nrf2 in high-glucose conditions. Following Nrf2 restoration, the binding of Nrf2 at the Gclc promoter and the expression of GCLC and HO-1 were enhanced, which is beneficial to rebalance the oxidative stress in a diabetic environment [96]. In addition, as epigenetic modifications can persist in a system, the above-mentioned alterations of the histone methylation level may continue to suppress the expression of antioxidant genes, resulting in high oxidative stress, even after the termination of the hyperglycemic challenge, known as metabolic memory phenomenon. The reestablishment of normal glucose conditions failed to reverse the abnormal methylation marks of H3K4 at the Sod2, Gclc-ARE4, and Keap1 promoters; therefore, the activity of Nrf2 and its downstream genes continues to be compromised [96-98]. According to these studies, the knockdown of the key enzymes that catalyze histone methylation appears to be effective in restoring the antioxidant defense system; thus, specific inhibitors of these enzymes may potentially protect diabetic retinopathy by regulating the Keap1-Nrf2 signaling pathway.

4.3. Histone readers and the Keap1-Nrf2 signaling pathway

Histone readers are the proteins that recognize the histone modifications that are deposited or removed by histone writers or erasers [100]. These readers play an essential role in the translation of a “histone code” for gene transcription. The bromodomain and extraterminal (BET) proteins are perhaps the most thoroughly characterized acetyl-lysine readers [101]. After binding to acetylated lysine residues, BET proteins may interact with transcription factors and chromatin remodeling complexes, recruiting them to gene promoters and thus activating or inactivating gene transcription [102]. It was recently found that BET proteins are involved in the regulation of antioxidant gene expression [103, 104]. BET proteins act as negative regulators of Nrf2 signaling; inhibition of BET proteins by genetic knockdown or the specific inhibitor JQ1 activates Nrf2-dependent transcription, increases the expression of the antioxidant genes HO-1, NQO1, and GCLC, and further ameliorates the ROS production induced by H_2O_2. BET proteins may interact directly with Nrf2 and are constitutively present at Nrf2-binding sites on the promoters of HO-1 and NQO1 [103]. In addition, Hussong et al. showed that BRD4, a member of the BET protein family, is a key mediator of Keap1 transcription under stress. However, under normal conditions, BRD4 appears to modulate anti-oxidative responses by directly targeting Sp-1 binding sites in the inducible heme oxygenase 1 (HMOX1) promoter [104]. However, the roles of histone readers in the interpretation of the histone code, chromatin remodeling, and recruitment of the repressive complex or co-activators to the Nrf2-regulated gene promoter remain unclear and need to be investigated in depth in the future.

5. Interaction of miRNAs and the Keap1-Nrf2 signaling pathway

miRNAs are endogenous short non-coding RNAs that usually contain 20-22 nucleotides. By complementary pairing with mRNA sequences, miRNAs inhibit the translation of mRNAs in ribosomes and/or facilitate the degradation of mRNA molecules. Thus, miRNAs represent another category of epigenetic mechanism, which regulates gene expression at the post-transcriptional level, mostly in a “fine-tuning” manner. During recent decades, increasing efforts have been made to profile miRNA expression patterns and characterize miRNA

functions to identify novel diagnostic markers and therapeutic targets. Among these studies, a number of miRNAs have been reported to affect the Keap1-Nrf2 signaling pathway at several nodes (summarized in Table 3).

5.1. miRNAs regulate Nrf2 activity by directly targeting the mRNA of Nrf2

As miRNAs function as post-transcriptional repressors of gene expression, miRNAs that directly target Nrf2 usually negatively regulate the Keap1-Nrf2 pathway. Notably, inefficient activation of Nrf2 results in alteration of Nrf2-dependent redox homeostasis, thus potentially triggering disease outcomes. Erythrocytes from patients with homozygous sickle cell disease (HbSS) have a reduced tolerance for oxidative stress. In a subset of HbSS patients with more severe anemia, higher erythrocytic miR-144 expression has been observed [105]. In the same study, Sangokoya et al. found that the 3′ UTR of Nrf2 is directly targeted by miR-144 in K562 cells and primary erythroid progenitor cells. Therefore, increased miR-144 may contribute to the attenuated Nrf2 levels in HbSS erythrocytes, which could account for the decrease in glutathione regeneration and impaired oxidative stress tolerance. By employing bioinformatic analysis of the human Nrf2 3′ UTR sequences for miRNA binding sites, Narasimhan et al. reported an in-silico prediction of 4 different miRNAs targeting human Nrf2, including hsa-miR27a, hsa-miR153, hsa-miR142-5p, as well as the already reported hsa-miR144 [106]. The direct interaction between the four identified miRNAs and Nrf2 was further validated using luciferase constructs carrying either the 3′ UTR of human Nrf2 or mutated miRNA binding sites within the Nrf2 3′ UTR. Moreover, ectopic expression of the corresponding miRNA mimics affected cellular Nrf2 mRNA levels as well as the nucleo-cytoplasmic concentration of the Nrf2 protein in a Keap1-independent manner, which consequently lessens GCLC and glutathione reductase (GSR) expression.

