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KEAP1-NRF2 Signaling and Autophagy in Protection against Oxidative and Reductive Proteotoxicity

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Abstract

Maintaining cellular redox status to allow cell signaling to occur requires modulation of both the controlled production of oxidants and the thiol reducing networks to allow specific regulatory post-translational modification of protein thiols. The oxidative stress hypothesis captures the concept that over production of oxidants can be proteotoxic but failed to predict the recent findings that hyper-activation of the KEAP1-NRF2 system also leads to proteotoxicity. Further, sustained activation of thiol redox networks by KEAP1-NRF2 induces a reductive stress, by decreasing the lifetime of necessary oxidative post-translational modifications required for normal metabolism or cell signaling. In this context it is now becoming clear why antioxidants or hyper-activation of antioxidant pathways with electrophilic therapeutics can be deleterious. Further it suggests that the autophagy-lysosomal pathway is particularly important in protecting the cell against redox stress-induced proteotoxicity, since it can degrade redox damaged proteins without causing aberrant changes to the redox network needed for metabolism or signaling. In this context, it is important to understand (i) how NRF2-mediated redox signaling or (ii) the autophagy-mediated anti-oxidant/reductant pathways sense cellular damage in the context of cellular pathogenesis. Recent studies indicate that the modification of protein thiols plays an important role in the regulation of both the KEAP1-NRF2 and autophagy pathways. Here we discuss evidence demonstrating that the KEAP1-NRF2 pathway and autophagy act in concert to combat the deleterious effects of proteotoxicity. These findings will be discussed with a special emphasis on their impact on cardiovascular disease and neurodegeneration.

Keywords

autophagy; mitophagy; post-translational modification; thiol; reactive oxygen species; antioxidants; redox signaling; NRF2; KEAP1; p62; NDP52

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Introduction

The redox dependent post-translational modification of proteins, most notably at thiol residues, are important cell regulatory and signaling mechanisms (1-4). In contrast, non-specific oxidative or nitrative damage to DNA, lipids or proteins is associated with diverse pathologies. However, contrary to the predictions of the oxidative stress hypothesis, that implies an oxidant-anti-oxidant imbalance causes non-specific accumulation of oxidative modification of protein, lipid and DNA this is not generally observed. In contrast, in living organisms, the level of modification of these biomolecules is often minor, with modifications occurring in the presence of cellular antioxidants rather than after their depletion. Importantly, non-specific accumulation of protein, lipid or DNA oxidative modifications rarely provides a specific molecular mechanism to explain toxicity or other biological effects. Furthermore, subtracting the total anti-oxidants from the total oxidants in a tissue is not predictive since biological redox regulation is inherently specific with respect both to the molecular species of antioxidant, their sub-cellular distribution and interactomes (5). These findings have led us and others to suggest that it is the loss of control of redox signaling and the gain of function associated with hyper-activation or suppression of redox cell signaling pathways or metabolism that underlies the cause of toxicity (5). Since the nuclear factor (erythroid-derived 2)-like 2 (NRF2) is important for transcriptional activation of electrophile response element-containing target genes, many encode antioxidant enzymes, a tight control of the NRF2 level itself is paramount. Indeed, the hyper-activation of the kelch-like ECH-associated protein 1 (KEAP1)-NRF2 system is not neutral or protective, as suggested by the oxidative stress hypothesis, but deleterious (6-9). Interestingly, proteotoxicity is a feature of both oxidative and reductive stress (10-12). This brings into focus the mechanisms in the cell which are designed to combat proteotoxic stress. The two most important of these are the proteasome and autophagy-lysosomal pathways (13-17). The mechanisms through which the proteasome processes oxidized proteins are well understood and have been discussed in a number of recent reviews (18-22). Here we will focus on the autophagy-lysosomal pathway and explore the recent concept that it contributes to the antioxidant protection of the cell through the removal of oxidatively damaged organelles and proteins (5;13;14;17;23) and extend this paradigm to the proteotoxicity associated with reductive stress (10-12).

How these antioxidant or anti-oxidative signals sense cellular damage is then critical to our understanding of cellular pathogenesis. It has been shown that in healthy cells, KEAP1 binds NRF2, and targets NRF2 to be ubiquitinated and degraded by the proteasome (24;25). Then in response to increased oxidative stress, cysteine thiols on KEAP1 are modified, allowing NRF2 to be released from KEAP1 and activate transcription of target genes (26). Although less well understood, it has been proposed that autophagy related proteins can also be redox regulated by cysteine thiol modifications. Recent evidence has also demonstrated significant cross regulation between KEAP1-NRF2 and autophagy activities (27-36). The redox regulation and cross regulation of KEAP1-NRF2 and autophagy activities will be reviewed below.

