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Beyond repression of Nrf2: An update on Keap1

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ABSTRACT

Nrf2 (*NFE2L2* – nuclear factor (erythroid-derived 2)-like 2) is a transcription factor, which is repressed by interaction with a redox-sensitive protein Keap1 (Kelch-like ECH-associated protein 1). Deregulation of Nrf2 transcriptional activity has been described in the pathogenesis of multiple diseases, and the Nrf2/Keap1 axis has emerged as a crucial modulator of cellular homeostasis. Whereas the significance of Nrf2 in the modulation of biological processes has been well established and broadly discussed in detail, the focus on Keap1 rarely goes beyond the regulation of Nrf2 activity and redox sensing. However, recent studies and scrutinized analysis of available data point to Keap1 as an intriguing and potent regulator of cellular function. This review aims to shed more light on Keap1 structure, interactome, regulation and non-canonical functions, thereby enhancing its significance in cell biology. We also intend to highlight the impact of balance between Keap1 and Nrf2 in the maintenance of cellular homeostasis.

1. Introduction

The fundamental role of defence pathways is to integrate adaptive cellular responses to stress events. These highly coordinated strategies aim to counteract internal disturbances, minimise acute damage, reset the homeostasis of the cell and sustain its survival. The Nrf2/Keap1 axis has been recognised as a central node for a cross-talk of cellular defence and survival pathways. Nrf2 (nuclear factor (erythroid-derived 2)–like 2) is a transcription factor, which transactivates expression of over 1000 protective genes [1,2], which comprise more than 1% of the human genome [3]. Its transcriptional activity is mainly regulated by a redox-sensitive repressor Keap1 (Kelch-like ECH-associated protein 1) [4]. Many excellent reviews deeply describe the exact mechanisms of Nrf2 regulation and its protective effects on the cellular homeostasis (exhaustively reviewed in Ref. [5]). Recent research has been broadly focused on the manipulation of Nrf2-Keap1 interaction, which enables modulation of Nrf2 activity in the therapeutic approaches [6]. The role of Keap1, however, has been attributed mainly to Nrf2 repressor function and redox sensor. Still, thanks to its extraordinary structure and reactivity, Keap1 undergoes numerous post-translational modifications, has a very rich interactome, and, thus, is involved in various cellular processes. We aim to underline this atypical biological significance of Keap1 in this review.

2. Keap1 résumé

Keap1, encoded by the *KEAP1* gene, was discovered in 1999. A two-hybrid screening assay identified it as a protein repressing Nrf2 transcriptional activity, sensitive to electrophiles [4]. It is also an intracellular sensor of oxidants [7]. *KEAP1* evolved as a product of gene duplication. In fish, two isoforms of *keap1* gene, *keap1a* and *keap1b*, are distinguished. During evolution, they separated, and *keap1b* was coevolving with vertebrate homologues [8,9]. Keap1 is a well-conserved protein, sharing around 95% of sequence homology between species (data from NCBI) and, importantly, possessing the same function throughout the phylogenic tree among the vertebrates [10]. The Ensembl database reports that there are 10 splicing variants of human *Keap1* gene, out of which 2 alternatively spliced transcript variants, possessing 6 exons and 5 introns, encode the same protein isoform, which is the full-length 624 amino acid long. Six other transcript variants encode potential isoforms (116, 129, 172, 212, 213 and 279 amino acid long), which were computationally mapped (uniprot.org). The last 2 transcript variants encode no protein. Recently 444 amino acid long Keap1 isoform, derived from the alternative splicing variant lacking the fourth and fifth exons, has been described in human highly-metastatic hepatoma cells and other cell lines. This isoform is missing the double glycine repeat (DGR, Kelch) domain, which is responsible

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Table 1
Keap1 is highly rich in cysteines. Comparison between human, mouse and rat.

	Human	Mouse	Rat
gene	*KEAP1*	*Keap1*	*Keap1*
aliases	iNrf2, KLHL19	iNrf2, mKIAA0132	iNrf2
chromosome	19	9	8
cysteines	27	25	25
amino acids	624	624	624

for interactions with Nrf2 [11].

Keap1 is expressed widely in various cell types and tissues [4]. It is mostly located in the perinuclear region of the cytoplasm, but also in the nucleus, endoplasmic reticulum and inclusion bodies [12–14]. Its absolute amount ranges between 50 000 to 300 000 molecules per cell, which corresponds to ~1 μM concentration [15]. That makes it a moderately abundant protein relative to another well-known repressor and a component of E3 ubiquitin ligase Hdm2 (up to 200 000 copies) [16] or transcription factors (up to 100 000 copies) [17]. It could partially explain how it preferentially is activated by Michael addition, its usual mode of redox activation [18]. Human Keap1 contains 27 cysteines (Table 1), which comprise 4.33% of all amino acids, whereas the average content of cysteines in human proteins is 2.26% [19]. Since the median length of coding sequences of human proteins is 375 amino acids [20], the average cysteine content counts for 8 per protein, which means that Keap1 contains over two times more cysteines than the average protein in human. This fact is of particular significance since the cysteine proteome plays a fundamental role in many biological functions (please refer to Ref. [21] for more details). A large part of Keap1 cysteines is highly reactive due to surrounding positively-charged, basic amino-acids [22]. The electrophile compounds activating Nrf2 modify directly critical Keap1 cysteine residues by alkylation and oxidoreduction reactions [23]. They can be divided into four classes, depending on Keap1 cysteine preferences [24]. It was shown that the oxidants, such as H_2O_2 and HClO, might induce Keap1 transient oxidation, but its mechanism remains elusive [25]. Interestingly, the study carried out it a zebrafish model showed that H_2O_2 is capable of Nrf2 target gene (*gstp1*) induction in an Nrf2-dependent manner in larvae but not in embryonic cells. Therefore, it was proposed that factors additional to Nrf2 and Keap1, absent at the embryonic stage, are required for sensing of H_2O_2 [23]. Keap1 is a metalloprotein binding zinc, which modulates the reactivity of Keap1 critical cysteines, thus influencing Nrf2-dependent response [26]. Coordination of zinc by Keap1 may explain how it can react with H_2O_2 preferentially versus most other protein cysteines under physiological conditions [27]. The breaking of the O–O bond in H_2O_2 cannot be significantly achieved under physiological conditions by cysteine alone, even in its thiolate (S^-) form, because OH^- is an extremely poor leaving group. Protonation of the leaving OH^- or the proximity of a metal, such as Zn^{2+}, to which the OH^- could bind, facilitates the oxidation of cysteine. It is well established that Keap1 represents a sensor of environmental stress [9], creates an adaptive interface between the exposome and genome [21], and is a target for pharmacological modification as it has been comprehensively reviewed in Ref. [6,7].

