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SUMO-Modification of Human Nrf2 at K[110] and K[533] Regulates Its Nucleocytoplasmic Localization, Stability and Transcriptional Activity

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Abstract

Background/Aims: Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that binds to the antioxidant response element(s) (ARE) in target gene promoters, enabling oxidatively stressed cells to respond in order to restore redox homeostasis. Post-translational modifications (PTMs) that mediate activation of Nrf2, in the cytosol and its release from Keap1, have been extensively studied but PTMs that impact its biology after activation are beginning to emerge. In this regard, PTMs like acetylation, phosphorylation, ubiquitination and sumoylation contribute towards the Nrf2 subcellular localization, and its transactivation function. We previously demonstrated that Nrf2 traffics to the promyelocytic leukemia-nuclear bodies (PML-NB), where it is a target for modification by small ubiquitin-like modifier (SUMO) proteins (sumoylation), but the site(s) for SUMO conjugation have not been determined. In this study, we aim to identify SUMO-2 conjugation site(s) and explore the impact, sumoylation of the site(s) have on Nrf2 stability, nuclear localization and transcriptional activation of its target gene expression upon oxidative stress.

Methods: The putative SUMO-binding sites in Nrf2 for human isoform1 (NP_006155.2) and mouse homolog (NP_035032.1) were identified using a computer-based SUMO-predictive software (SUMOplot™). Site-directed mutagenesis, immunoblot analysis, and ARE-mediated reporter gene assays were used to assess the impact of sumoylation on these site(s) *in vitro*. Effect of mutation of these sumoylation sites of Nrf2 on expression of Heme Oxygenase1 (HO-1) was determined in HEK293T cell.

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Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

Results: Eight putative sumoylation sites were identified by SUMOplot™ analysis. Out of the eight predicted sites only one ^{532}LKDE^{535} of human (h) and its homologous ^{524}LKDE^{527} of mouse (m) Nrf2, exactly matches the SUMO-binding consensus motif. The other high probability SUMO-acceptor site identified was residue K^{110}, in the motifs ^{109}PKSD^{112} and ^{109}PKQD^{112} of human and mouse Nrf2, respectively. Mutational analysis of putative sumoylation sites (human (h)/mouse (m) K^{110}, hK^{533} and mK^{525}) showed that these residues are needed for SUMO-2 conjugation, nuclear localization and ARE driven transcription of reporter genes and the endogenous HO-1 expression by Nrf2. These residues also stabilized Nrf2, as evident from shorter half-lives of the mutant protein compared to wild-type Nrf2.

Conclusion: Our findings indicate that SUMO-2 mediated sumoylation of K^{110} and K^{533} in human Nrf2 regulates in part its transcriptional activity by enhancing its stabilization and nuclear localization.

Keywords

Nrf2; SUMOylation; LKDE; PKSD; PKQD; SUMO-2; Sumoylation; Nuclear cytoplasmic localization

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor which is a member of a small family of basic leucine zipper (bZIP) proteins [1]. Multiple transcript variants for encoding different isoforms have been characterized for *Nrf2* gene. *Nrf2* gene expression is shown to be regulated in response to stress by different transcription factors including Nrf2 and NF-kB [2, 3]. In addition to gene expression, nuclear localization of Nrf2 is also regulated by various proteins and *via* its post-translational modifications (PTMs). Nrf2 is involved in regulating redox homeostasis in the cells by regulating gene expression of different target genes. Nrf2 is translocated into the nucleus following, or concomitant with its activation by electrophiles or reactive oxygen species (ROS) [4–9]. Upon nuclear translocation Nrf2 positively regulates genes which contain antioxidant response elements (ARE) in their promoters; many of these genes encode proteins involved in response to antioxidant defense, drug detoxification, cell growth, protein degradation, DNA repair, and many aspects of intermediary metabolism [6, 10–14]. Nrf2 has two nuclear localization signals one on the N-terminal and one on the c-terminal which help in its nuclear translocation in response to pro-oxidant stimuli and that the importin alpha-beta heterodimer nuclear import receptor system plays a critical role in the import process [15]. Activation of Nrf2 in the stressed cells involves PTM of its inhibitory protein Kelch-like ECH-associated protein 1 (Keap1), thereby impeding Keap1-mediated, Cul3/Rbx1-dependent ubiquitylation and subsequent proteasomal degradation of Nrf2 in the cytoplasm [16–19]. Inhibition of the action of Keap1 allows Nrf2 molecules to translocate into the nucleus and carry out the transactivation of its target genes [5].

PTMs occur to nearly all proteins and increase the functional diversity of proteins. PTMs that lead to the activation of Nrf2 have been extensively documented [5–7, 9, 12, 13], but information is limited on PTMs that impact Nrf2 after its release from Keap1. The most studied PTM is phosphorylation which influences not only Nrf2 translocation into the

nucleus [20–22] but also its export out of the nucleus leading to it being degraded by Keap1 in the cytosol [23]. We and others have shown that acetylation of Nrf2 enhances its transcriptional activity [24, 25] whereas deacetylation disengages it from the ARE and favors its cytoplasmic localization [25]. Ubiquitylation by SCF/β-TrCP has been reported to target Nrf2 for degradation [23] but the cellular locale for this effect has not been determined.

Sumoylation is a PTM that involves covalent attachment of small ubiquitin-like modifier (SUMO) protein(s) to target proteins that contain SUMO-binding motif(s) [26–30]. SUMO proteins are members of the ubiquitin-like family of proteins [31, 32]. Structurally, SUMO molecules resemble ubiquitin, but SUMO molecules contain approximately 11–35 additional amino acid residues at their N-terminus [32]. Sumoylation is known to regulate large variety of cellular processes including replication, DNA damage response, transcription, RNA maturation, cell cycle, by affecting protein stability, protein-protein interactions, trafficking and transcriptional activity [33]. The human genome encodes three different functional SUMO isoforms (SUMO-1, SUMO-2 and SUMO-3) that are conjugated to distinct but overlapping sets of target proteins [34]. Human SUMO-2 and SUMO-3 are ~97 % identical and cannot be distinguished by antibodies. Thus, they are often referred to as SUMO-2/3 [35, 36]. It has been shown that under physiological conditions SUMO-1 is constitutively conjugated to substrates, while SUMO2/3 paralogs are preferentially conjugated in response to stress [35, 37, 38]. Understanding the target-specific consequences of SUMO modification requires knowledge of the location of conjugation site(s) [39]. Sumoylation was first discovered in studies on RanGAP1 [31], a protein that is abundant in the nuclear pore complex and that plays a key role in transport events mediated by this complex. The covalent attachment of a either SUMO isoform to a target protein involves the lysine residue that lies within the amino acid sequence Ψ-K-x-(D/E) (where Ψ is a hydrophobic amino acid and x is any amino acid) [29], defined as consensus or canonical SUMO-binding motif. Sumoylation has not been extensively explored with respect to Nrf2.

Previous work from our laboratory has shown that Nrf2 undergoes sumoylation, both by SUMO-1 and SUMO-2/3 and that it traffics in part to promyelocytic leukemia-nuclear bodies [40]. In these nuclear bodies, RNF4, a poly-SUMO-specific E3 ubiquitin ligase, ubiquitylates poly-sumoylated Nrf2 leading to the degradation of Nrf2 [40]. We have also shown that another poly-SUMO-specific E3 ubiquitin ligase, Arkadia/RNF111 ubiquitylates Nrf2 but unlike RNF4, Arkadia/RNF111 stabilizes Nrf2 [41]. Recently it has been shown that K^{110} of Nrf2 is modified by SUMO-1 in hepatocellular carcinoma cells [42]. SUMO-2 acceptor lysine(s) for Nrf2 have not neen characterized. In this study, we identified two lysine residues (K^{110} and K^{533}) that are located in the ^{109}PKSD^{112} and ^{532}LKDE^{535} potential SUMO-target motif in human Nrf2 as bona fide SUMO-2 binding sites. K^{533} lies within the bZip region of the Neh1 domain, while K^{110} is adjacent to the activation domain in the Neh4 of Nrf2. We observed that Nrf2 is modified by SUMO-2 at K^{110} and K^{533} sites. Sumoylation on these sites, K^{110} and K^{533} are in part needed for the translocation and stability of human Nrf2 in the nucleus and for transactivation of the target genes upon oxidative stress. These findings could lead to valuable insight towards the regulation of Nrf2 and its impact in cancer, neurological and cardiovascular diseases.