In the context of cancer, a variety of miRNAs have been implicated in cell differentiation, cell proliferation/apoptosis, and tumor suppression [107, 108]. Specifically in the Keap1-Nrf2 pathway, miR-28 expression has been reported to be reversibly correlated with Nrf2 mRNA levels in human mammary epithelial cells and the breast cancer MCF-7 cell line [109]. Yang et al. also demonstrated that miR-28 regulates the Nrf2 pathway by targeting the 3′ UTR region, thereby facilitating the degradation of Nrf2 mRNA. In addition, Nrf2 shRNA or ectopic expression of miR-28 inhibited the anchorage-independent cell growth of MCF-7 cells, suggesting that miR-28 might influence breast cancer motility and growth by regulating the Nrf2 pathway. Similarly, in 17β-estradiol (E2)-induced rat breast carcinogenesis, an E2-mediated increase in miR-93 levels was associated with decreased expression of Nrf2 [110]. Furthermore, in human breast cell lines, miR-93 has been shown to have oncogenic potential, including the ability to increase colony formation, mammosphere formation, cell migration, and DNA damage and to decrease apoptosis.

5.2. miRNAs regulate Nrf2 activity by interacting with cellular Nrf2 regulators

On the other hand, those miRNAs that interact with cellular Nrf2 regulators are expected to influence Nrf2/ARE signaling as well. Keap1 has been most extensively studied as a cellular suppressor of Nrf2. In human breast cancer MDA-MB-231 cells, Eades et al. reported that miR-200a could interact with the Keap1 3′UTR, facilitating its mRNA degradation [111]. Therefore, the decreased miR-200a levels in breast cancer may provide a novel mechanistic

explanation for the deregulation of the Nrf2 pathway. By inducing the re-expression of miR-200a, the reduction in Keap1 levels subsequently enhances Nrf2 nuclear accumulation and NQO1 gene transcription. Furthermore, this study demonstrates that Nrf2 activation consequently inhibits the anchorage-independent growth of breast cancer cells in vitro and carcinogen-induced mammary hyperplasia in vivo. Cul3 is an important component of the Keap-1 protein complex that promotes Keap1-dependent Nrf2 ubiquitination and proteasomal degradation [112]. Kim et al. demonstrated that Cul3 is a target of miR-101. Under hypoxic conditions, miR-101 is up-regulated in a HIF-1a-dependent manner, thereby stabilizing Nrf2 protein and inducing HO-1. Local overexpression of miR-101 improves neovascularization and blood flow in a mouse model of hindlimb ischemia. A mechanistic study indicated that a positive feedback loop between the Nrf2/HO-1 and VEGF/eNOS axes is implicated in miR-101-mediated post-ischemic vascular remodeling and angiogenesis. Bach1 is a MAF-related transcription factor that plays critical role in specific of HO-1 gene regulation. In cells naïve to oxidative stress, Bach1 conceals the ARE sequences; thus, it antagonizes Nrf2 binding and represses HO-1 gene transcriptional activation [113]. In an earlier report, MacLeod et al. demonstrated the specificity attributes to that only HO-1 contains the necessary multiple *cis*-elements required for efficient Bach1 binding among human ARE-driven gene battery [114]. Hou et al. reported that let-7 miRNAs (let-7b, 7c) enhanced HO-1 gene transcription by down-regulating Bach1 protein levels [115]. Ectopic expression of the let-7 miRNA in Huh-7 cells resulted in increased resistance against oxidant injury induced by tert-butyl-hydroperoxide (tBuOOH), whereas the enhanced anti-oxidative capacity was counteracted by Bach1 over-expression. It is worth mentioning that the pro-inflammatory miR-155 also targets Bach1 degradation and induces subsequent elevation of HO-1 expression in endothelial cells [116]. It has been proposed that the cytoprotective response to inflammation results from the miR-155-mediated regulation of HO-1 rather than direct induction via the NF-κB pathway. This study provides a novel mechanistic insight by introducing miRNA in the cross-talk between the inflammatory and oxidative stress pathways. However, in lipopolysaccharide-stimulated murine RAW264.7 macrophages, either sulforaphane or allyl-isothiocyanate treatment leads to decrease in miR-155 levels, accompanied by HO-1 induction [117]. Given that each miRNA could have multiple target genes while being controlled by a variety of upstream signals, the precise mechanisms by which miR-155 affects the Nrf2-mediated cellular protective system remain to be fully elucidated.