3. Keap1 structure

Keap1 is a member of the BTB-kelch protein family, which comprises about 50 members classified as Kelch-like (KLHL1-42, Keap1 is classified as KLHL19) or Kelch, and possessing BTB domain 1-14 (KBTBD1-14). All of these proteins contain two canonical domains in their structure: BTB (Broad-Complex, Tramtrack and Bric a brac) and Kelch domain [28,29]. Five distinct regions can be distinguished in the structure of Keap1: (i) the N-terminal region (NTR), position 1–49 aa, (ii) BTB (also named poxvirus and zinc finger (POZ)) domain, position 50–179 aa, (iii) intervening region (IVR, also named BACK domain), position 180–314 aa, (iv) double-glycine repeat (DGR, Kelch) domain, position 327–611 aa, and (v) C-terminal region (CTR), position 612–624 aa [7], as depicted in Fig. 1A.

The BTB domain is an evolutionarily conserved motif with the multifunctional role, including mediation of Keap1 homodimerisation, its interactions with the Cullin-3-Rbx-1-E3 ligase complex, and sensing electrophilic compounds, including the most potent activators of Nrf2, the cyclic cyanoenones, and nitric oxide through a highly reactive cysteine residue (C151) [9,30–34]. The major determinants of the high reactivity of this cysteine are basic amino acids, K131, R135, and K150, and H154, localised in the spatial proximity to C151 [9,25,35]. Keap1 homodimerisation via BTB domain is required for the interaction with Nrf2 [36], which is achieved directly by Kelch domain of Keap1 and Neh2 domain of Nrf2 through its DLG and ETGE motifs (Fig. 1B) [37].

The IVR region (position 209–314 aa) is cysteine-rich. Two critical cysteine residues C273 and C288 are required for the repression of Nrf2 nuclear accumulation [38,39] and responsible for sensing of alkenals [9]. The IVR region contains the most reactive residues of Keap1 (C257, C272, C288, and C297), which are the direct redox sensors [18]. They are a target for pharmacological activation of this pathway as it is reviewed in Refs. [6,7].

The DGR domain contains six repeats of Kelch motif, which form a six-bladed β-propeller structure. Each blade of this structure (I-VI) is built of four-stranded antiparallel β-sheets (A-D), which enable Keap1 interactions with diverse proteins [40]. The sequences of Kelch repeats differ between particular KLHL1-42, providing the substrate selectivity [28]. However, some regions are conserved: DGR, present at the end βB strand along with individual tyrosine (βC) and tryptophan (βD) residues, arbitrates hydrophobic packing between blades. Thus, the Kelch domain is also referred to as DGR or DC (DGR and CTR) domain [41]. Those similar features enable the maintenance of overall “kelch” shape. The vast majority of kelch domains (just as in the case of Keap1) form six-bladed beta-propellers [29].

The X-ray structures of Kelch [40,42,43] and BTB domains [44] have provided essential insights in their detailed structure. The spatial organisation of all domains has been revealed by cryogenic electron microscopy. Keap1 resembles a cherry bob, where BTB domains form a stem, whereas IVR and Kelch domains are closely located and form the spherical “cherry” part (for the structural details, please refer to Ref. [45]).

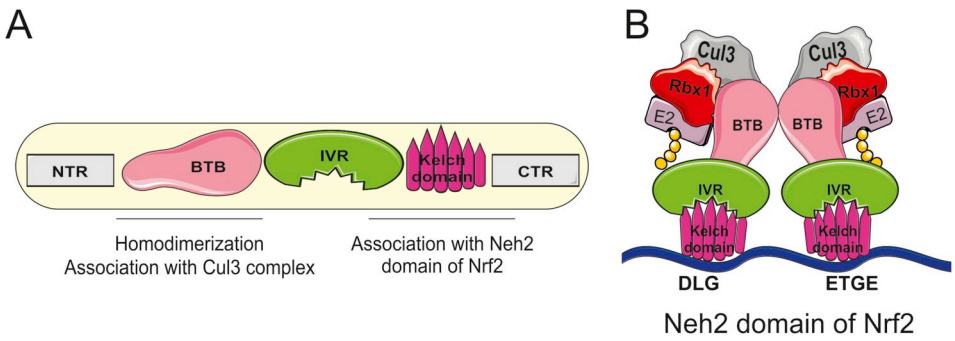

Fig. 1. Keap1 structure. (**A**) The organisation of Keap1 domains. NTR – N-terminal region. BTB - Broad-Complex, Tramtrack and Bric a brac. IVR-intervening region. DGR - double-glycine repeat region. CTR – C-terminal region; (**B**) Schematic illustration of the Keap1-Cul3-E3 ligase complex interacting with the Neh2 domain of Nrf2 via the DLG and ETGE motifs. Cul3 – cullin3; Rbx1 – RING box protein 1; E2 – ubiquitin ligase with ubiquitin (yellow circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

