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REVIEW

Mazes of Nrf2 Regulation

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Abstract—Nrf2 transcription factor plays a key role in maintaining cellular redox balance under stress and is a perspective target for oxidative stress-associated diseases. Under normal conditions, Nrf2 transcriptional activity is low due to its rapid ubiquitination and degradation in the 26S proteasome, as well as through various modifications of amino acid residues of this transcription factor that regulate its transport to the nucleus and binding to DNA. Continuous activation of Nrf2 is possible due to autophagy and epigenetic regulation that may underlie the increased resistance of tumor cells to radiotherapy and chemotherapy. This review deals with the mechanisms of regulation of Nrf2 transcriptional activity and its main elements, and pharmacological approaches to activation of the Keap1/Nrf2/ARE system.

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Transcription factor Nrf2 belongs to "leucine zipper"-containing factors of the CNC family that form homo- or heterodimers for DNA binding. The mammalian CNC family comprises six members: four NFE factors (p45 NF-E2, Nrf1, Nrf2, and Nrf3) and two BTB factors (Bach1 and Bach2). Nrf2 binds to DNA at the antioxidant response element (ARE), which contains the sequence 5′-A$^{/G}$TGAC$^{/T}$nnnGCA$^{/G}$-3′. All the factors of the CNC family can form regulatory-active dimers. However, depending on their ability to bind transcription cofactors, they either promote or inhibit transcription of ARE-dependent genes. Therefore, repression or induction of each leads to different effects on the cellular or organismal level [1-3]. In most cases, transcription factors Bach1 and Bach2 act as antagonists of Nrf2, competing for binding to ARE. Normally, Bach1 is localized mainly in the nucleus, which allows keeping some genes repressed [4]. Involvement of multiple components allows forming tissue- and organ-specific systems of regulation of gene expression that changes during ontogenesis [2, 5].

Transcription factor Nrf2 contains seven conserved domains Neh1-Neh7. Each of these domains performs a

Abbreviations: Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; aPKC, atypical protein kinase C; ARE, antioxidant response element); BTB, Broad-complex (Tramtrack, Bric-a-brac); bZip, basic leucine zipper DNA-binding domain; CNC, Cap'n'Collar; ERK1/2, extracellular signal-regulated protein kinase 1/2; FXR, farnesoid X receptor; GSK-3, glycogen synthase kinase 3; HER2/ErbB2/neu, tyrosine protein kinase of EGFR/ErbB receptor family; IKKβ, β-subunit of IκB-kinase; JAK/STAT, tyrosine kinase Janus kinase/signal transducer and activator of transcription; JNK, kinases of MAPK family (c-Jun N-terminal kinases); Keap1, Kelch-like ECH-associated protein 1; KIR, Keap-interacting region; LC3, microtubule-associated protein 1A/1B-light chain 3; LIR, LC3 interacting region, MAPK, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin (a serine/threonine protein kinase); mTORC1/2, mammalian target of rapamycin complex 1/2; Neh, Nrf2-ECH homology (ECH is a homolog of Nrf2 in chickens); NES, nuclear export signal; NFE, nuclear factor-erythroid derived; NLS, nuclear localization signal; Nrf2, NF-E2-related factor 2; PB1, Phox and Bem1; PERK, protein kinase-like endoplasmic reticulum kinase; PGAM5, serine/threonine phosphatase (phosphoglycerate mutase family, member 5); PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; p38 MAPK, p38 mitogen activated protein kinase; PPARγ, receptor binding peroxisome proliferators (peroxisome proliferator-activated receptor γ); p62/SQSTM1, ubiquitin-binding protein p62, same as sequestosome 1; PTEN, dual-specificity protein phosphatase (phosphatase and tensin homolog deleted on chromosome 10); Rbx1, RING-box protein 1; ROS, reactive oxygen species; RXRα, retinoid X receptor α; TRAF6, TNFα receptor-associated factor 6; β-TrCP, β-transducin repeat containing protein; UBA, ubiquitin association.

