Nrf2 Possesses a Redox-sensitive Nuclear Exporting Signal in the Neh5 Transactivation Domain

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NF-E2-related factor 2 (Nrf2) is the key transcription factor regulating the antioxidant response. Previous studies identified a nuclear localization signal (NLS) in the basic region and a nuclear exporting signal (NES) in the leucine zipper domain of Nrf2. In this study, we characterize a new functional NES (L175LLSPELQCLNI186) in the transactivation (TA) domain of Nrf2. A green fluorescence protein (GFP)-tagged Nrf2 segment (amino acids 162–295) called GFP-NESTATA exhibited a cytosolic distribution that could be disrupted by L184A mutation or leptomycin B treatment. Chimeric expression of this NESTATA with a nuclear protein GAL4DBD could expel GAL4DBD into the cytoplasm. A variety of oxidants, including sulforaphane, tert-butylhydroquinone, and H2O2, could effectively induce nuclear translocation of GFP-NESTATA. Mutational studies showed that cysteine 183 may mediate the redox response of NESTATA. The discovery of multiple NLS/NES motifs in Nrf2 and the redox sensitivity of NESTATA imply Nrf2 may be sufficient to sense and transduce oxidative signals into the nucleus, consequently initiating antioxidant gene transcription.

Antioxidant response is an important cytoprotective reaction. When exposed to oxidative stress, mammalian cells can respond with a rapid and coordinated expression of diverse antioxidant genes such as heme oxygenase 1 (HO-1)3 (1), γ-glutamyl-cysteine synthetase/ligase, NAD(P)H:quinone oxidoreductase 1 (2–4), and the phase II detoxifying enzymes such as glutathione S-transferase (GST) (2, 3) and UDP-glucuronosyltransferase (4). Pivotal to the antioxidant response is a transcription factor called Nrf2 (nuclear factor-erythroid 2-related factor 2) (5). Nrf2 knockout mice display reduced constitutive and inducible expression of HO-1 and GST (6–9). Nrf2 null mice are also more susceptible to the chemical toxin treatments (10, 11). Nrf2 is a member of the basic leucine zipper (bZIP) transcription factor subfamily featuring a Cap ‘n Collar motif (5). Like many transcription factors, Nrf2 signaling is regulated by subcellular localization (12). Under homeostatic conditions, Nrf2 molecules are predominantly sequestered in the cytoplasm. When stimulated with oxidative or electrophilic compounds, Nrf2 proteins can quickly translocate to the nucleus and form heterodimers with bZIP proteins called small Maf (musculoaponeurotic fibrosarcoma) proteins (13, 14). Nrf2/Maf heterodimer formation can enhance the specificity and binding of Nrf2 to a cis-acting enhancer called antioxidant-responsive element (ARE) (15) located in the promoter of many antioxidant and phase II detoxification genes (16–19).

Until now, Nrf2 signaling is generally considered to be regulated by a cysteine-rich protein called Keap1 (Kelch-like ECH-associated protein 1) (20, 21). Nrf2 forms a dimer with Keap1 in vitro (22) and probably in vivo as well. An ETGE motif (17) and a DLG motif (23) of the Neh2 (Nrf2 ECH homology) 2 domain of Nrf2 have been elucidated to mediate cooperative binding with the Kelch/double glycine repeat domain of Keap1 (19, 24). Keap1 is an actin-binding protein (19). Treatments with compounds to dissolve the cytoskeleton can result in Nrf2 nuclear accumulation (25). So the cytosolic sequestering of Nrf2 is attributed to Keap1 retention. Keap1 is also identified as a Cul-3-dependent adaptor protein for ubiquitin ligase ubiquitin-protein isopeptide ligase (26–28). Therefore, Nrf2 molecules may not only be sequestered by Keap1 but also subjected to constant degradation. In vitro, the Nrf2/Keap1 dimer can only be formed under reducing conditions (22) and can be disrupted by the treatment of phyto-oxidant sulforaphane or the phenolic compound tert-butylhydroquinone (tBHQ) (22). Some cysteine residues in Keap1, such as Cys-151, Cys-273, and Cys-288, are found to be critical in Nrf2 retention and release (24, 29). Based on these observations, it is hypothesized that Keap1 functions as a redox-switch for Nrf2 signaling (22). Whereas reducing conditions favor Keap1 retention of Nrf2, oxidative signals can induce Keap1 to quickly release Nrf2, leading to nuclear translocation of Nrf2 (20).

Although the Keap1 anchoring model seems to successfully explain the repression and activation of Nrf2 signaling in response to the changing redox conditions, some controversial observations are reported recently. It is reported that the cytosolic distribution of Keap1 is maintained by active nuclear...
Keap1-independent Nrf2 Signaling

export rather than cytoskeleton anchoring (30, 31). In certain cell lines, such as hepatoma HepG2 and H4IEC3 cells, remarkably high amounts of endogenous Nrf2 are found in the cell nucleus at unstimulated conditions (32). Even if the Keap1 anchoring model is still applicable in these cells, the amount or activity of cytosolic Keap1 proteins may not be sufficient to sequester Nrf2. Furthermore, the basal expression of Nrf2-driven antioxidant genes such as HO-1, the modifier subunit of glutamyl-cysteine ligase, and Prdx1 (peroxiredoxin I) remained unchanged in the livers of Keap1 knock-out mice (33). Conceptually, the necessity of Keap1 in Nrf2 signaling is based on the assumption that Nrf2 is unable to maintain cytosolic segregation by itself under quiescent conditions. In addition, the redox-switching role of Keap1 assumes that Nrf2 per se lacks redox responsiveness, or the redox responsiveness Nrf2 is functionally irrelevant to its nuclear translocation. To date, only one reactive cysteine is characterized in the basic region of Nrf2 (34). Mutation of this cysteine to a serine only alters its DNA binding affinity but fails to alter the subcellular localization of Nrf2 (34). These data therefore seem to provide further support to those assumptions. However, with the rapid progress in Nrf2 studies, those assumptions may merely reflect our limited knowledge about Nrf2. Recent studies have identified a nuclear export signal (NES) in the ZIP domain of Nrf2 (NESzip) and a bipartite nuclear localization signal (NLS) in the basic region of Nrf2 (bNLS) (35, 36). These discoveries raise the question whether Nrf2 possesses other functional NES or NLS motifs. In this study, we identify a new functional NES located in the transactivation (TA) domain of Nrf2. The existence of multiple NES and NLS motifs in Nrf2 enables Nrf2 to maintain cytosolic segregation by itself under quiescent conditions. Furthermore, unlike the NESzip, this NES TA possesses a reactive cysteine residue (Cys-183). An enhanced green fluorescence protein (EGFP)-tagged Nrf2 segment (amino acids 162–295), called EGFP-NES TA, exhibited a dosage-dependent nuclear translocation when treated with sulforaphane. Therefore, Nrf2 can sense the redox signal and translocate to the nucleus. These discoveries suggest Nrf2 may be able to transduce redox signals in a Keap1-independent manner. Keap1, however, may provide an additional regulation of the quantity of Nrf2 both at basal and at inducible conditions.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—Human cervical squamous cancerous HeLa cells were obtained from the ATCC (Manas- sas, VA). HeLa cells were cultured as monolayer using mini- mum essential medium supplemented with 10% fetal bovine serum, 2.2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 µg/ml streptomycin. Leptomycin B was purchased from Calbiochem. Reducing GSH, hydroperoxide (H2O2), diethyl maleate (DEM), and propidium iodide (PI) were pur- chased from Sigma. Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). Rabbit anti-lamin A (H-102), anti- Nrf2 (H-300), mouse anti-GFP (B-2), goat anti-Keap1, anti- HO-1 (C-20), and anti-β-actin (I-19) were all purchased from Santa Cruz Biotechnology (St. Cruz, CA).