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Redox regulation of the KEAP1-NRF2 pathway

Redox regulation of cysteine thiols has been implicated in controlling the function of a variety of regulatory processes mediated by thiol dependent signaling pathways, including electrophiles (2-5;37;38). Due to the reactive nature of the thiolate ($R\text{-}S^-$) group on these residues, even subtle changes to the redox balance of the cell can result in cysteine modifications that can alter protein function (2-5;37;38). One of the major sensors of cellular redox status is the KEAP1 protein, which binds NRF2 and constantly promotes its degradation (5;24;26;39). Under basal homeostatic redox conditions, NRF2 remains in the cytoplasm, where it is degraded by the proteasome with an approximate half-life of 20 minutes (25;40). NRF2 is signaled for degradation by ubiquitination via a complex consisting of KEAP1 and Cullen 3 (CUL3) E3 ligase. KEAP1 serves as a crucial adapter in this process, locking NRF2 in the cytoplasm via one high affinity and one low affinity binding site, while CUL3 serves to ubiquitinate NRF2 lysine residues between these KEAP1 binding sites, and shuttle NRF2 to the ubiquitin proteasome system (25;41;42). In response to both oxidant and electrophilic stress, the KEAP1-NRF2-CUL3 interaction is disrupted, allowing NRF2 to translocate to the nucleus and activate target genes containing electrophile response elements (EpRE also termed antioxidant response elements, AREs), including key antioxidant genes such as glutathione S-transferase (GST), NAD(P)H oxidoreductase (NQO1), and glutamate cysteine ligase (GCL) (43-46) (Figure 1).

Regulation of the NRF2 pathway is complex. Human KEAP1 contains 27 cysteine residues, and depending on specific electrophiles or thiol reactive species, unique sets of cysteine residues are modified to alter the protein's function (43;47-52). For instance, Cys151 has been shown to be important for electrophile and oxidant-induced KEAP1-mediated ubiquitination and degradation of NRF2 via a mechanism that disrupts KEAP1-CUL3 interaction by modification at this residue (53;54). Conformational changes in other cysteine residues, Cys273 and Cys288, are required for constitutive KEAP1-mediated ubiquitination of NRF2 via a mechanism that disrupts the “latch” of KEAP1-NRF2 binding at the low affinity site, thus preventing ubiquitination and degradation of NRF2, and freeing *de novo* synthesized NRF2 free from KEAP1 binding, allowing it to translocate to the nucleus (44;53;55-57). Stabilization, translocation and activation of NRF2 are highly dynamic, as both KEAP1 and NRF2 contain nuclear localization and export signals to facilitate their translocations in and out of the nucleus, which are important for initiation and termination of NRF2-dependent transcriptional activities (58-64).

Interestingly, NRF2 can also be regulated by redox-dependent post-translational modifications. There are 6 cysteine residues in human NRF2, and 7 in mouse and rat. Mutations in cysteine residues of NRF2 led to decreased reaction with electrophiles, an enhanced KEAP1-NRF2 interaction and decreased NRF2 half-life. Cys183 has been shown to be localized in the transactivation domain with a functional nuclear exporting signal sequence, its modification with oxidants inactivates its nuclear exporting signaling activity (65). Cys119, 235 and 506 are also required for NRF2 binding to EpREs (66;67). In addition to thiol modification on cysteine residues, phosphorylation of NRF2 on serine or threonine residues also plays a crucial role in binding to KEAP1 or EpREs, as well as in mediating the transcriptional activity of NRF2. For example, a NRF2 Ser40 mutation led to enhanced

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association with KEAP1 and therefore impaired EpRE-mediated transcription (68). Phosphorylation of NRF2 at multiple sites by MAP kinases does not significantly contribute to NRF2 stability, while moderately affecting NRF2 transcriptional activity (69).

It is clear that different electrophiles modify unique sets of cysteines on these proteins, and how cysteine modifications code for their function in different cellular contexts is still an important area of investigation.

Redox regulation of autophagy

Complementing the NRF2-dependent regulation of antioxidant responses, the autophagy-lysosomal pathway facilitates additional defense mechanisms against cellular oxidative stress by selectively removing misfolded or damaged intracellular proteins and organelles, and thereby attenuating and reversing the injury caused by oxidative stress. Mechanisms by which the autophagy pathway responds to oxidative and nitrosative stress signals are still largely unclear. Studies have suggested that mTOR and PI3K activities mediate some of the autophagy response to oxidative stress (5;14;16;17;70-72), although it is also highly conceivable that modification of autophagy proteins at cysteine resides may also play a role in modulating autophagy.