Materials and Methods

Plasmid Constructs, Cell Culture, and Transfection

Plasmid for the expression and purification of mouse Nrf2 as recombinant protein from *E. coli* as GST-mNrf2 was obtained from Dr. Michael Freeman (Vanderbilt University, Nashville, TN) [43]. Plasmid for ectopic expression of recombinant Flag-hNrf2, NC16 pCDNA3.1 FLAG NRF2 was a gift from Randall Moon (Addgene plasmid #36971, https://n2t.net/addgene:36971; RRID: Addgene-36971). HEK293T cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's minimum essential medium (DMEM) or (MEM), respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X DMEM nonessential amino acids (Invitrogen), and antibiotics (100 units of penicillin and 100 μg of streptomycin per ml) at 37 °C in 95% air, and 5% CO2 atmosphere. HEK293T cells were seeded and grown to ~ 50% confluence and were then transfected with Flag-hNrf2 plasmid wild-type, single mutants: K110R, K533R, or double mutant: K110R/K533R (referred to as 2K in the remainder of the document) and/or siRNA against Nrf2 using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Carlsbad, CA; cat no:11668019) as per manufactures instruction [44–46]. For activation studies the transfected after twenty-four hours of transfection the cells were treated with 10 μM arsenic trioxide ((As_2O_3; RICCA, Arlington, TX; cat no: R0817000–500A) and allowed to incubate for 4 hrs [41].

Site-directed Mutagenesis

Lysine (K) to arginine (R) mutations in the putative sumoylation sites was done in the pre-cloned mouse (mNrf2) or human (hNrf2) cDNA using QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA) and mutation carrying overlapping primer pairs as per the manufacture's instruction. cDNA for mNrf2 in plasmid GST-mNrf2 and hNrf2 in plasmid for Flag-hNrf2 were used as template.

Overlapping primer pairs used for creating K to R mutation in mouse Nrf2 at K110R:

5'-GCTACTCCCAGGTTGCCCACATTCCCAGACAAGATGCCTTGTACTTTGAAGACTG-3'/5'-CAGTCTTCAAAGTACAAGGCATCTTGTCTGGGAATGTGGGCAACCTGGGAGTAGC-3'

and K525R:

5'-GAGCAAGATTTAGGCCATTTGAGAGATGAAAGAGAAAAATTGCTCAGAGAAAA-3'/5'-TTTTCTCTGAGCAATTTTTCTCTTTCATCTCTCAAATGGCCTAAATCTTGCTC-3'.

For the human Nrf2 following primer pairs were used for mutation insertion at K110R:

5'-ACTACTCCCAGGTTGCCCACATTCCCAGATCAGATGCTTTGTACTTTGATGA

CTG-3’/5’-
CAGTCATCAAAGTACAAAGCATCTGATCTGGGAATGTGGGCAACCTGGGAG
TAGT-3’

and K533R:

5’-
GAGCAAGATTTAGATCATTTGAGAGATGAAAAAGAAAAATTGCTCAAAGA
AAA-3’/5’-
TTTTCTTTGAGCAATTTTTCTTTTTCATCTCTCAAATGATCTAAATCTTGCTC-
3’,

as guided by the corresponding nucleotide sequences. Mutant verified K110R constructs were used to generate the double mutant K110/K533 for human and K110/K525 for mouse, referred to as 2K in the manuscript. Plasmid DNA was isolated by using Qiagen QIAfilter kits. All mutations were confirmed by DNA sequencing at the Molecular Biology Core Facility at Meharry Medical College or Sanger Sequencing Facility at Vanderbilt University.

SDS_PAGE and immunoblot analysis

HEK293T cells were lysed in cell lytic reagent (Sigma Aldrich, St. Louis, MO; cat no: C2978) with protease and phosphatase inhibitor [25]. Total cell lysate or the purified proteins were resolved on 7% or 7.5 % SDS-PAGE (sodium-dodecyl sulfate poly-acrylamide gel electrophoresis) and were probed with the following antibodies: anti-Nrf2 (1:1000 dilution, Santa Cruz Biotechnologies, Dallas, TX; cat no: sc-13032), and anti-GST (1:1000 dilution, Santa Cruz Biotechnologies, Dallas, TX; cat no: sc-33613), anti-SUMO-2/3 (1:1000 dilution, Cell Signaling Technology, Boston, MA; cat no: 4971), anti-Actin (1:1000 dilution, Sigma Aldrich, St. Louis, MO; cat no: A3854), anti-FLAG (1:500 dilution, Sigma Aldrich, St. Louis, MO; cat no: A8592), anti-HO-1 (1:1000 dilution, Cell signaling technologies; cat no: 70081S), anti-Histone H3 (1:1000 dilution, Cell signaling technologies: cat. no: 12648S) and anti-Fibrillarin (1:1000 dilution, Santa Cruz biotechnology, Dallas, TX; cat no: sc253970). Signals were developed using chemiluminescent Western blot detection kit (Thermo Fisher, Waltham, MA). Western blotting analyses, using various antibodies, were performed as described previously [25, 40].

Purification of GST and GST-Nrf2/In vitro sumoylation assay

Using glutathione-agarose resin, GST-mNrf2 was purified as described by Rachakonda *et al.* [43]. The purified GST-mNrf2 wild-type, or mutant K110R, K525R, or 2K the fusion protein were used as a substrate for *in vitro* SUMO-2 conjugation assays, as described previously [40]. The products of the conjugation reaction were resolved on a 7.5% SDS-PAGE and analyzed by western blotting using either anti-SUMO-2/3 antibody or anti-Nrf2 antibody, as mentioned above.

siRNA mediated Knockdown of Nrf2 and Reporter Gene Assay

Nrf2 knockdown was done in HEK293T cells using siRNA targeted against the 3’UTR of human Nrf2. siRNA was procured from Sigma Aldrich, St. Louis (cat. No: SAS1_Hs01_00182401 and SAS1_Hs01_00182395). We used 100μM siRNA against Nrf2

(siN) or scrambled control (siC) as done previously [44]. Briefly HEK293T cells were seeded in 24-well plates and when they reached approximately ~50 % confluency the cells were transfected using Lipofectamine 2000 with either the Nrf2 siRNA (100 μM) or control siRNA (100 μM) for 48 hrs and cells were lysed for isolation of RNA and protein to validate the Nrf2 knock down [44]. Relative RNA levels of Nrf2 and HO-1 were measured by quantitative RT-PCR mentioned below. Relative level of Nrf2 Relative level of Nrf2 and HO-1 protein was monitored through immunoblot detection as mentioned above [41, 44, 45, 47]. To assess the transcription activity of the wild and mutant Nrf2 we used ARE-containing Heme oxygenase-1 (HO1) minimal promoter driven luciferase gene expression using *HO-1*-ARE-luc (*Heme Oxygenase-1*-Antioxidant Response Element-luciferase) as described previously [15, 41]. To determine the effect sumoylation has on the transcriptional activity of Nrf2, HEK293T cells were transfected with either the control siRNA or siRNA targeted against the 3'UTR of hNrf2 as mentioned above, and after 24 hrs of siRNA transfection cells were co-transfected with 0.4 μg of either wild-type or mutants (K^{110}, K^{533}, and 2K) Flag-hNrf2, 0.2 μg of experimental luciferase reporter plasmid (*HO-1*-ARE-luc) and 0.02 μg of control *Renilla* luciferase driven by thymidine kinase promoter plasmid pRL-TK (Promega Co., Madison, WI). To perform the Dual luciferase assay the cells were harvested 24 hrs after transfection, washed with 1 X PBS (phosphate-buffered saline) pH 7.4 (Thermo Fisher Scientific, Carlsbad, CA; cat no: 7001069) and lysed in passive lysis buffer (Promega Co, Madison, WI, cat no: E1960) and firefly and Renilla luciferase activities were measured as described previously [44–48]. Firefly luciferase activity was normalized with respect to *Renilla* luciferase activity and presented as a ratio (relative light units, RLU) [44, 45].

Nuclear and Cytoplasmic Fractionation

HEK293T cells were transfected with expression plasmid for either wild-type or mutant Flag-hNrf2. After 48 hrs, the cells were either control or treated with 10 μM As_2O_3 for 4 hrs. The nuclear and cytoplasmic fractionation was done according to the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Carlsbad, CA; cat no: 78833) [44].

Quantitative Reverse Transcriptase PCR (qRT-PCR) analysis

Post treatment of the HEK293T cells RNA isolation was done using Trizol (Thermo Fisher, Waltham, MA) and relative level of Nrf2, HO-1 and NQO1 RNA were measured through qRT-PCR analysis using iScript cDNA synthesis kit and SYBRgreen Taq Master mix (BioRad, Hercules, CA) [44, 45]. β-Actin or 18s-rRNA expression were used as normalizing control. The following primer pairs were used for amplification of

human Nrf2: (5'-CGTTTGTAGATGACAATGAGG-3'/5'-AGAAGTTTCAGGTGACTGAG-3'), HO-1: (5'-CAACAAAGTGCAAGATTCTG-3'/5'-TGCATTCACATGGCATAAAG-3'),

18s-rRNA: (5'-CGCGGTTCTATTTTGTTGTTT-3'/5'-GCGCCGGTCCAAGAATTT-3'), and

β-actin: (5'-GCTCGTCGTCGACAACGGCTC-3'/5'-GCTCGTCGTCGACAACGGCTC-3').

cDNA in qRTPCR using DDCt method [44, 45].