Plasmid Construction, Site-directed Mutagenesis, and Small Interfering RNA (siRNA) Knockdown—Full-length wild type human Nrf2 cDNA was kindly provided by Drs. Yuet W. Kan (University of California, San Francisco) and Jefferson Chan (University of California, Irvine). To examine the two NES-like motifs individually, two segments of Nrf2, NES TA (amino acids 162–295) and NES INT (amino acids 288–485), were PCR-amplified and subcloned into pEGFP-C1 vector (Clontech) by XhoI and BamHI digestion. The resulting plasmids were designated EGFP-NES TA and EGFP-NES INT, respectively. For fluorescent resonance energy transfer (FRET) examination, the PCR-amplified NES TA segment was inserted into the pEYFP-C1 vector (Clontech) by XhoI/BamHI digestion. The resulting plasmid was designated ECFP-NES TA. As a positive control, N-terminal segment (amino acids 1–174) of Nrf2 was also PCR-amplified and subcloned into the ECFP vector or inserted into ECFP-NES TA plasmid by PstI/BamHI digestion to form an ECFP-GAL4DBD or ECFP- NES TA-GAL4DBD chimera. For mutation studies, the key leucine residue of the NES TA (Leu-184) or the reactive cysteine 183 (Cys-183) was substituted with alanine. The mutagenic primers were synthesized and PAGE purified by Integrated DNA Technologies (Coralville, IA). Mutagenesis reactions were performed using QuikChange XL mutagenesis kit purchased from Stratagene (La Jolla, CA) according to the protocol described previously (35). Mutations were verified by DNA sequencing. The method of siRNA knockdown of Keap1 has been described in detail before (50) with few modifications. The sequence of siRNA primers is 5'-GCGCUUUGGCAUCAU- GAACCTT-3' (sense) and 5'-GUUCAUGGCAAGGC- CTG-3' (antisense). The primers were synthesized, and high pressure liquid chromatography-purified by Integrated DNA Technologies. The siRNA primers were annealed to form double strand Keap1 targeting siRNA with TT and TG overlaps and transfected into HeLa cells using the Lipofectamine method.

Transient Transfection and Reporter Gene Activity Assays—Transactivation activity assay has been described in detail before (37). Briefly, HeLa cells were plated in 6-well plates at ~4.0 × 10^4 cells/well. Twenty four hours after plating, cells were transfected using the Lipofectamine method according to manufactur- er’s instructions. For each well, 200 ng of ECFP-NES TA- GAL4DBD or its C183A mutant and 50 ng of GAL4-Luc reporter were added into 125 µl of Opti-MEM. Lipofectamine 2000 (Invitrogen) was added into another tube of 125 µl of Opti-MEM in a 1:2.5 ratio to the amount of plasmids and incubated at room temperature for 5 min. The plasmid solution was then mixed with Lipofectamine solution with vigorous agitation and incubated at room temperature for 30 min. Cells were incubated with transfection complexes for 3 h, changed to fresh minimum essential medium, and cultured for 16 h. Cells were treated with 5 and 12.5 µM sulforaphane for an additional 6 h.
before harvesting. Cells were washed twice with phosphate-buffered saline (PBS), scraped, and incubated in reporter lysis buffer (Promega) on ice for 30 min. After centrifugation, 10 μl of lysate was mixed with luciferase substrate (Promega), and the ARE-luciferase activity was measured using a Sirius luminometer (Berthold Detection System). Protein concentration was measured using the Bradford method. Luciferase activity was normalized by protein concentration.

**Cell Fractionation**—The protocol to extract nuclear and cytoplasmic proteins has been described before (38) with minor modifications.Briefly, HeLa cells were cultured in 60-mm Petri dishes and transfected with 5 μg of plasmids using the Lipofectamine method (Invitrogen). After 24 h, cells were rinsed with ice-cold PBS and harvested with cell lysis buffer A (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, pH 8.0). After incubation on ice for 10 min, the samples were centrifuged at 12,000 × g for 15 min. Supernatants (cytosolic extract) were collected. Nuclear pellets were washed twice with cell lysis buffer A and then resuspended in high salt buffer B (20 mM Tris-Cl, pH 7.9), vortexed, and centrifuged. Supernatants (nuclear extract) were collected. The protein concentration of each sample was measured. To generate homogeneous electrophoretic patterns, cytosolic proteins were diluted in buffer B. 20 μg of nuclear proteins and 10 μg of cytosolic proteins were loaded for immunoblot analysis.

**Immunoprecipitation and Western Blotting**—Twenty four hours after transient transfection of ECFP-Nrf2NT, ECFP-NES₅₁, and EYFP-Keap1, HeLa cells were harvested in cell lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM NaF, 100 μM Na₃VO₄, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor. The protein concentrations of cell lysates were determined by the Bradford method. For immunoprecipitation (IP), 50 μg of cell lysates expressing ECFP-Nrf2NT or ECFP-NES₅₁ were mixed with 100 μg of cell lysate expressing EYFP-Keap1. The mixture was incubated with 20 μl of protein A-conjugated Sepharose 4B beads (Zymed Laboratories Inc.) to remove endogenous IgG. Solutions were then incubated with 1 μg of mouse anti-GFP antibody at 4 °C for 1 h, subsequently mixed with 40 μl of protein A-Sepharose beads, and tumbled at 4 °C overnight. The IP solutions were then centrifuged. Pellets were washed three times with the lysis buffer. Protein complexes were eluted by loading buffer and heated to 80 °C for 5 min and subsequently analyzed by Western blotting. For Western blotting, either the IP products or lysates containing 20 μg of protein were resolved by 4–15% linear gradient SDS-PAGE (Bio-Rad) and transferred to polyvinylidene fluoride membrane using a semi-dry transfer system (Fisher). The membrane was blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) containing 20 mM Tris-HCl, 8 mg/ml NaCl, and 0.2% Tween 20, pH 7.6, at room temperature for 1 h. The membrane was probed with polyclonal rabbit anti-Nrf2 (H-300) (1:750), goat anti-Keap1 (1:750), anti-lamin A (1:250), and anti-β-actin (1:1000) in 3% nonfat milk TBS-T at 4 °C overnight. After washing three times with TBST, the membrane was blotted with peroxidase-conjugated secondary antibody (1:5000 dilution) at room temperature for 1 h. Proteins were visualized using the ECL mixture from Bio-Rad.