5.3. Transcriptional regulation of miRNAs by Nrf2

The biogenesis of miRNAs is similar to that of other RNA molecules and starts with the transcription of the genes that encode immature primary miRNA (pri-miRNA) by RNA polymerase II. It is possible that the transcription of pri-miRNAs could be regulated by transcription factors (TFs) such as Nrf2. Indeed, recent studies provide evidence that a number of miRNAs can be regulated by Nrf2, reviewed by [118]. In addition, a systematic analysis of the interactors and regulators of Nrf2 conducted by Papp et al. predicted 85 miRNA-Nrf2 mRNA interactions [119]. Interestingly, 35 TFs regulated by Nrf2 could increase the levels of 63 out of the 85 miRNAs mentioned above. This model indicates that miRNAs are involved in the fine-tuning feedback loops in Nrf2 signaling.

6. Cross-talk of epigenetic mechanisms in the modification of the Keap1-Nrf2 signaling pathway

Circumstantial evidence suggests that different epigenetic layers may be engaged in complex crosstalk to establish and maintain different chromatin states. Thus, the above-mentioned epigenetic modifications may not function alone, but they may be linked to each other and work in combination to regulate gene transcription [120]. Studies in our laboratory suggested that hypermethylated CpG islands in TRAMP C1 cells were associated with MBD2 and histone modifications, indicating interplay between DNA methylation and histone modification in the regulation of Nrf2 transcription activity. Chromatin immunoprecipitation assays showed that MBD2 and tri-methylated histone 3-lys9 (H3K9me3) are enriched in methylated CpGs in the Nrf2 promoter, whereas acetylated histone 3 (H3Ac) is associated with unmethylated CpGs [42]. Additionally, recent research has reported that nutritional phytochemicals, including sulforaphane, apigenin, 3,3′-diindolylmethane, and tanshinone IIA, epigenetically re-activate the expression of Nrf2 through the inhibition of both DNMT and HDAC [50, 51, 53-55]. Although these studies suggest an intimate communication and confounding actions between DNA methylation and histone acetylation/methylation in the silencing/activation of the Nrf2 gene, the fundamental question regarding which epigenetic event initiates and steers the crosstalk and Nrf2 silencing remains to be answered. Two different sequential models have been proposed to describe the interplay of DNA methylation and histone modification in gene silencing (reviewed in [121]). In one scenario, partial DNA methylation trigged by environmental and intrinsic signals attracts the binding of MePC2 and HDAC to the CpG sites, leads to deacetylated histone and inactive chromatin configuration, and further recruits DNMT1 to amplify the silencing signals. In another scenario, imbalanced HAT and HDAC activities induce chromatin hypoacetylation, a chromatin state that will be recognized by de novo DNMTs and result in a local hypermethylation state. Future studies are necessary to understand the mechanisms underlying the combination of the epigenetic events in the regulation of Keap1-Nrf2 signaling pathways.

These epigenetic modifications not only work in combination to interact with Keap1-Nrf2 signaling pathways, but are also known to cross-regulate each other in a manner that diversifies their functions and ultimately influences cellular activity. For example, the miR-200 family was previously shown to be aberrantly silenced by epigenetic mechanisms in breast cancer [122], and impaired miR-200a activity led to the overexpression of SIRT1 (class III HDAC) [123]. It is noteworthy that the epigenetic silencing of miR-200a by histone acetylation might contribute to the overexpression of Keap1 and loss of the Nrf2-dependent antioxidant pathway in breast cancer cells. Furthermore, Eades and coworkers found that treatment with the HDAC inhibitor suberoylanilide hydroxamic acid re-expressed miR-200a, which corresponded to decreased Keap1 expression, increased Nrf2 translocation, and elevated Nrf2-dependent NQO1 expression [111]. More recently, another study reported similar results: the HDAC inhibitor MS-275 efficiently reduces the deacetylation in the miR-200a promoter region and reactivates miR-200a. As a consequence, mature miR-200a destabilizes Keap1 mRNA, leads to enhanced translocation and binding of Nrf2 to the polyamine-responsive element of the spermidine/spermine N^1-acetyltransferase