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specific function (Fig. 1) [6-8]. The bZip-like domain Neh1 is responsible for Nrf2 dimerization with other proteins and DNA binding. In "leucine zippers", a leucine residue is located in every seventh position. These residues form a hydrophobic region allowing dimerization. At the same time, due to a specific structure of the bZip-domain, Nrf2 does not form homodimers. Instead, it interacts with bZip regions of auxiliary regulatory proteins that belong to the Maf and Jun families [9, 10]. The Neh2 domain is responsible for Nrf2 binding to the regulatory protein Keap1 (Kelch-like ECH-associated protein 1). For this purpose, this domain contains two motifs: ETGE and DLGex, which possess high ($K_a = 20 \cdot 10^7$ M^{-1}) and low ($K_a = 0.1 \cdot 10^7$ M^{-1}) affinities, respectively (Fig. 1). There are lysine residues between the ETGE and DLGex motifs that are subject to ubiquitination. Domain Neh3, situated at the C-terminal part of the protein, and tandem Neh4 and Neh5 provide transactivation activity of Nrf2. They bind to the transcription coactivators CBP/p300 and BRG1 in a synergetic manner. This leads to acetylation of histone proteins and chromatin decondensation allowing interaction of RNA polymerase complex with promoter regions of DNA, thus triggering synthesis of target gene mRNAs. The presence of domains Neh7 and Neh6 allows additional posttranslational negative regulation of Nrf2, which is redox-independent, in contrast to the one mediated by Neh2 and Keap1. Neh7 interacts with transcription factor RXRα (retinoid X receptor α) [11], and a poly-serine region of Neh6 contains DSGIS and DSAPGS motifs that are responsible for binding of substrate adaptor of E3-ubiquitin ligase complex $SCF^{\beta\text{-TrCP}}$ [12, 13].

Human Nrf2 contains six cysteines (seven in mice and rats), which are subject to oxidative modification [14], and numerous amino acid residues that can be phosphorylated [15]. Besides, Nrf2 protein has several nuclear localization signals (NLS) and nuclear export signals (NES) that are required for transport of the transcription factor into nucleus or cytoplasm, respectively. Due to its unique structure, the activity of Nrf2 as a transcription factor that determines expression of genes that it controls may be altered at the levels of transcription, translation, posttranslational modifications, nuclear translocation, and binding to promoters of regulated genes.

The importance of Nrf2 for life support processes becomes evident in experiments on animals with disrupted genes (knockouts). Though homozygous mice ($Nrf2^{-/-}$) do not show substantial deviations during birth and maturation compared to wild-type animals ($Nrf2^{+/+}$), different diseases develop by the age of two months in these mice. Starting from 20 weeks, high mortality caused mainly by autoimmune glomerulonephritis is observed [16]. Besides, in numerous studies it was shown that mice with *Nrf2*$^{-/-}$ genotype demonstrate increased severity of inflammatory response to different factors [17], probability of development of provoked tumors [18] and neurodegenerative diseases [19], ischemia and reperfusion damage [20], and lack of therapeutic effect of Nrf2 inducers under these conditions.

REGULATION OF Nrf2 ACTIVITY

Ubiquitination. A major mechanism for regulation of Nrf2 activity is changing stability of the protein at the posttranslational level. Nrf2 is a short-lived protein: its half-life ($t_{1/2}$) in murine hepatoma Hepa cells is 13 min, in rat hepatocytes – 22 min, in human HepG2 cells – 15 min, in murine peritoneal macrophages – 18.5 min [21, 22]. In the absence of activators, stability of Nrf2 in cells is determined by the enzymatic ubiquitination of its lysine residues and subsequent degradation by the 26S proteasome [7]. It was demonstrated in cell cultures that Nrf2 concentration in cells and nuclei grows upon inhibi-