**Epifluorescent Microscopy**—Expression and subcellular distribution of EGF-P-NES₅₁/H₂O₂ or its mutants in response to treatments of LMB or oxidative compounds was examined using a Nikon Eclipse E600 epifluorescent microscope and a Nikon C-SHG1 UV light source purchased from Micron-Optics (Cedar Knolls, NJ). HeLa cells were cultured on ethanol-sterilized glass coverslips and transfected with 1 μg of EGFP-NES₅₁ or its mutants by using the Lipofectamine method (Invitrogen) and cultured in minimum essential medium for 24 h. Prior to microscopic examination, some coverslips were briefly fixed in methanol for 2 min, rinsed three times in PBS, and then counterstained with PI (5 µg/ml PI in PBS solution supplemented with 100 µg/ml RNase A) to visualize the positions of cell nuclei. The EGFP signals were examined using a fluorescein isothiocyanate filter, and the PI signals were examined with a Texas Red filter. The epifluorescent images were digitized using a Nikon DXM1200 camera and Nikon ACT-1 software (version 2). Images were superimposed using Adobe Photoshop CS version 8.0. For the kinetics study, the cell numbers exhibiting whole cell or cytosolic distribution pattern in response to sulforaphane treatments were counted. The ratio of whole cell distribution was calculated and plotted against the durations of sulforaphane treatment using a curve fitting software (Origin 7.0) based on the Michaelis-Menten equation.

**Confocal Microscopy and FRET Assay**—To examine ectopic expression, HeLa cells were cultured in glass bottom dishes (MatTek, Ashland, MA) and transfected with plasmids expressing ECFP-GAL4DBD, ECFP-NES₅₁-GAL4DBD, or its L184A mutant. For FRET assay, HeLa cells were transfected with plasmids expressing ECFP-Nrf2NT, ECFP-NES₅₁, and EYFP-Keap1. Twenty four hours after transfection, cells were examined using a Zeiss LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY) with a ×63 water-immersion objective. We used a sensitized emission method for the FRET assay (39, 40). Three filter sets were used to detect the donor (ECFP), acceptor (EYFP), and FRET signals. The FRET signal is corrected for spectral bleed through and contamination of donor and acceptor fluorescence according to Youvan’s models (40) as indicated in Equation 1,

\[
F_c = \frac{(FRET - bg_{fret}) - cf_{don} \times (Don - bg_{don})}{1 - cf_{acc} \times (Acc - bg_{acc})} \quad (Eq. 1)
\]

The abbreviations uses are as follows: \(F_c\) is FRET concentration; \(bg\) is background intensity; \(cf\) is correction factor; \(fret\) is FRET signal; \(don\) is donor signal; and \(acc\) is acceptor signal.

The FRET concentration was normalized to donor and acceptor concentrations according to Equation 2,

\[
Fn = \frac{F_c}{\sqrt{(Don - bg_{don}) \times (Acc - bg_{acc})}}
\]

\[
Ac = \frac{F_c}{(Acc - bg_{acc})} \quad (Eq. 2)
\]

For data acquisition, the donor (ECFP) channel was excited with an argon laser line at 457 nm, and the emission was detected using a bandpass filter of 475–525 nm. The acceptor
To be a valid NES, the functional NES motifs are designated with filled circles. The pseudo-NES is designated with an open circle. Abbreviations used are as follows: + + , the basic region; bNLS, bipartite NLS motif localized in the basic region; CNC, the Cap’n Collar domain; GAL4DBD, the DNA binding domain of yeast GAL4 protein; LLLLIL, leucine zipper domain; Neh2, the Keap1 binding domain; Neh4 and Neh5, tandem of transactivation motifs; NESint, NES-like motif located in the intervening domain; NES	\textsubscript{TA}, NES motif located in the Neh5 domain; NES	\textsubscript{zip}, NES motif localized with the leucine zipper domain; Nrf2	\textsubscript{NT}, the N-terminal segment of Nrf2.

(EYFP) channel was excited at 543 nm and its emission was detected at 545–600 nm. The FRET channel was excited at 457 nm, and the emission was detected at 545–600 nm. For data analysis, we used the LSM510 SP2 software (version 3.2) to subtract donor and acceptor bleed through and normalize against acceptor (EYFP) and donor (ECFP) intensity.

**RESULTS**

**Dissecting Individual NES-like Motif of Nrf2—Cytoplasmic-Nuclear Transportations are Mediated by the Importin and Exportin Proteins.** They recognize a specific nuclear localization signal (NLS) or nuclear export signal (NES) on cargo proteins (41). Consensus leucine-rich NES motifs can be formulated as $\Phi^1(X)_2^3\Phi^1(X)_2^3\Phi^2\Phi^1$, where $\Phi$ represents hydrophobic amino acids, and $X$ represents any amino acid (42). To be a valid NES, $\Phi^1$ and $\Phi^2$ must be hydrophobic amino acids, and the positions of $\Phi^3$ and $\Phi^4$ may wobble (42). In Nrf2, besides the documented NESzip (35), there are several NES-like motifs. In this study, we examined one NES-like motif ($^{175}$LLLLIL$^{186}$) located in the Neh5 transactivation domain (TA) and one NES-like motif ($^{296}$LLLLIL$^{308}$) located in the intervening domain (INT), with the $\Phi$-corresponding residues underlined. Both NES-like motifs have legitimate hydrophobic residues at the $\Phi^1$, $\Phi^2$, and $\Phi^3$ positions. The $\Phi^4$ residue for NES\textsubscript{TA} is isoleucine 178. The $\Phi^4$ residue of NES\textsubscript{INT} is a glycine 299 residue or a proline 300 residue; none are long chain hydrophobic amino acids. Close to the Gly-299 residue, there are tandem leucines (residues 296–297) and an isoleucine residue (301). To examine whether these two NES-like motifs are functional, we made two enhanced green fluorescence protein (EGFP)-tagged chimeras, EGFP-NES\textsubscript{TA} containing the TA segment of Nrf2 (amino acids 162–295) and EGFP-NES\textsubscript{INT} containing the INT segment of Nrf2 (amino acids 288–485) (Fig. 1). These two constructs were so designed that they contained no other consensus NES, NLS, or NES/NLS-like motifs. In addition, the elucidated Keap1-binding motifs were also carefully excluded.

**Nrf2 Possesses a Functional NES Located in the TA Domain—**When expressed in HeLa cells, EGFP-NES\textsubscript{TA} exhibited a predominantly cytosolic distribution (Fig. 2A). The green fluorescence of EGFP-NES\textsubscript{TA} was found confined in the cytoplasm, with cell nuclei devoid of fluorescence (Fig. 2A). The positions of cell nuclei were also confirmed by PI counter-staining (Fig. 2A) superimposed with green fluorescent image (Fig. 2A). Cell percentage assay showed that EGFP-NES\textsubscript{TA} proteins were cytoplasmic in 83.5% of the transfected cells (Table 1). In the remaining cells (16.5%), an evenly distributed pattern was observed (Table 1). There was virtually no cell showing nuclear distribution. In contrast to NES\textsubscript{TA}, NES\textsubscript{INT} did not appear to be a functional NES. In HeLa cells, EGFP-NES\textsubscript{INT} is localized to both the cytoplasm and the nucleus (Fig. 2A). Therefore, despite the similarity of NES\textsubscript{INT} to the canonical NES, NES\textsubscript{INT} probably is a pseudo-NES. To determine whether the cytosolic distribution of EGFP-NES\textsubscript{TA} is mediated by the NES\textsubscript{TA} motif, we performed site-directed mutagenesis of NES\textsubscript{TA}. Substitution of leucine 184 with alanine (L184A) was sufficient to convert the cytosolic distribution of wild type NES\textsubscript{TA} into a whole cell distribution pattern (Fig. 2B). All HeLa cells expressing EGFP-NES\textsubscript{TA} L184A exhibited a whole cell distribution pattern (Table 1). Furthermore, we examined whether the function of NES\textsubscript{TA} is mediated by binding with nuclear exporting protein, CRM1 (chromosome maintenance region 1) (43). One hour of treatment with LMB, a specific CRM1 inhibitor (44), could convert the distribution of EGFP-NES\textsubscript{TA} into a whole cell pattern (Fig. 2B; Table 1). These data are in agreement with a previous study that CRM1 inhibited NES\textsubscript{TA} cytosolic localization (35). Combined, these data suggest that the observed cytosolic distribution pattern of EGFP-NES\textsubscript{TA} is likely maintained by active export in a CRM1-dependent manner.