(SSAT) promoter, and ultimately results in reduced polyamine synthesis and growth inhibition [124]. These studies suggested that cross-regulation of epigenetic modifications modulates Keap1-Nrf2 signaling. Therefore, more in-depth understanding will be necessary to exploit this complex regulatory network to combat dysregulation of the Keap1-Nrf2 pathway in chronic diseases.

7. Conclusions and perspectives

Keap1-Nrf2 signaling plays important roles in a variety of physiological, pathological, pharmacological, and toxicological processes and is subjected to multiple layers of regulation at transcriptional, translational, and post-translational levels. In recent years, the epigenetic regulation of Keap1 and Nrf2 expression in various oxidative stress-related diseases has begun to be unveiled. As depicted schematically in Figure 1, Keap1 and Nrf2 expression could be regulated by methylation/demethylation of CpGs in the promoter regions, acetylation/deacetylation and methylation/demethylation of histones, or targeting of mRNAs by miRNAs. In addition, Nrf2 has also been implicated in the transcriptional regulation of certain non-coding RNAs. However, although oxidative stresses are profoundly engaged in epigenetic modifications and chromatin organization, it is not clear whether Keap1-Nrf2 signaling directly or indirectly regulates epigenetic processes other than miRNA transcription.

To date, most epigenetic regulation of Keap1-Nrf2 signaling has been identified in the context of cancer. Because oxidative stresses and Keap1-Nrf2 signaling are widely involved in almost all major chronic diseases, the participation of epigenetic mechanisms in the regulation of Keap1-Nrf2 signaling in these diseases will be interesting to elucidate. Indeed, investigations have revealed the important roles of the demethylation of the Keap1 promoter in ARCs and cardiomyopathy and the reactivation of Nrf2 by HDAC inhibitors in neuroinflammation and cerebral ischemic injury, and further investigations in other oxidative stress-related diseases are guaranteed. Moreover, given the complexity of the crosstalk between genetic/epigenetic modifications and the Keap1-Nrf2 signaling networks, the exact mechanisms of epigenetic regulation of Keap1-Nrf2 signaling and their physiological significance remain open for further investigation.

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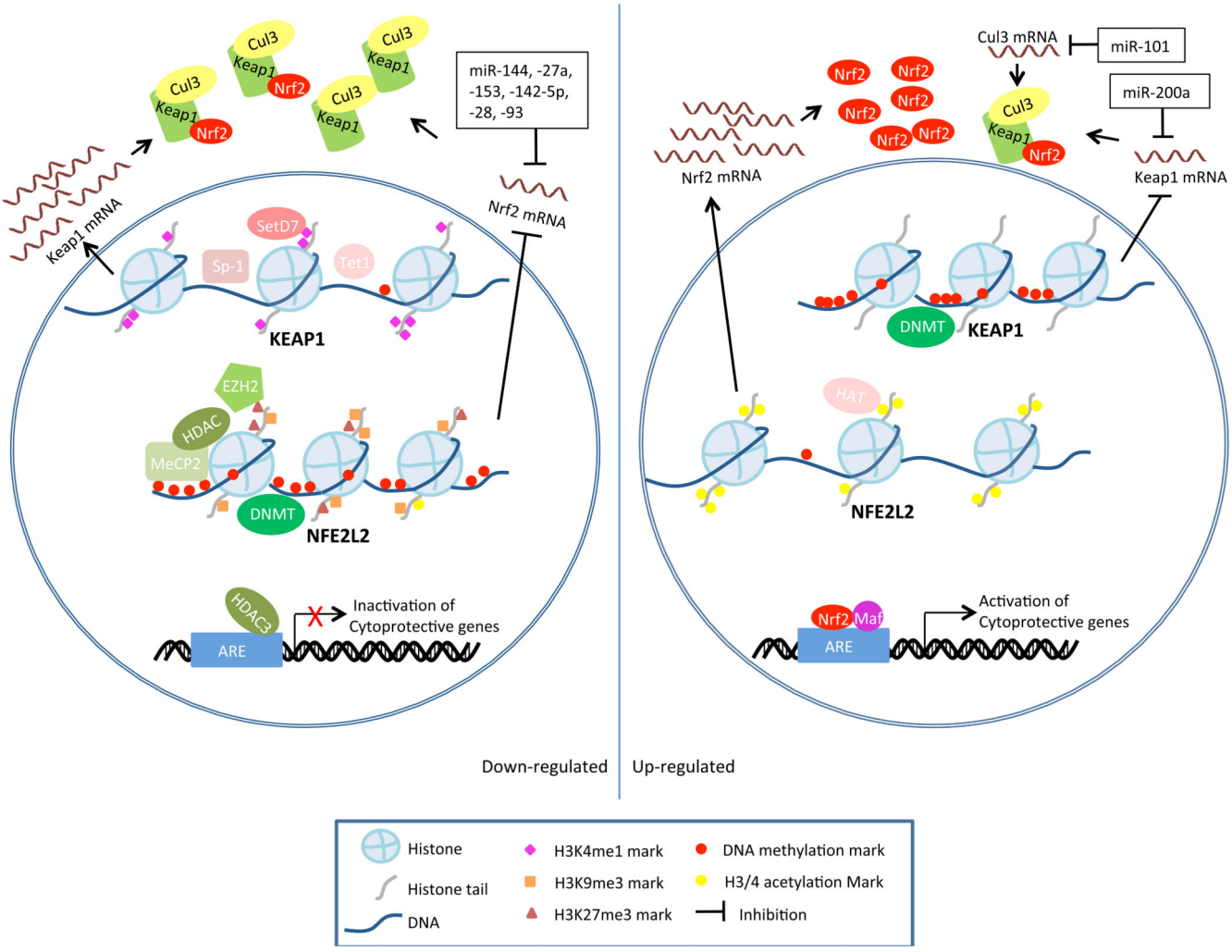