Immunoflourescence Analysis

The subcellular localization of the N-terminal FLAG-tagged Nrf2 was analyzed by confocal microscopy using anti-FLAG antibody conjugated with Cy3 (Sigma, St. Louis, MO) as previously described [25, 40–45]. Briefly HEK293T cells were grown to ~ 80% confluency on glass coverslips in 24-well plates and co-transfected with different Nrf2 siRNA and plasmids over expressing wild type or mutant Flag-tagged Nrf2 using Lipofectamine 2000. After 24 h, the transfected cells were treated with 10 μM As_2O_3 for 4 hrs. After the incubation control and treated cells were fixed and processed for antibody (Flag-Cy3) staining for detecting Nrf2. The coverslips were then mounted with ProLongTM Diamond Antifade Mountant containing DAPI (Invitrogen) to stain the nuclei blue. The stained, fixed cells were visualized and photographed using a Nikon TE2000-U C1 confocal laser-scanning microscope. To analyze Flag-Nrf2-Cy3 intensity, ROIs were drawn around the perimeter of the nucleus. The “Mean intensity” for the TRITC channel was exported and analyzed. Ten regions of interest (ROIs) were calculated for each condition. The mean intensity of each condition was normalized to wild type control in Microsoft Excel. GraphPad Prism (GraphPad Software, Inc, La Jolla, CA) software was used to establish statistical significance. To establish statistical significance, two-way ANOVA was performed and post-hoc Tukey’s test. To analyze the nuclear localization of wild type and mutant Nrf2, ROIs were drawn at puncta located within each nucleus. Pearson’s correlation coefficients for DAPI and TRITC channels were calculated using Nikon Elements software. Pearson’s correlation coefficients were extracted from each image with at least 10 ROIs calculated for each condition. GraphPad Prism was used to calculate statistical differences in Pearson’s correlation coefficients [47]. To establish statistical significance, Mann-Whitney u-test and one-way ANOVA analysis were performed.

Measurement of half-life of Nrf2

To measure the stability of Nrf2 we used recombinant wild type or mutant Flag-hNrf2. HEK293T cells were transfected with expression plasmid for either wild-type Flag-hNrf2 or Flag-hNrf2 mutants using lipofectamine [45, 47]. After 48 hrs, the cells were incubated with cycloheximide (100 ug/ml) for different time points maximum up to 60 min. At the end of each time point, the cells were washed once with 1X PBS and lysed, using non-denaturing lysis buffer [25]. The total cell lysate was subjected to electrophoresis on a 7% SDS-PAGE, followed by Western blotting with anti-Flag antibody to detect the Flag-hNrf2 or anti_HO1 antibody to detect HO-1 levels as mentioned above.

Statistical Analysis

*S*tatistical analyses were carried out using the Prism program (GraphPad Software, Inc, La Jolla, CA). Differences between multiple groups were tested using Dunnett’s multiple comparison test. The data are presented as means ± S.E..

Results

Locating putative SUMO-binding sites in Nrf2

Using an *in vitro* sumoylation assay kit our lab has previously shown that Nrf2 is covalently modified by SUMO [40]. The canonical SUMO-binding motif is defined as ψKX(D/E), where ψ represents an aliphatic or hydrophobic amino acid residue, 'K' in the consensus sequence is the SUMO acceptor lysine, and 'X' is any amino acid, which is adjacent to an acidic residue (Asp/Glu) [29, 49, 50]. It has been shown that SUMO may also bind to a lysine residue which is not in the consensus sequence [26–28, 30, 51, 52]. SUMO-binding site prediction algorithms have been developed to facilitate locating SUMO-binding sites in proteins [53]. To locate putative SUMO-conjugating sites in Nrf2 we used the amino acid sequence of human and mouse Nrf2. To find the sequence conservation between human and mouse NRF2 we did multiple alignment for mouse Nrf2 (597 amino acids, NP_035032.1) and human Nrf2 isoform 1 (605 amino acids, NP_006155.2), using NIH's Constraint-based Multiple Alignment Tool (COBALT; www.ncbi.nlm.nih.gov/tools/cobalt). The mouse and human isoform are highly conserved and have more than 98% identity (Supplementary Fig. 1A – for all supplementary material see www.cellphysiolbiochem.com). Using SUMOplot™ Analysis Program we located eight putative SUMO-binding sites in mouse and human Nrf2 isoform 1 as shown in Table 1. The human and mouse have the identical consensus motif except for the two located in the linker region, which are '^{109}PKQD^{112}' (K^{110}) and '^{429}QKAP^{432}' (K^{430}) for mouse and '^{109}PKSD^{112}' (K^{110}) and '^{393}RKTP^{396}' (K^{394}) for the human (Table 1). Out of the predicted eight sites the two putative sumoylation motifs that have a probability score of more than 50% are for K^{110} in 'PKQD' and K^{525} in 'LKDE' for mouse and K^{110} in 'PKSD' and K^{533} in 'LKDE' of human. The 'LKDE' canonical motif conserved in both mouse and human has the highest probability score of 90% for the consensus motif (ψKXE) for SUMO conjugation (Table 1). The Nrf2 K^{110} both for mouse and human is located in the Neh4 domain, while K^{525} for mouse or K^{533} for human within the 'LKDE' sequence is located in the bZIP motif of Neh1 domain involved in DNA binding, Nrf2 binding to its interactor and facilitating the transactivating function of Nrf2 (Supplementary Fig. 1B). These potential SUMO-binding sites were also predicted by SeeSUMO and SUMOsp SUMO-binding sites predication programs.

The K^{110} and K^{525} sequences are bona fide SUMO-2 conjugation sites in mouse Nrf2

To establish whether the K^{110} and K^{525} sites in mouse Nrf2 indeed binds SUMO-2, we mutated the lysine (K) residues to arginine (R). The wild type and mutant protein were expressed with N-terminal GST-tag in *E. coli*. We used the GST tag for purification of the recombinant wild-type and mutant GST-mNrf2 fusion protein from *E. coli*, and assessed their ability to bind SUMO molecules, using an *in vitro* sumoylation assay [40]. Mouse Nrf2 has predicted molecular weight is ~55–65 kDa but it is detected on a immunoblot between ~95–110 kDa in size [1, 54] and additional GST tag increased the size by ~26 kDa, thus the purified recombinant wild-type and mutant GST-mNrf2 migrate on 7% SDS-PAGE as ~130-kDa species as seen in the Coomassie blue stained gel for the purified wild type and mutant GST-mNrf2 protein (Fig. 1A, marked with *). Wild type and mutant GST-mNrf2 protein were purified near homogeneity from the bacterial lysate. Although majority of the purified protein was stable (as shown by the * in Fig. 1A), there was some break down of the protein

as seen by smaller bands in the Coomassie stained gel (Fig. 1A). The identity of the purified recombinant protein was validated by immunoblotting by anti-Nrf2 antibody (Fig. 1B) and anti-GST antibody (Fig. 1C). We chose to use SUMO-2, rather than SUMO-1, because SUMO-2 has been reported to be more abundant in the cells and is conjugated to the target protein upon stress [35, 40]. After in-vitro SUMO-conjugation assay the reaction product were resolve on SDS-PAGE and subjected to immunodetection with SUMO-2/3 antibody. In assay with wild type purified GST-mNrf2 as substrate, we observed higher molecular-size bands (shown by * in Fig. 1D) that migrated with slower mobility compared to control reaction (Fig. 1D, lane 2, upper panel), when the assay was performed in the presence of SUMO-2, indicating that GST-mNrf2 can be sumoylated (Fig. 1D*, lane 3, upper panel*). In comparison to the wild type GST-mNRf2 higher molecular size bands were detected at a lesser degree in the mutant GST-mNrf2: K110R, K525R or 2K (double mutant; K110/525) (Fig. 1D, upper panel). The higher molecular band of wild-type GST-mNrf2 was not detected in the absence of conjugatable SUMO (Fig. 1D, upper panel, lane 2). The differential sumoylation could be due to the difference in the levels of wild type and mutant GST-mNrf2. To address this concern, we stripped the blots in Fig. 1D, upper panel and performed the western blot with anti-Nrf2 antibody. We observed that the relative level of total GST-mNrf2 across the samples were comparable (Fig. 1D, lower panel). With purified GST-mNrf2^{K110R}, GST-mNrf2^{K525R}, and GST-mNrf2$^{K110R/K525R}$ (labeled 2K in all figures moving forward) mutants as substrates, the intensities of the slow migrating bands were much less than with the wild type, especially when the reaction products were blotted with anti-SUMO-2 antibody (Fig. 1D). To quantitate the difference in SUMO-conjugation we did densitometry of the immunoblots and plotted after normalization with the levels of corresponding mNrf2 (Fig. 1E). A significant lower sumoylation in the mutants compared to the wild-type GST-mNRf2 was observed. These data indicate that the mutations at K^{110} and K^{525} impaired sumoylation, and therefore both K^{110} in the ^{109}*PKQD*^{112} and K^{525} in the ^{523}*LKDE*^{527} sites of mNrf2 are SUMO-2 acceptor lysine residues.

