Kelch-like ECH-associated protein 1 (KEAP1) differentially regulates nuclear factor erythroid-2–related factors 1 and 2 (NRF1 and NRF2)

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Nuclear factor erythroid-2–related factor 1 (NRF1) and NRF2 are essential for maintaining redox homeostasis and coordinating cellular stress responses. They are highly homologous transcription factors that regulate the expression of genes bearing antioxidant-response elements (AREs). Genetic ablation of NRF1 or NRF2 results in vastly different phenotypic outcomes, implying that they play different roles and may be differentially regulated. Kelch-like ECH-associated protein 1 (KEAP1) is the main negative regulator of NRF2 and mediates ubiquitylation and degradation of NRF2 through its NRF2-ECH homology–like domain 2 (Neh2). Here, we report that KEAP1 binds to the Neh2-like (Neh2L) domain of NRF1 and stabilizes it. Consistently, NRF1 is more stable in KEAP1+/− than in KEAP1−/− isogenic cell lines, whereas NRF2 is dramatically stabilized in KEAP1−/− cells. Replacing NRF1’s Neh2L domain with NRF2’s Neh2 domain renders NRF1 sensitive to KEAP1-mediated degradation, indicating that the amino acids between the DLG and ETGE motifs, not just the motifs themselves, are essential for KEAP1-mediated degradation. Systematic site-directed mutagenesis identified the core amino acid residues required for KEAP1-mediated degradation and further indicated that the DLG and ETGE motifs with correct spacing are insufficient as a KEAP1 degron. Our results offer critical insights into our understanding of the differential regulation of NRF1 and NRF2 by KEAP1 and their different physiological roles.

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chemoresistance (27). Thus, a detailed understanding of the regulatory mechanisms of NRF2 is crucial for the development of therapies for either disease prevention or intervention (28).

NRF1 is arguably the most complex member of the family in terms of structure and regulation (29). The NFE2L1 (or Nrf1) locus encodes for several isoforms of NRF1, some of which are the result of alternative transcription or translation, as well as differential post-translational processing (29). Full-length NRF1 (TCF11 p140 or NRF1 p120 isoforms) localizes to the ER and is glycosylated (30). To yield a transcriptionally active protein, NRF1 has to be deglycosylated, extracted from the ER by the segregase p97 (VCP), and proteolytically cleaved (p110, p95, p85, and p65 isoforms) by the proteasome and several other proteases (31–33). Small molecular weight cleavage products (p65, p55, p46, p36, and p25 isoforms) act as dominant-negative transcriptional repressors (34, 35). NRF1 is important for differentiation, controls the expression of proteasome, antioxidant, and metabolic genes, and regulates inflammation (29, 36, 37). NRF1 is activated upon partial proteasomal inhibition, ER stress, oxidative stress, and hypoxia and in response to growth factors (30, 38–40). Under basal conditions, NRF1 is a short-lived protein that is negatively regulated by proteasomal degradation through the activity of several ubiquitin ligases, including β-TrCP, FBXW7, and HRD1 (41). Nrf1 gene deletion causes mouse embryonic lethality caused by anemia (42). Liver-specific Nrf1 deletion causes damage similar to human non-alcoholic steatohepatitis and hepatoma and is characterized by a reduced expression of several ARE-driven genes (43, 44). Moreover, conditional deletion of Nrf1 also causes bone abnormalities and neurodegeneration (42).

Because NRF1 and NRF2 are both responsive to oxidative stress and the main sensor of oxidative stress for the transcription of ARE-driven genes is KEAP1, a persisting question has been why NRF1 is not regulated by KEAP1. The Neh2-like (Neh2L) domain of NRF1 p140, p120, p110, p95, and p85 isoforms contains the DLG and ETGE motifs, and although previous studies have detected NRF1-KEAP1 interactions, NRF1 has not been found to be degraded by KEAP1 (45, 46). ER localization of NRF1 may prevent NRF1-KEAP1 interactions; however, because NRF1 conserves the Neh2L, suggesting that it could be a KEAP1 substrate, we investigated whether cytosolic NRF1 can be degraded by KEAP1. Furthermore, because the Neh2L is conserved but not identical to the Neh2, we sought to determine what are the critical amino acid residues necessary for effective KEAP1-mediated degradation. This information is necessary to deepen our basic mechanistic understanding of KEAP1-mediated degradation and to correlate this with the high mutation rate of the NRF2 Neh2 domain in cancer that leads to constitute NRF2 activation and resistance to treatments (47, 48), as well as the emerging role of NRF1 in cancer.