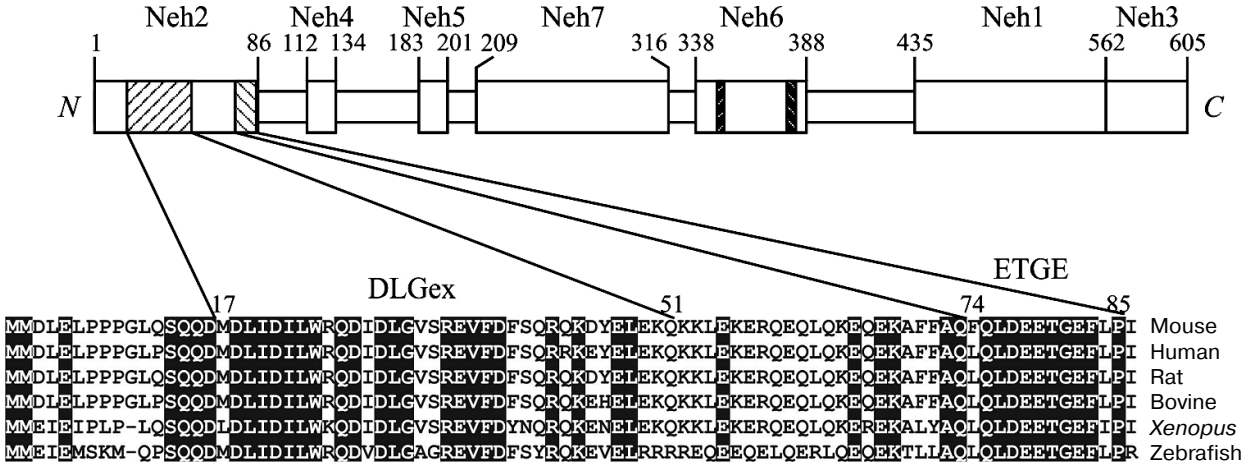

Fig. 1. Structural organization of transcription factor Nrf2 and its Neh2 domain interacting with Keap1 dimers.

tion of proteasome activity. Formation of complexes of Nrf2 with ubiquitin ligases requires adaptor proteins.

Keap1-dependent Nrf2 ubiquitination is the classical (canonic) Nrf2 ubiquitination pathway. Here, adaptor protein Keap1 promotes interaction of Nrf2 with Cullin-3-containing ubiquitin ligase complex E3 (further, Cul3-E3-ligase) [23]. Normal ubiquitination also requires protein Rbx1 (RING-box protein 1), which along with Cul3 forms a catalytic component of the enzymatic complex and interacts with E2 ubiquitin ligase for transfer of ubiquitin to Nrf2 (Fig. 2a). Keap1 contains five conserved domains: an N-terminal region (NTR), domains BTB, IVR, Kelch (consists of six repeats of Kelch motifs), and C-terminal region (CTR) (Fig. 3). The BTB domain participates in formation of Cul3-Rbx1-ligase complex [25]. The cysteine-rich domain IVR is a sensor for a broad spectrum of electrophilic compounds and regulates Nrf2 ubiquitination. The C-terminal Kelch domain interacts with ETGE and DLGex motifs of the Neh2 domain, thus providing regulation of Nrf2. It also interacts with cytoskeleton proteins actin and/or myosin VIIa, which causes mainly cytoplasmic localization of the Keap1/Nrf2 complex.

Oxidative/electrophilic modification of thiol groups of cysteine residues in Keap1 plays a key role in reducing