SUMO-2 acceptor K^{110} and K^{533} are needed for transcriptional activity of Human Nrf2

After the *in-vitro* validation that K^{110} and K^{525} in the mNrf2 are the SUMO-2 acceptor sites we wanted to assess the functional significance of these sumolyation, thus we switched to human Nrf2 (hNrf2) and used the expression plasmid for Flag-hNrf2. We used hNrf2 wild type, or mutant K110R (^{109}PKSD^{112}), K533R (^{532}LKDE^{535}) and 2K (K110R and K533R, double mutant) which are homologous to the mNrf2 sites as per SUMOplot™ and COBALT analysis (Table 1 and Supplementary Fig. 1). To assess the functional significance of Lysine K^{110} and K^{533} in hNrf2 that were observed to be involved in sumolyation we measured the ability of hNrf2 to transactivate an ARE-driven reporter gene in HEK293T cells. *Heme Oxygenase 1* (HO-1) is a ARE driven gene which is a well-established target for Nrf2 transactivation. We performed dual luciferase reporter assay and used an ARE-driven *Heme oxygenase*-1 promoter-firefly reporter constructs (HO1-ARE-Luc), as experimental reporter and thymidine kinase driven *Renilla* luciferase (TK-Luc) as normalizing control for dual luciferase reporter assay (Fig. 2A), along with plasmid expressing N-terminal Flag-tagged wild type or lysine mutant human Nrf2 protein. To analyze the impact of these mutant on the transcriptional activity of Nrf2 we knocked down endogenous Nrf2 transcripts in HEK293T cells using siRNA directed against the 3'UTR of Nrf2 mRNA. Therefore, during our rescue

experiments ectopic expression of our recombinant FLAG-hNrf2 transcripts which lack the hNrf2 3'UTR, will be resistant to the siRNA mediated knock down. We treated HEK293T cells with siRNA and evaluated the Nrf2 knockdown by quantitative RT PCR (Fig. 2B) and immunoblot (Fig. 2C). We were able to achieve more than 80% knock down of the endogenous hNrf2. Loss of endogenous Nrf2 affected the Nrf2-dependent gene transcription activity as was seen by normalized HO-1-ARE-luc reporter gene activity was 50% decreased in the hNrf2 knock-down cells (siNrf2) compared to cells treated with scrambled control siRNA (siC) (Fig. 2D). As the Nrf2 mediated gene expression is affected by change in the oxidation state of the cells, we stimulated the transfected cells with As_2O_3. Although there is some activation of endogenous hNrf2 transcriptional activity by the treatment of cells with As_2O_3 for 4 hrs the stimulation in control cells is 3-fold compared to its unstimulated control while with Nrf2 knock down it is only 2-fold compared to unstimulated cells with Nrf2 knock-down (Fig. 2D). In the hNrf2 knockdown cells even after stimulation with As_2O_3 the activation is 50% less compared to stimulated control cells (Fig. 2D). Thus, our knockdown of endogenous hNrf2 and stimulation is working and is a good background to study the significance of K^{110} and K^{533} on the transcriptional activity of Nrf2 we performed rescue experiments using the Flag-hNrf2 wild type and different lysine mutants (K110R, K533R and 2K). We studied the rescue of Nrf2-dependent gene transcription in Nrf2 knockdown by co-transfecting the FlaghNRF2 constructs either wild type or mutant along with HO1-ARE-Luc and TK-Luc plasmids in HEK293T cells with Nrf2 knockdown. We observed that the exogenous expression of wild type Flag-hNrf2 increased the reporter activity almost 2-fold compared to the control cells (pFlag; vector alone; Fig. 2E). There was slight increase in the reporter activity by the exogenous expression of the different sumolyation lysine mutants, K^{110}, K^{533} and 2K constructs compared to control but the level of activation was 1.5-fold, which is 50% less than the exogenous expression of wild type Flag-hNrf2 (Fig. 2E). The difference between the transcriptional activation using exogenous expression of wild type and mutants is not due to the differential expression levels of the recombinant Flag-tagged hNrf2 expression as can be seen by western blot with anti-Flag antibody all the wild type (WT) and different mutants (K^{110}, K^{533} and 2K) are expressed at identical levels (Fig. 2F).

siRNA mediated knock down of Nrf2 decreased the endogenous levels of its target HO-1 mRNA (Fig. 3A). We observed a 40% decrease in the HO-1 mRNA in Nrf2 siRNA treated cells compared to control siRNA treated cells. We show that ectopic expression of Nrf2 either wild-type or mutant was able to restore the levels of HO-1 mRNA compared to the control cells (pFlag, Fig. 3B). But we observed that the increase in the levels of HO-1 mRNA was 25% less with the K^{533} and 55% less with the 2K mutant compared to wild-type, while we noticed a slight increase with the K110 mutant. We saw a similar trend as reporter assay for endogenous levels of HO-1 protein after stimulation (Fig. 3C -lane labeled pFlag, & 3D). Our observations revealed that the level of HO-1 protein also followed a similar trend with Nrf2 knock down and rescue experiments (Fig. 3C & 3E). We observed that the level of HO-1 protein was increased 1.5-fold with wild-type Flag-hNrf2 compared to the control cells (pFlag; Fig. 3C & 3E) while it is decreased in the mutants with 60% decrease in the 2K mutant compared to control cells (Fig. 3C & 3E). Thus our observations

suggest that SUMO-2 conjugation sites (K^{110} and K^{533}) in hNrf2 are in part needed for the transcriptional activity of Nrf2.

SUMO-2 acceptor K^{110} and K^{533} are needed for efficient localization of human Nrf2 in the nucleus

In the previous observations it was validated that K^{110} and K^{533} are SUMO-acceptor lysine in hNrf2 and are needed for efficient transcriptional activity of Nrf2. The next question we asked why the K^{110} and K^{533} mutant hNrf2 have lower transcriptional activation, and we proposed that as transcriptional activation happens in the nucleus, the translocation of the mutants (K^{110}, K^{533} and 2K) lacking SUMO-2 conjugation site to the nucleus is compromised. To establish whether SUMO-2 conjugation site deficient Nrf2 would effectively translocate into the nucleus we isolated nuclear enriched and cytosolic fraction of the HEK293T cells transfected with either the wild type or mutant Flag-hNrf2 and performed western blot analysis. Prior to the fractionation the transfected cells were stimulated with 10 μM arsenic trioxide (As_2O_3) for 4 hrs. Untreated cells were used as controls. Arsenic trioxide was used to stimulate Nrf2, which is when larger amounts of Nrf2 have been shown to accumulate in the nucleus upon oxidative stress [55–58]. Even under non-stimulated conditions the level of the wild-type Flag-hNrf2 was relatively higher in the nucleus in comparison to the mutant Nrf2 (Fig. 4A). We observed that compared to unstimulated cells there is relatively more localization of the wild-type and mutant Flag-hNrf2 in the nuclear enriched fractions (Fig. 4B) upon stimulation by As_2O_3. On quantitation we found that upon activation by As_2O_3 the wild type Flag-hNrf2 has maximum localization to the nuclear enriched fraction while there was a 40% reduction in nuclear localization of K110R mutant and 20% reduction in K533R mutant compared to the wild-type Flag-hNrf2 (Fig. 4C). Fibrillarin was used as marker for nuclear enriched fraction validation.

To further validate our immunoblot data we performed immunoflourescnce experiment using confocal microscopy. *In-situ* immunofluorescence analysis of the Flagh-Nrf2 transfected cells using the anti-Flag-Cy3 antibody showed the presence of wild type, K110R, K533R and 2K mutants in the cytosol and the nucleus in HEK293T cells (Fig. 4D). In the same experimental condition, some Flag-Nrf2 also localized to the nucleus (Fig. 4D). However, when the cells were treated with 10 μM As_2O_3, nuclear localization of wild-type Flag-hNrf2 increased significantly (Fig. 4E). However, the localization of the K110R, K533R and 2K was relatively less than the wild-type (Fig. 4E) in the nucleus. The level of K110R was 60% and K533R was 20% less compared to wild-type hNrf2 in the nucleus (Fig. 4E). The 2K mutant had 70% reduction in its nuclear levels upon stimulation compared to the wild-type (Fig. 4E). These results show that the K^{110} and K^{533} residues are needed for efficient nuclear localization of hNrf2. This data suggests that the decrease in mutant hNrf2 transcription activity observed previously may be a result of decreased stability and compromised nuclear localization.

SUMO-2 acceptor K^{110} and K^{533} are needed for stability of Nrf2

To determine if sumoylation of Nrf2 effects not only its subcellular localization but also the its stability, we performed a cycloheximide chase experiment using the Flag-tagged wild

type and mutant (K110R, K533R and 2K) hNrf2. HEK293T cells transfected with Flagh-Nrf2 were treated with cycloheximide for different time points and analyzed through immunobloting. We observed that wild type hNrf2 was detected by anti-Flag antibody till 60 min, while there was no detection of mutants after 15min time-point (Fig. 5A). We also observed that compared to wild type and K533R mutant the K110R was most unstable (Fig. 5A). On quantitation we found that the SUMO-site deficient hNrf2 (K110R, K533R and 2K), reduced to its $t_{1/2}$ within ~15–20 min compared to the longer half-life of ~35–40 min for the wild-type protein (Fig. 5B). We also observed that the stability of Nrf2 affected the availability of its target gene HO-1 product under oxidative stress by reducing its half-life from 60 min in presence of wild-type hNrf2 to less than 10 min in the presence of the mutant hNrf2 (K110R, K533R and 2K; Supplementary Fig. 2A & 2B). We infer from these data that lysine K^{110} and K^{533} sumoylation not only affects the nuclear localization but also stabilizes hNrf2, which assists in the ability of Nrf2 to function as a transcription factor.