There are a number of autophagy proteins that can be modified at key cysteines. Recombinant ATG4 activity is regulated by hydrogen peroxide modification of Cys81 residue near the active site of this protease (70). TOR has conserved cysteines that can form a disulfide bond which decreases structural flexibility and may control its stability and function (73). High-mobility group protein B1 (HMGB1), which is normally involved in chromatin remodeling, has also been shown to have a cysteine (Cys106) that when modified by reactive species or mutated, localizes to the cytosol and interacts with the Beclin/VPS34 complex to activate autophagy (74). The tumor suppressor protein p53, which has been shown to play a role in cell cycle arrest, autophagy activation, and apoptosis, also has two core cysteines (Cys182 and Cys277) that are prone to oxidative modification (75). Proteins implicated in removal of mitochondria via autophagy or mitophagy, which include PARKIN and DJ-1, have also been shown to contain conserved cysteines that can be modified to alter protein function (76-79) (Figure 2).

As such, increased formation of reactive species could cause modifications of key cysteine residues in autophagy proteins, and provide a redox-based regulation of the autophagy pathway. Three additional autophagy related proteins, ATG3, ATG7, and ATG10, which are involved in the processing of microtubule associated protein light chain (LC3) and autophagosome synthesis, have all been shown to have key regulatory cysteines (70;80-83). RUBICON, a Beclin/VPS34 complex-interacting protein, has a cysteine rich domain that could be modified during increased oxidative or nitrosative stress to affect autophagy initiation (84). Depending on the orientation of reactive cysteine residues in the active site or binding motifs of ATG proteins, the degree of susceptibility to conformational changes in response to oxidative or nitrosative modification of the cysteine residues, could greatly alter the ability of the cell to initiate autophagy, thus warranting further investigation of these modifications (Figure 2).

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Cross regulation between the KEAP1-NRF2 pathway and autophagy

Recent evidence indicates a strong connection between the KEAP1-NRF2 antioxidant pathway and autophagy. p62/SQSTM1 (sequestosome 1), and NDP52 (nuclear dot protein 52) are adaptor proteins that possess both ubiquitin and LC3 binding sequences through a ubiquitin association domain (UBA) and an LC3-interaction region (LIR), and are therefore able to recruit ubiquitinated autophagy substrates to the autophagosome (13;85;86). Recent studies have also demonstrated that both p62 and NDP52 are NRF2 targets (27;33). Genetic ablation of KEAP1 led to the accumulation of ubiquitin aggregates and defective activation of autophagy, suggesting that KEAP1 binding to p62 may also be involved in p62 mediated autophagy of ubiquitinated proteins (31).

In addition to induced accumulation of p62 by KEAP1 ablation, inhibition of autophagy also results in accumulation of p62 and formation of cytoplasmic protein inclusions (32). Through a KEAP1 interacting region (KIR), p62 can bind and sequester KEAP1, blocking KEAP1-NRF2 binding, and allowing NRF2 to translocate to the nucleus, resulting in activation of EpRE containing genes, including antioxidants genes, p62 and NDP52 (Figure 3) (27-30;33). The further increase in p62 protein levels creates a positive feedback loop which leads to persistent activation of NRF2 (33;34).

p62 can also be phosphorylated in an mTOR-dependent manner, and phosphorylation of p62 increases its binding affinity to KEAP1 and subsequent induction of the expression of NRF2 target genes (35). The sulfinic acid reductases Sestrin1 and 2, have been shown to play an important role in promoting p62-dependent autophagic degradation of KEAP1 and upregulation of NRF2-dependent transcription (36). Knockdown or overexpression of p62 in a number of different mammalian cell lines, increased and decreased KEAP1 protein levels, respectively (30).

The integration of the KEAP1-NRF2 and autophagy pathways in response to oxidative and nitrosative stress may not be limited to p62 and NDP52 interactions. How exactly these two antioxidant pathways coordinate their responsibilities in maintaining cellular function and survival under different physiological and pathological conditions still needs further investigation.

Impact of redox regulation and cross-regulation of antioxidant and autophagy pathways on pathophysiology

The redox regulation and coordination of KEAP1-NRF2 and the autophagy pathway are essential for cell growth, differentiation, redox signaling and survival. *Nrf2* knockout mice exhibit blunted induction of EpRE containing genes encoding cytoprotective enzymes, as well as increased susceptibility to toxic chemicals in various tissues (87-93). Transgenic overexpression of *Nrf2* in cardiomyocytes suppressed cardiac proteotoxicity in a pressure overload study by transverse aortic arch constriction in mice, and the protective role may be mediated partially by upregulation of autophagy (94). Overexpression of *Nrf2* in astrocytes protects against MPTP toxicity (95) (Figure 4).

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While the concept that loss of function of antioxidant enzymes can be deleterious is consistent with the oxidative stress hypothesis in disease pathogenesis, the finding that over-expression of antioxidants can be deleterious is not (7). In support for a detrimental role of constitutive NRF2 activity, *Keap1* knockout in mice leads to constitutive NRF2 activity and postnatal lethality, this lethality can be rescued by a concurrent ablation of *Nrf2* (96). With the generation of *Keap1* knockout mice, the role of KEAP1 thiol modification in mediating its function has also been demonstrated by transgenic complementation rescue of *Keap1* knockout mice with transgenic expression of wildtype versus cysteine mutated KEAP1 (97).