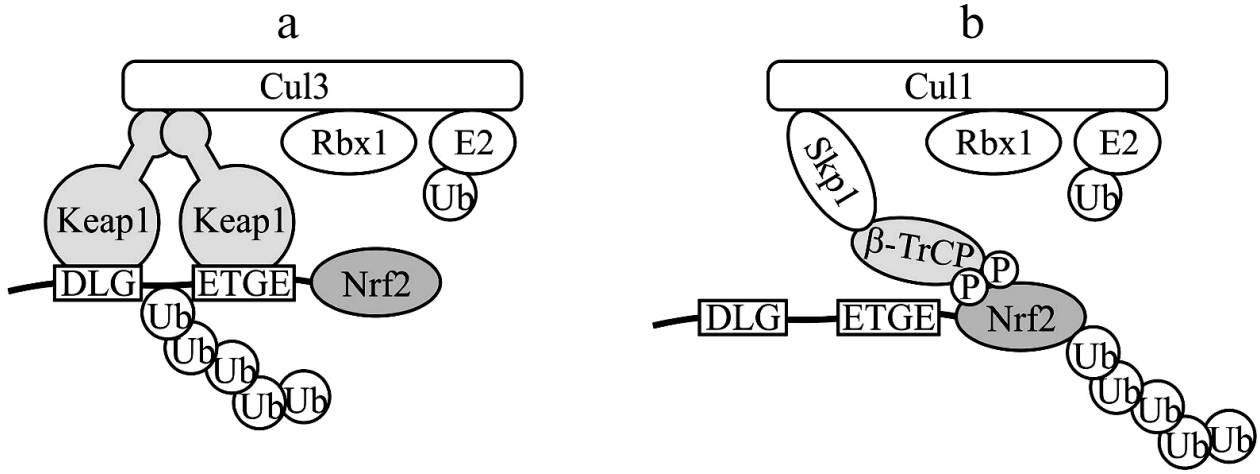

Fig. 2. Nrf2 ubiquitination with participation of adaptor proteins Keap1 (a) and β-TrCP (b) (according to Harder et al. [24]).

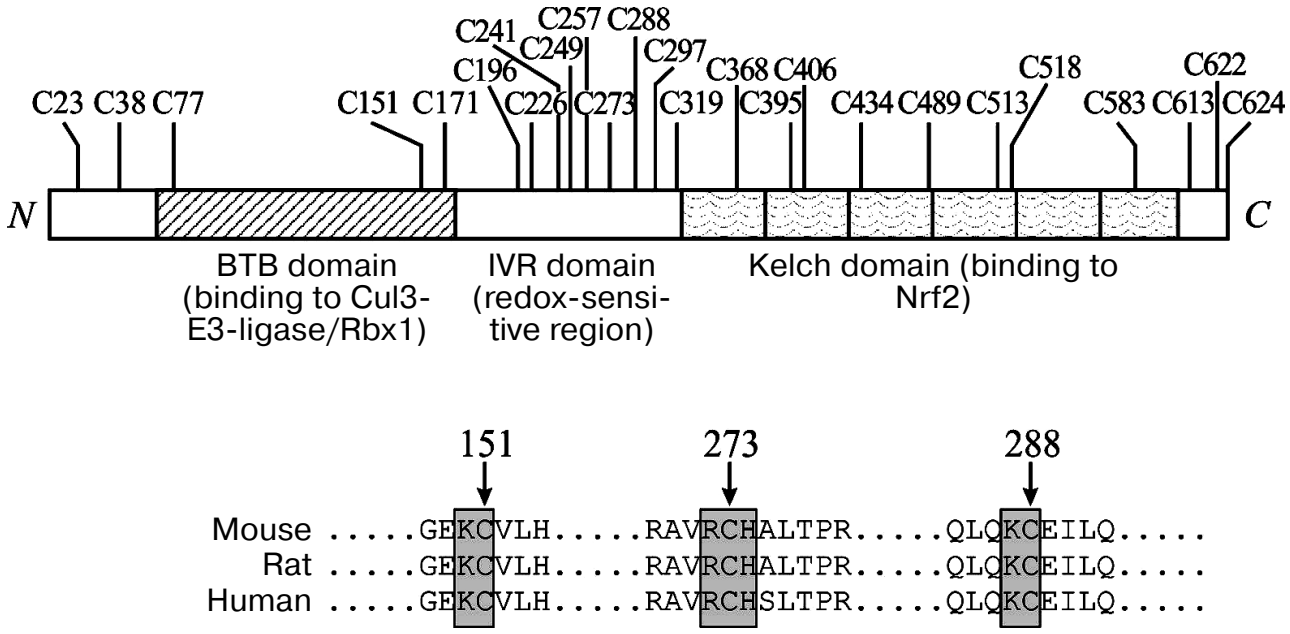

Fig. 3. Structural organization of Keap1 and location of its cysteine residues.

the ubiquitination-related repression of Nrf2 transcription factor. For instance, murine Keap1 contains 25 cysteines (in humans – 27; 4.3% of all amino acid residues), which are sensors for a broad spectrum of compounds that affect complex formation of Keap1 and Nrf2 or interaction with Cul3-E3-ligase. All cysteine residues in Keap1 are somewhat subject to oxidative modification.