Results

KEAP1 stabilizes NRF1 and prolongs its half-life

To understand how KEAP1 regulates NRF1 and NRF2, we generated H1299 KEAP1−/− cells using CRISPR-Cas9 technology to analyze endogenous protein levels of NRF1 and NRF2. In H1299 KEAP1+/+ cells, full-length NRF1 (TCF11) was detected as 140- and 120-kDa bands, whereas NRF2 expression was barely visible, as expected for cells in redox homeostasis. In H1299 KEAP1−/− cells, no KEAP1 protein was detected, which resulted in a high expression of NRF2. Surprisingly, NRF1 expression was greatly reduced upon KEAP1 knockout (Fig. 1A). We next tested whether this reduction in NRF1 protein levels was due to increased proteasomal degradation. Total levels of NRF1 were restored in H1299 KEAP1−/− cells upon treatment with MG132 (Fig. 1B), indicating KEAP1 might be protecting NRF1 from proteasomal degradation. Consequently, the half-life of NRF1 was measured in the presence or absence of KEAP1. H1299 KEAP1+/+ and KEAP1−/− cells were treated with cycloheximide (CHX) at different time points to block de novo protein translation, and protein abundance was quantified to determine half-life (Fig. 1C). Interestingly, in the absence of KEAP1, the half-life of both NRF1 isoforms decreases, because the half-life of NRF1 p140 was decreased from 36 to 29 min, whereas the half-life of NRF1 p120 was decreased from 147 to 92 min. These results suggest that far from mediating NRF1 degradation, KEAP1 stabilizes NRF1.

Neh2L has conserved DLG and ETGE motifs but is refractory to KEAP1-mediated regulation

NRF2 shares significant structural homology with full-length NRF1 isoforms (Fig. 2A). To understand what confers KEAP1 substrate specificity, the sequences of the human Neh2 domain of NRF2 and the Neh2L of NRF1 were aligned (Fig. 2B). We focused on the amino acid sequence flanked by the DLG and ETGE motifs, which comprises 54 amino acids, including the KEAP1-binding DLG and ETGE motifs, and the seven ubiquitin-accepting lysines in the Neh2 domain. Strikingly, less than 50% of the amino acids between the DLG and ETGE motifs were conserved (24 of 54), even though previous studies found 72% homology between the whole Neh2 and Neh2L domains (49). The other two major sequence differences were that Neh2L had only two Lys residues (versus seven in Neh2) and was one amino acid shorter (Arg in Neh2) (Fig. 2B). We generated a series of constructs to overexpress FLAG-tagged wild-type NRF2 and wild-type full-length NRF1 (bands observed at 140 and 120 kDa) in MDA-MB-231 cells. As shown in the left panel of Fig. 2C, NRF1 is resistant to KEAP1-mediated degradation, consistent with previous studies (45, 46). In contrast, overexpressed NRF2 is degraded by overexpressed KEAP1 (Fig. 2C, left panel). It has been speculated that NRF1 escapes from KEAP1-mediated degradation because of spatial constrains, because full-length NRF1 localizes to the ER, and its Neh2L domain remains hidden from KEAP1. However, overexpressed NRF1 from which 86 amino acids from the N-terminal region have been deleted (NRF1Δ86), resulting in a protein that localizes exclusively to the cytosol (data not shown) was still resistant to KEAP1 (Fig. 2C, right panel). These results indicate that the amino acid sequence of the DLG-ETGE region, and not ER localization, might dictate KEAP1-mediated degradation.