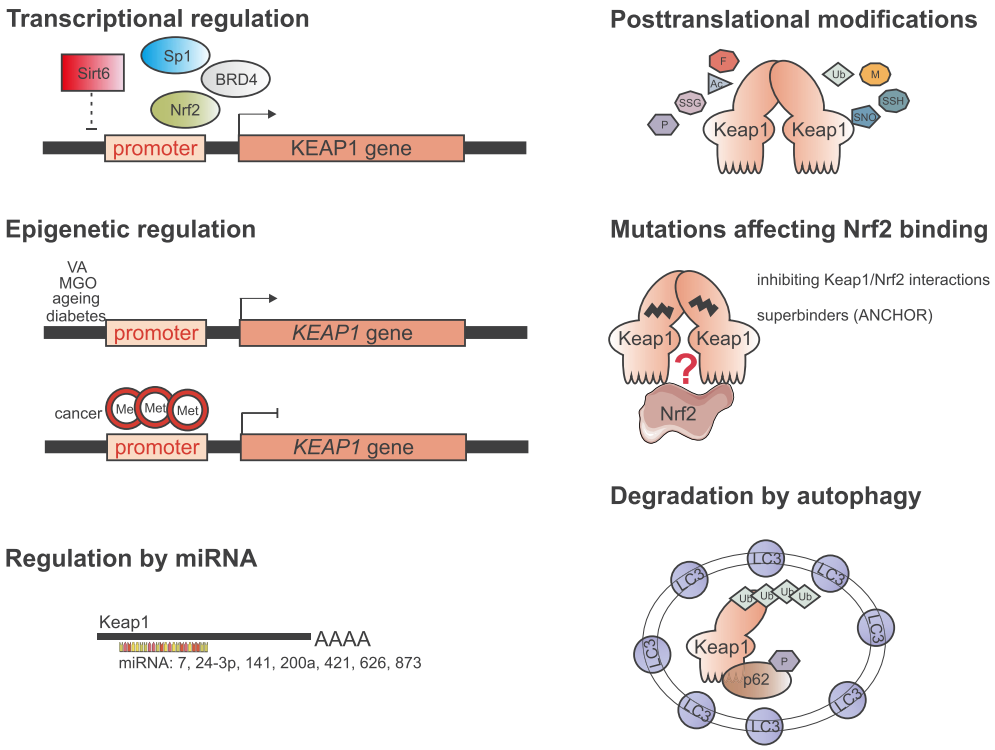

Fig. 2. The modes of Keap1 regulation. The level and activity of Keap1 are regulated via several mechanisms: transcription factors, epigenetic modifications, miRNAs, somatic mutations, post-translational modifications, and degradation. VA – valproic acid, MGO – methylglyoxal.

4. Regulation of Keap1 expression, level and activity

The level and activity of Keap1 are regulated via several mechanisms: (i) transcriptional regulation, (ii) epigenetic modifications, (iii) miRNAs, (iv) somatic mutations, (v) post-translational modifications, and (vi) degradation (Fig. 2).

KEAP1 is a transcriptional target of BRD4 [46,47], SP1 [48,49], Nrf2 [50] and possibly AP2 [49]. Also, the presence of putative binding sites for transcription factors: hepatic nuclear factor (HNF-1), signal transducer and activator of transcription-6 (STAT-6), CCAAT/enhancer-binding protein (C/EBP), OCT1, aryl hydrocarbon receptor (Ahr)/Arnt heterodimers, and hypoxia-inducible factor (HIF), has been identified in murine *Keap1* gene [50]. On the contrary, sirtuin 6 (Sirt6) deacetylase was shown to decrease *Keap1* transcript in cardiomyocytes. The molecular mechanism of this suppression remains, however, unknown [51].

The level of *Keap1* transcript is affected by differential methylation of its promoter. Keap1 increases with age in the human lens due to demethylation of its promoter, which results in higher gene expression [52]. Similar regulation is observed in diabetic conditions and in response to several compounds like methylglyoxal (MGO) [53] or valproic acid [54,55]. On the other hand, hypermethylation of the promoter is observed in many types of cancers, including lung, colon, prostate and gliomas. It also correlates with disease progression [48,49,56–59]. The promoter hypermethylation perturbs Keap1 transcription and significantly decreases its protein level [49,59]. Keap1 level can also be negatively regulated by miR-7 [60], miR-24-3p [61], miR-141 [62,63], miR-200a [64,65], miR-421 [66], miR-626 [67], and mir-873 [68].

Numerous mutations characterise the coding region of *KEAP1* gene in cancer cells [69,70]. They are associated with poor prognosis as they promote the proliferation of cancer cells and cause resistance to chemotherapy in different types of cancers [reviewed in Refs. [71,72]]. Interestingly, they are spanned uniformly across the whole gene and rarely cause protein truncation. Instead, the mutations result in altered ability to bind and regulate Nrf2 degradation, usually abrogating or decreasing its binding capacity. However, there are some mutations within *Keap1* which may facilitate Keap1 and Nrf2 interactions - called 'superbinders' (R320Q, R470C, G423V, D422 N, G186R, S243C and V155F). However, despite strong binding and enhanced ubiquitination, Keap1 mutant disables Nrf2 degradation, leading to increased Nrf2 transcriptional activity [73]. This group of mutants is also called 'ANCHOR' (Additionally NRF2-Complexed HypomORph). Interestingly, the anchor feature relates only to the increased interaction with Nrf2, but not with other Keap1-interacting proteins, for which the relation is comparable to the wild-type. The ANCHOR mutations stabilise Keap1 and phase-separate in a sequestosome (*SQSTM1,* p62)-dependent manner [74].