Cysteines Cys273 and Cys288 are crucial for Nrf2–Keap1 complex formation. These residues can form an intermolecular disulfide bond in the Keap1 homodimer [9]. Another residue required for proper functioning of the Nrf2/Keap1/ARE signal system is Cys151 in the BTB domain of the repressor protein. Under oxidative stress and the action of electrophiles, numerous low molecular weight components are covalently attached to this residue, generating modifications of Keap1 that are resistant to the action of reducing agents, hence disrupting the interaction with Cul3-E3-ligase [26]. Thus, Cys151, Cys273, and Cys288 may be considered as redox balance sensors responsible for induction of Nrf2/ARE-dependent genes. Sequences of 6-9 a.a. containing Cys151, Cys273, and Cys288 are identical in mice, rats, and humans, which indicates their physiological relevance (Fig. 3). Another physiologically relevant site for oxidative modification of Keap1 is cysteine residue Cys23 that forms an intramolecular disulfide bond with Cys38 upon interaction with oxidized glutathione [9], which reduces Keap1-mediated repression of Nrf2 [27].

A possibility was demonstrated of alternative (redox-independent) ubiquitination of Nrf2 after its phosphorylation by GSK-3β kinase (glycogen synthase kinase 3β). In this case, β-TrCP (β-transducin repeat-containing protein) plays a role of an adaptor protein, which forms E3-ubiquitin ligase complex β-TrCP–Skp1–Cul1–Rbx1 (Fig. 2b).

Existence of such an alternative process suggests a two-level ubiquitination system with the participation of Keap1 and β-TrCP [15]. In the first case, the main regulators of ubiquitination and degradation of Nrf2 are reactive oxygen species (ROS) and electrophiles modifying cysteine residues of Keap1. In the second case, the process is regulated by external signals (cytokines, growth factors, neuromediators, etc.) that induce phosphorylation and inhibition of GSK-3β. Recently, the possibility of Nrf2 ubiquitination by an endoplasmic reticulum E3-ligase was found. In this case, Hrd1 (synoviolin) acts as an adaptor protein [28]. However, this pathway is very specific and it is only activated in response to endoplasmic reticulum stress. It is worth mentioning that under normal conditions the main ubiquitination pathways are active. Therefore, the major part of Nrf2 is degraded by the proteasome. At the same time, determination of Keap1 and β-TrCP levels in animals having different lifespans revealed a reliable inverse correlation between their levels and maximum lifespan [29].

It should be mentioned also that adaptor proteins can be ubiquitinated by E3 ligases themselves. In particular, such autoubiquitination was demonstrated for Keap1. However, decrease in its levels did not depend on proteasomes [30].

Autophagy. After ubiquitination, Nrf2 proteins are digested by proteasomes, whereas Keap1 complexes may be eliminated by means of autophagy [31]. Autophagy is an important catabolic process for maintenance of cell homeostasis and protection against the invasion of a pathogen.

The multifunctional protein p62 (SQSTM1) plays a pivotal role in Nrf2 regulation via autophagy. This protein contains regions for binding to numerous proteins: ubiquitin, Keap1, LC3 (microtubule-associated protein 1A/1B-light chain 3), etc. It was shown that p62 actively interacts with ubiquitinated proteins; that is why it is named sequestosome-1. It was established that p62 is an autophagy receptor, as it binds (through its LIR domain) to the microtubule autophagy marker LC3 [32]. Now p62 has been shown to participate as a receptor in many kinds of selective autophagy [33].

Protein p62 contains six conserved domains (Fig. 4) allowing interaction with various proteins and thus realization of interconnection between various signaling systems. The N-terminal PB1 domain of p62 allows forming dimers and oligomers with other proteins: aPKC (atypical protein kinase C) and ERK (extracellular signal-regulated protein kinase). The ZZ domain is a "zinc finger" motif, which allows p62 to act as a transcription factor and to bind to DNA. Protein p62 binds to TRAF6 (TNF-α receptor-associated factor 6) through the TB domain and activates NF-κB. The C-terminal domains LIR, KIR, and UBA are required for binding Keap1 and ubiquitinated proteins and their degradation via autophagy. The KIR domain is structurally similar to the ETGE motif of Nrf2. This allows interaction of p62 with Keap1, thus interfering with binding to Nrf2 and its ubiquitination. At the same time, interaction of p62 with Keap1 leads to degradation of the latter via autophagy [34].