Substitution of the Neh2L for Neh2 renders NRF1 sensitive to KEAP1-mediated degradation

To test whether the amino acid sequence flanked by the DLG and ETGE motifs in Neh2 is the core KEAP1 degron, we
replaced the Neh2L domain of NRF1 with the Neh2 domain of NRF2 to generate a mutant NRF1 (mNRF1) (Fig. 2A). This construct was overexpressed in MDA-MB-231 cells, and its expression was compared with that of overexpressed FLAG-tagged wildtype NRF1 in the presence or absence of overexpressed wildtype KEAP1. Interestingly, mNRF1 became sensitive to KEAP1-mediated degradation (Fig. 2D). Of note, the p120 band was the predominant isoform detected when NRF1 was overexpressed, in contrast to the stronger endogenous p140 band (Fig. 1). Next, immunoprecipitation analyses were performed in HEK293 cells to test the interaction of KEAP1 with NRF1 and NRF2. Similar to NRF2, mNRF1 interacts with KEAP1 (Fig. 2E). Interestingly, wildtype NRF1 also binds to KEAP1, which is consistent with a previous study (45) (Fig. 2E).

Changing non-conserved amino acids of Neh2 in mNRF1 does not abolish KEAP1-mediated regulation

To determine which amino acids might be necessary for KEAP1-mediated degradation, we first focused on the non-conserved amino acids between Neh2 and Neh2L (Fig. 2B). A series of mutations in the Neh2 domain of mNRF1 were introduced to reverse those amino acids to the ones present in the Neh2L domain (Fig. 3A). As shown in Fig. 2B, mutation of groups of 2–4 amino acids in the Neh2 domain of mNRF1 was not enough to render this protein KEAP1-insensitive. Even the deletion of three ubiquitin-accepting Lys residues or two Lys residues was unable to prevent the degradation of mNRF1 by KEAP1 (Fig. 3B, right panel), indicating that the two Lys residues in the Neh2L of NRF1 should be sufficient to receive KEAP1-mediated ubiquitin addition. Therefore, the lack of five Lys residues in the Neh2L domain is unlikely to be the reason that NRF1 does not undergo KEAP1-dependent degradation.

Recapitulation of K7 in correct spacing in the Neh2L is not sufficient for KEAP1-mediated degradation

We next tried a different approach to elucidate the amino acid sequences that determine KEAP1 sensitivity by using wildtype NRF1 and mutating amino acids of its Neh2L to those of the Neh2 of NRF2 (Fig. 4A). Introducing ubiquitin-accepting K residues or the additional R residue to keep the correct spacing between the DLG and ETGE motifs was unable to render NRF1 a KEAP1 substrate (Fig. 4B, left panel). Furthermore, two independent mutants with all seven lysines in correct spacing still
did not become KEAP1-sensitive like the mNRF1 (Fig. 4B, right panel), indicating that other amino acids within the DLG and ETGE motifs might be necessary for KEAP1-mediated degradation.

**The minimal sequence requirement in Neh2 for KEAP1-dependent degradation**

We continued to perform a series of mutations in the Neh2L domain of NRF1 to introduce the amino acids found in the Neh2 of NRF2 (Fig. 5A). Mutations were done in blocks, and combinations of these blocks were tested in the presence or absence of KEAP1 (Fig. 5B–D). After testing numerous combinations, the minimal sequence required for KEAP1-mediated degradation was identified: 5K+R+QLDE+VS+2F+QE (Fig. 5D).

**KEAP1-dependent ubiquitylation and degradation of NRF1 is achieved with the minimal degradation sequence**

To confirm that the minimal sequence identified (Fig. 6A) is sufficient for KEAP1-dependent ubiquitylation and degradation, an ubiquitylation assay was performed (Fig. 6B). MDA-MB-231 cells were transfected with KEAP1, HA-tagged ubiquitin (HA-Ub), and the FLAG-tagged NRF constructs. The cells were also treated with the proteasome inhibitor MG132 to preserve ubiquitylated proteins. Immunoprecipitation was done using anti-FLAG beads, and immunoprecipitates were resolved by SDS-PAGE and immunoblotting with the indicated antibodies. Band intensities of three independent experiments were quantified for each mutant and normalized to the values of cells with endogenous NRF1. The results are presented as means ± S.D. *, p < 0.05.