Due to its reactive nature, Keap1 undergoes many post-translational modifications, including S-nitrosation, ubiquitination, alkylation, carbonylation, glycosylation, S-glutathionylation, oxidation, phosphorylation, succination, and sulfhydrylation. They serve as an alternative way of non-canonical Nrf2-activation (summarised in Table 2). Curiously, two of them, ubiquitination and S-nitrosation, not only modify the Keap1 residues but are also directly dependent on Keap1 [30,75]. In regard to ubiquitination, Keap1 is an adaptor protein for E3 ligase complex [30–33], which ubiquitinates cellular proteins, including Nrf2 [32], IKKβ [76,77], PGAM5 [78], Miro2 [79], MCM3, SLK, MAD2L1 [80], and possibly also Myo9b [81]. Keap1 itself also undergoes ubiquitination, which predisposes it to proteasomal-independent degradation and enables Nrf2 activation [82]. In accordance, the deubiquitinating enzyme USP15 negatively regulates Nrf2 through deubiquitination of Keap1 [83]. Concerning the S-nitrosation-Keap1 cross-talk, Keap1 may undergo S-nitrosation (SNO) at C151 and C273 residues [84–86], and recently it has been recognised as a crucial regulator of this post-translational modification [75] (further described in more detail). Keap1 also evolved as a sensor of nitric oxide [9]. Another type of feedback regulation is observed in case of MGO, which modifies Keap1 to form MICA (methylimidazole crosslink between proximal cysteine and arginine residues) and functionally inactivates it [87]. At the same time, treatment of cells with MGO induces Keap1 promoter DNA demethylation leading to overexpression of Keap1 mRNA and protein [88]. There are several post-translational modifications

Table 2
Post-translational modifications of Keap1. * - the modifications that are both mediated by Keap1 and exerted on Keap1 protein itself.

Modification	Residue	Increase in Nrf2 activity	Reference
Ubiquitination*	K39, K61, K84, K97, K108, K287, K312, K615	Yes (essential for Keap1 degradation)	[82,89]
S-nitrosation*	C151, C273	Yes	[9,84–86]
Alkylation (itaconate)	C151, C257, C273, C288, C297	Yes (modifies and lowers Keap1 level)	[90]
Carbonylation	C273, C288	Yes	[91]
Glycosylation	S104	No (crucial in mediating Nrf2 ubiquitination)	[92]
S-glutathionylation	C434	Yes	[93,94]
MICA (methylimidazole crosslink between proximal cysteine and arginine residues)	MICA crosslink between C151 and R135	Yes	[87]
Nitro fatty acids	C38, C226, C257. C273, C288, C489	Yes	[95]
Oxidation	disulphide between C226 and C613; intramolecular between C151	Yes	[25,26]
Phosphorylation	Y206, Y263, Y334,	Yes (enabling Keap1 nuclear export)	[96]
Succination	C151, C288	Yes	[97–99]
Sulfhydrylation	C151	Yes	[100]
Malonylation	K131	n.d.	[101]
Acetylation	K131	n.d.	[102]
Palmitoylation	n.d.	n.d.	[103]

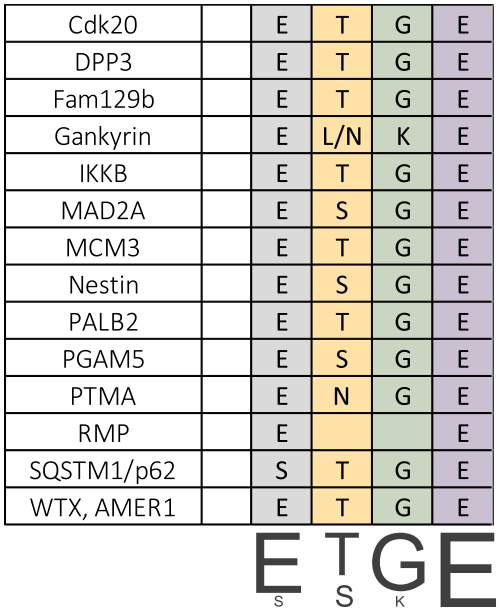

Fig. 3. Alignment of the confirmed Keap1 interaction motifs.

(malonylation, palmitoylation and acetylation), which have been reported, however, the exact impact on Keap1 was not fully elucidated [101–103].

Degradation of Keap1 occurs via p62-dependent autophagy [104], which is facilitated upon its preceding ubiquitination [82]. In basal conditions, the half-life of Keap1 is ~13 h, whereas electrophiles, such as tert-butylhydroquinone (tBHQ) or 1,2-naphthoquinone (1,2-NQ) can accelerate its degradation. The half-life of modified Keap1 is shortened to 3.4 h and 7.1 h by tBHQ and 1,2-NQ, respectively [104].