One of the first indications that the reductive stress is intimately linked to proteotoxicity was found in mice overexpressing mutant αβ-crystallin. These animals exhibit altered glutathione homeostasis due to changes in glutathione-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase activities, resulting in increased recycling of oxidized glutathione (GSSG) to the reduced form (GSH) (10). It has been shown that protein aggregates in these mice sequester KEAP1 and allow NRF2 activation of EpRE regulated genes (11). The role of NRF2-mediated reductive stress was demonstrated by the observation that crossing these mice to an *Nrf2*-deficient background significantly decreased proteotoxicity (12). These results indicate that protein aggregation can result in not only increased oxidative stress, but also increased reductive stress as a result of altered glutathione homeostasis and prolonged NRF2 activation and thus can be deleterious.

Mutations in NRF2 and KEAP1 are associated with human cancers, including cancers of the lung, head and neck, breast, liver, and skin (26;98), and mutations in p62 are found in adult onset Paget disease of the bone, amyotrophic lateral sclerosis and frontotemporal lobar degeneration (99). Constitutively high levels of NRF2 are associated with aggressive proliferation of cancer cells (100-102), and one important mechanism of NRF2 regulated cancer cell proliferation may be mediated by reprogramming metabolic activities (103). Highlighting the cross talk between the NRF2-KEAP1 and the p62 pathway is the recent demonstration that accumulation of p62 is also associated with poor cancer prognosis (102). When autophagy is blocked, for example due to *Atg5*, *Atg7*, or *Vps34* deficiency, p62 accumulates and is proposed to lead to KEAP1 sequestration and persistent NRF2 activation (104-106). While *p62* knockout mice develop adult-onset obesity and insulin resistance (107), impaired mitochondrial function with accelerated aging (108), high levels of p62 due to autophagy deficits have also been shown to be detrimental, as knockout of *Atg7* in the liver and brain results in the accumulation of p62 and ubiquitin conjugates, and liver tumors, which can be reversed by knockout of *p62* or *Nrf2* in the *Atg7* knockout background (29;32;109;110). p62 function also depends on the overall cellular context and circumstances. For example, p62 ablation is beneficial in a Huntington's mouse model, as it decreased nuclear inclusions and increased life span (111). On the other hand, p62 ablation in a spinal and bulbar muscular atrophy (SBMA) mouse model increased the levels of monomeric androgen receptor with polyQ expansion and exacerbated behavioral deficits such as performance on the rotarod, spontaneous motor activities and grip strength (112). p62 overexpression in transgenic mice increased inclusion body formation and ameliorated behavioral deficits (112), increased mitochondrial energy output, and improved behaviors associated with affective spectrum and anxiety disorders (113).

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The equilibrium between molecular couples, namely the oxidation-reduction ratio of nicotinamide adenine dinucleotide phosphate (NADPH to NADP), glutathione (GSH/GSSG) and cysteine:cystine (disulfide), control numerous signaling mechanisms including the activity of transcription factors (114-118). Since oxidative stress is no longer adequate to explain redox related pathologies then attention is now focusing on the broader concept of redox stress which includes the conditions or a hyper-reductive cellular state. For example in cardiac hypertrophy due to protein aggregation (10;114) increased activity of G6PD enhanced the levels of NADPH, leading to hyper-reduction of glutathione (GSH). In addition, a recent study has also described increased G6PD activity stimulating a hyper-reductive state in the distinct cortical regions of Alzheimer's brain from affected individuals (119). The inability to maintain thiols in the regulated oxidized state necessary for cell signaling or metabolic function in a highly reductive environment appears to be as deleterious as hyper-oxidation under conditions of oxidative stress. Remarkably, these findings may reconcile inconsistent data in the literature and also explain the failure of antioxidant therapies in different pathologies including those with cardiovascular or neurodegenerative diseases.

Conclusions and Future Directions

Many proteins contain redox modulatable cysteine residues that regulate protein conformation and function. This review highlights the significance of the KEAP1-NRF2 as a sensor and regulator of redox homeostasis and its cross-talk with the autophagy pathway. Although extensive studies have been performed in the past decades, 3 major gaps still persist concerning our knowledge of redox signaling, metabolism and their dysfunction which lead to redox pathologies. 1) Our understanding of the regulatory mechanisms and impact of KEAP1-NRF2 pathway in various forms of diseases are still incomplete; 2) Even less is known regarding the redox regulatory mechanisms that affect the key autophagy proteins and/or autophagy substrates such as damaged proteins with modifiable cysteines; and 3) It's not obvious whether there could be novel, but undiscovered cellular antioxidant mechanisms besides KEAP1-NRF2 and autophagy pathways, and how these mechanisms are coordinated in different cellular and disease contexts which may also provide new insights into disease mechanisms and therapeutic strategies.