Regulation of p62 is realized at both the transcriptional and posttranscriptional levels. Synthesis of gene *SQSTM1* mRNA is regulated by transcription factors Nrf2, NF-κB, AP-1, and FXR. A strong relationship between p62 and the Keap1/Nrf2/ARE redox-sensitive signaling system, whose activation causes increase in p62 synthesis, was revealed [31]. The affinity of Keap1 to p62-KIR is slightly lower than to the DLGex domain and significantly lower than to the ETGE domain of Nrf2. For this reason, such a structural and functional similarity between the domains of p62 and Nrf2 does not play a role in the classic model of activation of the Nrf2/Keap1/ARE system by electrophilic reagents. To trigger p62-mediated induction of Nrf2, an increase in p62 concentration is required. This enhances the probability of competitive displacement of Nrf2-DLGex from the complex with

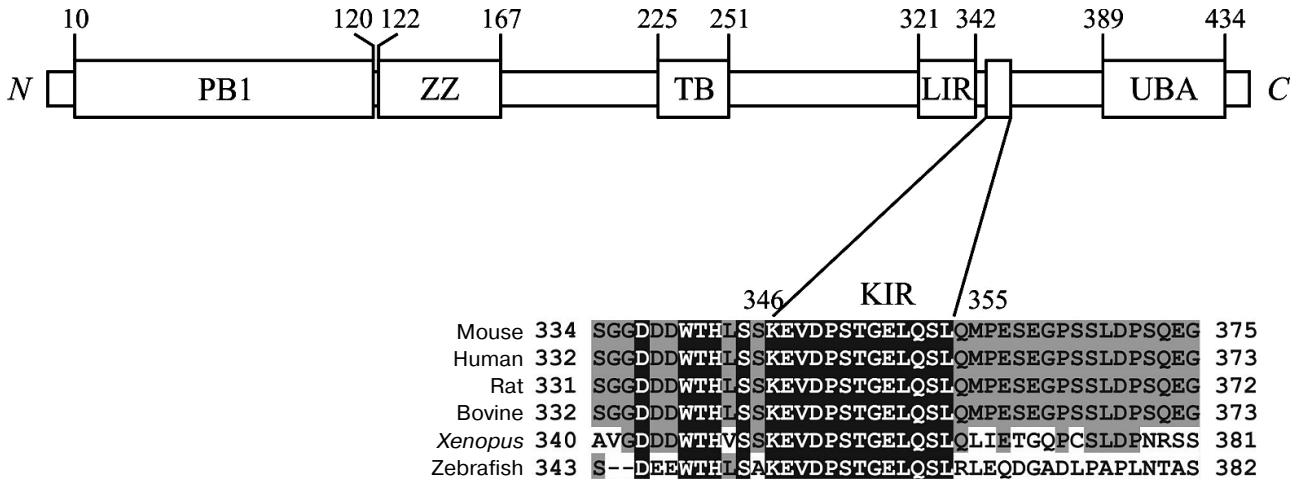

Fig. 4. Structural organization of p62 and its KIR domain interacting with Keap1.

Keap1. Such mechanism is probably realized upon prolonged activation of Nrf2, because Nrf2 raises its expression by binding to the promoter of the gene encoding p62. Thus, Keap1 is sequestered by increased p62 that in turn stabilizes Nrf2 and maintains its activity [35].

Phosphorylation of p62 at Ser349 in humans or Ser351 in mice significantly increases its affinity to Keap1, which exceeds that of Nrf2-ETGE (and even more that of Nrf2-DLGex) to Keap1 [36]. Such modification is only possible after preliminary phosphorylation of p62 at Ser403 by TBK1 kinase and subsequent formation of aggregates of p62 as such or with ubiquitinated targets [37]. On one hand, these aggregates are targets for autophagy. On the other hand, they are sites for Keap1 sequestering leading to induction of Nrf2/ARE [37].

Ubiquitination of Keap1 also strengthens its binding to p62. However, this interaction may be predominantly mediated by sestrins [38]. High antioxidant activity and ability to induce Nrf2 was discovered for Sesn2, which can form complexes with p62 and Keap1 and inhibit mTORC1 (mammalian target of rapamycin complex 1/2), thus activating autophagy. Presence of an ARE sequence in the promoter of the *Sesn2* gene enhances its activation effect on the Keap1/Nrf2/ARE system [38].