**Chimeric Expression of NES\textsubscript{TA} Can Expel GAL4DBD into the Cytosol—**To examine whether the NES\textsubscript{TA} alone is sufficient to cause nuclear export, we fused the NES\textsubscript{TA} to the DNA binding domain of the yeast GAL4 protein (GAL4DBD), a well known nuclear protein (45). We constructed an ECFP-tagged chimerical protein by inserting the NES\textsubscript{TA} segment to the N terminus of GAL4DBD (Fig. 1). When expressed alone, ECFP-GAL4DBD was nuclear in 65.1% of the transfected cells (Fig. 2C, arrowheads; Table 1). In contrast, more than 80% of cells expressing ECFP-NES\textsubscript{TA}-GAL4DBD chimera exhibited a cytosolic distribution pattern (Fig. 2C; Table 1). A point mutation in...
The NES$_{TA}$ Is Redox-sensitive—Because Nrf2 plays the pivotal role in transducing oxidative signals, we next asked whether NES$_{TA}$-mediated nuclear exporting activity can be modulated by redox compounds. Based on the chemical structures, oxidative compounds, also known as phase II inducers, can be divided into 10 classes (46). In this study, we examined the redox reactivity of EGFP-NES$_{TA}$ to the treatments of H$_2$O$_2$ (hydroperoxide), DEM (Michael acceptor), tBHQ (quinone), and sulforaphane (isothiocyanate). When EGFP-NES$_{TA}$-expressing cells were treated with solvent dimethyl sulfoxide (DMSO) as a negative control for 3 h, an uninterrupted cytosolic distribution was observed (Fig. 3A; Table 2). In contrast, 1 h of treatment with 1 mM H$_2$O$_2$ or 1 mM DEM could almost completely convert the cytosolic distribution of EGFP-NES$_{TA}$ to a whole cell pattern (Fig. 3A; Table 2). A similar disruptive effect on nuclear exporting activity of the redox-sensitive NES of the bZIP protein Bach2 was reported using similar dosages (35). Common to the treatments of all four oxidants, virtually no nuclear condensation of EGFP-NES$_{TA}$ was observed, probably reflecting the fact that EGFP-NES$_{TA}$ lacks any NLS motif. So the maximal inducing effect elicited by oxidants was a 100% whole cell distribution. We also examined the effect of reducing compounds on EGFP-NES$_{TA}$. After 3 h of treatment of 1 mM reducing GSH, a sulforaphane-conjugating agent, the cytosolic distribution of EGFP-NES$_{TA}$ was slightly increased (Fig. 3A; Table 2), probably reflecting inhibition of basal activation of EGFP-NES$_{TA}$. Co-treatment of 1 mM GSH could completely abolish the mobilizing effect of sulforaphane (Fig. 3A; Table 2).

Because there is a cysteine residue (Cys-183) embedded in the leucine residue (Leu-184) in the NES$_{TA}$ motif, we mutated the Cys-183 residue to examine whether this cysteine residue is a reactive cysteine mediating the redox reactivity of the NES$_{TA}$ motif. The C183A mutant significantly attenuated the translocation effect exerted by H$_2$O$_2$, DEM, tBHQ, and sulforaphane as compared with wild type EGFP-NES$_{TA}$. A considerable percentage of EGFP-NES$_{TA}$

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**TABLE 1**

| Constructs Subcellular distribution (%)$^a$ | N   | N + C | C |
|--------------------------------------------|-----|-------|---|
| EGFP-NES$_{TA}$ only                       | 0.0 | 0.0   | 100.0 |
| EGFP-NES$_{TA}$ L184A                      | 0.0 | 0.0   | 100.0 |
| EGFP-NES$_{TA}$ + LMB                      | 0.0 | 0.0   | 100.0 |
| ECFP-GAL4DBD                               | 65.1| 10.8 | 33.7 |
| ECFP-NES$_{TA}$-GAL4DBD                    | 0.0 | 0.0   | 100.0 |
| ECFP-NES$_{TA}$-(L184A)-GAL4DBD            | 41.7| 12.2 | 56.9 |
| EGFP-Nrf2                                  | 29.6| 2.0  | 84.4 |
| EGFP-Nrf2 (L184A)                          | 11.2| 3.9  | 79.6 |
| EGFP-bNLS-NEStip                           | 88.5| 5.4  | 11.5 |
| EGFP-Nrf2 (L544A)                          | 84.8| 5.0  | 10.8 |
| EGFP-Nrf2 (L154A)                          | 84.7| 7.6  | 15.3 |

$^a$ Percentages of HeLa cells that displayed nuclear (N), whole cell (N + C), and cytoplasmic (C) distribution of EGFP/ECFP-tagged proteins are presented. For each construct, around 300 cells were randomly chosen, counted, and pooled as a result of three independent experiments. The values were present as mean ± S.D.
transfection, cells were treated with 5 and 12.5 μM ECFP-NES TA-GAL4DBD or its C183A mutant (200 ng) with GAL4-Luc reporter (50 ng). Sixteen hours after nuclear and cytosolic proteins, respectively.

In comparison, treatment of 50 μM sulforaphane for 3 h failed to change EGFP-NESTA distribution. Co-treatment of 1 mM GSH effectively inhibited sulforaphane-elicited nuclear translocation of ECFP-NESTA(C183A). Treatment with 1 mM reducing GSH for 3 h failed to change EGFP-NESTA distribution. In HeLa cells, EGFP-NESTA exhibited cytoplasmic distribution when treated with solvent Me2SO (DMSO), Lamin A and β-actin were used as controls for endogenous nuclear and cytosolic proteins, respectively.