5. Keap1 interactome

Nrf2 is a hallmark interacting protein for Keap1. Many methods were implemented to study this interaction and to establish the minimal interacting fragment, including surface plasmon resonance [105], fluorescence polarisation assay [106], FRET [107], and ITC [108]. They revealed that Nrf2 binds to Keap1 in stoichiometry 1:2. Neh2 domain of Nrf2 is recruited to Keap1 by ETGE and DLG motifs [109]. Both motifs interact with the same residues of Keap1 dimer (DGR/Kelch), however with different binding affinities, probably due to different acidic residue composition. The ETGE motif has 100 times higher affinity than DLG [43,110]. This interaction can be mimicked by 16-aa long fragment, containing Nrf2 ETGE motif [107,111], or even 9 aa- (LDEETGEFL) or 7 aa-long (DEETGEF) sequences [105,106]). A minimal Nrf2 recognition sequence is of 6–7 amino acids [112].

The Nrf2/Keap1 complex adopts two different conformations, sequentially alternating in a cycle in basal conditions [3]. The open conformation is formed by newly synthesised Nrf2, which binds to one Keap1 molecule of the 'cherry bob' Keap1 dimer via high-affinity ETGE motif. This does not allow for Nrf2 ubiquitination and protects Nrf2 from proteasomal degradation. In the next step, low-affinity DLG motif of Nrf2 binds to the second member of Keap1 dimer. Thus, the complex adopts a closed conformational state, which predisposes Nrf2 for Keap1/Cul3/E3 ligase-dependent polyubiquitination and subsequent proteasomal degradation. The free Keap1 enters the next cycle then [3]. It is postulated that the inducers of Nrf2 activity block Nrf2/Keap1 complex in the closed conformation, but Nrf2 ubiquitination is not feasible then, and Nrf2 remains trapped by Keap1. Therefore, transactivation of Nrf2 target genes in achieved by *de novo* synthesised Nrf2, rather than through the liberation of Nrf2 from Keap1 complex [3].

The majority of Keap1-binding partners contain an evolutionally conserved ETGE motif, which is responsible for these interactions (Fig. 3) [113,114]. Direct interaction of Keap1 with other proteins via the non-canonical motifs, such as ELKE/ENKE, GLNLG, ARM domain, was also found (Table 3). Binding of the ETGE-containing proteins to Keap1 can displace Nrf2, preventing its degradation and enabling its translocation to the nucleus and the expression of cytoprotective genes. The interactions of Keap1 with other proteins and interaction motifs were discovered in the pull-down assay or in high-throughput studies and further confirmed by mutations and exchange of amino acids. Some of the interactors were also denoted in the Spotlite database [115]. The Keap1 interactors p62, DPP3 and Bcl-x_L were also determined in cDNA screenings based on ARE-driven transcriptional activity. Another, later neither described nor verified in context of Nrf2, were D-site of albumin promoter binding protein (DBP), kinesin family member 26B (KIF26B), cAMP-responsive element-binding protein-regulated transcription coactivator 1 (CRTC1), myeloid cell leukaemia sequence 1 (MCL1), and splicing factor, arginine/serine-rich 10 (SFRS10) [116]. Keap1 interactors verified by biochemical methods are presented in Table 3. All the depicted proteins, similarly to Nrf2, interact with Keap1 via its Kelch domains. The compounds disrupting the Keap1/Nrf2 interaction (e.x. sulforaphane, tBHQ or H_2O_2) were shown to have no effect or to increase interactions of Keap1 with its binding partners (Table 4). A significant part of Keap1-interacting proteins is characterised by the presence of disordered regions, which contain many acidic amino acids [117]. Kelch domain contains multiple positively-charged residues.

Table 3
Proteins interacting with Keap1, verified by biochemical methods. The location of interacting amino acids was shown for human (h) proteins. Presence of the interacting motif was also verified in mouse (m) and rat (r) proteins based on the sequence search. X – unspecified amino acid.

Protein name and symbol	Function	Motif interacting with Keap1	Reference
Actin	Cytoskeleton	n.d.	[128]
BPTF/FAC1	Chromatin regulation	n.d.	[129]
Cdk20	Cell cycle	^{25}ETGE$^{28,\ h\ m\ r}$	[130]
DPP3	Protein degradation	^{480}ETGE$^{483,\ h\ m\ r}$	[113,116,131]
EGFR	Growth factor signalling	n.d.	[96]
Fam129b	Apoptosis suppression, signalling	^{708}DLG$^{710,\ h}$ ^{718}ETGE$^{721,\ h\ m\ r}$	[132]
Gankyrin	Proteasome subunit	^{21}ELKE$^{24,\ h\ m\ r}$ ^{201}ENKE$^{204,\ h\ m\ r}$	[133]
GAPDH	Metabolism	n.d.	[75]
HBXIP	Amino-acid sensing, viral response	^{110}GLNLG$^{115,\ h}$	[134]
HSP90	Protein folding	n.d.	[135]
IKKB	Regulation of NFkB signaling	^{36}ETGE$^{39,\ h}$	[76,77]
KPNA6 (importin α7)	Nuclear import of Keap1	ARM domain	[136]
MAD2A	Cell division	^{92}ESGE$^{96,\ h\ m}$	[137]
MCM3	DNA replication	^{387}ETGE$^{390,\ h\ m\ r}$	[80,138]
Nestin	Intermediate filament	^{1414}ESGE$^{1417,\ h\ m\ r}$ DLG - predicted	[127]
NOS3	Production of nitric oxide	n.d.	[75]
p65	Inflammation	n.d.	[139]
PALB2	DNA repair	^{91}ETGE$^{94,\ h\ m\ r}$	[140,141]
PGAM5	Necrosis	^{79}ESGE$^{82,\ h\ m\ r}$	[78,79,142,143]
PIDD	Apoptosis	n.d.	[126]
PTMA	Chromatin remodeling	^{43}ENGE$^{46,\ h\ m\ r}$	[13,144,145]
RelA-associated inhibitor (iASPP)	p53 regulator	^{239}DLT$^{241,\ h}$	[146]
RMP	Gene transcription regulation	EXXE,$^{\ h\ m}$	[147]
p62	Autophagy	^{349}STGE$^{352,\ h\ m\ r}$ (phosphorylated)	[148,149]
WTX, AMER1	Regulation of Wnt signaling pathway	^{288}ETGE$^{291,\ h}$	[73,150]

Table 4
The effect of compounds activating Nrf2, tBHQ, sulforaphane and H_2O_2, on the interaction of Keap1 with its binding partners.