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Abbreviations

ARE	antioxidant responsive element
NRF2	nuclear factor (erythroid-derived 2)-like 2
KEAP1	kelch-like ECH-associated protein 1
P62	P62/SQSTM1, sequestosome 1

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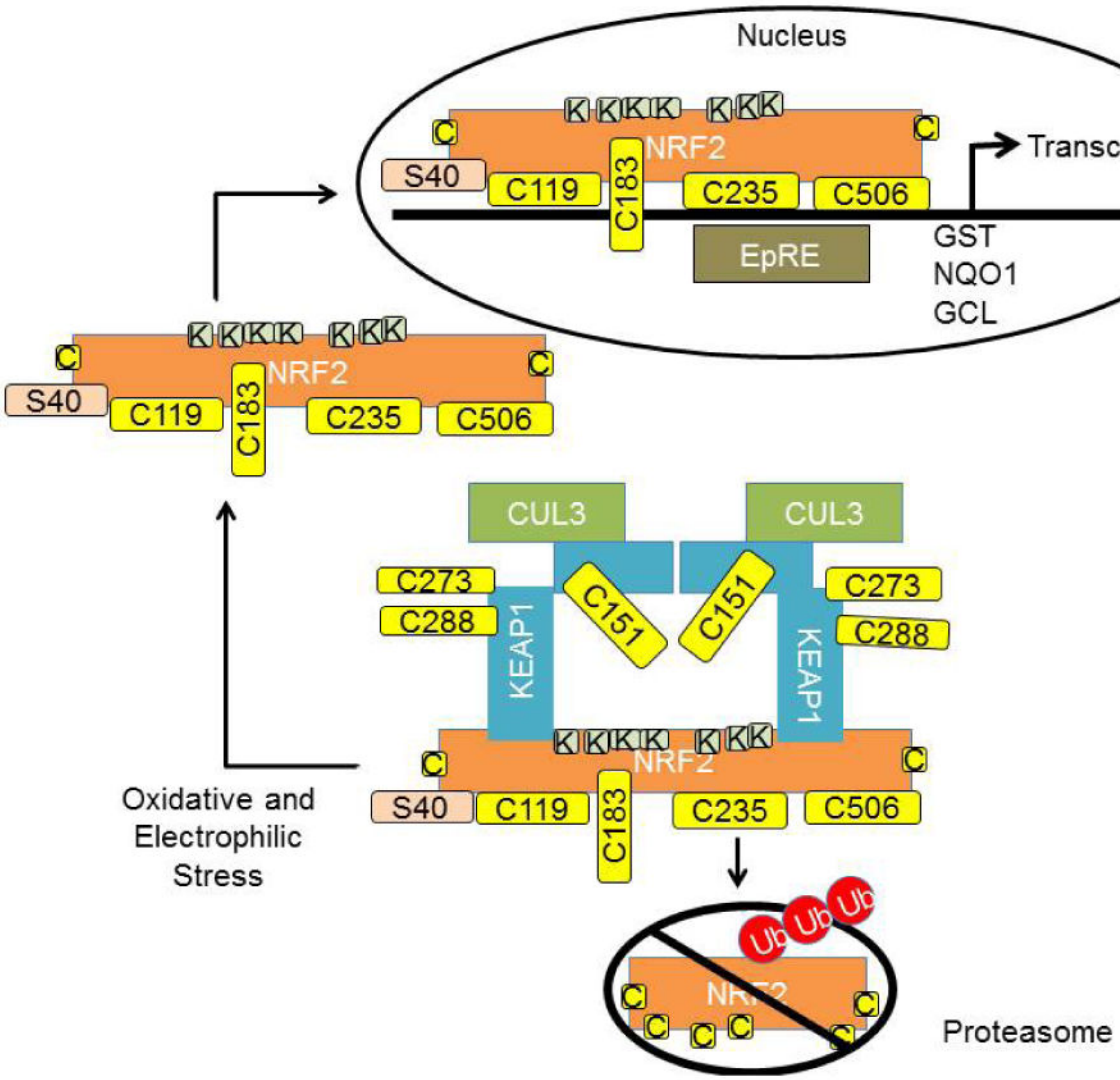

Figure 1. Redox regulation of the KEAP1-NRF2 pathway

Under basal conditions NRF2 is bound in the cytosol by interaction with KEAP1. This interaction is modulated via modification of cysteine thiol groups (yellow boxes) on both proteins. KEAP1 serves as an adaptor, allowing NRF2 to be ubiquitinated by Cullen 3 (CUL3). Once ubiquitinated, NRF2 is sent to the proteasome for degradation. Cysteine modification at C151, C273 and C288 impacts CUL3 or NRF2 binding at the lower affinity sites, thereby impacting NRF2 ubiquitination and degradation. Unbound NRF2 translocates to the nucleus and activates transcription of genes with AREs or EpRE, including glutathione S-transferase (GST), NAD(P)H oxidoreductase (NQO1), and glutamate cysteine ligase (GCL). NRF2 C183 modification is important for nuclear localization, C119, 235 and 506 are required for NRF2 binding to EpREs. Ser40 phosphorylation is important for disassociation from KEAP1.