### TABLE 2

| Constructs | Treatments | Duration (h) | N/C | C |
|------------|------------|--------------|-----|---|
| GFP-NESTA | Me2SO | 3 | 18.3 ± 1.7 | 81.7 ± 3.0 |
| GFP-NESTA(C183A) | Me2SO | 3 | 8.0 ± 0.2 | 92.0 ± 4.0 |
| GFP-NESTA | 1 mM H2O2 | 1 | 91.3 ± 0.5 | 8.7 ± 0.5 |
| GFP-NESTA(C183A) | 1 mM H2O2 | 1 | 76.0 ± 6.1 | 24.0 ± 4.4 |
| GFP-NESTA | 1 mM DEM | 1 | 98.6 ± 0.0 | 1.4 ± 0.3 |
| GFP-NESTA(C183A) | 1 mM DEM | 1 | 74.1 ± 0.0 | 25.9 ± 2.1 |
| GFP-NESTA | 200 μM tBHQ | 3 | 71.9 ± 2.9 | 28.1 ± 2.6 |
| GFP-NESTA(C183A) | 200 μM tBHQ | 3 | 21.0 ± 1.1 | 79.0 ± 3.7 |
| GFP-NESTA | 50 μM SFN | 3 | 83.5 ± 2.6 | 16.5 ± 0.2 |
| GFP-NESTA(C183A) | 50 μM SFN | 3 | 48.1 ± 2.4 | 51.9 ± 3.6 |
| GFP-NESTA | 1 mM GSH | 3 | 9.7 ± 0.9 | 90.3 ± 6.4 |
| GFP-NESTA(C183A) | 1 mM GSH + 50 μM SFN | 3 | 10.7 ± 0.8 | 89.3 ± 6.7 |

* Percentages of HeLa cells that displayed whole cell (N + C) and cytoplasmic (C) distribution of EGFP-tagged proteins in response to the treatments of redox compounds are presented. For each treatment, around 500 cells were randomly chosen, counted, and pooled as a result of three independent experiments. The values werepresent as mean ± S.D.
expressed the ECFP-NESTA-GAL4DBD chimera with the GAL4-luciferase reporter and measured the luciferase induction. At basal conditions, expression of wild type ECFP-NESTA-GAL4DBD elicited high constitutive luciferase activity that could be significantly down-regulated by sulforaphane treatment with 1 mM GSH (data not shown). The luciferase activities induced by wild type ECFP-NESTA-GAL4DBD were significantly higher than the C183A mutant (Fig. 3C), suggesting the C183A mutant may be less responsive at the basal conditions. When we treated cells expressing wild type ECFP-NESTA-GAL4DBD with sulforaphane (5 or 12.5 μM) for 6 h, there was pronounced luciferase induction in a dosage-dependent manner (Fig. 3C). The folds of induction (1.85 ± 0.10) mediated by the ECFP-NESTA-GAL4DBD chimera in response to 12.5 μM sulforaphane treatments appeared a little bit lower than the induction folds (~2.3) mediated by another Nrf2-GALDBD chimera N2 (amino acids 113–251) (37). The difference may be attributed to the fact that N2 contains both the Neh4 and Neh5 transactivation domains, whereas the NESTA only contains the Neh5 domain (48). In comparison with the wild type, only modest luciferase induction was observed for C183A mutant in response to sulforaphane treatment (Fig. 3C), consistent with the hypothesis that Cys-183 is a redox sensor.

**NES**<sub>TA</sub> **Nuclear Translocation Kinetics**—After observing NESTA was a redox-sensitive NES, we measured the kinetics of EGFP-NESTA nuclear translocation triggered by oxidants. Because the translocation effect exerted by sulforaphane was close to the physiologically achievable concentrations (49), we chose to study the translocation kinetics elicited by sulforaphane. As mentioned previously, the EGFP-NESTA construct lacks any NLS motif, and the maximal induction effect on EGFP-NESTA was a conversion from a cytosolic distribution to a whole cell distribution pattern. We employed the ratio of cells exhibiting whole cell distribution of EGFP-NESTA as an index of nuclear translocation. We treated EGFP-NESTA-expressing HeLa cells with 5, 12.5, 25, and 50 μM sulforaphane at 37 °C with 5% CO<sub>2</sub> supplement for various durations. We determined the ratio of cells showing uniform EGFP-NESTA localization patterns in response to sulforaphane treatments and plotted their values against the duration of treatments (Fig. 4A). The kinetics of sulforaphane-induced EGFP-NESTA translocation could be described by two parameters. One is the maximal accumulation ($A_{max}$) of EGFP-NESTA in the nucleus. The other parameter is the half-time to achieve maximal translocation ($t_{1/2}$). The $A_{max}$ values delineated the magnitude of EGFP-NESTA translocation, whereas the $t_{1/2}$ indicated the speed of translocation.

The EGFP-NESTA showed a dosage-dependent nuclear translocation in response to sulforaphane treatments (Fig. 4A). In the four tested concentrations, the $A_{max}$ values of EGFP-NESTA appeared to be positively correlated with the sulforaphane concentration (Fig. 4B; Table 3), i.e. the stronger the oxidative signal, the more EGFP-NESTA will translocate into the nucleus. The $t_{1/2}$ values are inversely correlated with sulforaphane concentrations (Fig. 4B; Table 3), suggesting that the stronger the oxidative signal, the faster the Nrf2 influx.

Consistent with the cysteine-mediated redox sensitivity, the C183A mutation significantly slowed down nuclear translocation (Fig. 4A). The $t_{1/2}$ value of the C183A mutant was almost two times longer than the wild type EGFP-NESTA when treated with 50 μM sulforaphane (Table 3). Unlike the $t_{1/2}$ value, the $A_{max}$ value of the C183A mutant was virtually unchanged compared with wild type EGFP-NESTA in response to 50 μM sulforaphane treatment (Table 3). The functional significance of this differential response of $t_{1/2}$ and $A_{max}$ of the C183A mutant is unknown.

To exclude the possibility that the observed sulforaphane-induced translocation effect is mediated by the EGFP tag, we also treated the EGFP-expressing cells with 50 μM sulforaphane for 3 h. EGFP protein was nonresponsive to sulforaphane treatment (data not shown). These data suggest EGFP-NESTA translocation is specifically mediated by the EGFP tag, whereas the EGFP-NESTA translocation is not affected by sulforaphane. As mentioned previously, the EGFP-NESTA construct lacks any NLS motif, and the maximal induction effect on EGFP-NESTA was a conversion from a cytosolic distribution to a whole cell distribution pattern. We employed the ratio of cells exhibiting whole cell distribution of EGFP-NESTA as an index of nuclear translocation. We treated EGFP-NESTA-expressing HeLa cells with 5, 12.5, 25, and 50 μM sulforaphane at 37 °C with 5% CO<sub>2</sub> supplement for various durations. We determined the ratio of cells showing uniform EGFP-NESTA localization patterns in response to sulforaphane treatments and plotted their values against the duration of treatments (Fig. 4A). The kinetics of sulforaphane-induced EGFP-NESTA translocation could be described by two parameters. One is the maximal accumulation ($A_{max}$) of EGFP-NESTA in the nucleus. The other parameter is the half-time to achieve maximal translocation ($t_{1/2}$). The $A_{max}$ values delineated the magnitude of EGFP-NESTA translocation, whereas the $t_{1/2}$ indicated the speed of translocation.
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## TABLE 3

| Kinetics of SFN-elicited NES_TA nuclear translocation |
|------------------------------------------------------|
| Kinetics study of sulforaphane (SFN)-induced nuclear translocation of EGFP-NES_TA or its C183A mutant. CRM1-specific inhibitor LMB was used as a positive control. \( A_{\text{max}} \) is the maximal accumulation of influx of EGFP-NES_TA or its mutant in the nucleus. The \( t_{1/2} \) is the time constant of half-maximal influx. \( R^2 \) is the regression coefficient. The values of \( A_{\text{max}} \) and \( t_{1/2} \) were calculated from more than 600 cells from three independent experiments. |
| LMB (nM) | SFN (mM) | EGFP-NES_TA | SFN (mM) | EGFP-NES_TA (C183A) |
|----------|----------|-------------|----------|---------------------|
| 10        | 100      | 1.000 ± 0.000 | 0.043 ± 0.020 | 0.991 |
| 5         | 0.5      | 1.000 ± 0.000 | 0.043 ± 0.020 | 0.991 |
| 12.5      | 25       | 1.000 ± 0.000 | 0.043 ± 0.020 | 0.991 |
| 25        | 50       | 1.000 ± 0.000 | 0.043 ± 0.020 | 0.991 |