Phosphatases and kinases. In view of the large number of serine, threonine, and tyrosine residues (17% in human Nrf2) that can be phosphorylated, protein kinases belonging to various families (PKC, JNK, PI3K, ERK, p38 MAPK, PERK, and GSK-3β) participate in regulation of Nrf2 stability, transport in/out of the nucleus, and binding to ARE [15, 39]. Serine/threonine PKC phosphorylates Ser40 in Nrf2, which weakens its interaction with Keap1 and increases stability and nuclear import. Besides, Cys151 modification is possible in Keap1 [40, 41]. Phosphorylation of Nrf2 significantly affects its nuclear import and export. For this reason, mitogen-activated protein kinases (ERK1/2, JNK2, p38 MAPK, and MEKK3/4) may (depending on the experimental conditions) induce rather different effects ranging from complete abolishment of Nrf2 activation to its high-level enhancement. In particular, JNK1 and ERK2 activate the Nrf2-dependent signal pathway, whereas p38 MAPK inhibits this pathway [40]; kinases PERK and PI3K enhance Nrf2 nuclear import, whereas Fyn-kinases phosphorylate Tyr568 and enhance Nrf2 export and degradation [42]. The adaptor protein Keap1 is also subject to posttranslational modification: phosphorylation of Tyr141 in the BTB fragment stabilizes the Keap1 dimer, whereas dephosphorylation stimulates degradation of the protein [43].

The serine/threonine kinase AMPK (adenosine monophosphate-activated protein kinase) is a main sensor for changes in cellular energetic balance [44]. Classical AMPK activators are AMP and ADP; it is also activated by Ca^{2+} and H_2O_2 [44]. AMPK phosphorylates Ser550, which is situated in the nuclear export domain of Nrf2; this facilitates accumulation of the transcription factor in the nucleus [45]. Besides, AMPK phosphorylates and inhibits the GSK-3β kinase, which enhances degradation of Nrf2 [45]. AMPK activation inhibits mTORC1 by phosphorylation of the Raptor protein, which is a part of mTORC1 complex. Thus, autophagy is induced [46]. Hence, in response to energy starvation, prolonged induction of Nrf2 may occur due to AMPK activation. In addition to phosphorylation, Nrf2 activity may be affected by other modifications (acetylation, methylation, sumoylation) [47-50].

Epigenetic regulation. Tight interconnection between the Keap1/Nrf2/ARE signaling system and epigenetic regulation mechanisms has been demonstrated [51-53]. Decrease in Nrf2 expression is related to hypermethylation of specific CpG sites in the Nrf2 promoter [54]. At

the same time, repression of Keap1 function is observed in many cancers. This causes significant elevation of Nrf2 activity. Methylation and demethylation of histones may also affect activity of Nrf2 and enhance its synthesis in tumor cells [56].

Acting directly on mRNAs of transcription factors Nrf2, Keap1, and Bach, microRNAs can enhance or inhibit expression of genes regulated by Nrf2 and affect cell resistance against stresses [51, 57]. Besides, microRNAs can bind to specific regulatory elements of genes and stimulate their transcription.

APPROACHES TO PHARMACOLOGICAL ACTIVATION OF THE Keap1/Nrf2/ARE SYSTEM

The Keap1/Nrf2/ARE system is activated by toxic compounds, especially by pro-oxidants and electrophiles [58] that cause oxidative stress. Therefore, unsurprisingly, the most known inducers of the Keap1/Nrf2/ARE system contain electrophilic groups (direct activators) or become electrophiles after metabolic conversions (metabolic activators). It is believed that they form covalent bonds with thiol groups of cysteine residues of Keap1, thus being indirect inhibitors of interaction between Keap1 and Nrf2. Direct activators include such compounds as Michael acceptors, isothiocyanates, vicinal dithiols, organosulfur and selenium-based compounds, electrophiles bearing a leaving group, trivalent arsenicals, heavy metal species, hydroperoxides, and nitrosative agents [21, 59].

Dissociation of Keap1–Cul3 complex [59] is an additional possible mechanism for Nrf2 activation by electrophiles. Such dissociation was shown for sulforaphane and *tert*-butylhydroquinone [60], but it did not occur in another study upon cell incubation with four different types of inducers [61].