Discussion

In this study we took up the challenge of characterizing the key lysine residue(s) within SUMO-conjugating motif(s) in Nrf2 that might be possible target for SUMO-2 modification and the impact of the SUMOylation of these residues on the subcellular localization, stability and transcriptional activity of Nrf2 in cellular context, as until now, identification of the SUMO-2 acceptor site(s) in the Nrf2 had not been undertaken. In our study, first we reveal that K^{110} and K^{525} of mouse Nrf2 and K^{110} and K^{533} of human Nrf2 are the SUMO-2 acceptor lysine in Nrf2. Second, we show that lysine (K^{110} and K^{533}) are needed for nuclear localization of human Nrf2 upon As_2O_3 induced oxidative stress. We also showed that K^{110} and K^{533} provide stability to human Nrf2 and are in part needed for transcriptional activation of its target gene; HO-1 upon oxidative stress. To our knowledge, this is the first study showing that K^{110} and K^{533} are SUMO-2 acceptor lysine in human Nrf2, that are needed for its stability and transactivation function upon oxidative stress induced by arsenic treatment in HEK293T cells.

The transcription factor NRF2 is the master regulator of the cellular antioxidant response. There are different models proposed for regulation of the protein levels of Nrf2 through E3 ubiquitin ligases mediated proteasomal degradation [59]. The most documented model is mediated by Keap1-Cul3-Rbx1 E3 ubiquitin ligase [16, 60, 61] or Keap1 independent mechanism mediate by beta-TcRP. Through its F box motif, β-TrCP binds to the SKP1-CUL1-RBX1 E3 ubiquitin ligase complex and ubiquitylates NRF2 in a KEAP1-independent manner [23, 62]. Then there is E3 ubiquitin-protein ligase HRD1-mediated degradation of Nrf2 under endoplasmic reticulum stress resulting due to unfolded protein response [63]. Upon activation and release from Keap1, Nrf2 translocates to the nucleus and acts as a transcription factor, regulating the transcription of various target genes via the ARE driven promoters to restore oxidative homeostasis.

Whereas post-translational modifications that mediate activation of Nrf2 have been extensively documented, relatively little is known about covalent modifications that impact its activity following activation. In this regards we and others have shown that acetylation and deacetylation [24, 25] regulate Nrf2 nucleocytoplasmic localization and transcriptional

activity. Other PTM that have been documented to affect Nrf2 function are phosphorylation [20–23] and SUMOylation [41]. Sumoylation is the most recent reported PTM of Nrf2 [40]. It has been shown that PML-NB are assembled under conditions of oxidative stress and participate in stress-regulated PTM of partner protein [64]. Work from our lab in past established that Nrf2 traffics, in part, to PML-NBs where we demonstrate that Nrf2 is a target for conjugation with SUMO and poly-sumoylated Nrf2 can be ubiquitylated by RNF4 and then degraded in PML-NBs in a proteasome-dependent manner [40]. Our lab has also documented that degradation of Nrf2 in the cytoplasm and nucleus is compartment specific and intact PML-NBs are essential for modification and degradation of nuclear Nrf2 [65]. Mcintosh et. al. have recently shown that after nuclear translocation Nrf2 undergoes poly-sumoylation and poly-sumolyated Nrf2 is stabilized in the nucleus by Arkadia, an E3 ubiquitin ligase, *via* K48-linked ubiquitylation [41]. Recent work by Guo *et al.* showed that K^{110} in human Nrf2 is a bona fide SUMO-acceptor for SUMO-1 and has a role in serine biosynthesis in hepatocellular carcinoma [42]. The sumoylation on Nrf2 by SUMO-1 has been shown to be necessary for Nrf2-MafG binding [66]. These studies show that Nrf2 is modified by SUMO. Thus, there are evidence that SUMO-modification of Nrf2 occurs. Given that the consequences of sumoylating a protein vary [29, 32, 49, 67–69] and are almost always context-dependent [70]. Sumoylation of transcription factors is a growing field of active investigation [71, 72]. It has been shown that the E3-ligase ZNF451–1, which exclusively conjugates SUMO-2/3 paralogs [33] is needed for stabilization of PML-NB upon oxidative stress during arsenic treatment [73], showing the importance of SUMO-2/3 conjugation under stress situations.

In the present work, we used a SUMO-site prediction algorithm SUMOplot and found that out of the eight putative, there are two most probable SUMO-conjugation motif in Nrf2. Through site directed mutagenesis and *in-vitro* SUMO-2 conjugation assay we validated putative SUMO-2 conjugating lysine, K^{110} within ^{109}PKQD^{112} and K^{525} within ^{524}LKDE^{527} SUMO-conjugation motif are indeed SUMOylated by SUMO-2 in mouse Nrf2. We also validated that homologous motif in hNrf2 for K^{110} within ^{109}PKSD^{112} and K^{533} within ^{532}LKDE^{535} are also SUMOylated. The following observations are noteworthy: the lysine (K) residue in the amino acid segment ^{532}LKDE^{535}, which exactly matches predictable canonical SUMO-binding motif, was found to be authentic for SUMO-2 conjugation. This site was predicted to be a SUMO-binding site by the SUMO-site prediction algorithm, SUMOplot™. We also confirmed one other lysine residue (K^{110}) to be *bona fide* SUMO-2 acceptor residues. K^{110} is also shown to be SUMO-1 acceptor lysine in hepatocellular carcinoma and the authors mentioned that this mutation does not affect the transcription of thioredoxin-dependent antioxidant systems upon serine starvation [42], but in our study we show that K^{110} is modified by SUMO-2 and is needed for nuclear localization and stability of Nrf2. We also showed that K^{110} is needed for transcriptional activation of HO-1 gene by Nrf2 upon arsenic treatment. This cell-type specific and context dependent transcriptional regulation by Nrf2 K^{110} SUMOylation requires further investigation.

SUMOylation is a PTM that primarily influences protein-protein interaction and has been reported to modulate nuclear and intra-nuclear localization of some proteins [4–9], antagonize ubiquitylation [8, 10], induce positive or negative regulation of transcriptional

activity, or engender targeting of proteins for degradation [29, 49, 50]. All these effects are not necessarily observed when a given protein is SUMOylated at a given site because the consequences of SUMOylation are often not predictable, rather, the effects [6, 10–14, 16, 17] appear to be context-dependent [13, 14, 70, 74]. Therefore, it was imperative in our study that we explore specific consequence(s) of the SUMOylation of Nrf2. Heme Oxygenase (HO) enzyme is involved in heme degradation to biliverdin, ferrous carbon monoxide, and its subsequent metabolite bilirubin. HO has three isoforms (HO-1 HO-2 and HO-3). HO-1 is the only inducible isoform. HO-1 expression in mammalian cells contributes to resistance to various types of free radical damages [75]. HO-1 is a Nrf2 target gene which mediates the antioxidant role of Nrf2 [76]. We used the HO-1 expression system as a way to monitor the significance of Nrf2 SUMO-2 modifications. We also observed that in the absence of SUMO-2 acceptor lysine the transcriptional activity of Nrf2 was compromised as shown by the effect on HO-1 mRNA, protein expression and HO1-ARE driven reporter gene assays. which were reduced in the mutants compared to the wild type Nrf2. It is known that Nrf2 interacts with its partner sMaf to activate Nrf2 ARE-driven target gene transcription [76]. It is possible that SUMO-2 conjugation may affect its interaction with sMafs, like SUMO-1 does with Nrf2 and MafG [66], which have been shown undergo SUMO modification [77]. This notion needs further validation. But to address the cause of reduced transcriptional activity we tracked subcellular localization, as Nrf2 has to be in the nucleus to act as a transcription factor. We noticed that subcellular localization of Nrf2 to the nucleus is compromised for the SUMO-2 acceptor site mutants (K^{110} and K^{533}) lowest being for the 2K (K110/533R) mutant. Through cycloheximide chase we found that the in the absence of either of the SUMO-2 acceptor lysine residues K^{110} and K^{533} the stability of the Nrf2 is compromised and half-life s reduced by ~50%, which also results in lower transcriptional activity. Further exploration is needed to find different interacting proteins that make complex with Nrf2 to facilitate its entry to nucleus and protect it from degradation, as consequences of SUMO-2 modification.