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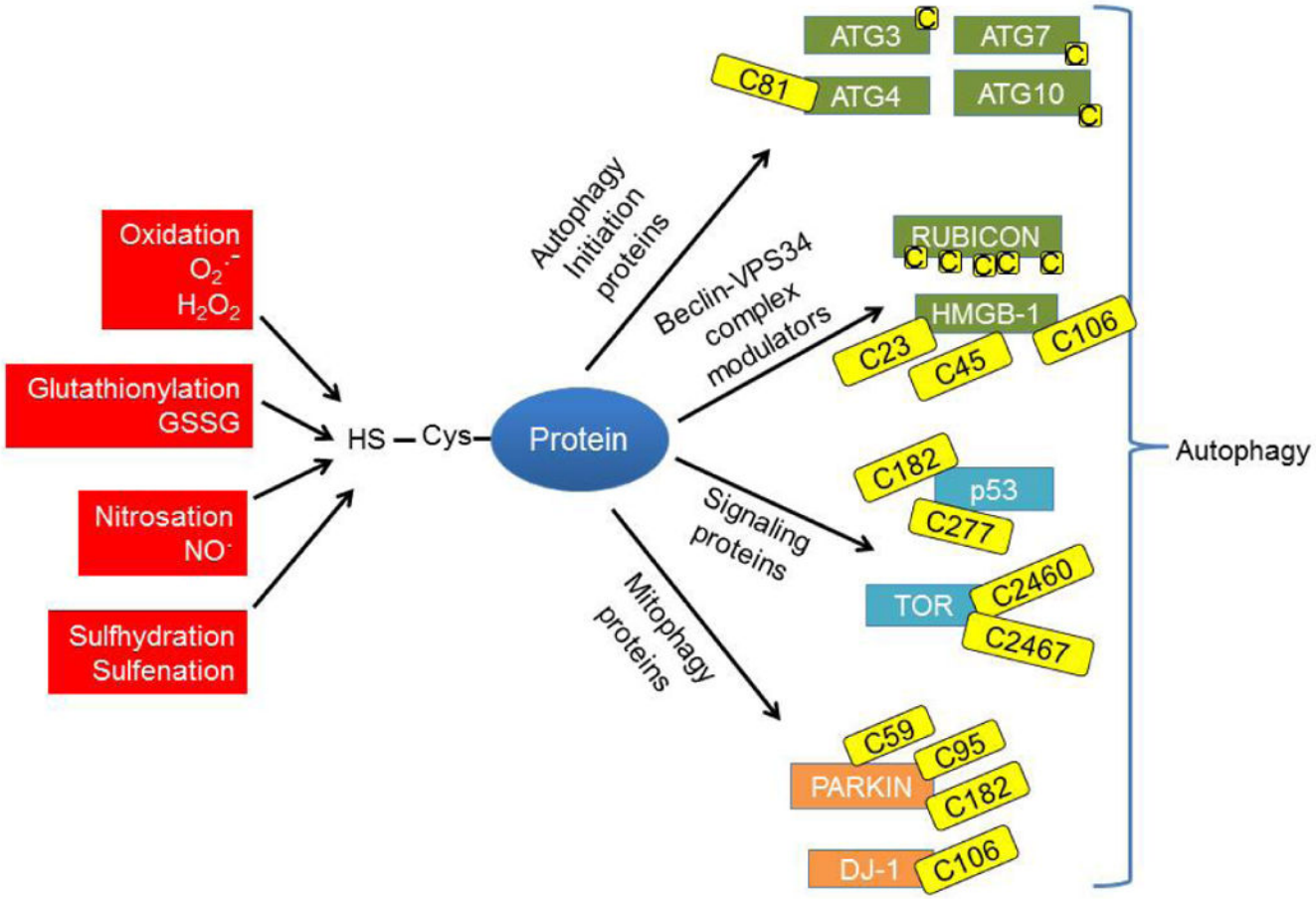

Figure 2. Redox regulation of autophagy

Proteins containing cysteine residues with a free thiol group can be modified through oxidation, glutathionylation, nitrosation, sulfhydration or sulfenation to modulate protein function. Many autophagy proteins have key modifiable cysteines including: 1) Autophagy initiation proteins (ATG 3, 4, 7 and 10); Recombinant ATG4 activity has been shown to be regulated by hydrogen peroxide modification of Cys81. 2) Modulators of the Beclin-VPS34 complex. RUBICON is a cysteine rich protein, and HMGB-1 is able to translocate to the cytosol, Cys106 modification is important for its translocation, Cys23-Cys45 disulfide bridge is important for its binding to BECN1. 3) The upstream autophagy signaling protein p53 has 2 cysteines that are prone to oxidative modification; and conserved cysteines in TOR can be oxidized. 4) Mitophagy-related proteins such as PARKIN, which is modifiable by nitrosation or sulfhydration, and DJ-1, which can be modified by oxidation. Modification of cysteines on these proteins may present a redox based regulation of the autophagy pathway during diseases associated with increased oxidative or nitrosative stress.