Compounds that modify thiol groups of Keap1 do not have sufficient selectivity and may affect other targets (PPARγ, IKK, JAK/STAT, HER2/ErbB2/neu, PTEN, PI3K/Akt, mTOR), and the whole thiol proteome [62, 63]. Lack of selectivity for Keap1 and pro-oxidant activity of these compounds explain a "U"-like dose dependence typical of inducers of this type, which indicates appearance of unwanted negative effects on increase in their concentration.

Direct inhibition of interaction between Keap1 and Nrf2 may have many advantages compared to using electrophilic activators of Nrf2; it has become of increased interest recently. Potential inhibitors of such protein–protein interaction have been designed [64-66], for instance, quinazoline-based nitrogen-containing heteroaryls whose biological activity is mainly associated with antioxidant activity [67], or short peptides [68]. Compounds of this kind interact with the Kelch domain of Keap1, which impedes its binding to Nrf2. Some cellular proteins including DJ-1 [69], p62 [36], and p65 [70] can also modulate protein–protein interaction between Keap1 and Nrf2.

On the other hand, such blockers of the protein–protein interaction do not selectively affect Nrf2, as Keap1 contributes to ubiquitination of many other substrates including IKKβ [71] and PGAM5 [72].

It is known that Nrf2 degradation may be facilitated by other proteins (β-TrCP, Hrd1, CRIF1). Particularly, CRIF1 participates in Nrf2 ubiquitination with subsequent proteasomal degradation. It interacts with the Neh2 domain and the C-terminal bZip domain of the transcription factor and can bind a mutant Nrf2 with changed ETGE motif [73].

A great body of data has accumualted concerning the participation of the Keap1/Nrf2/ARE system in pathogenesis of various diseases and experimental works that reveal therapeutic perspective of compounds (the vast majority of them are electrophiles acting on Keap1) that activate this system in neurodegenerative diseases, atherosclerosis, inflammation and inflammation-related tumors, diabetes and its complications, and malignant tumors [59]. At the same time, there are only a few completed clinical studies demonstrating efficiency and safety of these compounds. As an example, one may mention dimethyl fumarate (brand name Tecfidera) approved for application during relapsing-remitting multiple sclerosis. Although currently there are no data suggesting a relation between formation of stable "drug–protein" complexes and toxicity, one should not ignore the potential risk of such events, whereas biological effects of inhibitors of protein–protein interactions still should be determined *in vitro* and *in vivo*.

ROS are now considered as regulators of key cellular events such as proliferation, differentiation, and apoptosis [74-76]. For this reason, it is not surprising that ROS play the pivotal role in numerous pathological processes and conditions: inflammation [77], neurodegeneration [78], tumor growth [79, 80], and infectious and immune diseases. Thus, a multilevel system was developed in mammals for regulation of Nrf2, which is a key element of intracellular redox balance maintenance. One can claim that Nrf2 has acquired its versatility as a result of adaptation of living organisms to aggressive oxidative conditions [81] and the necessity of developing new mechanisms for regulation of cell differentiation [76] and their behavior within multicellular organisms [77].

It is worth mentioning that the search for compounds able to selectively activate the Keap1/Nrf2/ARE system via affecting the known mechanisms of its regulation is only begun to accelerate. Development of methods and approaches for designing chemicals *in silico*, including virtual screening procedures (search for quantitative relation between structure and properties, molecular docking, etc.), allow selecting a wide spectrum of compounds having desired properties that interact with certain molecular targets.

Effects on other regulatory regions, in particular Neh6 (a domain of redox-independent regulation) or Neh1 (contains DNA-binding motif and affects nuclear export and import), are also of great interest. Besides, the Neh2 domain still contains poorly studied sites for activators search. It is known that phosphorylation of Nrf2 by protein kinases (for instance, PKC) at Ser40 in the Neh2 domain (situated between DLGex and ETGE) impedes the interaction of Nrf2 with Keap1 (particularly, with Cul3-E3-ligase) and ubiquitination of Nrf2. Finding and designing inhibitors of a protein–protein interaction selective for these sites will allow obtaining several activators of the Keap1/Nrf2/ARE system that would lack disadvantages of electrophiles and inhibitors of the Kelch domain of Keap1. However, studies in this direction are limited due to lack of data regarding the three-dimensional structures for the regions of interest (particularly, crystallographic results).

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