Figure 2. The amino acid sequence comprised between the DLG and ETGE motifs determines KEAP1 sensitivity. A, schematic representation of the protein domains of full-length NRF1 (p140 TC11), an NRF1 where 86 amino acids were deleted from the N terminus that localizes exclusively to the cytosol (NRF1Δ86, p140), and NRF2. B, sequence alignment of the amino acids comprised between the DLG and ETGE motifs of the Neh2L domain of NRF1 and the Neh2 domain of NRF2. An asterisk denotes identical amino acid residues, a colon (:) denotes a conservative change, a period (.) indicates a semi-conservative change, and a space indicates non-conservative changes.

C and D, MDA-MB-231 cells were transfected with a KEAP1 construct and the indicated FLAG-tagged NRF constructs. Cell lysates were resolved by SDS-PAGE and immunoblotting with the indicated antibodies. C, KEAP1 degrades NRF2 but not wildtype full-length NRF1 or cytoplasmic NRF1 (NRF1Δ86). D, substitution of the Neh2L with Neh2 renders mutant full-length NRF1 (mNRF1) or cytoplasmic mutant NRF1 (mNRF1Δ86) sensitive to KEAP1-mediated degradation. The vertical line indicates that panels were obtained from different gels. E, both NRF1 and NRF2 interact with KEAP1. HEK293 cells were transfected with KEAP1 and the indicated FLAG-tagged NRF constructs. Cell lysates were immunoprecipitated using CBD beads, and immunoprecipitates were resolved by SDS-PAGE and immunoblotting with the indicated antibodies. Band intensities of three independent experiments were quantified for each mutant and normalized to the values of cells with endogenous NRF1. The results are presented as means ± S.D. *, p < 0.05.
firms that introducing the entire Neh2 domain or the minimal amino acid sequence into NRF1 restores sensitivity to KEAP1-mediated degradation.

Discussion

The CNC-bZIP transcription factors NRF1 and NRF2 are essential for maintaining redox homeostasis, metabolism, normal development, and proliferation and in coordinating other cellular stress responses. From an evolutionary standpoint, emergence of CNC-bZIP transcription factors was crucial for the adaptation of metazoans to increasing oxygen concentrations and protection from ROS (50). In Caenorhabditis elegans, Skn-1 controls the expression of antioxidant genes, but its regulation is independent of KEAP1-like proteins (51). In Drosophila, CNC activates transcription of antioxidant genes by dimerizing with small MAF protein and is negatively regulated by Kelch/Keap1 (52). Later, gene duplication and diversion events led to the appearance of new proteins with different functionalities that gave rise to the mammalian CNC-bZIP family of which NRF1 and NRF2 are members (53). Although both transcription factors are important for redox and protein homeostasis, metabolism, and proliferation, they also have non-overlapping functions. For example, whereas Nrf1 (Nfe2l1) deletion is embryonic lethal in mice, the phenotype associated to loss of Nrf2 (Nfe2l2) is very mild under basal circumstances (42, 54). It is plausible that although NRF2 evolved to specialize in the contention of oxidative insults, NRF1 diverted to fulfill other responses but maintained more “primitive” functions associated to hematopoiesis and development (55). Moreover, whereas both regulate antioxidant genes, the activity of NRF1 seems to be more important under basal conditions, and NRF2 is more prominent during induced conditions (56). These functional differences would thus require the emergence of different modes of regulation, which might explain some of the structural differences between them.