### FIGURE 5.

**FRET assay failed to detect NES_TA/Keap1 interactions.** A, confocal microscopy for FRET assay. The emitted fluorescence of donor (ECFP), acceptor (EYFP), and FRET were detected in three different channels. When 0.5 μg/ml ECFP-Nrf2_NT was co-expressed with 0.5 μg/ml EYFP-Keap1, intense FRET signals were detected. In contrast, virtually no FRET signals could be detected when ECFP-NESTA was co-expressed with EYFP-Keap1, indicating there is no interaction between NES_TA and Keap1. The fluorescence intensity was indicated by the color-coded bar. Scale bar indicates 10 μm. B, calculated FRET values after correction of spectral bleed through and contamination of donor and acceptor fluorescence. C, ECFP-Nrf2_NT could be immunoprecipitated with EYFP-Keap1. In contrast, ECFP-NESTA failed to be co-precipitated with EYFP-Keap1. Cell lysates (50 μg) expressing ECFP-NeSTa or ECFP-NESTA were mixed with 100 μg of lysate-expressing EYFP-Keap1. The protein complex was precipitated by mouse anti-GFP antibody and detected by anti-Nrf2 and Keap1 antibody, respectively.

Redox-sensitive Translocation of EGFP-NES_TA Is Keap1-independent—The EGFP-NES_TA construct did not contain any known Keap1-binding motif. However, to exclude the possibility that the observed redox-reactive NES_TA translocation is mediated by an unknown Keap1-binding motif, we performed FRET assay (39, 40). The FRET technique detects direct interaction between molecules. A fluorophore 1 (donor) is tagged on molecule A, and fluorophore 2 (acceptor) is tagged on molecule B, the putative binding partner of A. The emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. If molecule A and B physically bind, the complex will bring the donor and the acceptor in close proximity (i.e. nanometer range); the induced FRET signal can be detected when donor alone is stimulated. The FRET technique can discern molecules that co-localize in the same subcellular compartment but have no direct binding. In this study, we used an ECFP as donor and an EYFP as acceptor. We added an ECFP tag on the NES_TA segment (Fig. 1). As a positive control, we also added ECFP tag to an N-terminal segment of Nrf2 (Nrf2_NT, amino acids 1–174), which contains both the Keap1-binding ETGE motif (17) and the DLG motif (23). When expressed alone, EYFP-Keap1, ECFP-NES_TA, and ECFP-Nrf2_NT all exhibited cytosolic distributions (data not shown). Co-expression of ECFP-NES_TA with EYFP-Keap1 did not induce a FRET signal (Fig. 5, A and B). In contrast, co-expression of ECFP-Nrf2_NT with EYFP-Keap1 produced intense FRET signals treatments (Fig. 4A). The measured \( t_{1/2} \) of LMB treatment is also faster than the formerly reported time constant, measured at room temperature (35°C). The difference may due to the temperature (37°C) used in our experiments.

Multivalent NES/NLS Motifs Determined the Subcellular Distribution of Nrf2—The discovery of a new NES_TA motif, in combination with the previously characterized NESzip and bNLS, suggests Nrf2 possesses multiple NES and NLS motifs.
For a cargo protein containing multiple NES/NLS motifs, the tendency of its cytoplasmic-nuclear transportation is determined by the summation of the activity of each individual NES and NLS. Previous studies show that the driving force of the NESzip is weaker than the bNLS (35, 36). Indeed, an Nrf2 segment containing both the bNLS and the NESzip motif (Fig. 1, EGFP-bNLS-NESzip) exhibited a mainly nuclear distribution (Fig. 6A; Table 1). A cell fractionation assay also confirmed that nuclear immunoreactivity of EGFP-bNLS-NESzip was stronger (Fig. 6B). In comparison, the NES TA motif appeared to be a stronger NES. An Nrf2 segment containing both the NES TA and the bNLS motif (Fig. 1, EGFP-NESTA-bNLS) showed a predominantly whole cell distribution. More than 75% of cells showed whole cell distribution (Fig. 6A), and only 11.2% cells showed nuclear distribution (Fig. 6A, arrowheads), and 12% cells showed cytosolic distribution (Fig. 6A). There was a higher percentage of EGFP-Nrf2-expressing cells showing nuclear distribution in comparison with EGFP-NESTA-bNLS-expressing cells (Table 1). The NES TA-bNLS segment lacked the 162 amino acids in the N terminus of Nrf2. Whether this area contains any unidentified NLS is unknown. In addition, this N-terminal region also appeared to have strong redox reactivity4; therefore, it may be more sensitive to basal oxidative stress. These observations may explain why there appears to be a higher percentage of nuclear distribution in EGFP-Nrf2 than in EGFP-NESTA-bNLS-expressing cells.

In previous studies (35, 36) and in this study, a point mutation of a single key leucine residue, i.e. Leu-544 in the NESzip motif and Leu-184 in the NES TA motif, was sufficient to nullify their nuclear exporting activity. Mutation of either the Leu-184 residue or the Leu-544 residue in full-length Nrf2 was sufficient to convert the heterologous distribution pattern (Fig. 6, A and B; Table 1). In other words, both NES TA and NESzip motif are indispensable to counterbalance the nuclear importing activity mediated by the bNLS motif alone or with other unidentified NLS motifs.

Loss of function of the NES TA motif could naturally occur in the cell. Because the NES TA motif is redox-sensitive, the increased oxidative stress may disable the NES TA motif. However, the redox reactivity of NES TA as observed in segment studies, may not prove functional in the full-length Nrf2 due to the difference of protein folding and solvent accessibility of oxidants. We further examined whether oxidants could mobilize wild-type Nrf2 into the nucleus. At a low concentration of 50 μM, a 10-min treatment of tBHQ or H2O2 caused pronounced nuclear translocation of EYFP-Nrf2 (Fig. 7A; Table 4). Similar results were reported in Hepa-1 cells that treatments with 50 μM tBHQ can elicit GFP-Nrf2 nuclear translocation within 15 min (36). In MDA-231 cells, 16 h of treatment with 25 μM tBHQ can elevate nuclear immunoreactivity of Nrf2 (24). When endogenous Keap1 proteins were knocked down (supplemental Fig. 1) by siRNA (50), the tBHQ-induced nuclear translocation of EYFP-Nrf2 appeared to be uninterrupted (Table 4), suggesting that tBHQ-induced Nrf2 nuclear translocation is mainly

4 W. Li, unpublished data.
independent of Keap1. In comparison to the wild type Nrf2, the C183A mutant exhibited an attenuated translocating response. Under basal conditions, nuclear localized Nrf2 (Fig. 7A, arrowheads) decreased remarkably from 24.3% in wild type Nrf2 to 7.8% in the C183A mutant (Table 4). In parallel, significantly more C183A mutant-expressing cells (27.3%) exhibited the cytosolic localization (Fig. 7A, arrows) than cells expressing wild type Nrf2 (10.9%) (Table 4). AttenuatedARE-luciferase induction was also observed in the C183A mutation (Fig. 7B).