Figure 1.

Schematic model depicting epigenetic modifications of Nrf2 and Keap1. Left and right panel shows epigenetic modifications that lead to down- and up-regulation of Keap1-Nrf2 signaling, respectively.

Table 1

DNA methylation regulates Keap1-Nrf2 signaling pathway.

Target	Diseases	Methylation	Experimental Model	Impact on Keap1-Nrf2	Outcomes	Ref
Nrf2	Prostate cancer	↑	TRAMP mice and TRAMP-C1 cells	↓ Nrf2, NQO1, GST mu	Prostate carcinogenesis	[40-42]
		↑	Human prostate cancer tissues	↓ Nrf2	Advanced stages	[43]
		↓	TRAMP-C1 or LNCap cells treated by 5-Aza/TSA	↑ Nrf2, NQO1, HO-1 ↓ DNMTs, HDACs	Not Applicable	[42, 43]
		↓	TRAMP-C1 cells or TRAMP mice treated by γ-TmT, curcumin, sulforaphane, 3,3′-diindolylmethane, or Z-Ligustilide	↑ Nrf2, NQO1, HO-1 ↓ DNMT, HDAC	Inhibition of carcinogenesis	[44, 49-52]
	Skin cancer	↓	Mouse skin epidermal JB6 P+ cells treated by sulforaphane, apigenin, or Tanshinone IIA	↑ Nrf2, NQO1, HO-1 ↓ DNMT, HDAC	Inhibition of TPA-induced transformation	[53-55]
Keap1	Lung cancer	↑	Human lung cancer tissues and cell lines	↓ Keap1	Not Applicable	[56, 57]
			Human non-small cell lung cancer	Not Applicable	Worse prognosis	[58]
	Gliomas	↑	Human malignant gliomas	↓ Keap1	Better prognosis	[59]
	Breast cancer	↑	Primary breast cancers and pre-invasive lesions	↓ Keap1	Higher mortality in triple-negative; reduced relapse	[60]
	Colorectal cancer	↑	Colorectal cancer cell lines and surgical specimens	↓ Keap1 ↑ Nrf2, NQO1, AKR1C1	Not Applicable	[61]
	Prostate cancer	↑	Prostate cancer cell lines	↓ Keap1; ↑ Nrf2, HO-1, NQO1, Gclc	Increased tumor growth, enhanced chemo- and radio-resistance	[63]
	Thyroid cancers	↑	Papillary thyroid carcinoma	↓ Keap1 ↑ Nrf2-regulated genes	Not Applicable	[64]
	Age-related cataracts	↓	Cataractous lenses with increasing age or in diabetes patients; human lens epithelial cells treated by homocysteine, valproic acid, methylglyoxal or selenite	↑ Keap1, TET1 ↓ Nrf2, CAT, GST, DNMT	Elevated ROS, age-related cataracts	[65, 67-72]
	Diabetic cardiomyopathy	↓	Myocardial biopsies of non-diabetic and type-2 diabetic cardiomyopathy patients	↓ Keap1 ↑ Nrf2-regulated genes	Failure of Nrf2 mediated antioxidant system	[73]

Table 2

Histone modifications regulate Keap1-Nrf2 signaling pathway.