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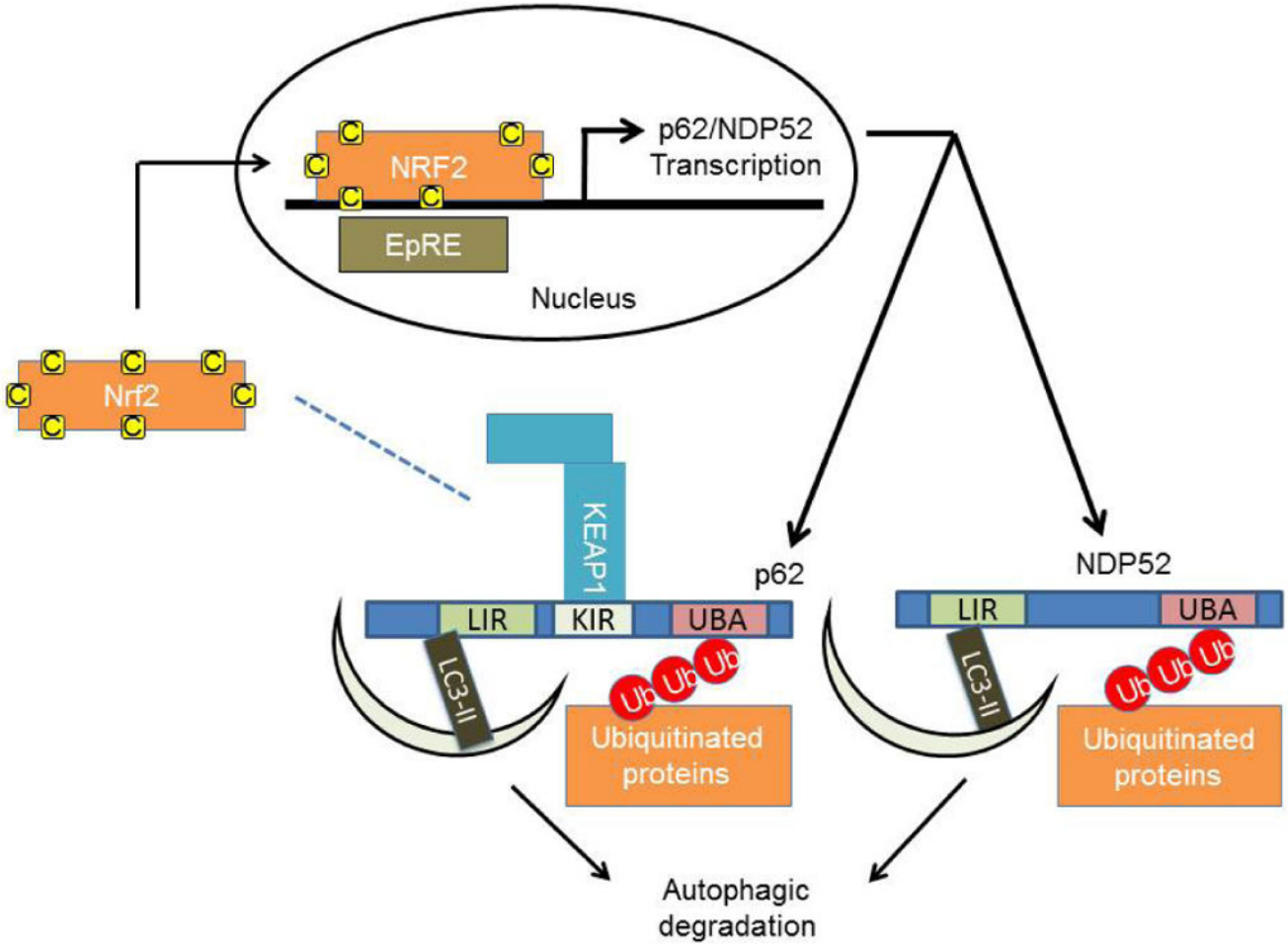

Figure 3. Cross regulation of autophagy and KEAP1-NRF2

KEAP1 can also interact with p62. This interaction sequesters KEAP1 away from NRF2, preventing NRF2 ubiquitination and degradation. NRF2 stabilization and translocation to the nucleus allows its transcriptional activity. One of the NRF2 target genes is p62 which sequesters KEAP1 and brings it to be degraded by autophagy. This positive loop plays a role in coordinating autophagy and NRF2 activities.