Protein name and symbol	tBHQ	sulforaphane	H_2O_2	Reference
DPP3	–	–	increase	[131]
gankyrin	no effect	no effect	increase	[133]
MCM3	no effect	no effect	–	[80]
PALB2	no effect	–	no effect	[140]
PGAM2	–	increase	no effect	[142,151]
iASPP	increase	–	–	[146]
RMP	–	–	increase	[147]
WTX	no effect	–	–	[150]

Thus, such an interaction is favoured [118]. Additionally, previously reported Keap1-interacting proteins, like myosin VIIa [119], cortactin [120,121], RhoGAP1 [122,123], contain SH3 domains. Apart from interaction with eukaryotic proteins, Keap1 was shown to interact with Marburg virus proteins [124]. Additionally, there are also small molecules that bind to the Kelch domain [125]. Mass spectrometry-identified proteins interacting with Keap1 are listed in Ref. [80,113,126,127].

6. Keap1 and NO-based regulation

Nitric oxide and reactive nitrogen species (RNS) induce Nrf2 nuclear translocation. It is achieved due to the S-nitrosation [9,84–86] or oxidative modification [25,26] of Keap1. However, the NO-related functions of Keap1 go beyond its post-translational modifications, aimed at the release of Nrf2.

A sensor of nitric oxide in Keap1 is cysteine C151 located in the BTB domain, which interestingly emerged in coincidence with the expansion of NOS family of proteins. NO sensing capacity of C151 is dependent on the presence of basic amino acids K131, R135 and K150, which spatially are present in the direct vicinity of C151. Perceiving of NO is accomplished by S-nitrosation of this cysteine residue [9]. However, Keap1 is not only a sensor of NO and RNS. Recently, it has also been recognised as a crucial modulator of S-nitrosation [75]. S-nitrosation it is an oxidative chemical reaction that generates a nitroso group on a cysteine thiol. Throughout years, the mechanism of formation of the nitrosothiol in the biological systems was elusive and thought to be non-enzymatic. Especially that such a reaction would require the formation of an intermediate NO^+ [152], and the kinetics of such mechanisms raises constraints in *in vivo* conditions [153,154]. Keap1 directly interacts with nitric oxide synthase (NOS) and transnitrosating protein GAPDH (glyceraldehyde 3-phospho dehydrogenase) to govern S-nitrosation in mammalian cells. Depriving the cells of any protein of this complex inhibits protein S-nitrosation [75]. Keap1/GAPDH/NOS enzymatic complex resembles the bacterial one, which comprises SNO synthase (Hcp), transnitrosylase GAPDH and NarGHI, as NO source [155]. It suggests that Keap1 can be a mammalian SNO synthase. It could be speculated that NO sensing by C151 and the requirement of positively charged amino acids for this process could account for such enzymatic activity of Keap1. Importantly, Keap1-related S-nitrosation of NOX4 has a fundamental significance in endothelial cells (ECs), protecting them from oxidative damage and apoptosis in the absence of Nrf2. Moreover, S-nitrosation determines EC fate, balancing between premature senescence and cellular death [75].

7. Keap1 in cytoskeleton regulation

Just upon discovery of Keap1 as Nrf2-interactor [4], the similarity of Keap1 to *Drosophila* Kelch protein was raised. Kelch is required to organise the ovarian ring canal cytoskeleton through F-actin binding and cross-linking [156]. Keap1 was shown to colocalise with a variety of both integrin and cadherin-based adhesion assemblies [119,157]. Subsequently, due to the resemblance to *Drosophila* Kelch protein, the interaction between Keap1 and actin was investigated. Keap1 colocalises and interacts with actin via its DGR domain. Disruption of actin leads to the release of Nrf2 from Keap1 interaction and its nuclear translocation [128]. Keap1 is present in focal adhesions and adherent junctions, but not in stress fibres [158]. It stabilises F-actin and restricts focal adhesion turnout. Additionally, its overexpression weakens the

migratory capacity of cells and triggers thick stress fibres formation due to RhoA overactivity. The latter is caused by the enhanced Myo9b degradation [81]. Furthermore, Keap1 regulates actin organisation, through associations with actin-regulatory proteins, such as myosin VIIa [119]. More complex regulation was reported in case of cortactin, which subcellular localisation is regulated by Keap1 binding [121]. Finally, Keap1 leads to the overabundance of RhoGAP1, the protein regulating Cdc42 activity. It impairs podosome formation and disrupts actin rearrangements, thus preventing endothelial cell migration and angiogenesis [123]. Keap1 may also interact with the other components of the cytoskeleton, tubulin [159], intermediate filament lamin [131] and nestin [127]. However, the functional significance of these interactions remains unknown.