Target	Diseases	Modifications	Enzymes	Experimental Model	Impact on Keap1-Nrf2 and outcomes	Ref
Nrf2	Neuroinflammation and neurodegenerative diseases	Deacetylation of histones H3 and H4	HDACs	Astrocyte-rich cultures exposed to conditioned medium	High level of HDAC activity leads to: ↓Nrf2 and γGCL-M; ↓Nrf2-mediated antioxidant defense	[78]
	Chronic obstructive pulmonary disease	Histone acetylation	HDAC2	Human airway epithelial BEAS2B cells, monocyte-derived macrophages from COPD patients	Treatment of HDAC inhibitor leads to: ↓Nrf2 stability; ↓Nrf2-regulated HO-1 expression; ↑sensitivity to oxidative stress	[80]
	Human non-small cell lung cancer	H3K27me3	EZH2	A549 cells, human non-small cell lung cancer patients, nu/nu mice	Low expression of EZH2 leads to: ↑Nrf2, NQO1, and HO-1	[93]
Keap1	Cerebral ischemic injury	Histone acetylation	HDACs	Permanent middle cerebral artery occlusion model in mice, cortical neuronal cells, and RAW 264.7 cells	Treatment of HDAC inhibitor leads to: ↓Keap1; ↑Nrf2 nuclear translocation; ↑Nrf2-ARE binding; ↑HO-1, NQO1, and GCLC; ↑neuronal cell viability; and ↓cerebral ischemic injury	[79]
	Diebetic retinopathy	H3K4me1	SetD7	Bovine retinal endothelial cells, retina from rats and human donors	Hyperglycemia leads to: ↑SetD7; ↑binding of Sp1 at Keap1; ↑Keap1; and ↓Nrf2, Gclc, and HO-1.	[96]
HO-1 E1 enhancer	Pathological processes of inflammation and cancer	Histone hypoacetylation	HDAC3	HepG2, HEK293, and L929 cells	↓ ARE-dependent gene expression	[77]
Sod2	Diebetic retinopathy	H3K4 methylation	LSD1	Bovine retinal endothelial cells, retina from rats and human donors	Hyperglycemia leads to: ↑binding of LSD1 and Sp1 at Sod2; ↓Sod2 expression.	[97]
Gclc-ARE4	Diebetic retinopathy	H3K4 methylation	LSD1, KDM5A	Bovine retinal endothelial cells, retina from rats and human donors	Hyperglycemia leads to: ↓binding of Nrf2 at Gclc-ARE4 and Gclc transcripts	[98]

Table 3

miRNAs regulate Keap1-Nrf2 signaling pathway.

Target	miRNA	Experimental model	Impact on Keap1-Nrf2	Outcomes	Ref
Nrf2	miR-144	K562 cell line, primary erythroid progenitor cells	↓Nrf2 levels, ↓glutathione regeneration ↓antioxidant capacity	Associated with anemia severity in sickle cell disease	[105]
	miR-27a,-142-5p,-144, -153	SH-SY5Y cells	↓Gclc, Gsr levels	Not applicable	[106]
	miR-28	MCF-7 cell line	↓Nrf2 mRNA	Increased anchorage-independent growth	[109]
	miR-93	E2-induced breast carcinognesis in ACI rats	↓Nrf2 and Nrf2 regulated genes	Decreased apoptosis, increased DNA damage	[110]
Keap1	miR-200a	MDA-MB-231 cell line	Keap1 mRNA degradation, ↑Nrf2 nuclear accumulation, ↑NQO1	Inhibits anchorage-independent growth	[111]
Cul3	miR-101	hypoxic condition	↑Nrf2 nuclear accumulation, ↑HO-1	Improves neovascularization and blood flow in ischemia	[112]
Bach1	let-7	Huh7 cell line	↑HO-1	Increased resistance against oxidant injury against tBuOOH	[115]
	miR-155	primary HUVECs	↑HO-1	Cytoprotective during inflammation	[116]