In the present study we analyzed the differences in the NRF1 Neh2L domain and the NRF2 Neh2 to elucidate the minimal amino acid sequence requirements that confer KEAP1-mediated degradation. Consistent with previous studies, the presence of DLG and ETGE motifs in Neh2L and the interaction of NRF1 with KEAP1 are not sufficient to mediate ubiquitination and degradation of NRF1 by KEAP1. The inability of KEAP1 to
degrade NRF1 is not only due to the localization of NRF1 to the ER but can be explained by the amino acid sequence comprised between the DLG and ETGE motifs. In addition to the enrichment of ubiquitin-accepting lysines and maintenance of the proper DLG-ETGE spacing, other amino acids are crucial for KEAP1 sensitivity. Here, we defined the minimal amino acid sequence that renders NRF1 sensitive to KEAP1-mediated degradation. A great challenge in the analysis of these results is the lack of a crystal structure resolution for both NRF1 and NRF2. A previous study has determined that the Neh2 domain of NRF2 is intrinsically disordered but contains a central α-helix that is downstream of the DLG motif and spans the ubiquitin-accepting lysines and a short anti-parallel β-sheet spanning the ETGE motif (18). Interestingly, the region that comprises the α-helix (Phe39–Phe71, which in Fig. 2B correspond to Phe11–Phe42) in the Neh2 has the lowest amino acid homology with the Neh2L, which could explain the insensitivity of NRF1 to KEAP1-mediated degradation. In the future, incorporation of our results to the 3D models of the proteins will offer valuable information to understand the interactions of Neh2 and Neh2L with KEAP1 and the requirements for a KEAP1 degron. Our study suggests that the amino acid sequences of the Neh2/Neh2L domains diverged during evolution, resulting in opposite regulation by KEAP1.

Consistent with the opposite regulation of NRF1 and NRF2 by KEAP1, several studies indicate that these proteins may have opposing functions. A study found that NRF1 overexpression reduces the NRF2-mediated induction of glutamate-cysteine ligase genes GCLC and GCLM (9). Another report indicates that NRF1 impairs glucose metabolism and causes insulin resistance in mice (57), whereas NRF2 promotes insulin sensitivity and prevents diabetes (58). Moreover, it has been stipulated that p65 NRF1 might act in a dominant-negative fashion to repress the transcriptional activity of NRF2 (9). These different roles might have determined the divergence in the regulatory mechanisms of NRF1 and NRF2. However, certain degradation mechanisms are shared between them: both proteins are substrates of HRD1 and GSK-3-β-TrCP degradation (23, 24, 41), and both proteins are also substrates of p97, although in different contexts (32, 59). NRF1 needs p97 to be extracted from the ER and delivered to the proteasome, both for partial cleavage (release of transcriptionally active peptides) and for degradation (32). NRF2, on the other hand, is extracted from the KEAP1–CUL3 complex by p97 and delivered to the proteasome for degradation, as we recently described (59). It remains to be determined whether the interaction of p97 with NRF1 and NRF2 shares cofactors and interaction domains, but this is another example of the complexities of NRF1/2 regulation.

**Figure 4. Recapitulation of K7 in correct spacing in the Neh2L is not sufficient for KEAP1-mediated degradation.** A, amino acid sequence alignment showing the lysines (K) and arginine (R) introduced in the Neh2L of NRF1 mutants by site-directed mutagenesis. B, FLAG-tagged mutant K and R NRF1 mutants were coexpressed with KEAP1 in MDA-MB-231 cells to test whether these amino acid substitutions sensitized NRF1 to KEAP1-mediated degradation. Two independent clones of 5K+R are shown in the right panel. The vertical line indicates that panels were obtained from different gels. Band intensities of three independent experiments were quantified for each mutant and normalized to the values of cells with endogenous KEAP1. The results are presented as means ± S.D., *p < 0.05.
vious studies have also suggested that the NRF1 N-terminal domain that is absent in NRF2 might further dictate regulatory differences (60). Although the sequence determinants in the Neh2 domain governing KEAP1-dependent degradation of NRF2 have been identified here, the functional significance for the differential regulation of NRF1 and NRF2 by KEAP1 remains a mystery. Surprisingly, we identified that the binding of KEAP1 with NRF1 might actually protect NRF1 from degradation, possibly by masking NRF1 from other E3 ubiquitin ligases.