In summary, these data clearly show that under normal physiological conditions, the redox reactivity of the NES$_{TA}$ motif enables Nrf2 to detect oxidative signals and transmit them to the nucleus.

**DISCUSSION**

In this study, we identified a novel redox-sensitive NES$_{TA}$ motif located in the transactivation domain of Nrf2. The discovery of NES$_{TA}$ may draw a quite different picture of Nrf2 signaling.

Previous studies identified a NESzip and a bNLS motif in Nrf2 (35, 36). Because the driving force of the bipartite bNLS is stronger than the NESzip (35, 36), if Nrf2 only possesses a bNLS and a NESzip, it has a stronger tendency driving toward the nucleus and indeed would need Keap1 to anchor it in the cytoplasm during the unstimulated state. The existence of an extra NES$_{TA}$ motif with a strong driving force, however, may reverse the cytoplasm-nuclear transport tendency of Nrf2. Our present study shows that the NESzip and the NES$_{TA}$ in combination can effectively counterbalance the activity of bNLS and provide sufficient nuclear export driving force to hold Nrf2 in the cytoplasm (Fig. 6). If this is true, Keap1 may be dispensable to maintain cytosolic sequestering of Nrf2.

Unlike the NESzip motif, this newly identified NES$_{TA}$ motif appeared to be redox-sensitive. Treatments with diverse oxidative compounds elicited EGFP-NES$_{TA}$ nuclear translocation (Fig. 3; Table 2). There is a Cys-183 residue embedded in this NES$_{TA}$ motif, bearing resemblance to the reported redox-sensitive NES motifs of the yeast AP-1-like transcription factor (YAP-1) (51) and mammalian bZIP protein Bach2 (47). Our mutation analyses showed that NES$_{TA}$ redox sensitivity may be mediated by the Cys-183 residue. C183A mutation could remarkably slow down translocation kinetics (Fig. 4, A and B; Table 3) and attenuate the luciferase reporter gene activity (Fig. 3C). It is possible that direct sulfhydryl modification of the Cys-183 res-
induce inhibits the access and binding of nuclear exportin CRM1 to the NES$_{TA}$ motif and consequently results in nuclear accumulation of EGFP-NES$_{TA}$. Similar results have been reported in YAP-1 protein (51). Alternatively, intramolecular disulfide bond formation may also disable the NES activities (52). In our EGFP-NES$_{TA}$ construct, there is a highly conserved Cys-226 residue (Fig. 9). Whether the Cys-226 residue functions as a disulfide bond partner of Cys-183 requires further study. To examine these possibilities, it is necessary to detect the presence and type (53) of sulfhydryl adducts of NES$_{TA}$ using the liquid chromatography and tandem mass spectrometry method, which has been successfully used in characterizing the reactive cysteine residues of Keap1 in response to the treatments of tBHQ (54) and sulforaphane (55).

Unlike the CRM1-specific inhibitor LMB, which can covalently modify exportin CRM1 (44) and exert a nearly all-or-none effect on NES$_{TA}$ nuclear translocation, sulforaphane could elicit NES$_{TA}$ nuclear translocation in a dosage-dependent way (Fig. 4B). Presumably due to high concentrations of endogenous glutathione, sulforaphane may only partially and reversibly disable the NES$_{TA}$. The dosage-dependent response of NES$_{TA}$ to sulforaphane suggests that Nrf2 cannot only sense oxidative signals but also precisely transmit the “intensity” of oxidative signals to the nucleus and up-regulate gene transcription accordingly, particularly at low stress conditions.

Based on these observations, we propose a new Nrf2 signaling model (Fig. 8). The Nrf2 molecule possesses multivalent NES/NLS motifs, and their relative driving forces are represented by the size and direction of the arrows (see Fig. 8). Under the unstimulated conditions (Fig. 8A), the combined nuclear exporting forces of NES$_{TA}$ and NESzip could counteract the nuclear importing force of the bNLS. As a result, Nrf2 exhibits a predominantly whole cell distribution (Fig. 6A). Although the majority of Nrf2 molecules remain in the cytoplasm, the residual nuclear Nrf2 may account for the basal or constitutive Nrf2 activities. The observation of a small percentage of cells exhibiting nuclear and cytosolic distribution of Nrf2 (Fig. 6A) may reflect the hyper- and hypo-oxidative condition of individual cells, respectively. When challenged with oxidative stress (Fig. 8B), the redox-sensitive NES$_{TA}$ is disabled, but the redox-insensitive NESzip remains functional (35), and the bNLS motif may remain functionally uninterrupted (34). Because the driving force of NESzip is weaker than the bipartite bNLS motif (Fig. 6), the nuclear importing force mediated by the bNLS prevails and triggers Nrf2 nuclear translocation (Fig. 8B).

This model may not only account for the repression and activation of Nrf2 signaling but may also account for the inactivation of Nrf2 signaling. Accumulating evidence shows that Nrf2 activation can consequently elevate the expression and enzymatic activities of γ-glutamyl-cysteine synthetase/GST as well as the GSH level in cells (56, 57). Considering the reversible nature of sulfhydryl modification by sulforaphane, the elevated GSH levels may favor the restoration of NES$_{TA}$ activity and trigger Nrf2 nuclear export. Further studies are needed to examine this possibility.

Unlike the previous Keap1 model, this new model hypothesizes a Keap1-independent Nrf2 signaling. Neither FRET nor the IP assay could detect NES$_{TA}$/Keap1 interaction. Inhibiting endogenous Keap1 activity with siRNA also failed to change tBHQ-induced nuclear translocation of Nrf2 (Table 4). Definitive proof

[TABLE 4: Nuclear translocation of full-length Nrf2 induced by oxidants]

| Constructs                  | Subcellular distribution* |
|-----------------------------|---------------------------|
|                             | N  | N + C | C   |
| EYFP-Nrf2                   | 24.3 ± 2.1                  | 64.8 ± 4.7 | 10.9 ± 0.9 |
| EYFP-Nrf2 + Keap1 siRNA     | 23.0 ± 1.3                  | 71.6 ± 0.2 | 5.3 ± 0.7  |
| EYFP-Nrf2 + 50 μM tBHQ (10 min) | 45.5 ± 3.6                  | 50.0 ± 3.6 | 4.5 ± 0.0  |
| EYFP-Nrf2 + Keap1 siRNA + 50 μM tBHQ (10 min) | 45.0 ± 0.2                  | 49.8 ± 0.9 | 5.2 ± 1.8  |
| EYFP-Nrf2 + 50 μM H$_2$O$_2$ (10 min) | 33.1 ± 1.6                  | 55.7 ± 3.2 | 11.2 ± 0.2 |
| EYFP-Nrf2 (C183A)           | 7.8 ± 0.7                   | 64.9 ± 0.8 | 27.3 ± 1.6 |
| EYFP-Nrf2 (C183A) + Keap1 siRNA | 4.6 ± 0.2                   | 70.4 ± 4.0 | 25.1 ± 1.7 |
| EYFP-Nrf2 (C183A) + 50 μM tBHQ (10 min) | 19.9 ± 2.9                  | 67.9 ± 0.8 | 12.2 ± 2.1 |
| EYFP-Nrf2 (C183A) + Keap1 siRNA + 50 μM tBHQ (10 min) | 8.3 ± 0.5                   | 80.5 ± 1.8 | 11.2 ± 0.5 |
| EYFP-Nrf2 (C183A) + 50 μM H$_2$O$_2$ (10 min) | 8.9 ± 1.3                   | 62.1 ± 1.0 | 29.0 ± 0.4 |

*Percentages of HeLa cells that displayed nuclear (N), whole cell (N + C), and cytoplasmic (C) distribution of EYFP-tagged proteins are presented. HeLa cells were transfected with 0.5 μg/ml EYFP-Nrf2 or EYFP-Nrf2 (C183A) plasmids with or without 125 nM Keap1 siRNA. Sixteen hours after transfection, cells were treated with tBHQ or H$_2$O$_2$ and subjected to microscopic examination. For each construct, around 600 cells were randomly chosen, counted, and pooled as a result of three independent experiments. The values were present as mean ± S.D.