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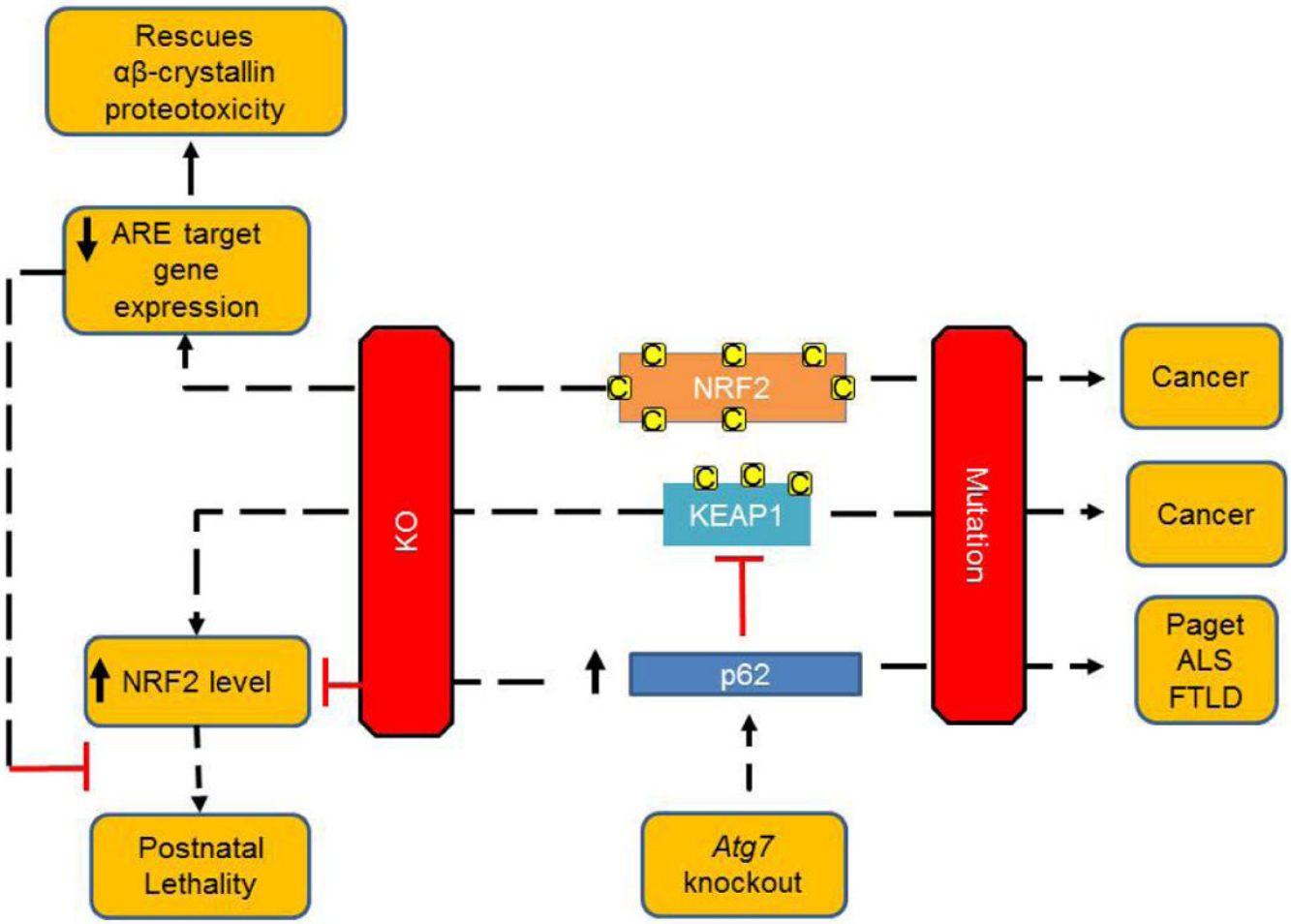

Figure 4. NRF2, KEAP1 and p62 in disease pathogenesis

NRF2, KEAP1 and p62 play important roles in cellular redox, protein and organelle homeostasis. Mutations in NRF2 and KEAP1 are associated with human cancers, and mutations in p62 are associated with adult onset Paget disease of the bone, amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *Nrf2* knockout mice exhibit blunted induction of EpRE containing genes encoding cytoprotective enzymes, and increased susceptibility to toxic chemicals in various tissues, but also exhibit decreased proteotoxicity induced by overexpression of mutant αβ-crystallin through a redox-mediated mechanism. *Keap1* knockout in mice is also detrimental and leads to constitutive NRF2 activity and postnatal lethality, this lethality can be rescued by a concurrent ablation of *Nrf2,* further demonstrating the mechanisms of KEAP1 in regulating NRF2 stability and function. Autophagy deficit-induced liver damage can be ameliorated by *p62* or *Nrf2* deletion, supporting the interaction of p62-NRF2 pathways. Overexpression of *Nrf2* or *p62* has been shown to lead to tissue specific and disease model specific consequences.