In recent times Nrf2 has become a debatable molecule in cancer field accumulating evidences have established Nrf2 driven pathways as driver of cancer progression, metastasis and resistance to therapy [59, 78]. Understanding modifications like SUMOylation of K^{110} and K^{533} that are important in stabilizing Nrf2, understanding how these modifications stabilize Nrf2 and in some cases how they can be destabilized to tackle cancer progression and increase the efficacy of therapeutic agents. It is also important to state that the Cancer Genome Atlas Research, Network has characterized as SNP (single nucleotide polymorphism) at K^{533} in urothelial bladder carcinoma [79], which may result into loss of Nrf2 function and contribute to cancer.

Recent studies have identified new functions for NRF2 in the regulation of metabolism and other essential cellular functions, establishing NRF2 as a truly pleiotropic transcription factor. Recently it was shown that loss of SUMO-E2 conjugatig enzyme Ubc9 causes severe diabetes in mice due to loss of Nrf2 mediated protection against oxidative stress in pancreatic beta-cells [80]. Authors also showed that loss Nrf2 was due to lack of SUMOylation of lysine residues K^{525} and K^{595}, which stabilizes Nrf2 [80]. Our study also finds that SUMO-2 modification of K^{533} in human Nrf2 homologous to mouse K^{525} also stabilizes Nrf2 in human embryonic kidney cells.

Some studies have shown that Nrf2 is involved in cross-talk with NF-kB, p53 and AhR [81], which are involved in inflammation, immune response, Alzheimer disease, cancer, diabetes and arthritis [82–86]. Identifying K^{110} and K^{533} in Nrf2 as SUMO-2 binding sites adds to the understanding of how Nrf2 is regulated and the downward effects with other proteins. Through our observations with some limitations as we are using artificial constructs to express recombinant Nrf2 with either Flag- or GST-tag we want to propose a model involving SUMO-2 modification Nrf2. We want to propose that Nrf2 upon oxidative stress is translocated to the nucleus where it is conjugated with SUMO-2 at K^{110} and K^{533} using the ZNF451–1, which stabilizes the Nrf2 and increases its ARE-mediated transcriptional activation of its target gene HO-1. Nrf2 K^{110} and K^{533} are needed for efficient nuclear localization at this point molecular players involved in K^{110} and K^{533} mediated nuclear translocation are not known. We speculate that these lysine residues are involved in some kind of PTM in the cytosol that facilitate the stability of Nrf2 and its nuclear translocation via interacting with other proteins. The molecular mechanism involved in this process need to be explored and further validated. Thus from our observations we can conclude that SUMO-2 modification of Nrf2 happens at the K^{110} and K^{533} residues and is needed in part for Nrf2 nuclear localization, stability and transcriptional activity of Nrf2 ARE-driven target gene transcription.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2hNrf2	human Nrf2
3’_UTR	3’ untranslated region
ARE	antioxidant response element
cDNA	complementary DNA
GFP	Green fluorescence protein

GST	Glutathione S-transferase
HO-1	heme oxygense gene
Keap1	Kelch-like ECH-associated protein 1
Maf	musculoaponeurotic fibrosarcoma
mNrf2	mouse Nrf2
Neh	Nrf2-ECH-homology
Nrf2	nuclear factor erythroid 2-related factor
ORF	open reading frame
RT-PCR	reverse transcriptase polymerase reaction
SDS-PAGE	Sodium-dodecyl Sulfate poly acrylamides gel electrophoresis

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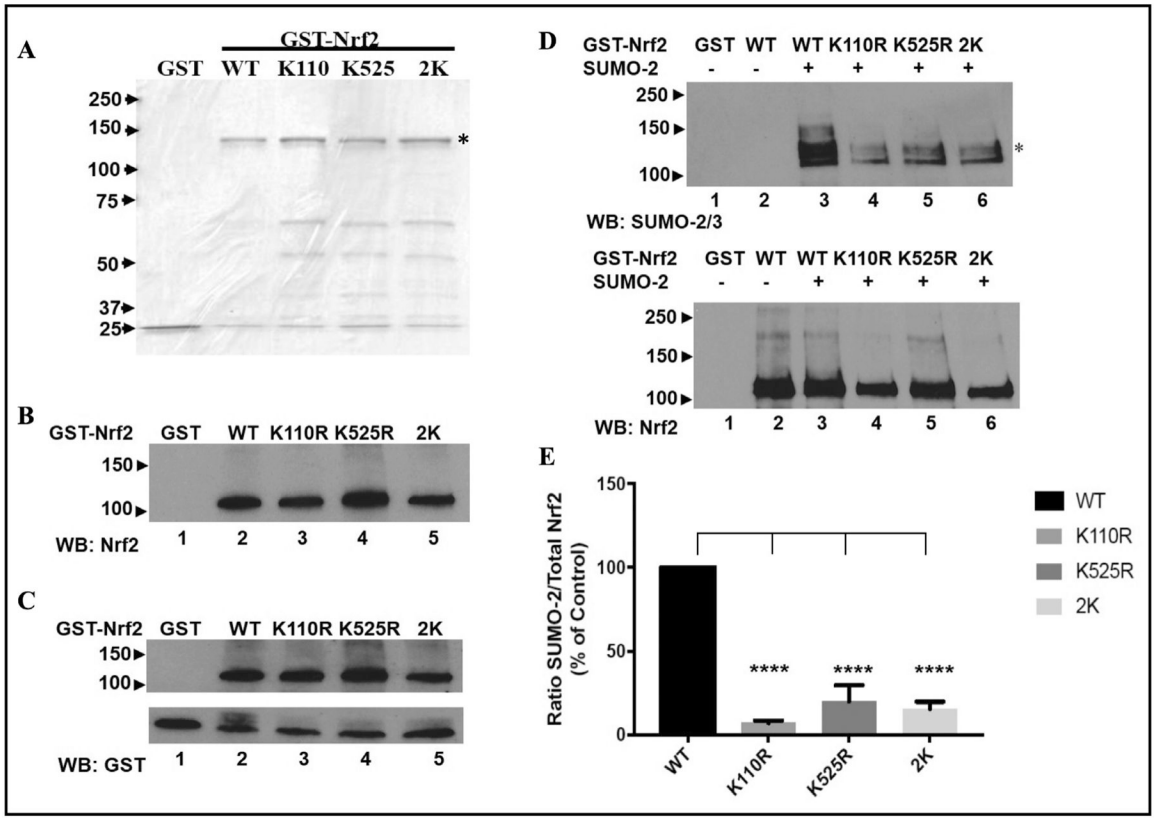

Fig. 1.

K^{110} and K^{525} are SUMO-acceptor lysines in mouse Nrf2. Wild-type and mutant K110R, K525R, 2K (double mutant; K110R/K525R), GST-mNrf2 fusion proteins were purified from E. coli as described under Experimental Procedures. After purification, purified protein was resolved on 7.5% SDS-PAGE. A. Comassie brilliant blue stained gel picture. The purified protein migrates at ~130 kDa marked by *. The identity of the purified protein was validated further with immunoblot analysis. B. Immunoblot analysis of purified protein from panel A with anti-Nrf2 antibody. C. Immunoblot analysis of purified protein from panel A with anti-GST antibody. In vitro sumoylation assay was performed with kits (Active Motif, Carlsbad, CA), using 0.5 μg of the purified fusion proteins as substrates and products of the reaction were separated on 7.5% SDS-PAGE and analyzed by western blotting using the indicated antibodies. The lower panel is showing the bottom part of the same Western blot reacting with anti-GST antibody showing GST protein (~32 kDa). D. SUMO-2 conjugated GST-mNrf2 were detected with anti-SUMO-2/3 antibody (upper panel) and detected with anti-Nrf2 antibody (lower panel). E. Densitometric analysis of the immunoblots in panel D was done and normalized values were plotted to find relative SUMOylation level with respect to total Nrf2. Values plotted (E) (K110R 7%, K525R 20% and 2K 16%) are the ratio of SUMO-2 conjugated mNrf2 to total mNrf2. Results are mean ±S.E. (n=3). Statistical analysis was performed with one-way analysis of variance with Dunnett's multiple comparison test. ****, statistically different ($p<0.001$). The data in B-E are representative of results from three separate experiments.