[FIGURE 8: Hypothetic model for Nrf2 signaling. The identified NES and NLS motifs of Nrf2 are symbolized by filled circles and boxes, respectively. Their driving forces are designated by the direction and size of arrows. During the unstimulated condition (A), two NES motifs can counterbalance the driving force of the bNLS motif and sequester Nrf2 in the cytoplasm. When challenged by oxidative stress (B), the reactive cysteine in the NES$_{TA}$ can detect the presence of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and disable the NES$_{TA}$ (plain line). As a result, the driving force of bNLS motif prevails and causes Nrf2 nuclear translocation.]}
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may require replicating EGFP-NES<sub>TA</sub> translocation experiments in Keap1 null mouse embryonic fibroblast cells. Although Nrf2 appears to be self-sufficient to transduce redox signals, this new Keap1-independent model does not exclude the involvement of Keap1. Keap1 may provide an additional regulation controlling the Nrf2 availability, especially via the constant ubiquitination and proteosomal degradation. Therefore, this new model favors the debate that Keap1 function is alternative but not exclusive in regulating Nrf2 signaling (58). The importance of Keap1 may vary in a spatial- and temporal-dependent fashion. To some Nrf2-driven structural proteins, such as keratin K6 and loricin of epidermal tissue (59), rigid Keap1 sequestering of Nrf2 appears to be vital during development. The Keap1 null mice were found to die of hyperkeratosis of the esophagus and forestomach before weaning (59). In contrast, the liver-targeted Keap1 knockout appeared to incur no severe phenotypic deficit. Although the activities of some phase II genes such as GST<sub>μ</sub> subunits were indeed up-regulated (33), the expression of some other antioxidant and phase II genes such as HO-1, modifier subunit of glutamyl-cysteine ligase, and peroxiredoxin I were unaffected (33). The importance of Keap1 regulation in Nrf2 signaling may depend on its actual expression level. Unfortunately, so far it is unknown whether there is tissue- or cell type-specific difference in the expression ratio of Nrf2 over Keap1. This will require further study.

If the new NES<sub>TA</sub> plays an important function of redox switch for Nrf2 signaling, we asked whether this NES<sub>TA</sub> motif is conserved across species. When the amino acid sequences of all cloned Nrf2 molecules were aligned with the key Φ positions underlined, this NES<sub>TA</sub> motif is found conserved only in human, dog, bovine, chicken, and Zebrafish (Fig. 9). To our surprise, this NES<sub>TA</sub> motif appeared not to be conserved in the p45 protein, the rodent, and frog Nrf2 orthologs. In the Φ<sup>1</sup> position, a hydrophilic threonine was found in the p45 protein and rodent Nrf2. A polar asparagine residue was found in the frog Nrf2 (Fig. 9, light gray box). According to the formulation of consensus NES motifs (42), the presence of threonine or asparagine may be sufficient to disable the NES activity. Re-examining the human NES<sub>TA</sub> sequence, we found the NES<sub>TA</sub> motif may also orient in a reversed direction, as the key Φ positions are illustrated by italicized fonts and underlined by dotted lines (Fig. 9). In the reversed orientation, the NES<sub>TA</sub> motif appears to be conserved in all cloned Nrf2 molecules, including p45, rodent, and frog Nrf2 molecules (Fig. 9). Because the Φ<sup>1</sup> residues of functional NES motifs are usually leucine or isoleucine (42), one concern is that in p45 and rodent Nrf2, the Φ<sup>1</sup> residue is a phenylalanine (Fig. 9). Occasionally, atypical hydrophobic residues are found at the Φ<sup>1</sup> position of functional NES, such as a methionine was found at the Φ<sup>1</sup> position of RXRα (60). Future expressive assays are needed to examine whether these NES<sub>TA</sub>-like motifs are functional.

The Nrf2 molecule shares homology and probably similar origin with Nrf1, including the N terminus (61). In contrast, the Nrf2 molecule shares poor homology with Nrf3 in the N terminus (62). It is very interesting to find that the NES<sub>TA</sub> motif is also strictly conserved in all cloned Nrf1 molecules, including rodent Nrf1 (Fig. 9).

With regards to the redox sensitivity, the residues corresponding to the reactive Cys-183 in human Nrf2 are conserved in almost all cloned Nrf2 molecules (Fig. 9), underscoring its potential importance for Nrf2 signaling. In contrast, this cysteine residue is absent in Nrf1 molecules (Fig. 9). The Cys-226-corresponding residue is highly conserved in Nrf1 and Nrf2 molecules (Fig. 9), with the exception of frog Nrf2 and Zebrafish Nrf1. However, the reactive cysteine residue corresponding to the Cys-183 in human Nrf2 is conserved in all cloned Nrf2 molecules (black box) but not in Nrf1 molecules. In comparison, the residue corresponding to Cys-226 in Nrf2 is conserved in nearly all cloned Nrf2 and Nrf1 molecules with the exception of frog Nrf2 and Zebrafish Nrf1 (unknown). In comparison, the NES-like motif of NES<sub>INT</sub> does not match the NES formula in both orientations.

FIGURE 9. Sequence alignments of the NES<sub>TA</sub> motif. Positions of key residues (Φ) in the consensus NES motif, as formulated by Φ<sup>1</sup>(X)2–3Φ<sup>2</sup>(X)2Φ<sup>3</sup>, are denoted by the underlined bars. The newly identified human NES<sub>TA</sub> motif appears to be conserved (transparent boxes) in dog, bovine, chick, and Zebrafish Nrf2. However, the NES<sub>TA</sub> motif is not conserved (shaded box) in p45 molecule and mouse, rat, and frog Nrf2. In comparison, this NES<sub>TA</sub> motif is strictly conserved in all cloned Nrf1 molecules. However, the NES<sub>TA</sub> motif may be conserved in a reversed orientation as designated by dotted underlines and italicized Φ positions in all cloned Nrf2, including p45 and rodent Nrf2. The reactive cysteine residue corresponding to the Cys-183 in human Nrf2 is conserved in all cloned Nrf2 molecules (black box) but not in Nrf1 molecules. In comparison, the residue corresponding to Cys-226 in Nrf2 is conserved in nearly all cloned Nrf2 and Nrf1 molecules with the exception of frog Nrf2 and Zebrafish Nrf1 (unknown). In comparison, the NES-like motif of NES<sub>INT</sub> does not match the NES formula in both orientations.

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