KEAP1 has other degradation substrates in addition to NRF2 (61). The inhibitor of nuclear factor κB kinase subunit β, a positive regulator of NF-κB, is another protein that contains ETGE and DLG motifs and is a KEAP1 substrate (62). However, it only contains three lysine residues and has very little homology with the Neh2. Phosphoglycerate mutase family member 5 is a mitochondrial serine/threonine phosphatase that interacts with Bcl-xL and has an ESGE motif that allows it to bind to KEAP1 and be ubiquitylated and degraded by the proteasome (63). Degradation of phosphoglycerate mutase family member 5 by KEAP1 is inhibited by oxidative stress and sulforaphane, consistent with the mode of regulation of NRF2 (63). The partner and localizer of BRCA2 (PALB2) is a critical protein for the DNA damage response and homologous recombination that is also a KEAP1 ubiquitylation substrate (64). PALB2 is a lysine-rich protein that contains an ETGE motif near its N terminus. KEAP1-mediated ubiquitylation of key lysines upstream of the ETGE in PALB2 restricts its interaction with BRCA1 without promoting proteasomal degradation, but this interaction can be restored by deubiquitylation (64). Recently, a new KEAP1 substrate, has been identified. The helicase minichromosome maintenance 3 is ubiquitylated by KEAP1 but not degraded by the proteasome (65). These studies suggest that KEAP1-mediated ubiquitylation might not be restricted to protein degradation but that it could also affect signaling events, because ubiquitylation is a complex post-translational modification with different outcomes based on mono- or polyubiquitination and the ubiquitin-linkage in the case of polyubiquitination (66). Therefore, it will be interesting to consider the biochemical properties dictating the interaction of these and other yet unidentified KEAP1 substrates in comparison with NRF2.

Mutations in the NRF2–KEAP1 pathway are common events in cancer. A study by the Cancer Genome Atlas Research Network reported that >30% of squamous lung carcinomas have alterations in the NRF2–KEAP1–CUL3 pathway that result in high, constitutive expression of NRF2 (47). Somatic mutations...
Minimal sequence for KEAP1-mediated degradation

in NRF2 occur almost exclusively at the DLG or ETGE motif, which disrupt its interaction with KEAP1. KEAP1 is also significantly mutated in lung adenocarcinoma (67). Interestingly, none of the mutations in KEAP1 occur in amino acids that directly interact with NRF2 (68). However, some of these mutations enhance the interaction between KEAP1 and NRF2, and although they did not affect ubiquitylation, they prevented proteasomal degradation of NRF2 (68). Mutations, KEAP1 allelic losses, or NRF2/NFE2L2 gene amplifications cause nuclear accumulation (activation) of NRF2, increased expression of antioxidants, and drug metabolism and excretion enzymes, metabolic reprogramming, and expression of anti-apoptotic genes that cause malignancy and resistance to anticancer therapies (69). In contrast, the role of NRF1 in cancer has barely been explored, and there are very limited data available. Few NFE2L1 mutations have been reported in cancer, with no obvious hotspots as seen for NRF2. Liver-specific NRF1 disruption causes oxidative stress and liver cancer (43). In breast cancer, overexpression of NFE2L1 is associated with chemoresistance (70). Although it could be argued that NRF1 could play a role in cancers treated with proteasome inhibitors for its ability to induce the expression of proteasome subunits, studies suggest that NRF2 mediates resistance to proteasome inhibitors (71). Collectively, these studies and ours highlight the need for further research to understand the biochemical, structural, and functional basis of the NRF2–KEAP1 interaction and regulation, as well as the relevance of cancer-related mutations in the NRF2–KEAP1 pathway for the development of better cancer biomarkers and therapies. Additionally, future research should characterize in detail the role that KEAP1 plays in enhancing NRF1 stability.