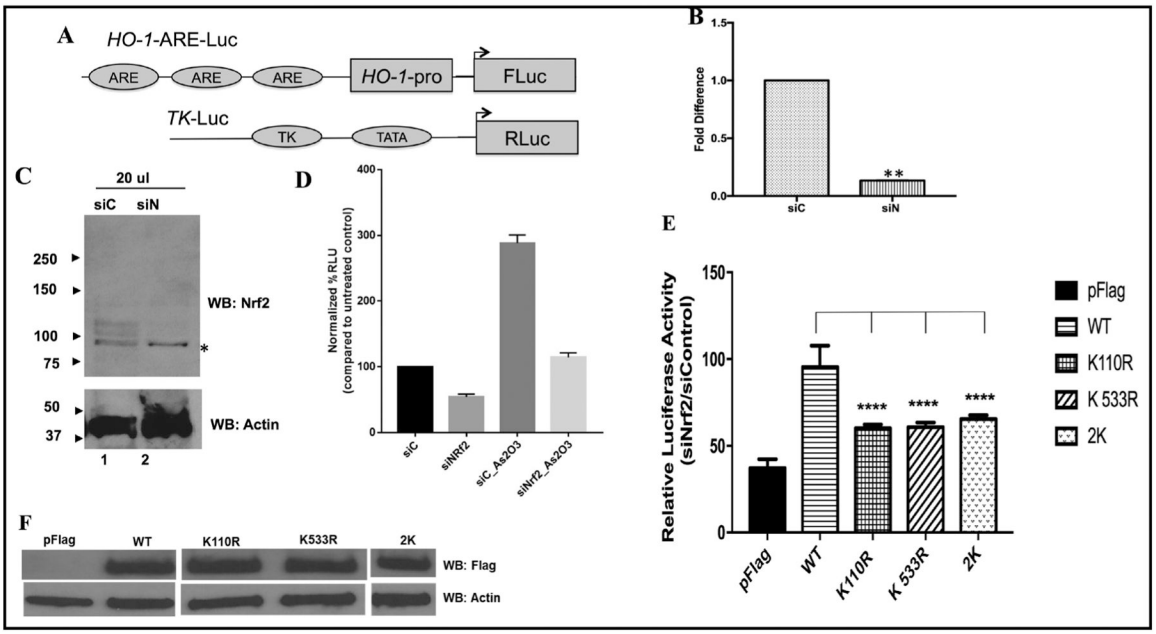

Fig. 2.

SUMO-2 acceptor K^{110} and K^{533} are needed for efficient transcriptional activity of Nrf2. A, Schematic of the HO-1-ARE-Luc (firefly luciferase, experimental) and Tk-Luc (control) promoters used in the luciferase reporter assay. HEK293T cells were transfected with 100 ηM control siRNA or Nrf2 siRNA for 48 hrs. B. Quantitative RT-PCR analysis of the levels of Nrf2 mRNA in HEK293T cells after knockdown with siC: scrambled control siRNA, siN: siRNA against 3'UTR of endogenous Nrf2. Results are mean ±S.E. (n=3). ** indicate there is a statistically significant difference. C. Western blot analysis of Nrf2 protein levels after siRNA mediated knockdown (* denotes the non-specific reactivity of the antibody). D. To monitor Nrf2 mediated transcriptional activation shown is the relative luciferase activity driven by HO1-ARE promoter in HEK293T cells treated with either control siRNA (siC) or siRNA against Nrf2 (siN) without activation, or with siRNA and activation by treatment with As2O3 (siC_As2O3 and siN_As2O3). Results are mean ±S.E. (n=9). ** indicates there is a statistically significant difference in all observations compared to siC. E. To study the significance of the SUMO-acceptor lysine in Nrf2 rescue experiments were performed using plasmid expressing either wild type Flag-hNrf2 (WT) or different mutant Flag-hNrf2 (K110R, K533R or 2K, K110R/K533R double mutant) in the HEK293T cells treated with siRNA against Nrf2. pCMV-Flag vector (pFlag) alone was used as a control. Shown is the relative luciferase activity driven by HO1-ARE promoter mediated by Flag-tagged hNrf2 wild type or mutants. Results are mean ±S.E. (n=9). ****, statistically different ($p<0.001$), indicates there is a statistically significant difference in all observations compared to empty vector (pFlag) transfected cells. F. immunoblot showing the expression of recombinant Flag-tagged Nrf2 wild type (WT) and mutant (K110R, K533R AND 2K) proteins after transient transfection in HEK293T cells using Flag antibody. Empty Flag-vector (pFlag) is used as a negative control. β-Actin is used as a loading control.

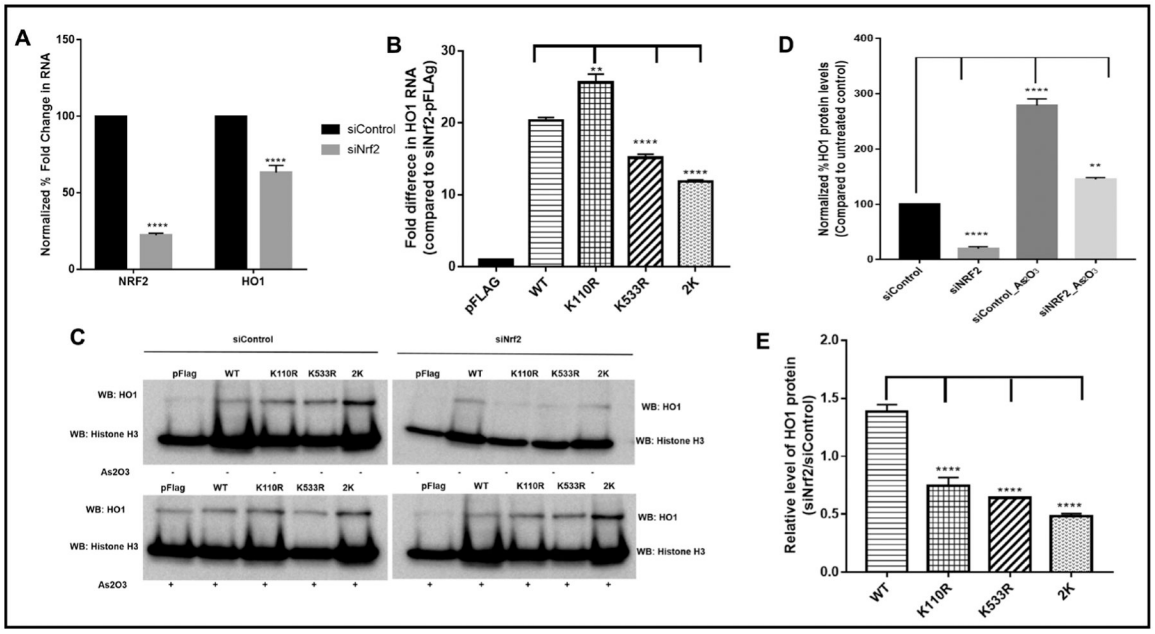

Fig. 3.

Nrf2-dependent gene transcription of Heme Oxygenase 1 (HO-1) gene is reduced in the absence of K^{110} and K^{533} of Nrf2 protein. To study the significance of the SUMO-acceptor lysine in Nrf2 rescue experiments were performed using plasmid expressing either wild type Flag-hNrf2 (WT) or different mutant Flag-hNrf2 (K110R, K533R or 2K (K110R/K533R) double mutant) in the HEK293T cells treated with siRNA against Nrf2. pCMV-Flag vector (pFlag) alone was used as a control. A, shown is real-time RT-PCR analysis of the levels of Nrf2 and HO-1 mRNAs in HEK293T cells treated with control siRNA or Nrf2 siRNA. 18s rRNA mRNA was used as a normalization control. Results are the mean ± S.E. (n = 3). **** indicates statistical significance, $p<0.001$. B, shown is real-time RT-PCR analysis of the levels of HO1 mRNAs in HEK293T cells post rescue with WT or mutant Nrf2 after siRNA mediated knock down of endogenous Nrf2 for 48 hrs,. 18s rRNA mRNA was used as a normalization control. Results are the mean ± S.E. (n = 3). **** indicates statistical significance, $p<0.001$ for all observations compared to that for vector-only control. C, Western blot analysis shows the effect of the wild type and mutant Nrf2 on the HO-1 protein level in HEK293T treated with either control siRNA or Nrf2 siRNA without activation from As_2O_3 (−) and with activation from As_2O_3 (+). Histone-H3 is used as a loading control. D, shown is the densitometric analysis of the pFlag lanes from panel C to show the relative levels HO-1-protein in HEK293T cells treated with either control siRNA (siControl) or siRNA against Nrf2 (siNrf2) without activation, or with siRNA and activation by treatment with As_2O_3 (siControl_As_2O_3 and siNrf2_As_2O_3). Histone-H3 is used as a normalization control. Results are mean ±S.E. (n=4). **** indicates there is a statistically significant difference in all observations compared to siControl. E, shown is densitometric analysis of the Western blot in panel C to evaluate the relative levels of HO-1 protein after rescue experiment. Results are shown as fold change over the empty vector transfected cells post activation by As_2O_3. Results are the mean ± S.E. (n = 4). **** indicates statistical significance $p<0.001$.