8. Keap1 in proteostasis

The role of Nrf2 in proteostasis is mostly attributed to the transcriptional regulation of the ubiquitin-proteasome system and autophagy [reviewed in Ref. [160,161]]. Keap1 is an essential component of Rbx1-E3-ubiquitin complex, which marks various proteins for degradation, including Nrf2 [32], but also IKKβ [76,77], PGAM5 [78], Miro2 [79], MCM3, SLK, MAD2L1 [80], and possibly Myo9b [81]. Additionally, Keap1 interacts with DPP3, which participates in the hydrolysis of peptides formed during proteasomal degradation [113,116,131]. However, there is no data on how Keap1 affects DPP3 enzymatic activity. Keap1 was reported to assist in ubiquitin aggregate clearance through autophagy, where it interacts with p62 and LC3 [162]. In response to selective autophagy, it relocalises to inclusion bodies following interaction with p62 [12]. Keap1 colocalises with p62 in puncta to foster its degradation [148,162–164]. The interaction between Keap1 and p62 occurs following phosphorylation of STGE motif of p62, which can be executed by mTORC1 [165] or TAK1 [166]. Thus, an increase in the negative charge of this sequence enables interaction with positively charged residues of the Kelch domain of Keap1. The binding affinity of this pair is similar to the one observed for the DLG motif of Nrf2 and Kelch [149]. The interaction between p62 and Keap1 is enhanced when p62 undergoes phase separation (and appears as puncta) driven by cytoplasmatic DAXX (death-associated protein 6) [167]. Importantly, mutation in the KIR domain of p62, which abrogates the interaction of Keap1 with p62, has been associated with amyotrophic lateral sclerosis (ALS) [168]. High-throughput data also indicate for Keap1 interaction with essential autophagy regulator Atg5 [169]. Co-immunoprecipitation revealed direct interaction of Keap1 with proteasomal subunits PSMD2, PSMD4 and segregase Vcp/p97 [74], the latter, interestingly, had been previously reported to negatively regulate Nrf2 stability [154].

9. Keap1 at the mitochondrial interface

There are some contradictory data on the localisation of Keap1 in mitochondria, depending on the experimental setup and method used [14]. However, there is a growing body of evidence indicating the presence of Keap1 in the proximity of mitochondria, where it interacts with mitochondrial protein PGAM5 [78,79,143]. This interaction tethers Nrf2 at the mitochondria, thus abrogating its transcriptional activity [143]. However, a decrease either in Nrf2 or PGAM5 causes impairment of retrograde mitochondrial signalling, due to hyperactivity of Keap1-Cul3 complex and enhanced degradation of Miro2 [79]. Moreover, during an elevated generation of oxidants, Keap1/PGAM5 mediates caspase-independent cell death, called ‘oxeiptosis’ [142]. Conversely, Keap1 is crucial for the maintenance of proper mitochondrial homeostasis, as p62 recruits it to mitochondria, and together with Rbx1, mediates mitochondrial ubiquitination, thus, preventing the formation of dysfunctional megamitochondria and mitigating non-alcoholic fatty liver disease [170].

10. Keap1 and cell cycle progression

Apart from the cytoplasmatic localisation, Keap1 is also present in the nucleus [131,136]. The Kelch domain-derived interaction with importin α7/karyopherin α6 (KPNA6) is required for the nuclear import of Keap1, which is crucial in the fine-tuning of the antioxidant response [136]. Similarly, p65 interacts with Keap1 and promotes its nuclear translocation [139]. These nucleus-related functions of Keap1 were investigated mainly in the context of Nrf2 activation. However, Keap1 may also be a crucial regulator of cell cycle progression, independently of Nrf2. Recently, Keap1 was shown to negatively regulate endothelial cell proliferation and induce senescence in those cells [75,123]. Moreover, Keap1 knockout mice fail to properly execute the S-phase of the cell cycle during liver regeneration [171]. This impairment was associated with deregulation among cell-cycle mediators and mitogenic pathways. Keap1 knockout mice were also characterised by better genome integrity and stability, possibly due to increased expression of Rad51 [171]. BRCA1 and PALB2 are essential regulators of DNA damage response, also through the recruitment of Rad51. Notably, Keap1, through sequestration of PALB2, impedes the cell-cycle dependent BRCA1-PALB2 interaction [141]. Furthermore, more in-depth studies revealed that Keap1 interacting motif in PALB2 is central to PALB2-BRCA1 complex formation and facilitation of homologous recombination in G1 cells. Keap1 recruits CRL3 ubiquitination complex to PALB2. The ubiquitin moiety on lysine 20 of PALB2 suppresses its interaction with BRCA1. Finally, inactivation of Keap1 leads to the formation of stable BRCA1-PALB2-BRCA2 complex in both G1 and S phases [141].

MCM3 (DNA replication licensing factor, mini-chromosome maintenance protein), involved in DNA replication, interacts similarly to MCM7 with Keap1-Cul3 complex in cell-cycle dependent manner. However, this interaction does not lead to MCM degradation. Instead, it regulates MCM complex dynamics [80]. Keap1, together with MCM3 and MCMBP, were shown to form a ternary complex. The interaction with MCMBP was also reported in high throughput study [172]. Interestingly, Keap1 may interact with Cdk20 [130], Chd6 [131], Cdc34 [173], Sass6 and many other [174]. Keap1 forms also complex with ProTα [13,144,145], which regulates the compaction state of chromatin. However, this interaction was not studied in the context of proliferation and cell cycle progression.