Experimental procedures

Antibodies, reagents, and cells

Primary antibodies against NRF2, KEAP1, tubulin, and GAPDH, as well as horseradish peroxidase–conjugated sec-
EcoRV restriction site directly upstream of the DLG motif and site-directed mutagenesis was performed to introduce an ETGE motifs in the Neh2L of NRF1 for that of the Neh2 of NRF2, (Sigma). To replace the region comprised between the DLG- and NRF2

Cloning and mutagenesis

Skeletal muscles were isolated from C57BL/6 mice 

Cloning and mutagenesis

Skeletal muscles were isolated from C57BL/6 mice

The restriction mutated Neh2 DLG-ETGE region was ampli-

Table 1

Codons are shown in the top row, and corresponding amino acids are shown below.

| Codon | EcoRV | Agel |
|-------|-------|------|
| Original | 5’ GAT ATT GAT D I D CTG GGG 3’ | 5’ GAG ACT E T GGG GAG 3’ |
| Mutated | 5’ GAT ATC GAT D I D CTG GGG 3’ | 5’ GAG ACC E T GGT GAG 3’ |

Cloning and mutagenesis

The coding sequence of human wildtype NRF1 (NFE2L1) and NRF2 (NFE2L2) was cloned into the pFLAG-CMV vector (Sigma). To replace the region comprised between the DLG-ETG motifs in the Neh2L of NRF1 for that of the Neh2 of NRF2, site-directed mutagenesis was performed to introduce an EcoRV restriction site directly upstream of the DLG motif and an Agel restriction site within the ETGE motif (Table 1). Parental (methylated) DNA was digested using DpnI for 1 h at 37 °C. The restriction mutated Neh2 DLG-ETGE region was amplified by PCR using Pfu (Thermo Scientific) according to the manufacturer’s instructions. Purified Neh2 amplicons were ligated into the restricted NRF1 to replace its Neh2L to yield the FLAG-tagged mNRF1 construct. All the other mutations were performed using the wildtype NRF1 or NRF2 constructs as templates and specific primers for site-directed mutagenesis.

Transfection and immunoblotting

Lipofectamine 3000 (Invitrogen) was used according to the manufacturer’s instructions. The cells were transfected with the indicated plasmids and 24 h later were harvested in sample buffer (62.5 mM Tris-HCl, pH 6.9, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromphenol blue). After sonication, the cell lysates were resolved by SDS-PAGE and subjected to immunoblot analysis. For protein half-life calculations, HEK293 cells were cotransfected with KEAP1 and with the indicated NRF2/NRF1 plasmids for 24 h, treated with 50 μM CHX at the indicated time points, and then harvested in 1× sample buffer and resolved by SDS-PAGE. H1299 WT and KEAP1−/− cells were treated with 50 μM CHX at the indicated time points and then harvested in 1× sample buffer and resolved by SDS-PAGE. To block proteosomal degradation of NRF1, H1299 WT and KEAP1−/− cells were treated with 10 μM MG132 for 4 h and then harvested in 1× sample buffer and resolved by SDS-PAGE. Densitometry analyses were done in ImageJ, normalizing to their respective control sample.

Immunoprecipitation

The cells were transfected with the indicated constructs for 24 h. Cell lysates were collected in radio immunoprecipitation assay (RIPA) buffer containing 10 mM sodium phosphate (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma) were also added to the RIPA buffer. Cell lysates were incubated with anti-FLAG M2 affinity gel (Sigma) or with chitin beads (New England Biolabs) on a rotator at 4 °C overnight. To detect protein expression in the total cell lysates, 10 μl of the cell lysates in RIPA buffer were mixed with 10 μl of 2× sample buffer (62.5 mM Tris-HCl, pH 6.9, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue) and boiled for 5 min. The immunoprecipitated complexes were washed with RIPA buffer with DTT, phenylmethylsulfonyl fluoride, and protease inhibitor mixture three times and eluted in sample buffer by boiling for 5 min. Samples were then resolved by PAGE and subjected to immunoblot analysis.

Statistical analysis

Experiments were performed in triplicate, unless otherwise indicated. The results are presented as means ± S.D. Paired Student’s t test was used to analyze changes in protein expression, and a p < 0.05 was deemed significant.

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