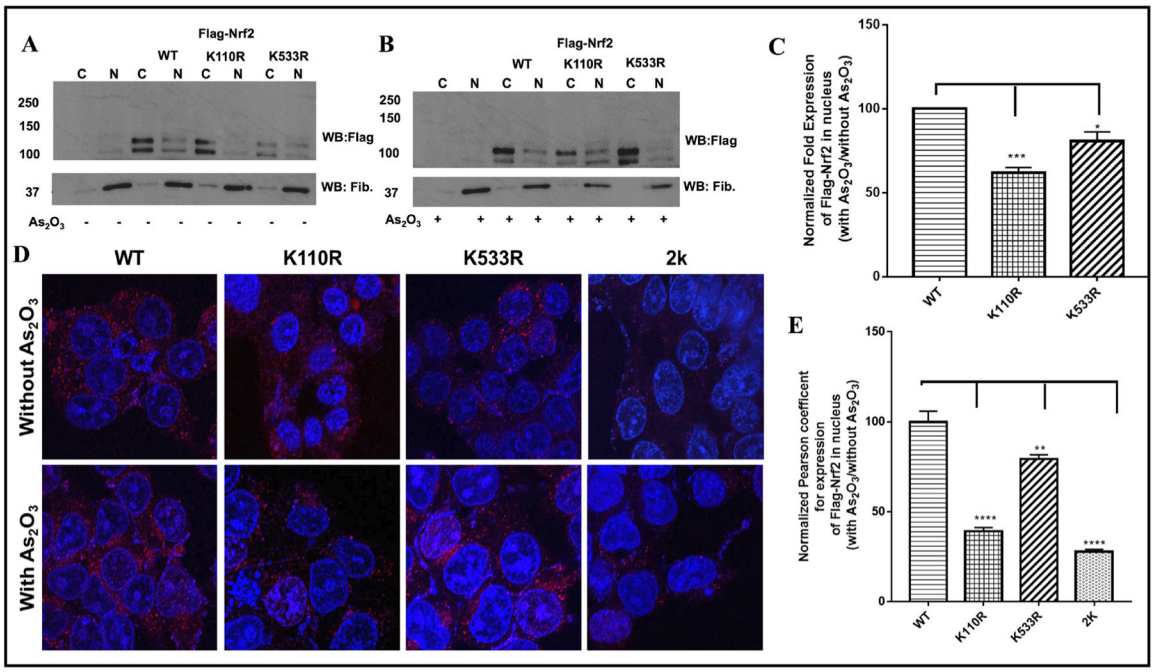

Fig. 4.

SUMO-acceptor lysines are needed for efficient nuclear localization of Nrf2. HEK293T cells were transfected either with the empty Flag-vector (pFLAG), or Flag-Nrf2 wild-type (WT) or mutant (K110R, K533R) and levels of Flag-tagged Nrf2 were monitored in the cytoplasmic and nuclear enriched factions with or without activation with As_2O_3. A. Immunoblot analysis using anti-Flag antibody on cytoplasmic (C) and nuclear enriched (N) factions for cells without activation. B. with activation by 10 μM As_2O_3 treatment for 4 hrs. Antibody against nuclear protein Fibrillarin was used to validate the enrichment of nuclear proteins in nuclear fraction. C, graphs representing the relative level of nuclear Nrf2 post activation with As_2O_3 compared to WT. Density of both the bands in the panel A and B with anti-Flag antibody were quantitated for Flag-Nrf2 levels in the nuclear enriched fraction, normalized by Fibrillarian. The ration of normalized value of with As_2O_3 over without As_2O_3 are plotted. Results are mean ±S.E. (n=3). *** indicates statistical significance $p<0.001$ in mutants compared to wild type. C: cytoplasmic fraction, N: nuclear enriched fraction. C: cytoplasmic fraction, N: nuclear enriched fraction. D. immunofluorescence confocal microscopy analysis of Flag-hNrf2 WT or mutant protein with or without activation by As_2O_3 on its nuclear localization. Anti-Flag-Cy3 (red) antibody was used for detection of recombinant Nrf2 WT or mutant, Cell nuclei are painted blue with DAPI. E. Images such as those in D were used to obtain the Pearson coefficients for subcellular colocalization to determine colocalization of Flag-hNrf2 with the nucleus. Normalized values with As2O3 over without As2O3 are plotted. ** indicates statistical significance $p<0.001$ in mutants compared to wild type.

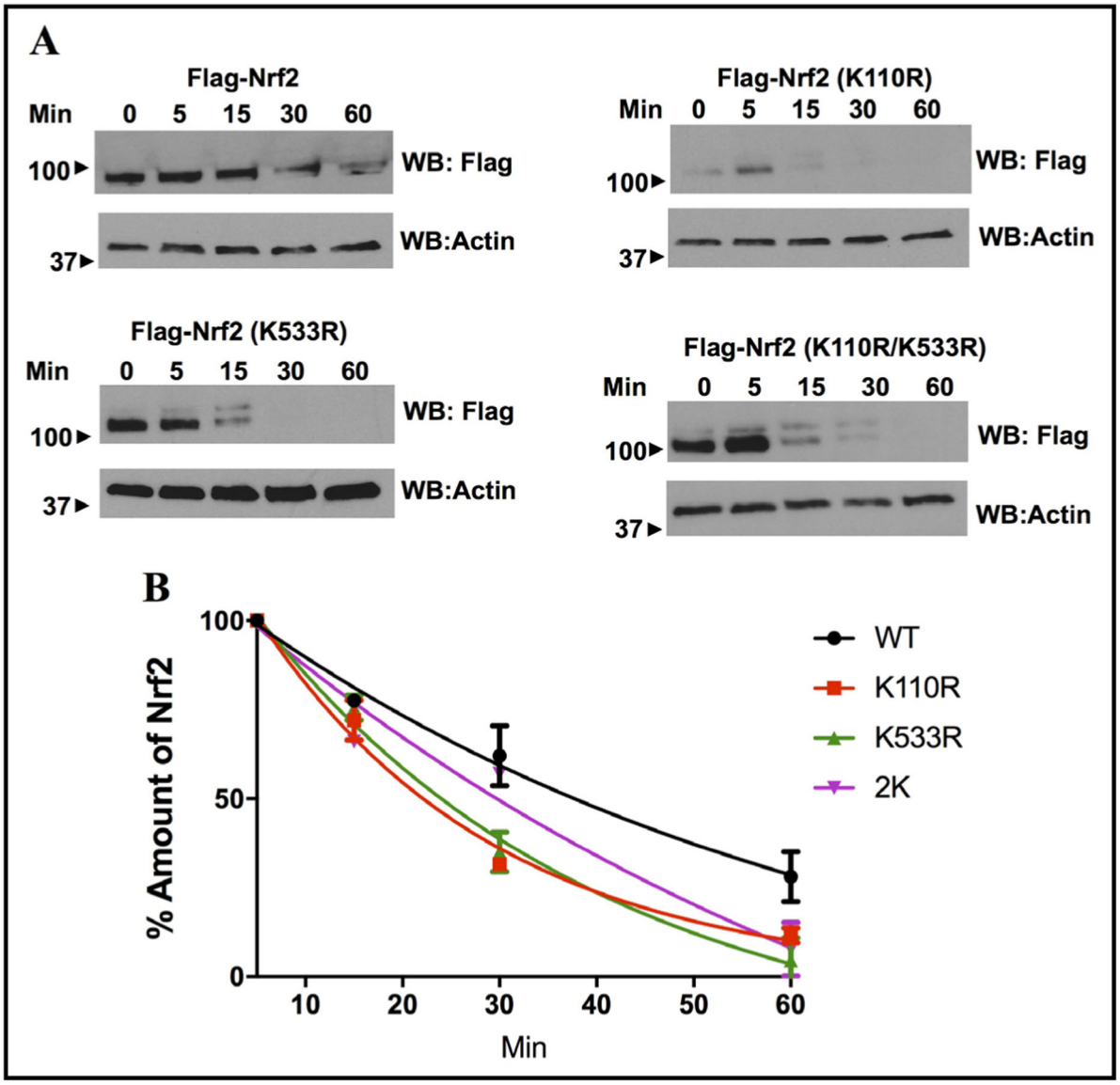

Fig. 5.

SUMO-acceptor lysines are needed for stability of Nrf2. HEK293T cells were transfected with either wild-type Flag-hNrf2 (WT), or mutant FLAG-hNrf2: K110R, K533R, or 2K (double mutant, K110R/K533R). Twenty-four hours after transfection the cells were treated with 100 μM cycloheximide (CHX) for 0, 5, 15, 30 and 60 min. Total cell lysate were prepared and separated on a 7.5% SDS-PAGE and analyzed by Western blotting using anti-Flag and anti-β-Actin antibodies. A, Representative blots are shown. B. Densitometric quantitation of the blots in A. Values for Flag-Nrf2 were normalized with actin and normalized percent values plotted to find the half-life of mutant Flag-hNrf2 with respect to wild type. UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT) was used for quantitation. The values plotted are means ± S.E (n=3).

Table 1.

Putative SUMO acceptor sites in mouse and human Nrf2. SUMOplot™ predicted. Putative, sumoylation sites along with the probability score

Domain in Nrf2	Predicted SUMO-binding site (mouse/human)	SUMO-acceptor Lysine (K) residue (mouse)	SUMO-acceptor Lysine (K) residue (human)	Prediction probability score (mouse/human)
Neh2	KKLE	K53	K53	0.48
	EKAF	K68	K68	0.15
None	PKQD/PKSD	K110	K110	0.61
	QKAP/RKTP	K430	K394	0.39/0.5
Nehl	RKLE	K510	K518	0.44
	LKDE	K525	K533	0.91
	EKGE	K535	K543	0.5
Neh3	KKPD	K591	K599	0.48