11. Keap1 repression and the significance of Nrf2/Keap1 balance

The unique structure of Keap1 and the resulting diversity of interacting proteins may suggest that the interplay between Keap1 and its interactome should be tightly controlled. The function of Keap1 as an Nrf2 repressor has been very well established; the issue of Keap1 repression, however, has been beyond the research mainstream until now. Recent papers have recognised that the presence of unrestrained Keap1 is detrimental for cellular homeostasis [75,79,123] and emphasised the role of Nrf2 as the repressor of Keap1 [75,123], which implicates that Nrf2 and Keap1 mutually inhibit each other. Nrf2-depleted cells exhibited substantial deregulation of mitochondrial homeostasis due to the Keap1-mediated degradation of Miro2 [79]. The function of endothelial cells devoid of Nrf2 is aggravated due to the overabundance of unrestrained Keap1, which stabilises RhoGAP1 and potentiates protein S-nitrosation, inhibiting angiogenesis and evoking premature senescence of ECs [75,123]. The role of Nrf2 as Keap1 repressor is further supported by the phenotype of Nrf2 transcriptional knockout mice, in which the sequence coding the DNA binding domain has been disrupted by LacZ, resulting in the formation of fusion protein N-terminal Nrf2-β-gal. Unless challenged by detrimental factors, these animals develop well and do not exhibit abnormal phenotype. It is likely achieved by the presence of the Neh2 domain in these animals, capable of Keap1 binding [123]. The clinical significance of Nrf2/Keap1 imbalance can be implicated in age-related diseases [reviewed in Ref. [175]]. Keap1 is reported to increase during ageing [176] or in the models of premature

senescence [75]. However, the exact role of Keap1, in contrast to that of Nrf2, in ageing has not been addressed yet.

Keap1 inactivation, thus defined by Nrf2 hyperactivity, is characterised by multiple disorders, including hyperkeratosis of the digestive tract [177], bone hypoplasia [178], abnormal kidney development and function [179,180], and pancreatic atrophy [181]. Nrf2 serial activation was also associated with hyperproliferation of keratinocytes and pathogenesis of psoriasis [182]. Additionally, such cells (keratinocytes with Nrf2 overactivity) are featured by sebaceous gland enlargement and seborrhea, together with thickening and hyperkeratosis of hair follicle infundibula [183]. Furthermore, when mice were expressing a constitutively active Nrf2 (caNrf2) mutant in hepatocytes, liver regeneration was impaired due to delayed proliferation and enhanced apoptosis [184]. Finally, recently it was demonstrated in adult flies, that while mild Nrf2 activation extended lifespan, a high Nrf2 expression level led to developmental lethality or, after inducible activation, it altered mitochondrial bioenergetics, led to the onset of diabetes type 1 hallmarks and ageing acceleration [185]. The adverse effects of Nrf2 hyperactivity often result from the overabundance of its target genes or through metabolic reprogramming [186]. Accordingly, Keap1 loss promotes Kras-driven lung cancer and results in dependence on glutaminolysis [187,188] and consequently on exogenously delivered non-essential amino-acids (NEAA) [189]. Detrimental effects of Nrf2 hyperactivity have also been described in 4 cases of human mutations in *NFE2L2*, which disable the binding of Nrf2 to Keap1. The patients exhibited developmental delay, failure to thrive, immunodeficiency, leukoencephalopathy, and hypohomocysteinaemia [190]. Remarkably, as described above, the lack of either Nrf2 [75,79,123] or Keap1 [177] results in detrimental effects for the cells and organisms. Dysfunction of cells devoid of Nrf2, exhibiting impaired angiogenic potential and migration, senescence onset and increased S-nitrosation of proteins in case of endothelial cells and aberrant mitochondrial trafficking in case of retinal pigment epithelial cells, can be fully rescued by concomitant deletion of Keap1 [75,79,123]. In accordance, detrimental effects of Keap1 knockout, hyperkeratosis and constriction of oesophagus accounting for juvenile lethality of these mice, are reversed in Nrf2/Keap1 double knockout animals [177]. These results evidence that Nrf2/Keap1 balance is crucial to maintain cellular homeostasis.

The physiological and clinical relevance of Nrf2 hyperactivity models, Keap1-null [177] and caNrf2 [184] mice, might be questionable, taking into consideration that human Keap1 deficiency has not been reported so far and in caNrf2 mice Neh2-deficient caNrf2 is driven by a powerful CMV promoter [184]. To address this issue, we compared the fold change of Nrf2 target gene expression in these mice, patients with mutations within Neh2 domain of Nrf2 [190] and human tumours with loss of *NFE2L2* exon 2 [191]. The expression of target genes in caNrf2 mouse is usually increased by ~10-fold up to ~100-fold [184], in Keap1-null mouse by ~10-30-fold up to ~400-fold [180], in Keap1 floxed mouse, which revealed the hypomorphism of floxed Keap1 allele and milder phenotype than Keap1-null animals, by ~10-fold [192]. Expression of Nrf2 target genes in human cells isolated from patients with Neh2 mutations was increased usually by ~2-fold up to ~60-fold [190], while in human tumour samples deficient in exon 2 of *NFE2L2* by ~2-fold up to ~30-fold [191]. These limitations might be relevant, taking into account 'the hormetic characteristics' of Nrf2 activity [193], which also supports the significance of Nrf2/Keap1 balance.

12. Concluding remarks

The function of Keap1 as an Nrf2 repressor has been established exceptionally well and in detail. However, Keap1 is not only a repressor of Nrf2. The non-canonical functions of Keap1 should be explored further, for example, in the context of age-related dysfunctions, when an imbalance between Nrf2 and Keap1 occurs. Moreover, these atypical functions of Keap1 and the role of Nrf2 as a Keap1 repressing protein should be taken into consideration during data interpretation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.03.023.

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